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TECHNICAL MANUSCRIPT 44

DISINFECTION OF
AEROSOLIZED PATHOGENIC FUNGI
ON LABORATORY SURFACES
I. TISSUE PHASE

MAY 1963

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
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ABSTRACT

The effect of several different fungicides on laboratory surfaces contaminated with the tissue phase of aerosolized Blastomyces dermatitidis, Coccidioides immitis, Cryptococcus neoformans, and Histoplasma capsulatum was ascertained. A statistical analysis of the data shows the correlation between fungi, surfaces, time, and concentration of disinfectant. All fungicides were effective at established times and concentrations and the type of contaminated surface affected the fungicidal efficacy. By interpolating plotted graphs, laboratory personnel may determine, with a given fungicide, the concentration and time required to disinfect instruments, pipettes, gloves, bench tops and floors contaminated with the tissue phase of pathogenic fungi.

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I. INTRODUCTION

Review of the literature has revealed no report on the efficacy of disinfectants for laboratory surfaces contaminated with aerosolized pathogenic fungi such as Blastomyces, Coccidioides, Cryptococcus, and Histoplasma. There seems to be no universally accepted test for fungal disinfectants.

The first well-known method of testing germicides employed silk threads impregnated with spores of Bacillus anthracis.¹ An improvement was made by Rideal and Walker.² It and other modifications are still in use today. The phenol coefficient test is recommended, but is not an official test of the Food and Drug Administration. Phillips³ states that the phenol coefficient method is the one most widely used and misused. Reddish⁴ lists the limitations of the phenol coefficient test as (a) limitation to phenol-like compounds, (b) variability in resistance to the test culture, (c) necessity of repeated tests to obtain a final phenol coefficient, and (d) difficulty in obtaining consistent results. Stedman and co-workers⁵ regard much of the controversy as arising from the innate complexity of the disinfecting procedure resulting from the composition of the surface, the technique used in applying the disinfectant, and the particular organism. Sykes⁶ sees no future for the phenol coefficient type of test and believes that an entirely fresh and untrammelled approach to the problem is needed. Rogers and co-workers⁷ reviewed the various swabbing techniques, agar-contact methods, rinsing processes, tracer techniques, and in-use testing and concluded, as Walter⁸ did, that the method that best serves the purpose of the individual should be selected.

Testing a disinfectant on surfaces is not new; in fact it was the first method used by Koch.¹ Different methods of using surfaces to evaluate disinfectants have been reported.^{5, 9-18}

In this investigation, the tissue phase of four fungi, Blastomyces dermatitidis, Coccidioides immitis, Cryptococcus neoformans, and Histoplasma capsulatum, was aerosolized on surfaces of five materials commonly used in laboratories on the work bench, floor, or equipment. Aerosolization was selected because an earlier study¹⁷ demonstrated that many laboratory mycological procedures create aerosols that may cause laboratory-acquired infection.¹⁸⁻²¹ Candidate fungicides were evaluated by concentration and time for their ability to kill the fungi on different surfaces. This study, as will a future study on cultural phases of the test fungi, attempts to approximate conditions that may occur when infectious aerosols are created by mycological laboratory techniques. The aerosolized cells or spores settle and dry on the surfaces. Further investigation to evaluate the action of these fungicides on the cultural phase will be reported at another time.

II. MATERIALS AND METHODS

A. TEST FUNGI

Spherules of *C. immitis* (strain M-11) were grown by the method of Converse²² and filtered by gravity through six layers of sterile surgical gauze to remove trace amounts of hyphae. The Yeast cells of *H. capsulatum* (strain 3021), *B. dermatitidis* (strain 3110), and *C. neoformans* (strain C-1-in) were grown in 50 milliliters of Pine's medium,²³ modified brain-heart infusion broth,* and enriched nutrient broth** respectively. Purity and viability of the fungi were ascertained by microscopic examination and serial dilution culture prior to and after aerosolization and drying.

B. TEST SURFACES

The following materials (1.0-inch squares) were used in this investigation: (a) wood painted with two coats of Plicoat,** (b) glass, (c) stainless steel, (d) neoprene, and (e) asphalt floor tile. The surface was cleaned with detergent and water, thoroughly rinsed with distilled water, and dried. The stainless steel and glass surfaces were sterilized by autoclaving at 15 pounds of pressure for 15 minutes. Painted wood, rubber, and asphalt tile surfaces were sterilized with ethylene oxide.²⁴

C. GENERAL PROCEDURE

Oster and Golden²⁵ state that there are three basic conditions for a fungicidal test: (a) contact of the fungus and fungicide for a limited time, (b) complete removal of the fungicide from the fungus by a suitable wash, and (c) the basic structure of the colony unaltered by the compound or test.

In the present investigation, 1.0-inch-square sections of test materials were placed in an aerosol chamber (Figure 1). The test fungus was introduced into the chamber with a Vaponefrin nebulizer*** that had been modified to produce fungal-bearing particles with diameters ranging from 1 to 30 microns.

* Bacto brain-heart infusion broth (Difco Laboratories, Detroit 1, Michigan), 37 grams; dextrose, 10 grams; and distilled water, 1 liter. Autoclave at 15 pounds of pressure for 15 minutes.

** Bacto nutrient broth (Difco Laboratories, Detroit 1, Michigan), 8 grams; thiamin hydrochloride, 1 gram; and distilled water, 1 liter. Autoclave at 15 pounds of pressure for 15 minutes.

*** Fisher Scientific Co., New York, N.Y.

**** Vaponefrin Company, Metuchen, New Jersey.



Figure . Chamber Used for Aerosolizing Fungal Particles. (FD Neg 6967)

After aerosolization, the residual aerosol was evacuated through a fiberglass filter. Then dry sterile air was admitted to the chamber to dry the seeded surfaces. No drying menstruum, which by its nature can be expected to exert a greater or less degree of protection against attack by the disinfectant,⁶ was used.

The test procedure is outlined in Figure 2. The seeded surface was immersed in the disinfectant. A disinfectant should achieve its objective in as short a time as possible, and a contact time of not less than two nor more than ten minutes should be used.²⁶ Sykes⁶ regards five or ten minutes contact time too short and prefers a 30-minute time with no advantage in prolonging the time beyond 30 minutes. We chose times of 1, 5, 10, 20, 30, and 60 minutes so that at a given time and concentration the disinfecting efficiency could be determined by graphic interpolation. At each of the six selected times a sample surface was removed and immersed for 30 seconds in 50 milliliters of an aqueous solution of a neutralizer that was specific for the test disinfectant. The entire surface sample was swabbed with a Calgiswab.* Calcium alginate soluble wool was preferred to cotton, as Higgins²⁷ showed that cotton did not recover as many organisms as did soluble wool. After dissolving the Calgiswab in four milliliters of 1.0 per cent sodium citrate, 0.5-milliliter aliquots of the resulting suspension were plated in triplicate on appropriate media.

* Calcium alginate soluble wool. Consolidated Laboratories, Inc., Chicago Heights, Illinois.

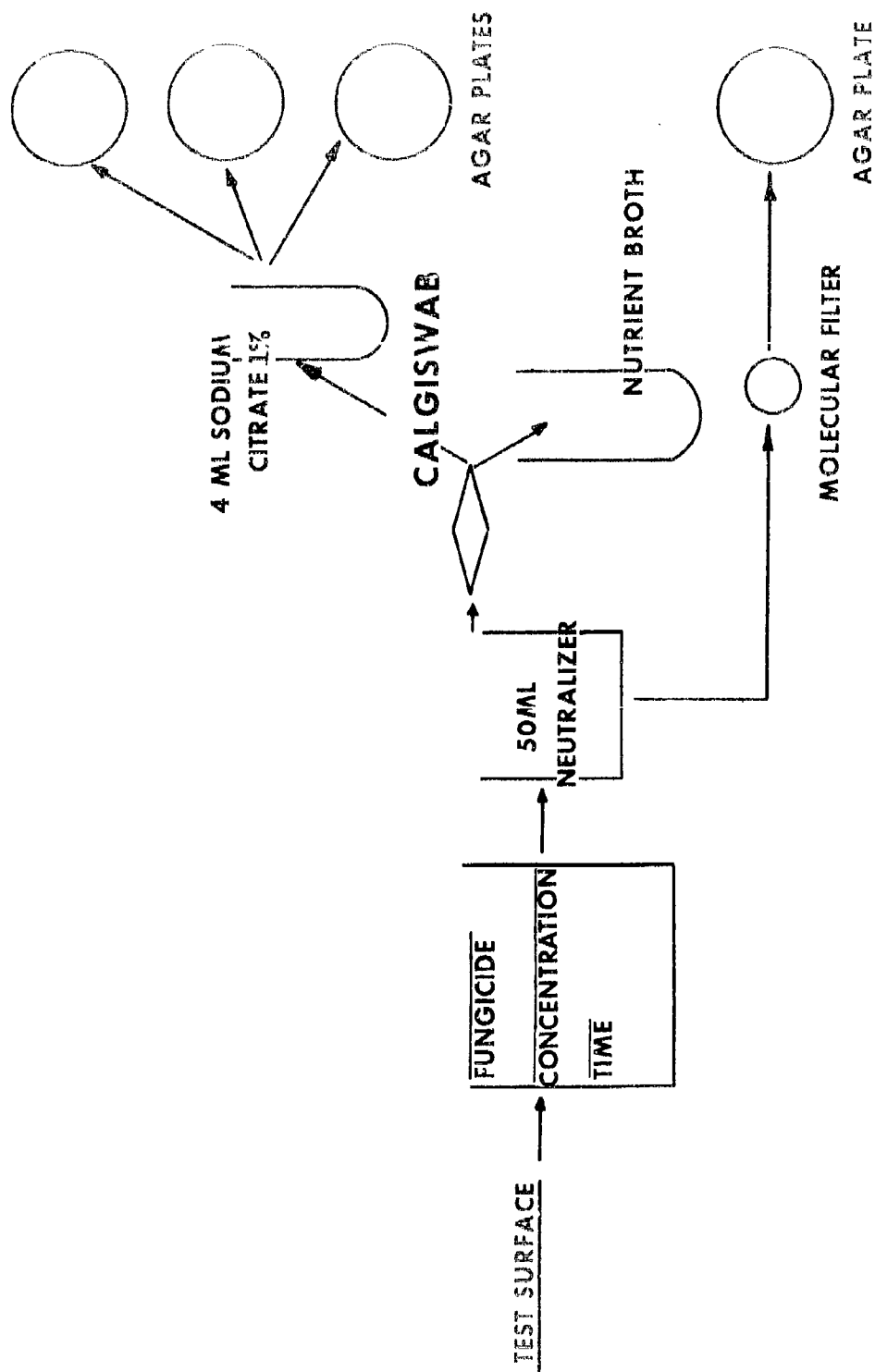


Figure 2. Flow Chart for Test Procedures in Fungus Disinfection.

To assure complete recovery of any fungus particle that may have remained on the seeded surface after swabbing, or may have washed off into the neutralizing solution, the surface was immersed in appropriate broth and the neutralizing solution was passed through a membrane filter²⁸ and cultured by Gordon's technique.²⁹ Petri plates, tubes, and membrane filters were incubated at 37°C for ten days. An exception to this was made for H. capsulatum because Pine³⁰ reported that on a blood medium³¹ at 27° to 30°C this organism would convert from yeast cells to mycelial colonies and better recovery would result.

A control was tested concurrently by immersing the seeded surface in sterile 0.85 per cent saline and proceeding as with a test surface. All controls showed growth except for H. capsulatum. Cysteine, 0.1 per cent, was incorporated in the saline solution because Rowley and Huber³¹ reported that this combination maintained the viability of H. capsulatum yeast cells. With this modification, H. capsulatum grew in the controls.

D. SPECIAL PROCEDURES

For use in statistical analysis, as later described, an additional set of tests was made employing C. neoformans (because it was the most resistant of the four organisms to fungicides), asphalt tile (because it was the most difficult to disinfect), four disinfectants (phenol, "Cresylic," "Phenolic A," and "Iodenic"), at fungicidal concentrations of 0.4, 0.6, 1.0, 1.5, 2.5, 4.0, 6.0, and 10 per cent, and at times of 0.5, 1.0, 1.5, 2.5, 4.0, 6.0, 10, 15, 25, 40, and 60 min.

E. CANDIDATE FUNGICIDES

(a) A liquid n-alkyl (50% C₁₂, 30% C₁₄, 17% C₁₆, 3% C₁₈) dimethyl benzyl ammonium chloride (designated "Quat.").

(b) Ethyl alcohol

(c) Phenol

(d) Formaldehyde

(e) Peracetic acid

(f) Sodium hypochlorite

(g) A cresylic product (o-phenylphenol) containing soap and alcohol (designated "Cresylic").

(h) A phenolic formulation containing o-benzyl p-chlorophenol, p-tertiary amyl phenol, and o-phenylphenol (designated "Phenolic A").

(i) A phenolic formulation containing *o*-benzyl *p*-chlorophenate and potassium ricinoleate (designated "Phenolic B").

(j) An iodophor containing polyethoxy polypropoxy ethanol-iodine complex, and nonyl phenyl ether of polyethylene glycol-iodine complex (designated "Iodenic").

The disinfectants were evaluated at concentrations of 0.1, 0.5, 1.0, 2.0, 5.0, and 10 per cent, with the exception of ethyl alcohol (30, 50, 70, and 90 per cent). The disinfectant concentrations were prepared on a volumetric basis in distilled water, without regard for the specific gravity. However, "Quats" (50 per cent), formaldehyde (37 per cent), and peracetic acid (40 per cent) were prepared on an active ingredient basis. The disinfectants were prepared immediately before use.

F. CULTURE MEDIA

The following media were used: (a) substrate for the membrane filter and agar for plating the resulting suspensions of the dissolved Calgiswab, and (b) liquid broth for incubation of the swabbed surface.

1. B. dermatitidis

a. Substrate and Plating Medium

Bacto brain-heart infusion broth, 37 grams; dextrose, 10 grams; agar, 20 grams; and distilled water, 1 liter. Autoclave at 15 pounds of pressure for 15 minutes.

b. Broth Medium

Same as plating medium, without the addition of agar.

2. C. immitis

a. Substrate and Plating Medium

Bacto peptone, 10 grams; dextrose, 20 grams; and distilled water, 1 liter. Autoclave at 15 pounds of pressure for 15 minutes.

b. Broth Medium

Same as plating medium, without the addition of agar.

3. C. neoformans

a. Substrate and Plating Medium

Bacto peptone, 10 grams; dextrose, 20 grams; and distilled water, 1 liter. Autoclave at 15 pounds of pressure for 15 minutes.

b. Broth Medium

Same as plating medium, without the addition of agar.

4. H. capsulatum

a. Substrate and Plating Medium³¹

Bacto casamino acids, 10 grams; dextrose, 20 grams; calcium pantothenate, 1.0 milligram; biotin, 0.5 milligram; and distilled water, 1 liter. Adjust pH to 6.5. Add agar, 20 grams. Autoclave at 15 pounds of pressure for 15 minutes. Cool to 45°C and add aseptically defibrinated sheep blood, 60 milliliters.

b. Broth Medium³²

Bacto casamino acids, 10 grams; dextrose, 3 grams; yeast extract dialysate, 3 grams; sodium chloride, 2.5 grams; cysteine hydrochloride, 0.5 gram; potassium chloride, 2.5 grams; disodium phosphate, 4 grams; and distilled water to make 1 liter. Autoclave at 15 pounds of pressure for 20 minutes.

G. NEUTRALIZING SOLUTION FOR DISINFECTANTS

1. Cresylic, Phenolic A, Phenolic B, Formaldehyde, and Phenol

Broth medium for specific fungi (described in Section II, F) plus 1.0 per cent Tween 80.*

2. Iodenic, Sodium Hypochlorite, and Peracetic Acid

Specific broth media plus 0.5 per cent (dry weight) sodium thiosulfate.

3. "Quat" and Ethyl Alcohol

Specific broth media plus 0.07 per cent azolectin dissolved in 0.5 per cent aqueous Tween 80.

In the three solutions listed above, the specific neutralizer was added to the distilled water used to prepare broth media.

* Tween 80 - polyoxyethylene sorbitan monooleate, Atlas Powder Company, Wilmington, Delaware.

III. STATISTICAL EVALUATION OF RESULTS

When tests are performed in which many factors are involved, it is essential to standardize or eliminate variables to determine the reliability of the test procedure. Because the seeded surfaces are necessarily observed at specific times, the exact time required for disinfection could not be estimated. Previous experience in statistical analysis of the effect of disinfectants on bacterial aerosols has shown that when log transformation is applied to concentration of disinfectant, responses of the bacteria to the disinfecting process are often linearized. To test the hypothesis that the regression of "time required for disinfection" on "concentration of disinfectant" is linear in the log scale, samples of disinfectants were observed at a greater number of times and concentrations, as described in Section II, D. Concentrations of 0.4, 0.6, 1.0, 1.5, 2.5, 4.0, 6.0, and 10 per cent were tested at 0.5, 1.0, 1.5, 2.5, 4.0, 6.0, 10, 15, 25, 40, and 60 minutes to give approximately equal intervals on the log scale for both variables. Times and concentrations were converted to logarithms, and for a given log concentration the corresponding log time was estimated by linear interpolation between two or more log times for which both positives and negatives resulted. For example, with 1.5 per cent cresylic disinfectant all replications resulted in negatives at 60 minutes; three positives and one negative occurred at 40 minutes. It was therefore assumed that the true time for disinfection is between 40 and 60 minutes. The following equation was used to determine the time required for disinfection:

$$\text{Log } t = \text{Log Lower } t + \left(\frac{\text{No. of positives}}{\text{Total observations in } \Delta \text{ Log } t} \right) \Delta \text{ Log } t$$

where $\Delta \text{ Log } t$ = shortest log time in which all negatives occurred minus the next shortest log time, in which at least one positive was observed. A probability level of 0.01 permitted reasonable conclusions concerning the homogeneity of slopes. It was concluded that: (a) regression for "time required for disinfection" on "concentration of disinfectant" is accurately represented as

$$Y = 10^{ax^b} \text{ or } \log Y = a + b (\log X)$$

where Y = time required for disinfection
 X = concentration of disinfectant,
 a = intercept
 b = slope

(b) the parameter b could be treated as constant over all disinfectant-fungus-surface combinations, and (c) the disinfectants could be ranked according to effectiveness in terms of time with a given concentration of disinfectant.

Notice (Figures 3 through 6) that this equation does not apply to the use of ethyl alcohol. These curves were fitted by eye to observed results rather than by any computed equation.

The data presented in Figures 1 through 4 are plotted in semilog scale and rank the fungicidal efficiency with regard to time and concentration on different laboratory surfaces.

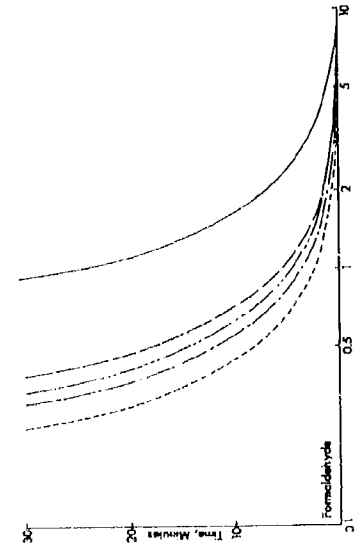
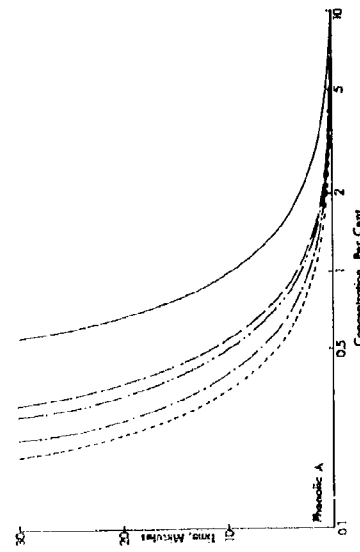
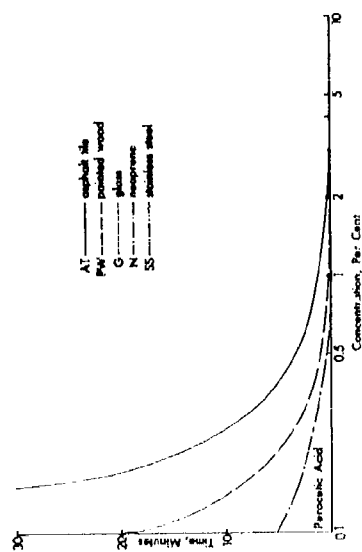
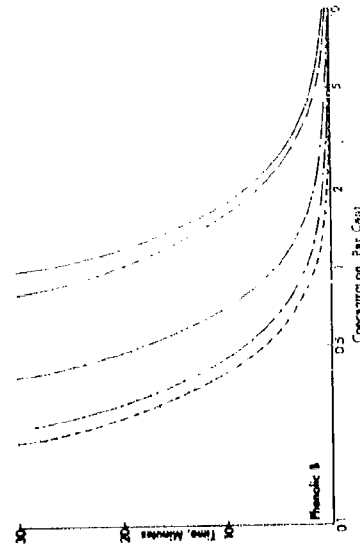
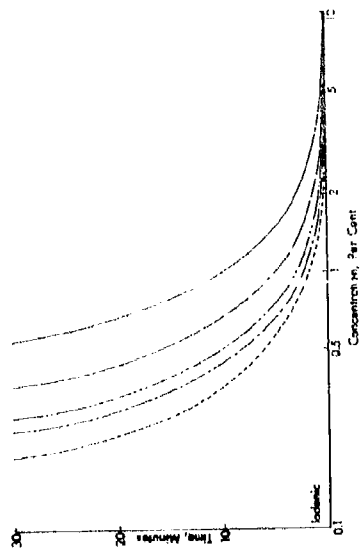
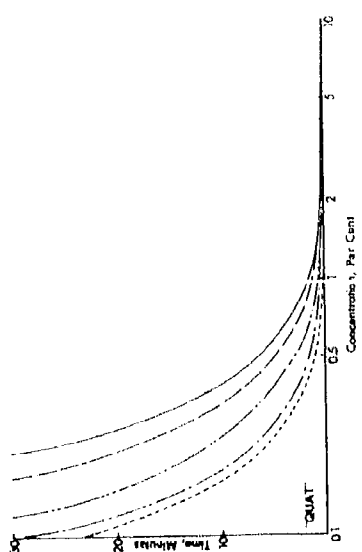
IV. DISCUSSION OF RESULTS

A. COMPARISON OF THE FOUR FUNGI

The tissue phases of B. dermatitidis, C. immitis, and H. capsulatum react comparably when subjected to a given fungicide. However, when C. neoformans is subjected to the same fungicide, a greater time is required for disinfection. In all probability the capsule of C. neoformans impedes the fungicide's penetrating the cell wall.

B. EFFECT OF THE SURFACE MATERIAL

The data in Figures 1 through 4 indicate that when time and concentration of a fungicide are standardized, the nature of the test surface determines the fungicidal efficiency. A disinfectant will behave differently on a hard, impervious surface such as glass or metal than it will on rubber, and even more differently on a porous surface such as wood.⁶ Since there is no method of comparing the degree of porosity of test surfaces,¹⁵ the surfaces were examined microscopically. It was determined that the order of decreasing porosity was: asphalt tile, painted wood, stainless steel, neoprene, and glass. The significant effect of surfaces is well known. Stedman and co-workers¹⁶ state that higher concentrations of disinfectants are required to disinfect porous surfaces than nonporous surfaces in a given time. They show that phenolic, cresylic, and quaternary disinfectants were more effective against Staphylococcus aureus spread on stainless steel than on asphalt tile. Experiments show that a cresylic disinfectant was more effective on Escherichia coli spread on glass, less so on rubber, and least effective on asphalt tile.³³ Microorganisms were more difficult to recover from unpainted oak surface than from stainless steel.³⁴ Vashkov and Nekrasova³⁵ report that painted wood surfaces were disinfected more rapidly than unpainted wood surfaces when using Staphylococcus aureus and E. coli.



AT ——— aluminum
 PE ——— painted wood
 G ——— glass
 N ——— neoprene
 SS ——— stainless steel

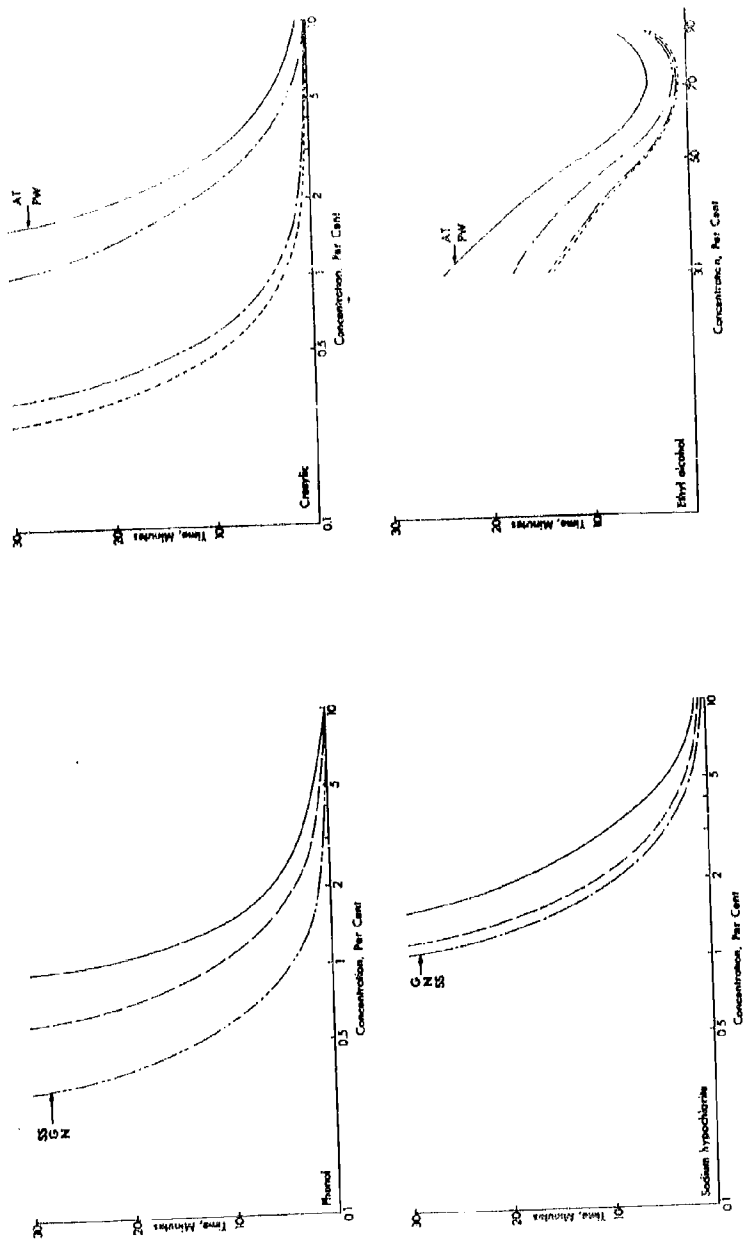
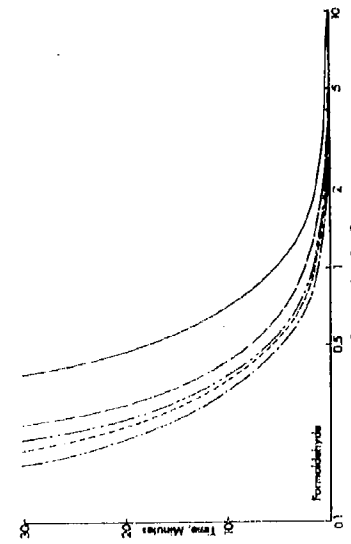
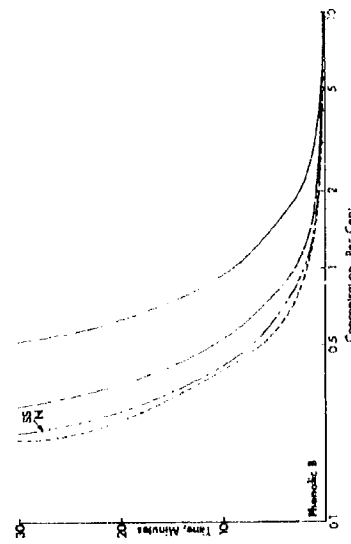
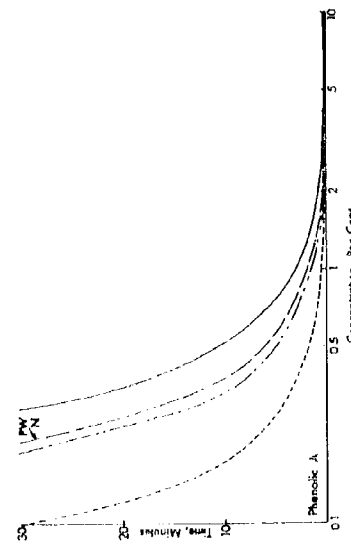
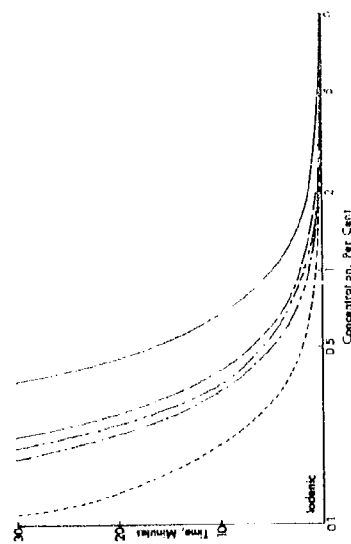
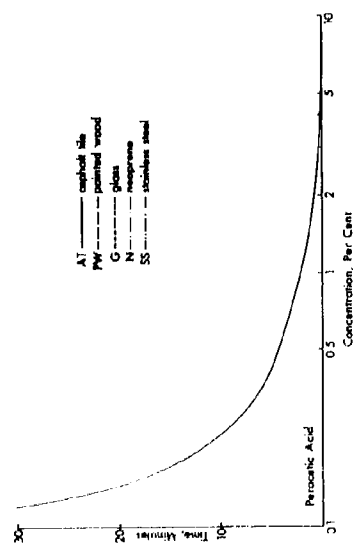
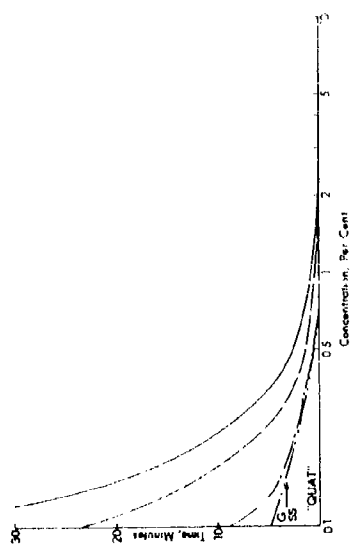


Figure 3. Effect of Fungicides on Cryptococcus neoformans.



AT — cathodic tile
PW — painted wood
G — glass
N — neoprene
SS — stainless steel

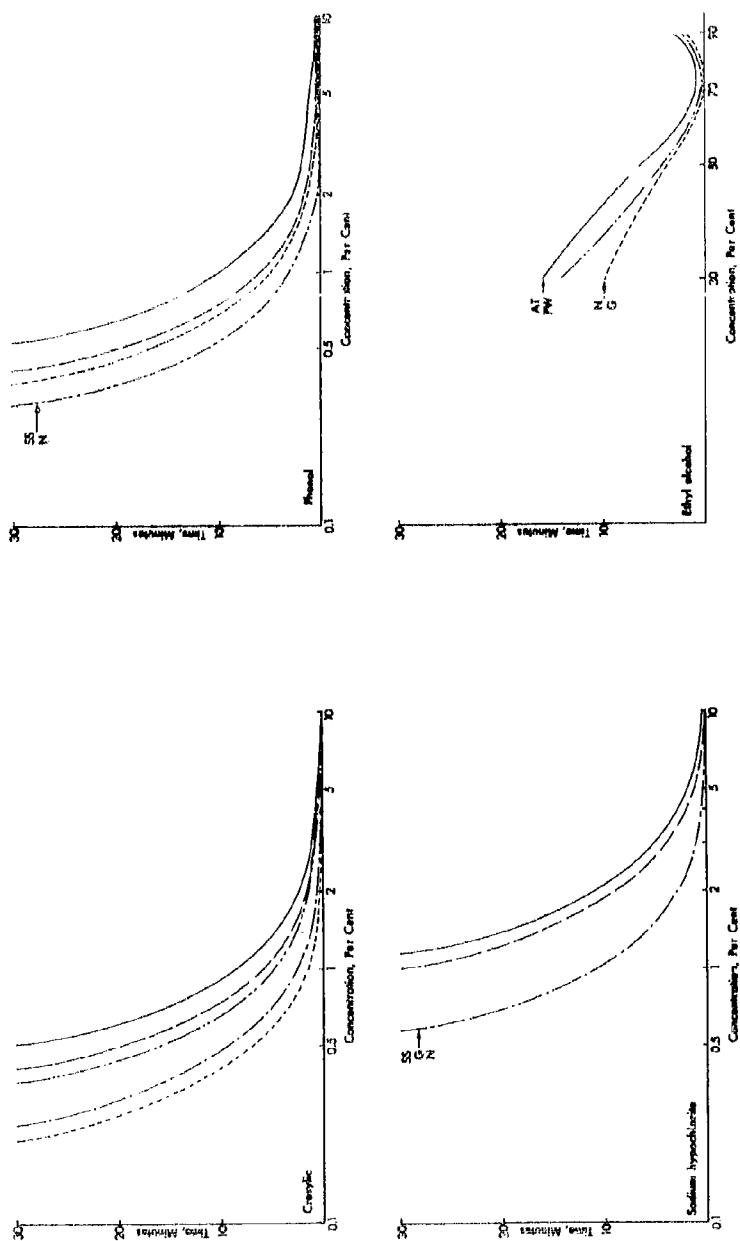
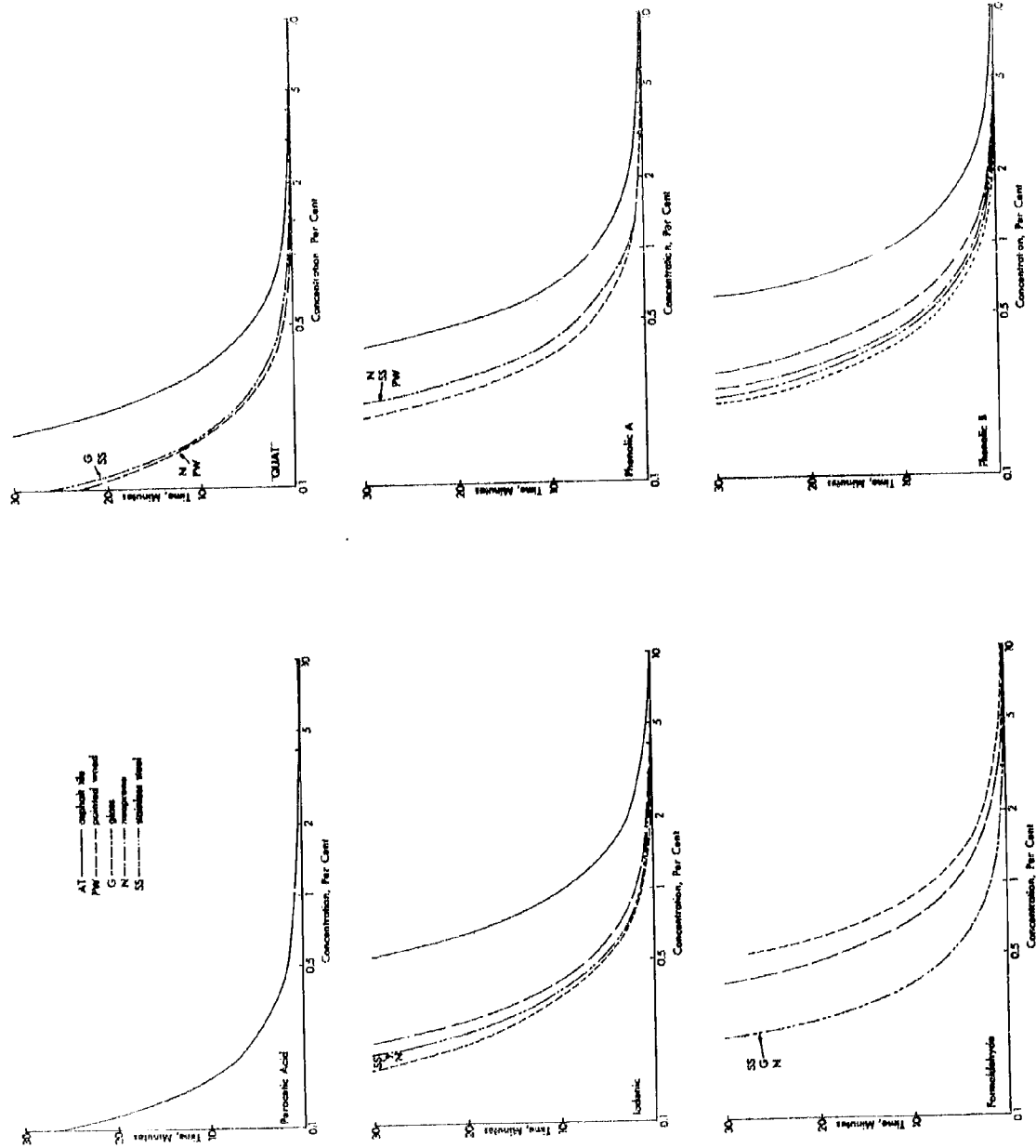


Figure 4. Effect of Fungicides on *Histoplasma capsulatum*.



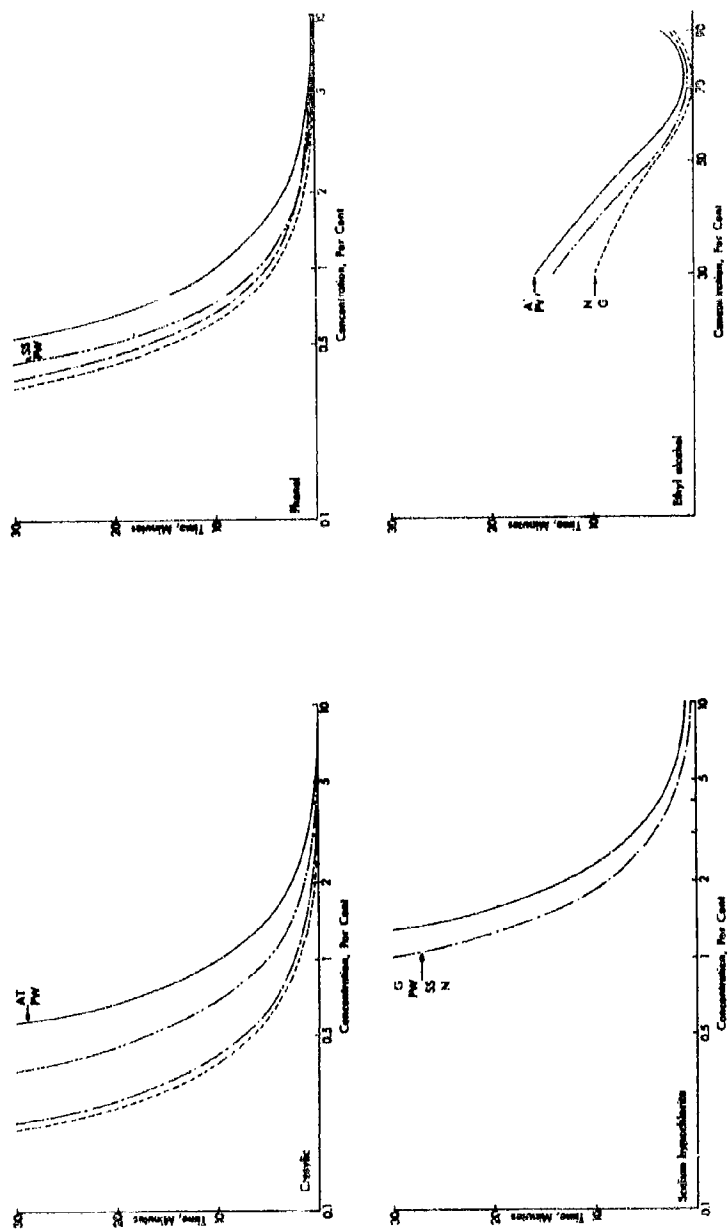
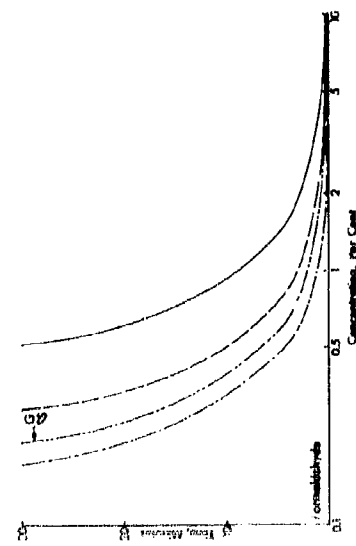
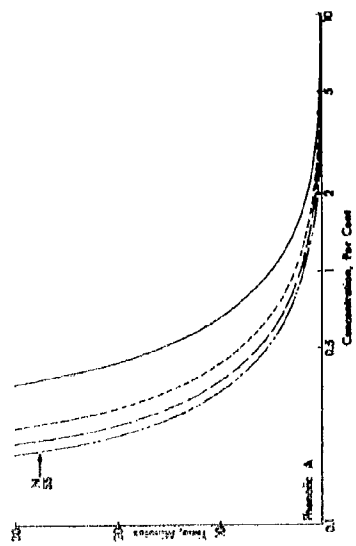
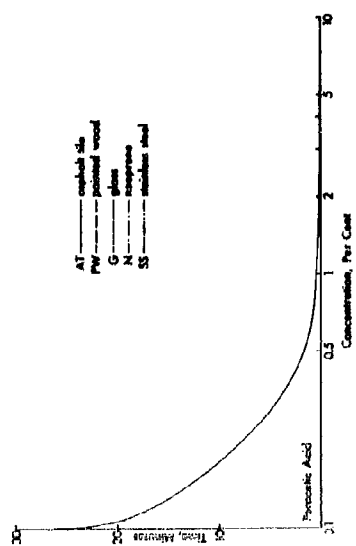
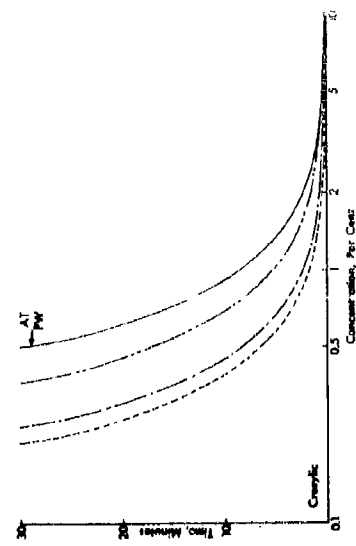
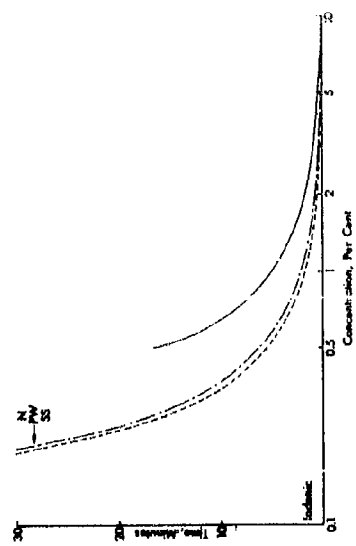
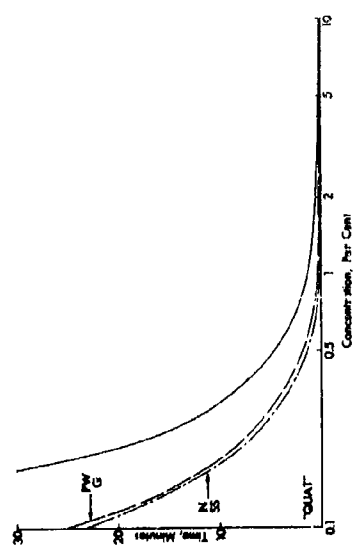


Figure 5. Effect of Fungicides on *Blastomyces dermatitidis*.



AT — carbon 10
PV — carbon 10
G — carbon 10
ZK — carbon 10
SI — carbon 10

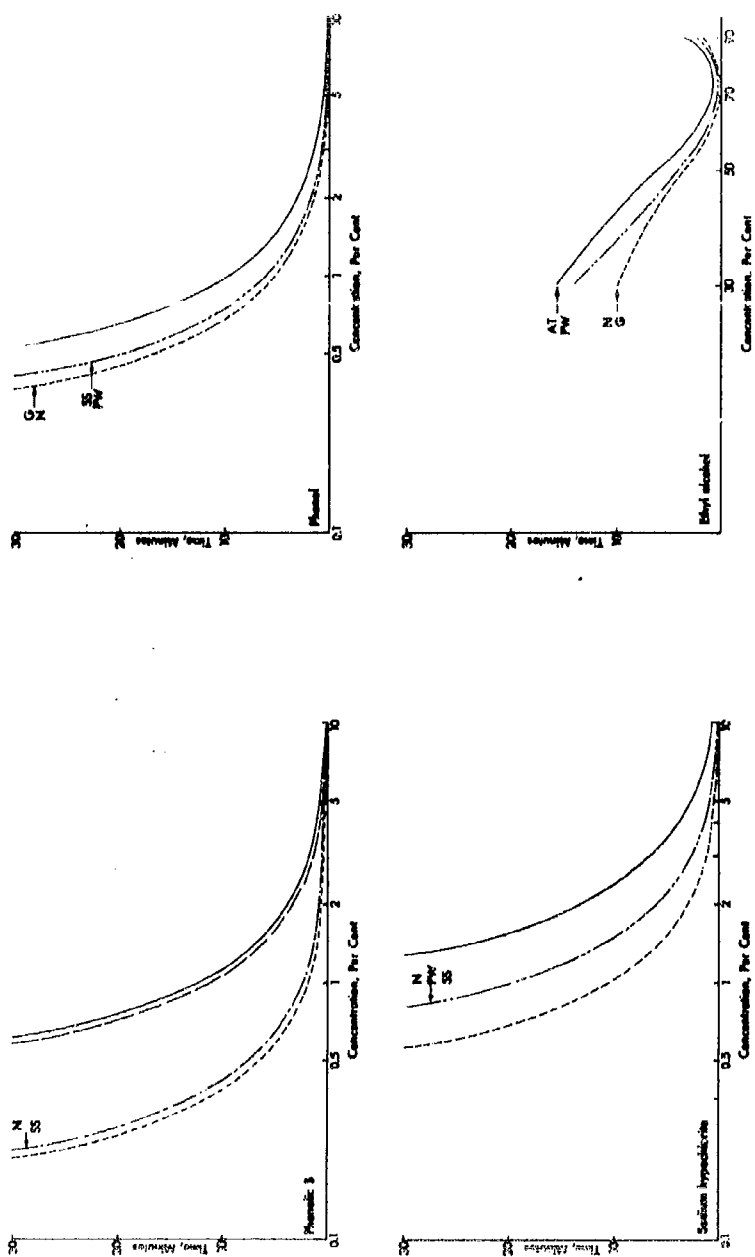


Figure 6. Effect of Fungicides on *Coccidioides immitis*.

C. CORRELATION WITH PERTINENT REPORTS BY OTHER INVESTIGATORS

Peracetic acid, quaternaries, iodenic solutions, and sodium hypochlorite are less effective when prepared on a volumetric basis^{36, 37} than when prepared on a basis of active ingredients present.³⁸⁻⁴¹ Throughout this study, peracetic acid and "Quat" were prepared according to the amount of active ingredients present. The hypochlorite and iodenic solutions were prepared on a volumetric basis because the amount of active ingredient varies with the manufacturer, and because in common laboratory disinfectant use the solutions are prepared by simple volumetric dilution. To permit easier comparison of the present results with those of other investigators, the amount of active ingredient for these four fungicides is presented in Table I.

TABLE I. ACTIVE INGREDIENT AVAILABLE IN PPM OF TEST FUNGICIDES

Tested Per Cent	Iodenic	Quat	Hypochlorite	Peracetic Acid
0.1	16	500	52.5	400
0.5	80	2,500	262.5	2,000
1.0	160	5,000	525.0	4,000
2.0	320	10,000	1050.0	8,000
5.0	800	25,000	2625.0	20,000
10.0	1,600	50,000	5250.0	40,000

Cantor and Shelanski⁴² report iodenic compounds to be more effective against yeast than hypochlorite and quaternaries on a basis of active ingredients present. Dunn⁴³ found that in the test tube one to two per cent active-ingredient quaternary killed C. neoformans, Candida albicans, and Saccharomyces cervisiae in ten minutes but not in five minutes. In one minute, 0.025 per cent peracetic acid is said to have killed completely a concentration of 42×10^6 E. coli per ml.⁴⁴ In the present study, peracetic acid was found to act on the fungi so rapidly that time/concentration relationships could not be clearly plotted except with capsular C. neoformans on three surfaces.

Dunn⁴³ has shown that 1.0 per cent phenol did not kill C. albicans or C. neoformans in five minutes, but that 1.0 per cent was effective in ten minutes. Other investigators^{45, 46} report that 1.0 per cent killed C. albicans and C. tropicalis in 30 minutes. Vashkov and co-workers⁴⁷ state that compounds of phenol in which the halogen is in the para position to the

hydroxyl group are more effective than compounds in which halogen is in the ortho position. Stedman and co-workers^{5, 15} state that cresylic and phenolic B disinfectants are more effective than phenol when vegetative microorganisms are dried on steel and tile surfaces. Other experiments^{46, 48} resulted in a fungicidal efficiency for a cresylic disinfectant of approximately two times greater than phenol, and for phenolic B of approximately four times greater. Our data (Figures 1 through 4) indicate that phenolic A is more effective than cresylic or phenolic B disinfectant.

Formaldehyde in aqueous solution has been reported effective in two to five minutes against C. albicans.^{38, 49, 50}

Ethyl alcohol has been the subject of conflicting reports. With vegetative bacteria dried on threads, concentrations of 40 to 70 per cent were germicidal but 90 per cent was not.^{51, 52} With wet surfaces, 95 per cent ethyl alcohol was effective, but with dry surfaces 70 per cent was better.^{53, 54} Our data (Figures 1 through 4) verify the findings of previously mentioned experiments in regard to the action of ethyl alcohol on the tissue phase of the test fungi air-dried on surfaces.

V. SUMMARY OF RESULTS

Statistical analyses of the data in Figures 1 through 4, correlating the four fungi, five surfaces, and time and concentration indicate that heterogeneity exists among the fungicides, and ranks them in order of decreasing effectiveness as follows: (a) peracetic acid, (b) "Quat," (c) iodenic and phenolic A, (d) formaldehyde, (e) phenolic B, (f) cresylic, (g) phenol, (h) sodium hypochlorite, and (i) ethyl alcohol. However, with the exception of the rapid activity of peracetic acid and "Quat," and the greatly reduced activity of sodium hypochlorite and ethyl alcohol, the fungicidal efficiency of the remaining fungicides is approximately equal and they can be substituted for each other. All the fungicides at a proper time and concentration are effective. Laboratory personnel using data in Figures 1 through 4 can, by interpolation, determine, with a given fungicide, what time and concentration are required to disinfect instruments, pipettes, gloves, and, in case of a laboratory accident, bench tops and floors, when mycological procedures involve the tissue phase of Blastomyces, Coccidioides, Cryptococcus, and Histoplasma.

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