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A MODIFIED REDDISH CUP TECHNIQUE FOR EVALUATING THE GERMICIDAL ACTIVITY OF "LIQUID ANTISEPTICS."*

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DISCUSSION.

Comparative test determinations of the bactericidal activity of antiseptics usually present difficulties in the laboratory because so many variable physical and bacterial growth factors tend to influence the results. Phenol coefficient tests based upon the time and concentration of any antiseptic to kill eighteen to twenty-four-hour cultures of the E. typhi are still used where antiseptic strength is required, but the results are subject to deviations or errors, and by many authorities are not considered very reliable. A check test using an entirely different determinative technique would therefore appear advisable. Recently Reddish evolved a method for testing antiseptic and germicidal ointments by the cup and smear method in which ointments were dropped into a cup, cut into plated culture media and seeded

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with *staphylococci* or mixed infection pyogenic cocci. After twenty-four hours' incubation the zones of inhibition and diffusion were noted.

The writer in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION, Vol. 25, 606 (1936), published a paper evaluating the antiseptic or bactericidal activity of 85 U. S. P. and N. F. ointments based on comparative inhibition and diffusion zones in culture media seeded with *staphylococci*, or mixed infection cocci obtained from boils or pimples. The comparisons were formulated from the actual width of the respective inhibition and diffusion zones. The apparent success of this method in evaluating ointments suggested that a similar technique might be attempted and adapted to testing the *germicidal activity of liquid antiseptics and disinfectants*, with the results also interpreted visually in terms of comparative inhibition and diffusion zones by actual measurement.

Standardization of Culture Media.—In order to get uniform data for antiseptic coefficients, based on inhibition of viable seeded organisms and diffusion into the surrounding medium, the consistency, density, viscosity, moisture content, temperature and surface tension of the culture medium should be kept constant by careful weighing of all ingredients and also the final product according to the U. S. P. H. or "Standard Methods Formulæ" for nutrient agar medium. The sterilization time, pressure and temperature should be regulated and standardized according to the amount prepared. In our experiments one liter of nutrient agar was sterilized in the autoclave at 120° C., for half an hour with steam pressure 15 lbs. per square inch. The nutrient agar formula was prepared according to exact weight and content.

Agar	25 Gm.
Peptone	10 Gm.
Meat extract	5 Gm.
Sodium chloride	5 Gm.
Water	1 liter

The heat of autoclaving tends to increase the acidity of the medium, so the final post autoclaving p_H should be 7.4. Before inoculating the tube culture media with the test organism, the media should be cooled in the hot water-bath to the usual 45°–50° C. Hot media attenuates or limits the growth rate of the thermolabile cocci making them less resistant to germicides and in doing so increases the inhibition zone.

Several preliminary batches of antiseptics were tested varying the technique for preparing the culture media. When 15 Gm. of agar were used instead of 25 Gm. the diffusion and inhibition zones were greater for all antiseptics tested. When a very hard media with high viscosity and surface tension was made containing 30 Gm. of agar the diffusion zones were lessened. Prolonged autoclaving increased the concentration of the medium and thus decreased the inhibition zones. A batch of watery medium which barely jelled gave inhibition zones which covered the entire plate when used as a test medium for strong antiseptics, particularly when the blotter impregnation technique was used. Variations in the surface tension of agar and density of the medium clearly influence the end results, so that every phase of the medium preparation should be standardized.

Even with this standardization results tend to vary in different laboratories. It is suggested that as an alternative method all batches of tests be run on the basis of comparison with 10% phenol using the same sterilization time, culture media content, technique and incubation period for each batch of germicides to be tested. Technicians may, therefore, set up their own comparative standards when batches of tests are run by comparing unknown antiseptic inhibition zone

widths with the phenol zones run at the identical time and using exactly the same technique and culture media; 10% phenol and salicylic acid 1/4% strength gave fairly constant inhibition zones of 1 and 0.1, and may be conveniently used as the basis for standards of comparison.

Modified Technique for Liquid Germicidal Coefficient Determinations. Method I.—Five loopfuls of eighteen-hour-old cultures of mixed *staphylococci* and *streptococci* were inoculated into tubes of 15-cc. sterile liquid nutrient agar which was cooled to between 45°–50° C. The seeded culture media was then poured into large-sized petri dishes and allowed to harden. Next, circular cups were cut by pressing down the open end of a test-tube through the center of solid culture media, one cut for each test plate, and the circular plugget of culture media so cut was lifted out with a spatula and discarded. In addition a drop or two of agar was placed on the bottom of each cup. The liquid antiseptic to be tested was then poured in up to the brim of the cup with a pipette or eye dropper. Incubation at the usual 37° C. for forty-eight hours followed, after which the final plate readings and measurings were made with calipers. The resultant widths were indicated in terms of centimeters of both the inhibition and diffusion zones.

Difficulties were encountered immediately, in that antiseptics such as alcohol soon evaporated in the cups, culminating in results that were confusing. We therefore modified the Reddish technique by placing the antiseptics in the cups and covering the bottom as usual with agar, but sealing the cups with either wax paper, cellophane or melted wax, so that evaporation of the antiseptic was retarded or prevented. The results so obtained were more uniform and the inhibition zones could be measured accurately with calipers and compared.

Method II.—The most satisfactory method resorted to, however, involved the use of 1/2–1-inch diameter circles or squares of blotting paper (size immaterial), which were then soaked to saturation in the antiseptics to be tested and placed on the surface of the seeded culture medium with a pair of forceps. To prevent evaporation of volatile antiseptics, such as alcohol, re-moistening of the blotting paper with drops from a pipette during the incubation period was necessary in order to keep the blotter fully impregnated until the final reading. If further standardization is required the white blotting paper usually used in bacteriological laboratories may be cut into squares of two-centimeter dimensions when 2 cc.'s of the test antiseptic will saturate the blotter.

TABLE I.—APPROXIMATE AVERAGE AGAINST MIXED INFECTION STAPHYLOCOCCI AND STREPTOCOCCI.

Liquid Antiseptic Inhibition Zones.		Phenol Coefficient.
Phenol 10%	1.0 cm.	1.0
Phenol 5%	0.5 cm.	0.5
Phenol 1%	0.1 cm.	0.1
Tinct. iodine U. S. P.	3.0 cm.	3.0
Mercury oxycyanide	2.5 cm.	2.5
Mercurochrome	1.8 cm.	1.8
1/1.000, HgC12	1.1 cm.	1.1
Argyrol 20%	0.7 cm.	0.7
Argyrol 10%	0.3 cm.	0.3
Protargol 1/2%	0.8 cm.	0.8
Silver nitrate 2%	0.4 cm.	0.4
U. S. P. antiseptic solution	0.4 cm.	0.4
Comp. cresol 1/100	0.7 cm.	0.7
Comp. cresol 1/1000	0.05 cm.	0.05
Salicylic acid 1/4 aqueous	0.1 cm.	0.1
Alcohol 95%	0.5 Results doubtful	0.5
Alcohol 50%	0.2	0.2

In this case because Phenol 10% was one, the coefficient equals the width of the inhibition zone.

Against Bacillus Subtilis Cultures.		Phenol Coefficient.
Phenol 10%	0.6 cm.	1.0
Mercurochrome	1.1 cm.	1.8
Tinct. iodine	2.7 cm.	4.5
Comp. cresol 1/100	0.5 cm.	0.8
Protargol 1/2%	0.5 cm.	0.8
Mercury oxycyanide	2.0 cm.	3.3

Using 10% phenol as the standard with an approximate inhibition zone of 1.0 cm., the phenol coefficient of germicidal activity may readily be computed by dividing the inhibition zone width of the antiseptic to be tested by 1.

Example: Mercury oxycyanide 2.5
Tincture of iodine 3.0
Silver nitrate 2% 0.4

The results obtained with the modified impregnated blotter technique were reasonably uniform for evaluating the germicidal strength of the various liquid antiseptics under test. One advantage of this test method is the very clear visualization possible for photographic record of bactericidal activity. In order to make comparative evaluations of several antiseptics tenable, it was necessary to set up tentative standards for comparison. We therefore prepared according to U. S. P. formulas, solutions of 5% and 10% phenol; tincture of iodine; mercurochrome; 50% and 90% alcohol; 10% argyrol; compound cresol solution, and several other U. S. P. or N. F. antiseptics and disinfectants (see Table I). The relative antiseptic strengths were determined by measuring the width of each inhibition and diffusion zone in terms of centimeters or fractions thereof. Photographs and charts were made in order to clearly show the comparative antiseptic activity of standard antiseptics when compared with 10% phenol which had a fairly constant inhibition zone on standard culture media of approximately 1 cm.

SUMMARY AND CONCLUSION.

(1) Liquid antiseptics may be check tested for germicidal strength with visual interpretation in terms of the width of the zones of inhibition against pyogenic cocci or other tests organisms. (2) The blotter impregnation technique gives uniform results provided the culture media preparation is standardized. (3) The U. S. P. arsenicals, iodides, coal tars and silver preparations have high germicidal coefficients when compared with 10% phenol. (4) 10% phenol gave a zone of 1 cm., so that phenol coefficients equaled the actual zone widths in these particular tests.

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A PROPOSAL FOR AN ALTERNATIVE PERMISSIBLE PROCESS FOR THE MANUFACTURE OF THE TINCTURE OF SWEET ORANGE AND OF LEMON.

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Observation of the inconvenient features of the official processes for the manufacture of the tinctures, has prompted the following comments and suggestions.

The official processes, while feasible on a small scale in the retail pharmacy, are not very practical in the manufacturing laboratory since in order to obtain the outer rind grated from the fresh fruit, as the U. S. P. specifies, comparatively large quantities of the latter must be employed with the resulting expense of material and labor, particularly where no immediate use can be found for the edible portion of the fruit. Since approximately seven Gm. of outer rind are obtainable from a lemon and eighteen Gm. from an orange, a liter of tincture requires about seventy of the former and twenty-eight of the latter. Add to this the necessary tedious hand labor, and the cost becomes quite impressive.

A simple expedient, therefore, suggests itself, which is amply justified by experiment and reason. The official tinctures of sweet orange and of lemon are essentially acoholic solutions of the volatile oils and coloring principles obtained by maceration of the outer yellow rind grated from the fresh fruit.

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