Quantitative Methods.—The red palladium (II)–carphenazine color complex developed in the colorimetric assay forms immediately on addition of the test solution to the palladous chloride reagent and is stable for about 15 minutes. The color fades slowly thereafter, and the absorbance of the solution decreases about 2.5% in 3.5 hours. With reagent containing 0.015% palladous chloride, the colors produced by solutions of carphenazine maleate obeyed Beers law in the concentration range of 0.0 to 0.3 mg. per ml. Reagents containing 0.005 and 0.010% palladous chloride failed to produce linear concentration-absorbance plots with the same concentration range of carphenazine maleate. The acidity of the reagents was kept constant throughout these tests (pH 2.1 to 2.2). Analysis of commercial tablets labeled to contain 25 mg. of carphenazine maleate gave an average value of 96.7 ± 1.0%.[2]

[2] Maximum deviation from the mean value.

Repetitive analyses performed on the same tablet extracts were more precise so that the gross deviation may be attributed mainly to variations in the extraction procedure.

Samples of carphenazine maleate dissolved in glacial acetic acid and in a 4:1 mixture of chloroform and acetonitrile were titrated potentiometrically with 0.1 *N* perchloric acid in glacial acetic acid and in dioxane, respectively. The former system gave a sharp break (about 100 mv.) in the curve, equivalent to 98.1% of the amount of carphenazine maleate taken; the latter solvent system was unsatisfactory. In both systems a small amount of a dark gummy precipitate (soluble in acetone) formed as the titration progressed.

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Technical Articles

Improved Viable Counting Method for Petrolatum-Based Ointments

By W. T. SOKOLSKI and C. G. CHIDESTER

A filtration method for recovery of viable micro-organisms from petrolatum-based ointments is described. In this method the ointment is dissolved in isopropyl myristate and passed through a filter. Advantages of the method are that all the micro-organisms in the ointment sample are concentrated on the filter pad, and the filter pad can be rinsed to remove antibacterial agents and traces of petrolatum. Markedly higher recoveries were obtained by the proposed method than by methods in common use.

Current viable counting methods for petrolatum-based ointments involve either (*a*) smearing the ointment directly on the surface of an agar plate (1, 2) or (*b*) extracting the micro-organisms from ointment by shaking with water (3, 4) and plating the aqueous phase. There have been conflicting reports concerning the sterility of ophthalmic ointments. Using an extraction method, Vander Wyk and Granston (3) found that 85% of the ointments they tested were contaminated with micro-organisms; Bowman and Holdowsky (4) found only 10% contaminated in a similar method. Neither report included a test of the effectiveness of the extraction method—such as an attempt to recover a known inoculum from ointment.

One objection to the use of aqueous extraction methods for ointments that contain antimicrobial agents is that the resulting concentrations

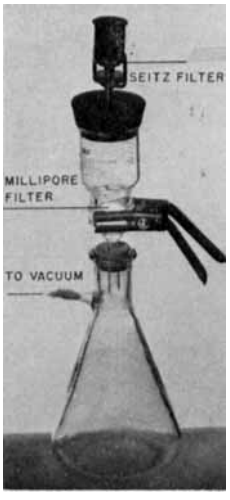

Fig. 1.—Filter unit for viable count or sterility testing of petrolatum-based ointments.

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TABLE I.—RECOVERY OF *Bacillus subtilis* FROM SEEDED CORTISONE ACETATE OPHTHALMIC OINTMENT

Experiment	Viable Count of Inoculum (Calcd. per Gm. Ointment)	Method of Viable Cells Recovered per Gm. Ointment: Proposed (Filtration)	I Agar Smear, 0.5 Gm./Plate (two plates)	II Water Extract	III Broth Extract
	32	22	0, 2	0	4
	29	11	0, 0	0	3
	36	9	1, 1	1	7
1	..	9	..	..	3
	39	26	1, 2	35[a]	..
	..	22	..	0	2
	..	..	..	1	4
Av. per Gm.	34	16	2	0–1	4
	54	39	3, 0	2	2
	48	4	1, 0	1	1
2	54	53	1, 0	4	4
	60	98	0, 0	2	1
	..	79	3, 1	0	0
	..	30	..	0	0
Av. per Gm.	54	50	2	1–2	1–2
	51	17	0, 0	4	2
	34	25	1, 0	1	0
3	30	15	0, 0	0	1
	34	24	1, 0	3	1
	..	44	0, 0	3	1
	..	..	..	0	1
Av. per Gm.	37	25	0–1	2	1
	43	50[a]	2, 0	0	0
	44	37	1, 0	1	3
4	..	35	0, 0	0	0
	..	35	1, 0	0	9
	..	..	1, 0	1	3
	..	..	..	..	2
Av. per Gm.	44	36	1	0–1	3
	98	42	1, 0	0	3
	..	52	0, 0	2	10
5	99	47	1, 0	3	2
	..	65	0, 0	1	22
	..	64	0, 0	3	2
	..	252[a]	0, 0	0	4
Av. per Gm.	99	54	0–1	1–2	7

[a] Aberrant observations according to U.S.P. XVI, Table I, p. 874. Responses not included in average.

of these agents in the aqueous phase and in the final growth medium could be high enough to inhibit growth. It is likely that many of the organisms become enveloped in petrolatum without access to nutrients with either an extraction method or an agar smear method. They may be viable but unable to multiply.

An effective viable counting method for ointments would be desirable—first, to determine whether the incidence of contamination in ophthalmic ointments is high enough to be a serious problem—and second, because the method could possibly be adapted to the sterility testing of ophthalmic ointments if this should become a requirement.

The purpose of this paper is to describe a filtration method similar to the one used by Holdowsky in a sterility test for antibiotics (5). In this method the entire ointment sample is dissolved and filtered, and the filter pad is rinsed for the removal of antimicrobial agents and traces of petrolatum. Comparisons of recoveries obtained by the proposed method and by current methods are made.

PROPOSED METHOD

Materials and Equipment.—Isopropyl myristate;[1] brain heart infusion broth (BHI) and nutrient broth;[2] polysorbate[3] 80; 47 mm., 0.45 μ, type HA filter pads, 47-mm. absorbent pads, and 250-ml. filter holders;[4] No. 13½ rubber stoppers; Seitz filter pads and filter holders; fine-tipped forceps; 50 mm.-Petri dishes with covers, were utilized.

Preparation of Materials and Equipment.—Prepare rinse and growth media. The basic rinse medium is nutrient broth with 1% polysorbate 80 added. The basic growth medium is BHI with

[1] Marketed as Deltyl Extra by Givaudan-Delawanna, Inc., New York, N. Y.
[2] Baltimore Biological Laboratories of Difco Laboratories, Baltimore, Md.
[3] Marketed as Tween 80 by Atlas Powder Co., Wilmington, Del.
[4] Millipore Filter Corp., Bedford, Mass.

TABLE II.—RECOVERY OF BACTERIA FROM OPHTHALMIC OINTMENTS AFTER SEEDING WITH APPROXIMATELY 70 CELLS/GM.

Ointment[a]	Active Ingredients per Gm. of Ointment	Viable Cells Recovered/ Gm.
Neo-Medrol	Neomycin sulfate, 5 mg. Methylprednisolone, 1 mg.	10
Neo-Delta-Cortef	Neomycin sulfate, 5 mg. Prednisolone acetate, 2.5 mg.	32
Neo-Cortef	Neomycin sulfate, 5 mg. Hydrocortisone acetate, 0.5 mg.	68
Baciguent	Bacitracin, 500 units Phenacaine hydrochloride, 20 mg.	21
Myciguent	Neomycin sulfate, 5 mg.	26
Mycitracin	Neomycin sulfate, 5 mg. Polymyxin B sulfate, 5000 units Bacitracin, 500 units	40
Penicillin G potassium	Potassium penicillin G, 100,000 units	19
Sulfathiazole	Sulfathiazole, 50 mg.	18
Neosone	Cortisone acetate, 15 mg. Neomycin sulfate, 5 mg.	35
Neo-Cortef with tetracaine	Neomycin sulfate, 5 mg. Tetracaine hydrochloride, 5 mg. Hydrocortisone acetate, 5 mg.	93
Mercury yellow oxide	Mercury yellow oxide, 10 mg.	0

[a] Trade names of The Upjohn Co., Kalamazoo, Mich.

0.1% polysorbate 80 added. For neomycin ointments, add 3% sodium chloride to the rinse and growth media; add 0.1% ascorbic acid to the growth medium. For penicillin ointments, add 200 kinetic units penicillinase per milliliter to the rinse and growth media after they have been autoclaved. For sulfonamide ointments, add 100 mcg. *p*-aminobenzoic acid per milliliter to the rinse and growth media.

Sterilize 100-ml. volumes of isopropyl myristate by dry heat at 200° for 2 hours. Autoclave the following for 20 minutes at 121°: growth medium; 500 ml. volumes of rinse medium; covered Petri dishes, each containing 2 absorbent pads; and individually wrapped forceps (for use in aseptic handling of bacterial filters). Sterilize filter pads by exposure to ethylene oxide. Assemble filter units as shown in Fig. 1 but do not include filter pads. Place absorbent pads between the sintered glass supports and the funnels for protection, and autoclave the complete unit at 121° for 30 minutes.

Procedure.—For each ointment to be tested, bring 500 ml. rinse medium and 100 ml. isopropyl myristate to temperature in a 47° water bath. Place a filter pad in a filter unit and moisten it with 0.3 ml. rinse medium; dissolve 1 Gm. of the ointment in the 47° isopropyl myristate. The sample should not be dissolved very long in advance of filtration because prolonged exposure to a temperature of 47° may be damaging to vegetative cells (6, 7).

Add the isopropyl myristate–ointment solution to the funnel over the filter pad. Apply a vacuum capable of pulling 550 mm. of mercury (ordinary house vacuum in our tests) until all the solution has passed through the filter pad. Rinse twice with 250-ml. aliquots of rinse medium. Allow all the liquid to pass through the filter pad, but do not let the filter pad become dry. With ointments containing antibacterial agents, the rinse medium should be added in five or more aliquots to ensure adequate rinsing. With neomycin ointments, the second or third rinse should be allowed to stand in the funnel for about 15 minutes.

Add 4 ml. of growth medium to a Petri dish containing absorbent pads. Aseptically remove the filter pad and place it on the absorbent pads. All the micro-organisms which were originally in the ointment sample should now be on the filter pad. Incubate the Petri dish at 32–35° and examine the filter pad for growth after 24 and 48 hours.

EXPERIMENTAL

Comparison of Methods

In five experiments, the proposed method was compared for efficiency in recovery of a known inoculum to three other procedures commonly used for the microbial examination of ointments. Since the time of exposure to the ointment environment necessarily varied between methods, conditions favoring survival of the inoculum in the ointment seemed desirable. For this reason we decided to inoculate with spores and to use an ointment which did not contain antibacterial agents.

Procedure for Each Experiment

Preparations for the proposed method (*see above*) were made. In addition, a Waring Blendor, a 30-ml. syringe, and 50-ml. Erlenmeyer flasks containing glass beads and either (*a*) 7 ml. distilled water or (*b*) 7 ml. BHI broth with 0.1% polysorbate 80 were sterilized by autoclaving for 20 minutes at 121°. Twenty by 100-mm. Petri dishes containing 28 ml. sterile BHI agar with 0.1% polysorbate 80 were prepared, and melted tubes of the same agar (for use in making poured plates) were placed in a 47° water bath. Twenty-eight grams of 1.5% cortisone acetate[5] ointment were thoroughly blended with 1 ml. of an aqueous *Bacillus subtilis* UC 564 spore suspension containing approximately 10^8 spores/ml. Poured-plate viable counts of 0.1-ml. aliquots of the spore suspension were made. The seeded ointment was heated to 47° and distributed from a syringe.

The proposed method, as described previously, was followed.

[5] The Upjohn Co., Kalamazoo, Mich.

TABLE III.—RECOVERY OF VEGETATIVE CELLS FROM ISOPROPYL MYRISTATE

Organism	Viable Count of Inoculum	Viable Cells Recovered
K. pneumoniae	72	22
P. vulgaris	Spreaders[a]	Spreaders[a]
Ps. aeruginosa	*ca.* 560	Spreaders[a]
S. aureus	*ca.* 1000	*ca.* 800
S. lutea	46	29
C. albicans	52	43
S. cerevisiae	61	52
Penicillium sp.	Dense growth[a]	Very dense growth[b]

[a] Unable to count because colonies grew together. [b] Colonies were too numerous to count.

Method I.—Each 1-Gm. sample of seeded ointment was divided between two Petri plates. The ointment was added directly to the surface of the agar and spread with a sterile glass rod.

Method II.—Each gram of seeded ointment was added to 7 ml. water. The flasks were heated for 10 minutes in a 47° water bath, then shaken vigorously for 1 hour on a wrist action shaker at room temperature. The entire aqueous phase was plated with 28 ml. of agar.

Method III.—This was the same as *Method II*, except that broth was used to extract the ointment instead of water and the flasks were shaken at 35°. This method was included in the comparison because some oil and petrolatum products have been tested for sterility by incubation in nutrient media (8–10).

All plates were incubated at 35° and examined for growth after 24 and 48 hours. Table I gives 48-hour counts.

Tests of Unseeded Cortisone Acetate

The cortisone acetate ointment used in the comparison of methods was not sterilized. To determine the number of organisms present in the ointment prior to seeding, seven 1-Gm. samples were tested by the proposed method.

Tests of Additional Ointments

Recovery of a known inoculum by the proposed method was also tested with 11 additional ophthalmic ointments, all containing antibacterial agents. Because it would have been difficult to blend an inoculum uniformly in 1-Gm. quantities of ointments, the ointment-isopropyl myristate solutions were inoculated and mixed by vigorous shaking. Each solution was inoculated with 0.1 ml. of an aqueous *B. subtilis* spore suspension. Poured-plate viable counts were made to determine the number of spores in the inoculum.

RESULTS AND DISCUSSION

The data in Table I show that markedly higher recoveries of bacteria from seeded ointment were obtained with the proposed filtration method than with the other methods. This was probably due to the removal of the organisms from the petrolatum environment. Poor recoveries with *Method I* were not surprising since one would expect most of the organisms to be coated with petrolatum. However, recoveries with extraction *Methods II* and *III* were not much higher. Possibly the majority of organisms were not extracted. If they were extracted they may have retained a petrolatum coating.

The test ointment used in the recovery study (Table I) did not contain antibacterial agents. If sulfonamides or water-soluble antibiotics had been present, they would have been transferred to the viable count media in *Methods I, II,* and *III*. Since most antimicrobial agents are more active against growing cells than against dormant cells, cells which could survive in ointment might be inhibited in the viable count medium. Although the proper additives can antagonize the activity of some drugs, it would be advantageous to remove the viable cells from the drug environment.

In viable counts of unseeded cortisone acetate, an average of seven bacteria per gram were recovered of which six resembled *Bacillus sp.* The recoveries given in Table I include all colonies resembling the inoculum.

Recoveries obtained by the proposed method from seeded ointments containing antibacterial agents are given in Table II. Viable cells were recovered from all ointments except mercury yellow oxide.[6] The addition of thioglycollic acid to the medium for antagonizing the mercury salt (11) was not attempted. In preliminary tests of neomycin ointments, recoveries were very low. This may have been due to adsorption of neomycin to the cellulose ester filter pads. Sodium chloride, which is known to desorb neomycin from cellulose (12), was added to the rinse medium and the second or third rinse was allowed to stand in the funnel for 15 minutes. As an additional precaution, two known inhibitors of neomycin activity, sodium chloride (12) and ascorbic acid (13), were added to the growth medium. In subsequent tests of neomycin ointments, recoveries were satisfactory.

The next consideration was the possibility that isopropyl myristate would be toxic to vegetative microbial cells. Isopropyl myristate is reported to be nontoxic to humans since "drugs and cosmetics containing this substance may be safely applied topically and parenterally" (14). This oil is the isopropyl ester of a saturated fatty acid and should be less dehydrating than unsaturated vegetable oils. Table III indicates that recoveries of viable vegetative cells from isopropyl myristate were comparable to recoveries of spores.

The proposed method could be used for sterility testing of ointments with simple modifications. As described by Holdowsky (5), a 25-mm. disk (approximately one-half the filtering area) could be cut from the filter pad for incubation in fluid thioglycollate medium while the remainder is incubated in Sabouraud's medium. An apparatus consisting of a series of filter units is now commercially available.[7] The units are fitted so that rinse medium may be supplied aseptically. However, it is necessary to remove the rubber stopper from the funnel to add the test solution. To reduce the risk of contamination from air, an additional opening could be made in the rubber stopper to accommodate a hypodermic needle. The test solution could then be added with a syringe.

SUMMARY

A filtration method for the recovery and enu-

[6] The Upjohn Co., Kalamazoo, Mich.
[7] Millipore Filter Corp., Bedford, Mass.

meration of viable organisms from petrolatum-based ointments was described and shown to be more efficient than methods now commonly used. An application of the method to the sterility testing of ointments was discussed.

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Notes

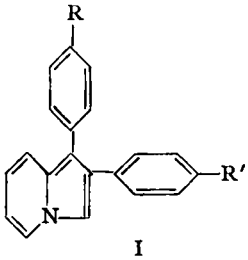
Preparation of *m*- and *p*-Nitrobenzoyl Derivatives of Some Arylindolizines

By VINCENT S. VENTURELLA†

Investigations into the benzoylations of substituted arylindolizines has shown that the reaction is reduced but not prevented by steric or electronic effects.

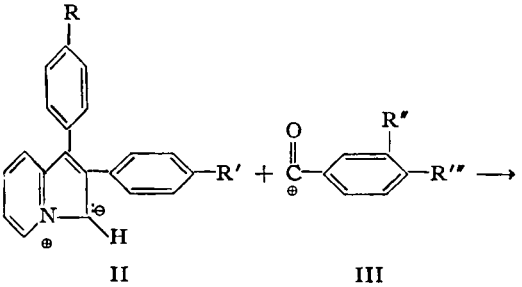
IN A PREVIOUS REPORT (1) it was found that the benzoylation of compounds of structure I proceeded easily and in fair yields except with Compound I *d*. At the time it was indicated that the failure of the reaction to occur with this compound could be attributed to the conjugative and inductive effects of the substituents present in the phenyl rings. Since the benzoylation of the open 1 position is said to occur easily (2), and since the reaction depends upon the contributions from structures II and III, it was desirable to test the effects of the presence of nitro groups on the formation of the benzoylium ion (III) and hence on the formation of a stable benzoyl derivative.

Since hydrolysis of the end product was previously (1) found not appreciable if the reaction was performed in benzene solution below 60° for 1 hour, followed by allowing the mixture to stand 48 hours at room temperature, any change in the formation of the benzoyl derivatives would presumably be because of an increase or decrease in the formation of III.

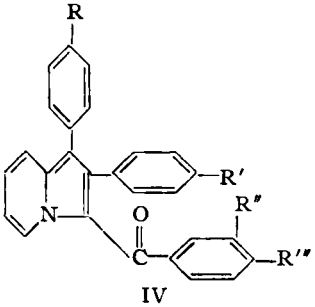


I

I *a*, R = H, R′ = Br
b, R = Cl, R′ = Br
c, R = H, R′ = NO_2
d, R = Cl, R′ = NO_2



II III



IV

IV *a*, R = R″ = H, R′ = Br, R‴ = NO_2
b, R = R‴ = H, R′ = Br, R″ = NO_2
c, R″ = H, R = Cl, R′ = Br, R‴ = NO_2
d, R‴ = H, R = Cl, R′ = Br, R″ = NO_2
e, R = R″ = H, R′ = NO_2, R‴ = NO_2
f, R = R‴ = H, R′ = NO_2, R″ = NO_2

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