Method for Sterility Testing of Oils

By R. RUSSOMANNO and E. G. WOLLISH

A reliable procedure was needed for the sterility testing of oils since the present U.S.P. method, primarily designed for aqueous solutions, did not always yield unequivocal results. A membrane filtration method together with the necessary equipment was developed. The procedure, which is simple and rapid, is useful for the detection of contaminations of oils (except those of high viscosity) with viable organisms, even at low count levels.

THE STERILITY testing of oils used as a vehicle for drugs has presented problems for the bacteriologist for quite some time. Eisman *et al.* (1) have studied the preservation of vegetable oils by chemical agents and determined the number of surviving microorganisms by plate counts. In the present investigation, the U.S.P. XVI method (2), designed chiefly for aqueous preparations, has not proved very satisfactory for the sterility testing of oils; another procedure using detergents (3) has shown limitations due to poor growth conditions. In several publications, Gay (4) has demonstrated the advantages of membrane filtration for the sterility testing of injectables. Membrane filtration, proposed by Holdowsky (5), has now become the official procedure for the testing of antibiotics (6). At a November 1963 meeting in London, Gay presented details of a recent modification of a membrane filtration apparatus which completely eliminates manual transfers (7). Sokolski and Chidester (8) described a membrane filtration method for petrolatum-based ointments. This unique method utilizes isopropyl myristate as solubilizer for the ointment base.

EXPERIMENTAL

A simple method has been developed which permits the separation of viable microorganisms by membrane filtration. The filtration is followed by washing and removal of the oil with an aqueous solution of Triton X 100, a detergent that was found nontoxic in contact with several types of microorganisms. The membrane filter is punched out, and parts of it are incubated in both media fluid thioglycollate U.S.P. and fluid sabouraud U.S.P.

The method has been applicable to a variety of oils (sesame, peanut, mineral, and liquid coconut oil), except for those of high viscosity, like castor oil. The procedure has been found dependable in about two years of usage in our laboratory.

Equipment.—The assembled filter unit (Millipore or equivalent) (Fig. 1) without membrane is autoclaved for 30 minutes at 121° or 10 minutes at 132°. A Cornwall pipeting syringe (BD) (Fig. 1) is assembled as a pumping device to transfer oil samples into the filter unit. The pumping apparatus is sterilized in a manner as above. The sterile units are dried before use. (Our laboratory utilizes a high-vacuum sterilizer.) The bacteriological membrane filters (Millipore), 0.45-μ porosity, 47-mm. diameter, are sterilized for 10 minutes at 121°. Before the test, a filter membrane is aseptically placed in the unit. Membrane cutting devices and forceps are prepared

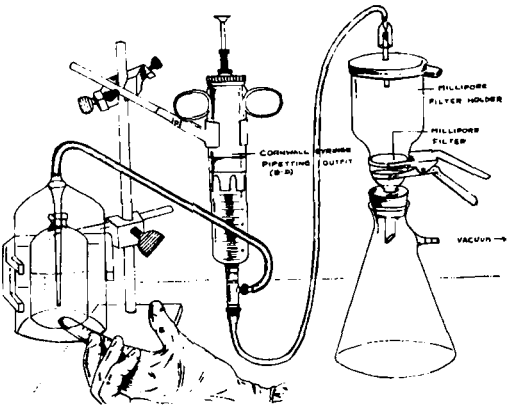

Fig. 1.—Assembled filtration unit.

and sterilized prior to testing. An approximate quantity of 100 ml. of fluid thioglycollate and sabouraud liquid medium (Difco or Baltimore Biological Labs.) with 0.1% polysorbate 80 U.S.P.[1] added is dispensed into 38 × 200-mm. test tubes. The tubes are closed with metal closures and sterilized for 20 minutes at 121°. Aqueous solutions of the detergent Triton X 100 (Rohm and Haas), 0.1%, and of peptone (Difco or BBL), 0.1%, are prepared and sterilized for 20 minutes at 121°.

Procedure.—A 200-ml. sample of oil is pumped aseptically (Cornwall syringe BD) into a filter unit (Fig. 1). A vacuum source (approximately 550 mm. Hg) is attached to the filter unit. The oil sample is then filtered through the bacteriological membrane. The membrane is washed by filtration with two 200-ml. portions of 0.1% Triton X 100 to remove residual oil. The Triton washings are immediately followed by a single wash with 200 ml. of 0.1% peptone. With a sterile, circular punch, the center area (approximately 17.5 mm. diameter) is cut out, as described by Holdowsky (5), and transferred aseptically to a 38 × 200-mm. tube containing approximately 100 ml. of fluid thioglycollate medium with 0.1% polysorbate 80.

The outer portion of the membrane is transferred aseptically into a similar tube containing approximately 100 ml. of sabouraud liquid medium with 0.1% polysorbate 80. A fluid thioglycollate medium tube containing the center portion of the membrane is incubated at 30–32° for 10 days. The tube containing the outer portion of the membrane in sabouraud liquid medium is incubated at 22–25° for 10 days. The culture tubes are gently agitated and observed for growth at regular intervals throughout the incubation period.

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Note added in proof: The following additional references are pertinent to the text: Avis, K. E. ,and Gershenfeld, L., THIS JOURNAL, 44, 682(1955); Avis, K. E., and Gershenfeld, L., *Am. J. Pharm.*, 129, 11,234(1957).

[1] Marketed as Tween 80 by the Atlas Chemical Industries Wilmington, Del.

TABLE I.—FILTRATION RESULTS OF OIL CONTAMINATED WITH LOW COUNT LYOPHILIZED CULTURES

Microorganism	Days 1	3	5	7
S. aureus	0[a]	+[b]	+++	+++
E. coli	0	0	+	++
B. subtilis	0	+	++	+++

[a] 0, No growth. [b] +, Growth.

DISCUSSION

Toxicity Studies on Triton X 100.—The detergent Triton X 100 at 0.1% concentration was nontoxic when in contact with vegetative forms of bacteria for several hours. The surfactant may be used at room or temperatures of 45 ±1° during the membrane washing phase. An additional wash with peptone 0.1% is used to remove the excess detergent and sustain viability of microorganisms that were impinged on the membrane, thereby maintaining the sensitivity of the method (9, 10).

Bacteriological Validity of the Proposed Procedure.—Low count (approximately 25–125 in number), lyophilized cultures of *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis* spores were inoculated into 200 ml. of liquid coconut oil. The contaminated oils were treated as described under *Procedure*. (See Table I.)

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Convenient Synthesis of *N,N,N'*-Trisubstituted Formamidines

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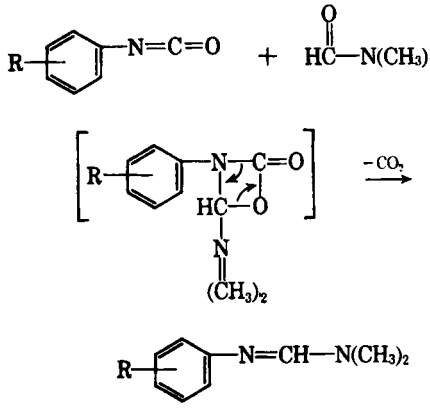
The applicability of a method for preparing trisubstituted formamidines by treating isocyanates with an excess of formamides under neutral conditions at 150° was examined. This amidine-forming reaction was limited to the use of aryl isocyanates when treated with *N,N*-dimethylformamide as the solvent.

RECENT DISCLOSURE of the reaction of *p*-toluenesulfonyl isocyanate (1) and phenyl isocyanate (2) with *N,N*-dimethylformamide (DMF) to form amidines prompted a further investigation into the scope of this facile method for the preparation of trisubstituted formamidines. The synthesis of amidines and formamidines by the interaction of substituted amides with anilines in the presence of phosphorus pentachloride (3–5) or phosphorus oxychloride (6) has been reported previously. More recently, symmetrical *N,N'*-diarylacetamidines were prepared by heating 2 moles of an aromatic amine with 1 mole of ethyl orthoacetate in the presence of *p*-toluenesulfonic acid (7) or, more effectively, acetic acid (8).

In the present investigation, the applicability of a method for preparing trisubstituted formamidines by treating isocyanates with an excess of formamides under neutral conditions at 150° was examined. This method was limited to the use of aryl isocyanates when treated with DMF as the solvent at 150° under neutral conditions. One mole of carbon dioxide was evolved per mole of isocyanate consumed in the reaction. By the removal of unreacted DMF, the *N,N*-dimethyl-*N'*-aryl formamidines could be isolated in 75–98% yield in a high state of purity.



The cyclic mechanism postulated by King (1) for the formation of the sulfonylamidine and by Weiner (2) for formamidine is supported by observations that groups on the benzene ring which tend to increase the electrophilicity of the isocyanate increase the rate of the reaction, whereas electron-donating groups have a tendency to decrease the rate of formation of the formamidine. 2,4,5,6-Tetrachlorophenylene diisocyanate readily formed the bis formamidine under the same reaction conditions. However, formamide failed to yield a formamidine with phenyl isocyanate under the conditions of our experiment, since formanilide was the isolated product.

Similarly, the treatment of alkyl isocyanates with *N,N*-dimethylformamide or aryl isocyanates with

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