aglycone quercetin, from *A. tataricus* roots and pollen (7, 8), and the pollen of *A. ageratoides* var. *ovatus* (8), as well as *A. yomena* (8).

The identity of additional flavonoids present in this plant will be reported at a later date.

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Keyphrases

Aster pilosus leaves
Quercetin-3-β-D-mono-galactoside—isolated
TLC—separation, identity
Column chromatography—separation
IR spectrophotometry—structure

Bacterial Contamination in Some Nonsterile Antibiotic Drugs

By MACK WHITE, FRANCES W. BOWMAN, and AMIEL KIRSHBAUM

Methods have been developed for the determination of microbial contamination in some nonsterile antibiotic preparations. Microorganism counts have been performed on both antibacterial and antifungal antibiotic powders. The highest counts were found in samples from the antifungal antibiotic.

In 1964, an extensive investigation into microbiological contamination of medical preparations was conducted in Sweden (1). A report of these findings was submitted to the Royal Swedish Medical Board, including a proposal for an upper limit of 100 bacteria per gram in nonsterile medicinals.

The *Antibiotic Regulations* of the Food and Drug Administration (FDA), the USP, and the NF require that antibiotics for injection be sterile, and they specify sterility tests for these preparations. None of these compendia, however, require sterility for oral or topical antibiotics, except for surgical powders and some ophthalmic and otic solutions. Tolerances are not specified in the official compendia for microbial contamination of nonsterile drugs except for one topical antibiotic preparation; the *Antibiotic Regulations* limits the contamination of this preparation to not more than 10 microorganisms per Gm. (2). No other oral or topical antibiotics undergo routine microbial examination as part of the antibiotic certification program.

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Recently, several batches of Agent F, an antifungal antibiotic, were found to be grossly contaminated with microorganisms. Although sterility is not required for the powder or dosage forms of this antifungal antibiotic, gross contamination would indicate a possible violation of the good manufacturing practices required by FDA (3). This possibility led to a study of the problem and to the development of suitable methods for the determination of microbial contamination in nonsterile antibiotic preparations.

EXPERIMENTAL

Materials—Prepare the following nutrient agar: peptone, 6.0 Gm.; yeast extract, 3.0 Gm.; beef extract, 1.5 Gm.; agar, 15.0 Gm.; and enough distilled water to make 1000.0 ml. The pH should be 6.5 to 6.6 after sterilization.

Prepare peptone water by dissolving 1 Gm. of peptic digest of animal tissue, USP or equivalent, in sufficient distilled water to make 1,000 ml. Dispense 99-ml. portions into flasks and sterilize in an autoclave at 121° for 20 min. The final pH should be 7.1 ± 0.1.

Method I—Powder—Place 1.0 Gm. of powder into 99 ml. of sterile peptone water. From this sus-

pension, prepare a number of 10-fold serial dilutions by aseptically transferring 1 ml. to 9 ml. of sterile peptone water. From each dilution remove 1 ml. and add to separate sterile 20 × 100 mm. Petri dishes. Add 15 ml. of sterile liquid nutrient agar which has been cooled to 48–50°. Swirl the agar and allow to harden. Then add a 5-ml. overlay of nutrient agar and allow to harden. Invert the Petri dish and place in a 37° incubator for 48 hr. Remove and count colonies, using a Quebec colony counter.

Method II—Powder—Place a 0.45-μ solvent-resistant membrane filter, 47 mm. diameter, in a filter funnel fitted into a vacuum flask. Aseptically place 0.1 Gm. of powder into 50 ml. of sterile dimethylsulfoxide (DMSO) and allow to dissolve. Immediately filter the solution, using vacuum, through the membrane. Wash the membrane with 100 ml. of sterile peptone water, and allow to dry by continuing vacuum for 5 min. after filtration.

Place the membrane on the surface of 15 ml. of agar which has been previously allowed to harden in a Petri dish. Overlay with 5 ml. of agar and allow to harden. Incubate the plate in an inverted position for 48 hr. at 37°. Count the colonies that appear on the pad. (Ordinarily the agar overlay is not necessary, since the membrane pad is dry, and spreading growth will not occur. However, it is used here to prevent curling of the pad caused by the DMSO.)

Method III—Tablets—Use Method I, except use a sterile mortar and pestle to reduce the tablets to a powder for initial dilution. If 1 Gm. will not suspend properly, reduce initial concentration to 0.1 Gm.

Method IV—Ointment—Place 1 Gm. of ointment into 99 nl. of sterile *n*-hexane in a conical flask and shake until the ointment dissolves. Prepare a number of 10-fold serial dilutions by transferring 1-ml. aliquots to 9 ml. of sterile *n*-hexane. Filter each dilution and further proceed as described for filtration under II, omitting the 5-ml. agar overlay.

RESULTS AND DISCUSSION

Four different batches of contaminated powder were received for examination. Agar plate counts performed by conventional emulsion or surface swab methods gave unreliable results without any diminution in organisms per plate from lower to higher dilutions. The predominating contaminant was an aerobic, motile Gram-variable bacillus that resembled *Bacillus sphaericus* in its morphological and biochemical characteristics. According to Bergey's Manual (4), this organism usually grows in motile microcolonies of various shapes that move in large arcs from the point of inoculation and cover the plate in 1 day. The surface of poured agar plates should be dried by holding at room temperature for 2 or 3 days before use.

Therefore, unless extreme precautions are taken, the colonies do not remain as separate entities which can be related to a single parent; instead they will run together and counts will be erroneous. As an example, one count from another laboratory was reported to us as 2×10^{12} per Gm. Pelczar and Reid (5) report that it has been calculated that 1 Gm. of bacteria contains approximately 10^{12} bacteria. Therefore, a count of 2×10^{12} bacteria per Gm. of powder was unlikely even if the contaminating microorganisms were extremely minute. Such an inaccuracy results not from an arithmetic error, but from an illusion due to the spreading growth of the contaminant.

The use of the 5-ml. agar overlay described in Method I contains the individual colonies and prevents their spreading. Repeated tests showed good reproducibility with excellent arithmetic correlation of total counts obtained from different dilutions.

Because Agent F is relatively insoluble in water, the lowest dilution that was feasible was 10^{-2} as described in Method I. However, if the sample contains less than 100 organisms per Gm., the dilution in this technique precludes the recovery of microorganisms. Therefore, for samples contaminated with relatively few organisms, Method II should be used. In evaluating the results, the number of colonies are counted and multiplied by a factor of 10, since only 0.1 ml. is used, to obtain the number of organisms per Gm. Although the sample is dissolved in 50 ml. of DMSO, this dilution does not affect the final count because, during the filtration, the entire volume (50 ml.) passes through the membrane and the undiluted microorganisms are retained. Thus, if 8 colonies were observed on the membrane pad, the final count would be $8 \times 10 = 80$ organisms per Gm.

Using these techniques, counts for Agent F powder have ranged from 30 organisms per Gm to 4×10^8. The use of a grossly contaminated powder in the formulation of a dosage form such as a tablet or ointment resulted in a highly contaminated product. When the powder containing 4×10^8 microorganisms was used to make a tablet weighing 1 Gm. the tablet contained 9.4×10^7 microorganisms per Gm. The final dosage form was almost as heavily contaminated as the bulk although only a few mg. of the contaminated antibiotic was used in the formulation for each tablet. The excipient ingredients used in the tablet were also tested and less than 10 microorganisms per Gm. were found in each ingredient. Since the contamination of the tablet was greater than that originally introduced by the raw materials, the production procedures probably favored bacterial growth or additional contamination occurred during the manufacturing process. In any event, it was later shown that acceptable antibiotic preparations could not be made from heavily contaminated antibiotic powder.

After the manufacturing procedures were changed to yield a less contaminated powder, two batches of tablets were examined and found to be satisfactory. The antibiotic powder manufactured by the revised procedures contained 3×10^3 microorganisms per Gm. and the samples of the two batches of tablets made from this powder contained less than 50 microorganisms per Gm. An ointment was prepared from a powder having a count of 4×10^8 organisms per Gm. as an ingredient; the final product had a count of 4×10^5 per Gm. When a powder with a count of 1.2×10^2 was used in the formulation of an ointment, counts ranging from zero to a maximum of 100 organisms per Gm. were found in the ointment.

Because of the experiences with Agent F, other antibiotics were screened for bacterial counts. Among these were candicidin, kanamycin, bacitracin, neomycin, griseofulvin, nystatin, amphotericin, penicillin G, nafcillin, tyrothricin, and gramicidin.

TABLE I—VIABLE MICROORGANISMS PER GRAM FOUND IN NONSTERILE ANTIBIOTICS

Antibiotic Powders	Bacteria/Gm.	Molds/Gm.
Antifungal		
Antibiotic A	6,700	None detected
Antibiotic B	400	None detected
Antibiotic C	200	None detected
Antibiotic D	80	15
Antibiotic F (old process)	9,400,000	2
Antibiotic F (old process)	1,000,000	None detected
Antibiotic F (new process)	3,000	None detected
Antibiotic F (new process)	2,000	None detected
Antibacterial		
Nafcillin	None detected	None detected
Bacitracin	None detected	None detected
Neomycin	18	None detected
Zinc bacitracin	2	38
Penicillin	12	None detected
Tyrothricin	None detected	3

No other counts approached those of Agent F. As shown in Table I, the highest counts were found in samples of the antifungal antibiotics, indicating that they may present more of a problem than the antibacterial antibiotics.

The methods described here can be applied to bacterial counting of most antibiotics. However, the solvent systems should be modified according to the solubility characteristics of the antibiotic or antibiotic formulation under test. Any organic solvent used should, of course, be nontoxic to the contaminating microorganisms. Since the problem encountered with Agent F was gross bacterial contamination of a spore-forming organism, the tests were developed specifically for spore-forming bacteria. The techniques described may also be used for fungal contamination by using Sabouraud's agar in place of nutrient agar, incubating at 25° instead of 32°, and extending the incubation time from 2 to 5 days.

Antibiotic drugs, even those that are represented as being nonsterile, should be relatively free of microorganisms. Gross contamination is an indication of poor manufacturing practices. In addition, Curtis *et al.* (6) have stated that any organism, under certain conditions, can be considered pathogenic.

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Keyphrases

Antibiotic preparations—bacterial contamination
Antifungal preparations—bacterial contamination
Agar overlay—test procedure

Synthesis of Imidazolthiones from 3,4-Diphenyl-4-oxazolin-2-thione

By S. CARBONI, E. GROTHE, and M. F. SAETTONE

The synthesis of some 4-hydroxyimidazolidin-2-thiones (VI) and 4-imidazolin-2-thiones (VII) by a method involving the reaction of 3,4-diphenyl-4-oxazolin-2-thione (IV) with primary amines is described.

A METHOD FOR THE preparation of imidazolones, based on the reaction of 5-unsubstituted 4-oxazolin-2-ones (such as V, Scheme I) with primary amines, was recently reported (1). In view of the interesting bacteriostatic (2, 3), fungistatic (3, 4), and CNS-depressant (5) activities reported for some 4-imidazolin- and imidazolidin-2-thiones, the authors have applied their method to the preparation of imidazolthiones from 4-oxazolin-2-thiones; some preliminary results are reported herein.

3,4-Diphenyl-4-oxazolin-2-thione (IV), prepared either from phenacyl alcohol (I) and phenyl isothiocyanate (II) through the intermediate 3,4-diphenyl-4-hydroxyoxazolidin-2-thione (III), or from V and P_2S_5, was chosen as a model compound for this study.

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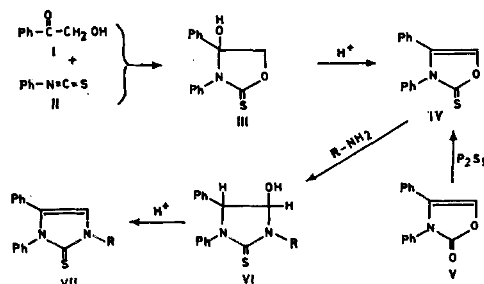
Treatment of IV with some primary amines gave good yields of 3,4-diphenyl-5-hydroxyimidazolidin-2-thiones (VI, see Table I). These could be easily dehydrated to afford the corresponding 4-imidazolin-2-thiones VII (Table II). In order to secure a structure proof for compounds of type VII, 1-



Scheme I