Small Tube Method for the Evaluation of Antifungal and Antibacterial Activity

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Small tubes containing a thin layer of agar medium have been used in a method for screening pure chemicals for antifungal and antibacterial activity. Employing known agents, this method provided results comparable to those obtained by other methods. In addition, the method affords the following advantages which make it suitable as a screening procedure: a no-growth end point which may be readily determined, it is effective for nonaqueous as well as aqueous solutions, it utilizes inexpensive apparatus, a minimum quantity of media and test agents are required, and a relatively small amount of working area is needed for conducting the tests.

ALTHOUGH there are numerous techniques available to test for antimicrobial activity, none of them appear suitable for the simultaneous screening of a large number of agents. The various modifications of these methods have been reviewed and evaluated by Reddish *et al.* (1), Skinner (2), and Heatley (3). Vanbreuseghem (4) recently considered the merits of those methods which determine antifungal activity.

The *in vitro* methods generally are classified as either dilution or diffusion methods. The former are best suited for assay procedures, but the methods are time-consuming for screening purposes, and many of them are not satisfactory to determine antifungal activity when filamentous fungi are used as test organisms. This is particularly so if partial inhibition is studied because it is difficult to determine the amount of growth of these fungi. Diffusion methods, such as represented by the use of filter paper disks on an agar plate, find limited value with water-insoluble agents and with organic solvents having antimicrobial activity. Also, the determination of the end point, usually the measuring of clear zones of inhibition, is tedious and somewhat arbitrary, especially with filamentous fungi.

The above factors, plus certain other innate disadvantages of existing methods, prompted this study. A technique has been developed which appears to be suitable for screening, qualitatively and semiquantitatively, large numbers of agents for antifungal and/or antibacterial properties.

EXPERIMENTAL AND RESULTS

Materials and Methods.—The fungal species used were *Candida albicans*, ATCC 10231, and *Trichophyton mentagrophytes*, ATCC 9972. The bacterial species employed were *Escherichia coli* and *Staphylococcus aureus* from the collections maintained at the Department of Microbiology, Oregon State University.[1]

The fungal organisms were maintained on Sabouraud dextrose medium Difco (liquid and agar), and the bacteria were maintained on nutrient medium Difco (broth and agar). The liquid cultures also served as a source of inocula. The experimental studies were conducted in flatbottom, screw-capped vials (17 × 60 mm.) containing 0.5 ml. of the desired medium. This volume provided a layer of medium approximately 2 mm. in depth. Antifungal activity was determined on Sabouraud dextrose agar medium and antibacterial activity on nutrient agar medium.

Actively growing 2- to 4-day-old liquid cultures (10 ml./culture tube) of bacteria and *C. albicans* were used as inocula. The small experimental tubes were inoculated by adding 1 drop of a suspension of the respective organisms utilizing a sterile standard medicine dropper (about 20 drops/ml. of water). To facilitate obtaining inocula with a medicine dropper, all organisms were transferred to sterile 15-ml. wide-mouth containers.

For studies using *T. mentagrophytes*, transfers were made from agar slants to 125-ml. conical flasks containing 30 ml. of Sabouraud medium. The mycelial growth from 5- to 7-day-old stationary cultures was homogenized in a sterile semimicro Waring blender for 30 sec. and added as inocula to the experimental tubes as described previously. All incubations were conducted at 37°.

Solutions of the agents to be tested were prepared in acetone, water, or 95% ethanol, depending on their solubility, and placed in dropper bottles. Unless otherwise indicated, dilutions of 1:100, 1:500, 1:1,000, 1:5,000, and 1:10,000 were tested for all compounds. Prior to inoculation with microorganism, 2 drops (approximately 0.04 ml.) of the various freshly prepared solutions were added to the tubes, and the solvent was allowed to evaporate or be absorbed into the agar medium.

The tubes were examined for growth after 24–48 hr. of incubation for bacteria and *C. albicans* and 48–72 hr. for *T. mentagrophytes*. In certain instances incubation was allowed to continue for 5–7 days in order to determine if the method might be suitable to indicate fungistatic *versus* fungicidal activity.

Antimicrobial Studies.—The agents used to test antifungal activity included salicylic acid, salicylanilide, griseofulvin, nystatin, tolnaftate,[2] and penicillin V. Antibacterial determinations were made with penicillin V, streptomycin sulfate, sulfadiazine, sulfathiazole, and sodium sulfathiazole. The series of dilutions previously described were prepared as follows: acetone–salicylic acid, salicylanilide, griseofulvin, penicillin V, and sulfadiazine; water–streptomycin sulfate and sodium sulfathiazole; 95% ethanol–nystatin and tolnaftate. Replicates of 3 tubes per dilution for each organism

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[2] Marketed as Tinactin by the Schering Corp.

TABLE I.—ANTIFUNGAL ACTIVITY

Compd. and Dilutions	*C. albicans*	*T. mentagrophytes*
Salicylic acid		
1:100	±[a]	
1:500	+	
1:1,000	+	
1:5,000	+	
1:10,000	+	
Salicylanilide		
1:100	−	−
1:500	−	−
1:1,000	±	−
1:5,000	+	−
1:10,000	+	−
Penicillin V acid		
1:100	+	+
1:500	+	+
1:1,000	+	+
1:5,000	+	+
1:10,000	+	+
Griseofulvin		
1:100	+	−
1:500	+	−
1:1,000	+	−
1:5,000	+	−
1:10,000	+	−
Tolnaftate[b]		
1:100	±	−
1:500	+	−
1:1,000	+	−
1:5,000	+	−
1:10,000	+	−
Nystatin[c]		
1:100	−	−
1:500	−	−
1:1,000	−	−
1:5,000	±	−
1:10,000	+	+

[a] +, growth; −, no growth; ±, equivocal growth. [b] Readings taken at 72 hr. [c] After 72 hr., growth of *T. mentagrophytes* was noted at all dilutions except 1:100.

were prepared. Controls consisted of tubes inoculated with the organism only, and blanks consisted of solvent and the respective microorganism. The results are summarized in Tables I and II. Unless stated otherwise, the final results are based on 48-hr. readings. The data reported here are consistent with those well documented for the agents listed (5, 6).

At the higher concentrations (1:100 and 1:500) the compounds which were relatively water-insoluble and tested as nonaqueous solutions tended to crystallize on the surface of the medium after the solvent evaporated. To determine if the presence of surface crystals affected the growth of the organism, or the activity of the agent, a modified procedure was tested. The compounds were suspended in the modified agar media (diluted 50%) in dilutions of 1:100, 1:500, and 1:1,000. Two drops of the suspension was added to the small tubes providing them with a thin film of medium containing the uniformly dispersed compound. The compounds tested in this manner were salicylanilide, griseofulvin, and penicillin V, with fungi; and penicillin V and streptomycin sulfate against bacteria. All except the last one are not soluble in water. Since the results did not vary from those obtained in the original study, they were not recorded here.

The study utilizing nystatin (Table I), comparing the effects of this agent on both fungi, was allowed to continue for 7 days. During this period, and commencing at 72 hr., growth of *C. albicans* developed in those tubes containing the 2 lower concentrations. Growth of *T. mentagrophytes* was noted at dilutions of 1:500 and higher at 72 hr., and which appeared completely uninhibited at the seventh day. Thus, it was shown that nystatin most likely acts as a fungicide against *C. albicans* in all but the extremely diluted concentrations and as a fungistat against *T. mentagrophytes* in all but the highest concentration, while having a low order of effectiveness. These data are in general agreement with previously recorded findings (7).

Growth of the microorganisms in those tubes designated as controls and blanks was luxuriant in all but 1 instance. Alcohol appears to have a slight fungistatic action against *T. mentagrophytes*. In those cases where alcohol was used as the solvent (nystatin and tolnaftate) readings were taken only after 72 hr.

DISCUSSION

A simplified method for the simultaneous screening of a relatively large number of pure chemicals for antimicrobial activity has been devised. Using established antifungal and antibacterial agents, it was demonstrated that the method provided results consistent with those obtained by other techniques. Thus, it would appear that other pure chemicals may be screened in a similar manner. Extended studies have indicated that this method is further applicable to the screening of crude plant extracts (8).

Since a no-growth end point is used and determined by mere visual inspection of the small tubes, the tedious task of measuring clear zones of inhibi-

TABLE II.—ANTIBACTERIAL ACTIVITY

Compd. and Dilutions	*E. coli*	*S. aureus*
Penicillin V acid		
1:100	−[a]	−
1:500	+	−
1:1,000	+	−
1:5,000	+	−
1:10,000	+	−
Streptomycin sulfate		
1:100	−	−
1:500	−	−
1:1,000	±	+
1:5,000	+	+
1:10,000	+	+
Sulfadiazine		
1:100	−	−
1:500	+	−
1:1,000	+	±
1:5,000	+	+
1:10,000	+	+
Sulfathiazole		
1:100	+	−
1:500	+	−
1:1,000	+	−
1:5,000	+	+
1:10,000	+	+
Sodium sulfathiazole		
1:100	+	+
1:500	+	+
1:1,000	+	+
1:5,000	+	+
1:10,000	+	+

[a] +, growth; −, no growth; ±, equivocal growth.

tion is avoided. This factor bears particular significance with microorganisms that produce irregular defined zones, *i.e.*, many filamentous fungi. According to Cavillito (9), complete absence of visual growth is the most reproducible and satisfactory end point in an inhibition test.

The effectiveness of most diffusion methods is dependent on those factors which govern the ability of the agent to diffuse both horizontally and vertically throughout the medium. Cooper (10) indicates the importance of these factors in critical assay procedures. They are less important when applied to general screening procedures which are primarily designed to merely distinguish between active and nonactive agents. Through the use of small tubes and thin layers of media, the extent of horizontal and vertical diffusion has been limited, thus rendering diffusion factors negligible.

Those compounds which were highly water insoluble and tested as nonaqueous solutions tended to crystallize on the surface of the medium after evaporation of the solvent. The occurrence of these crystals had no apparent effect on the growth of the microorganisms or on the suspected activity of the antimicrobial agents. For example, penicillin V, having the properties of low water solubility, antibacterial but not antifungal activity, demonstrated in this experiment no effect on the growth of the fungi while it did inhibit growth of the bacteria. Furthermore, when griseofulvin was tested it was shown that the surface crystals had no effect on the growth of *C. albicans*, against which this agent is ineffective, but it did exhibit its usual inhibitory effect on *T. mentagrophytes* (11). These findings were also substantiated in the experiment where the compounds were uniformly dispersed and added to the tubes in a thin film of the respective medium. In this instance the results did not vary from those observed in the original study.

A slight antifungal effect was noted when alcohol was used as a solvent for those agents tested against *T. mentagrophytes*, but this was easily overcome by merely extending the incubation period slightly and providing adequate control tubes for comparison.

The method appears to distinguish between fungistatic and fungicidal activity. This may be accomplished by extending the incubation period to 7 days or longer but not beyond 10 days, since the medium begins to show signs of drying. It would also be a simple matter to attempt to reculture the microorganism to determine the nature of the activity of the agent being studied.

Additional advantages afforded by this method are a minimum of expenditure for materials or apparatus, and that a relatively small amount of working area is required to test a series of compounds when compared to the space needed if Petri dishes are employed.

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Inhibition of Replication of Lee Influenza Virus in Tissue Culture by Puromycin

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Puromycin, an inhibitor of protein synthesis with general growth inhibitory properties, was found to inhibit replication of influenza virus in tissue culture in low concentrations without destroying all metabolic activity of the tissue. The aminonucleoside of puromycin, reported to be as effective as the complete compound against trypanosomes, was inactive as an inhibitor of the virus. Evidence suggests the mechanism of the virus inhibition is probably interference with protein synthesis, and that the antitrypanosomal activity has a different mechanism.

PUROMYCIN (I) is an antibiotic whose structure, proved by total synthesis by Baker *et al.* (1), is that of a nucleoside bound to an unusual amino acid.

Puromycin has been found to inhibit the growth of a variety of cells, including bacteria (2), protozoa (3, 4), and animal tumors (5). This compound has been shown to inhibit protein synthesis in several biological systems, including a cell free rat liver extract (6), Ehrlich ascites tumor cells, and rabbit reticulocytes (7). It was effective in curing experimental infections of mice and rabbits with several species of trypanosomes (3, 8), and also in the therapy of human trypanosomiasis (9).

Puromycin's mechanism of action seemed to offer a possible new approach to the inhibition of virus protein formation. During the course of this study, the inhibition of poliovirus replication in tissue culture by puromycin was reported (10).

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