A MODIFIED AGAR TEST METHOD FOR WOOD PRESERVATIVES

by

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A thesis presented to the Department of Chemistry of Union College in partial fulfillment of the requirements for the degree of Bachelor of Science in Chemistry.

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I. INTRODUCTION AND HISTORY

The General Problem of Preserving Woods

The principal criteria for a good wood preservative involve the following considerations:

1. The preservative must be sufficiently toxic to curb the action of the wood rotting fungi and wood attacking insects.
2. The preservative must not attack wood.
3. The preservative must not undergo a chemical or physical change brought upon by contact with the wood cells so as to render the chemical agent non-toxic or less toxic.
4. The preservative must not be a substance which will attack metals.
5. The preservative must be of such a nature as to make it easily injected into wood.
6. The leachability, volatility, chemical stability, penetrability, cleanliness, cost, toxicity to human beings and other similar factors must conform to the practical conditions under which the preservative is to be used.

Although all of the above mentioned conditions are of high importance, the fact remains that the first consideration in selecting a wood preservative must be given to its ability to kill or to vitally inhibit the biological organisms which attack the wood. "Biological organisms" can be correctly construed as pertaining to both plants and animals. However, this discussion will be confined purely to the harmful effect of fungi upon spermatophytic plants and to the means by which the toxic action of chemical agents upon the saprophytic growth can be studied.

The General Method of Protecting Wood from Saprophytic Fungi

There are two possible methods by which wood could be protected from the attack of saprophytic fungus growth. Either the conditions can be so controlled as to be unfavorable for the growth of the attacking organism, or the wood can be permeated with a poisonous substance which will in some way disrupt the vital processes of the fungus. It is obvious that the first suggested preventative would not at all be practical due to the wide geographical range over which wood is used and to the various types of weather conditions to which it may be subjected. Consequently, the latter approach to the problem has been the one followed by the wood preservation industry and by scientific investigators in the field.

The Biological Significance of Wood Decay by Fungi and Its Retardation

The fungi are a subdivision of the plant phylum Thallophyta which includes the free living algae as well as the fungi. Unable to manufacture their own food material due to lack of chlorophyll, the fungi depend the higher plants for survival and growth. There are two types of fungi which attack trees. The parasitic fungi, the result of which is evidenced by the American Chestnut blight and the Dutch Elm disease, are those that obtain their nutrition from living hosts. The saurophytic fungi, the result of which is evidenced by the rotting of telephone poles and railroad ties, are those which obtain their food from dead organic matter. It is with the latter group that this paper will be concerned.

Lentinus lepideus, classified by workers in the field as Madison 534, was the fungus employed in this research. This fungus belongs to the higher order of the fungi or the mushrooms. It is a member of the class Basidiomycetes. The main body of growth consists of a mass of microscopic strands, or hyphae; the entire network is referred to as a mycelium. This main growth is seldom seen in nature unless one rips away a slab of bark from an old rotting log. The part of the organism generally observed by the layman is the fruiting body or the "mushroom". These structures periodically shoot up from the mycelial growth with unbelievable speed when the conditions are propitious. The fruiting body can be differentiated into a stem (stipe) and a cap (pileus). Gills on the underside of the cap contain basidia which in turn contain the spores. The elliptical spores of lentinus lepideus are approximately 5x12 μ in size. These spores are responsible for the asexual reproduction of a new mycelium. Of course, the plants also propagate asexually by extension and subbranching of the hyphae.

Lentinus lepideus has gained wide infamy in the railroad industry due to its destruction of railroad ties. In fact, the fungus has received the name in railroad circles as the "train wrecker".

According to Procter (10), the hyphae of the growth penetrate the cell walls of the wood by enzyme secretion from the tips of the advancing strands, the stimulation for secretion being possibly given by contact of the hyphae with the cell wall. The wood cell carbohydrates are then hydrolyzed by acid and enzyme secretions from the growth, and the simple sugars

are absorbed into the hyphae for metabolic use (14).

It appears then, that the function of a wood preservative is to either inhibit or negate the enzyme action of penetrating the host cells, or to render the hydrolytic enzyme action on the cell carbohydrates ineffective, or to be absorbed into the hyphae of the growth and to vitally upset the fungus's metabolic processes. It is conjected that the attack of preservatives by the last two functional means can be studied by employing an agar-malt medium for fungi growth. However, the fact that a true insight cannot had by using this method can be surmised from the findings of Hirkinshaw and Findlay (7). They discovered the metabolic product methyl p-methoxycinnimate was common to both agar and actual coniferous growth of Lentinus lepideus. The products methyl anisate and methyl cinnimate were found, on the other hand, only in infected wood and not in the agar cultures.

The Need for Laboratory Test Methods

As was stated previously, the problem of wood preservation has many complicating factors. The old standby of the wood preservation industry - coal-tar creosote - is by no means the ideal preservative. It is black and smeary to work with; paint cannot be effectively used over it; it increases the fire hazard of the wood; and burns which workers contract while handling treated products are the curse of the telephone industry (5). Other good prospects such as zinc chloride leach away in use. Then too, some of the really super poisons such as mercuric chloride are also too toxic to man as to merit consideration. Hence, the need

areas for testing many chemicals in an effort to locate some which would comply with all the demands which would be put upon them. Since the first consideration which must be given to a wood preservative is to its toxic effect on fungi growth, it was at first necessary to make numerous tests with different chemicals. It can be imagined that field tests, lasting over periods of several years, greatly slow down the research. Thus, from this obstacle, several different laboratory methods were developed which simulated the actual conventional field tests, and yet yielded experimental results in a fraction of the time.

Laboratory Methods for Testing Wood Fungi

There are two general laboratory test methods which have been employed to determine the toxicity of wood preservatives. The one first developed (in the 1880's) involved the use of small samples of impregnated wood (12). The samples were either in the form of small wood blocks or as sawdust or wood flour.

A newer method which was developed in the early 1900's involved the use of an agar-malt growth medium. American investigators have tended to favor this method, whereas German workers have predominantly used the wood media. The Agar dish check has the greater advantage of being simpler, more convenient, and faster, whereas the slower wood method has been said to give truer results (8). However, neither procedure strictly correlates with the actual field test, since the use of different fungi and fungi strains as well as manipulation techniques, uncontrollable conditions and artificial environment do not make laboratory methods simulate in full the natural conditions.

The Agar-Salt Method

During the first fifteen years of development and use of the agar-salt dish test many different media were employed and interpretation of results with each individual investigator varied considerably. Hence, there arose much confusion in the field, the the need became apparent for a standardization of procedure and of interpretation. A move in this direction was taken by Schmitz and his associates in 1930 (13) when they undertook to correlate the various ideas and opinions on the subject. This group recommended the agar-nutrient as consisting of:

Difco bacto-agar	15 g.
Trommers plain diastasic extract of malt	25 g.
Distilled water	1000 cc.

Among other things they also defined the killing point and the inhibition point of growth on poisoned samples and prescribed a detailed method to follow in preparation, poisoning and planting of the cultures.

In short, the following method was suggested:

1. Gels were to be prepared according to the prescribed formula in a suitable container such as a glass-stoppered flask by steaming at atmospheric pressure.
2. The gel preparation flask was to be sterilized by steaming at 10 lbs. pressure for 20 minutes.
3. The preservative was to be added either directly to the hot sterile agar-salt mixture or in a sealed ampule, later broken by sterile tongs. The mixture was then to be shaken to insure even dispersement. After being shaken, approximately 25 g. of the poisoned mixture was to be poured into each petri dish (15x90 mm.).

Immediately after cooling, the culture plates were to be inoculated with the test fungus. The inoculum was to be approximately one cm. square and was to be taken from 14 day old growth.

4. The test plates were to be incubated for 14 days at 28°. The amount of radial growth was to be measured daily for 6 days and every other day from 7 to 14 days.

5. In the case of volatile poisons, glass-stoppered flasks were recommended to replace petri dishes for cultures.

6. If no growth was observed after 14 days, the original plug was to be transferred to a test tube agar-salt slant medium. If after 14 additional days on the slant medium, no growth was again observed, the inoculum was to be considered killed. The lowest concentration of poison necessary to kill the inoculum was considered the killing concentration of the preservative. The inhibition point was defined as the highest concentration of poison which would still allow a planted inoculum to produce growth on the 14 day slant-gel.

This procedure and variations of this procedure are now generally followed by researchers in the field of wood preservatives.

II. THE EXPERIMENTAL DEVELOPMENT OF A NEW TEST METHOD

A Modified Agar-Malt Test Method

The purpose of this thesis is to present a new modification of the old agar-malt dish check method. The new test provides for fungus to grow from a planted plug over a pure agar-malt medium and then to grow up over paper cups which contain poisoned nutrient-agar. In this way each poisoned sample is uniformly subjected to a frontier of new growth. By the old method, as described in the last section, a 14 day old growth inoculum was planted on a single poisoned gel.

In this study it has been observed that conditions cannot be practically controlled to infallibly give uniform planting inocula, even if the requirements for temperature, nutrient uniformity, and time are rigidly adhered to. It has been observed, that even when preparing pure cultures of Lentinus lepideus on pure agar-malt media, despite painstaking care to take inocula from the same growth source; to pour samples from the same nutrient-agar batch; to sterilize the petri dishes and their contents together in the same operation; to plant the gels at the same time; and to keep the gels in the same constant-temperature oven - in spite of all these precautions, both the nature and the radius of fungus growth on the various plates after given periods of time almost always differed.

It was also thought that by using numerous small test samples in one central container, a quicker and more convenient study could be made.

Thus, for the advantages of having a front of new fungus growth attack each poisoned gel and of having a more convenient centralized testing of individual samples, the new test method was developed.

A. The Test Pan

The Development of a Test Pan

The first idea in making a test container was to use a plastic ice cube tray and to let new growth progress over increasing poisoned agar-nutrient concentrations in the cubes until the growth stopped at the inhibition point. Polystyrene trays from different manufacturers were tested. However, it was found that all of the trays shriveled up in the autoclave sterilization under pressure and heat.

Next, metal ice cube trays were used, but the fungus growth would not span the metal partitions. At the same time, a type of partitioned pan was made by soldering metal strips to the pan bottom and sides, but the same difficulty developed.

The fourth trial container consisted of a metal pan bottom and a glass top. (The previous tops had been sheets of cellophane which were somewhat stretched under the heat of sterilization.) Crucibles were arranged around the inside circumference of the bottom in much the same manner as the paper cups used in the present testing pans. Pure agar-malt was poured around the crucibles and into the crucibles themselves. Again, the fungus would not pass over the porcelain cups. Moreover, the glass tops cracked under the rapid heating incurred in the sterilizer. Pyrex

glass could not be used due to the difficulty of drilling holes in the top which were necessary to introduce preservatives and fungus plugs.

The problem, then, was to develop a top which take the heat and pressure of the autoclave, and yet of such a material which would permit holes to be drilled easily. Then too, the receptacles containing the wood preservative mixed with nutrient-agar had to be of such a nature as to allow the approaching fungus growth to pass over the tops. To fulfill these conditions, an improved bottom and top were developed.

The Final Improved Testing Pan

The final testing pan consisted of a shallow circular baking tin which fitted over a deeper circular baking tin. The top had cut-out sections to allow for visibility and a cellophane cover to protect the culture against contamination. The cellophane was fastened to the pan with "Scotch Tape", and the cellophane, tape and pan were treated with bekelite spar varnish to protect the pan from rusting and the cellophane and tape from stretching and becoming soggy when subjected to the steam treatment. One hole was drilled in the center, into which was introduced the fungus innoculum. Another hole was drilled closer to the circumference of the top to provide for the introduction of the preservative. The top tin was slightly larger than the bottom so that the top rim of the bottom rested on the inside bottom of the top. The top could be rotated over the bottom so that the outside aparature could be placed over any cup in the bottom. The bottom tin had paper cups

arranged in a circle around the inside circumference. The fungus grew readily up the paper cups and down inside to attack the cup ingredients. A detailed procedure used to prepare these testing pans follows.

The Procedure Used in Preparing Testing Pans
(See Drawing Plates)

Key

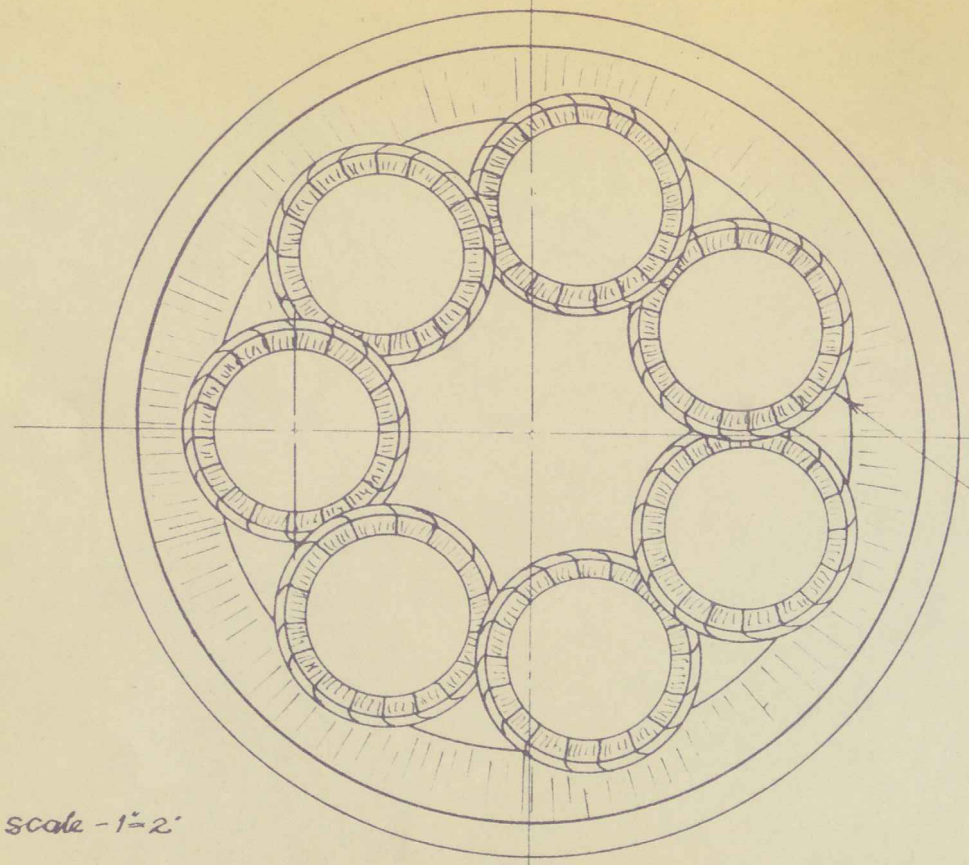
1. Sections were cut from the top tin as shown on the plate by using a jig saw with a metal-cutting blade. The holes were made with a drill press.
2. The tin, with the indicated removed sections and holes, was given a coat of bakelite spar varnish. (Gold Brand bakelite spar varnish is manufactured by the Brooklyn Varnish Company, Brooklyn 1, N. Y.)
3. A circular piece of celluloid or cellophane was then fastened to the overside of the top with "Scotch Tape". The celluloid piece was slightly smaller in circumference than the top to allow the tape to grip the tin on top as well as on the sides. Tape was also used on the underside of the top to secure the celluloid to the inside sections of the pan.
4. Holes were made in the celluloid above the pan holes and the margins taped.
5. Varnish was then applied over celluloid, tape and coated metal on the inside and outside of the tin. After the first coat dried, a second coat was put on.
6. After the varnish dried, the cover was ready for use. The celluloid sometimes sagged a little due to heat treatment, but this effect was not appreciable. The

covers were maintained in good shape by applying a fresh coat of varnish after each five autoclave treatments.

Bottom

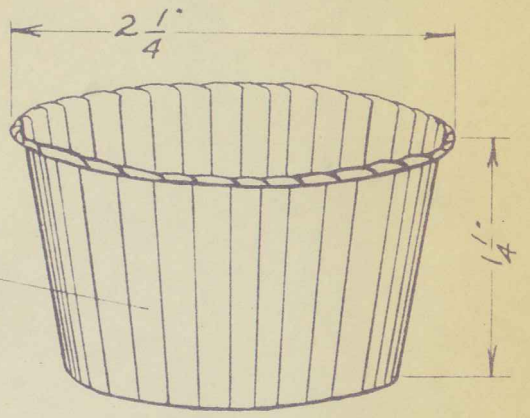
1. Inasmuch as the bottoms used are of aluminum, varnish is not necessary on the bottom pans. (The only tops available at a reasonable cost were made of tin.) The use of a tin bottom, however, would probably entail varnish protection.
2. Seven paper cups were arranged in a circle about the inner circumference of the pan. In order to fit all the cups in, it was necessary to overlap the cup tops. This practice, however, did not result in any experimental difficulty.
3. Each cup was secured to the bottom of the pan by a strip of masking tape in order to minimize movement. (The paper cups used are manufactured unde r the name of Sunshine Souffle Cups #4B, by the Hers Cup Company, New York City. The cup capacity is 2 oz.)
4. The bottom tin with cups fastened in was then ready for immediate use.

PAN WITH CUPS



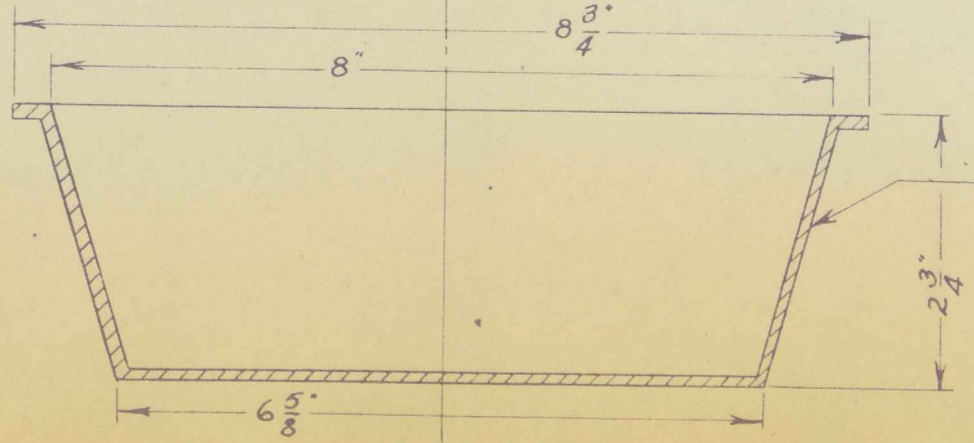
scale - 1" = 2"

DETAIL - PAPER CUP
- full size -

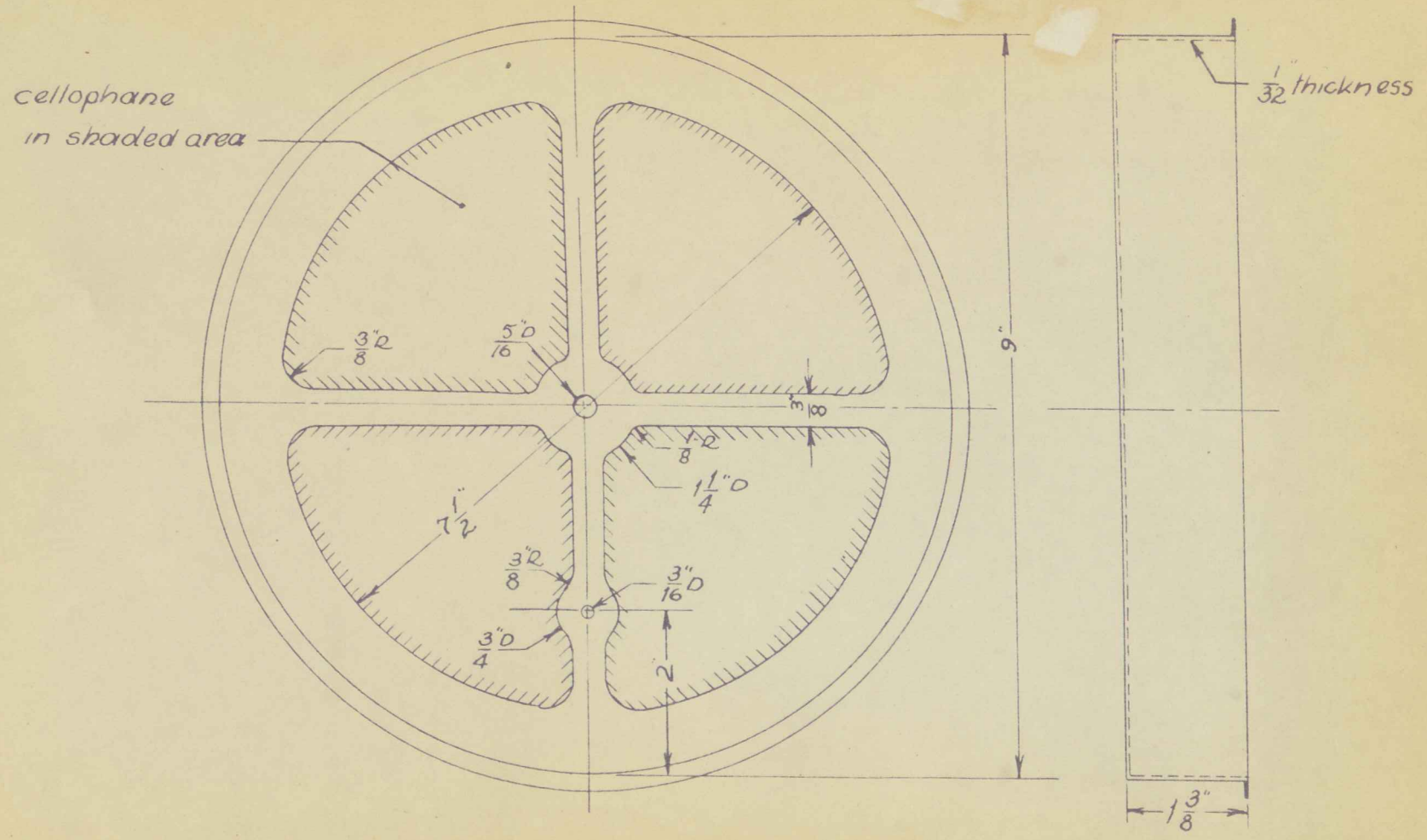


TYPE USED —
"SUNSHINE" SOUFFLÉ CUP #4B

Capacity - 2 oz.



MODIFIED AGAR TEST PAN		
DEPT OF CHEMISTRY UNION COLLEGE		
BY ~~Albert~~ Robert E. Hayes J.A.T.		DATE 5/18/50



COVER FITTING

Scale - 1" = 2"

MODIFIED AGAR TEST PAN	
DEPT OF CHEMISTRY UNION COLLEGE	
BY Robert E. Hayes, J.A.T.	DATE 5/18/50

B. Preparation, Poisoning, Innoculation and Incubation of Test Cultures

Remarks on General Procedure

In general Schmitz's procedure was followed, but several important ramifications should be observed.

To begin with, a sterile poison mixture was not prepared in one central flask and then transferred to the different culture receptacles. Instead, molten gel was first poured into the cups and the pan bottom before sterilization. After sterilization, a poisoned mixture was then introduced into the cups containing pure agar-salt.

In the second place, it is significant to note that a special technique was employed to obtain a mixture of the poison with the agar-salt. The technique was recently developed by R. W. Pinholt, reviewer of this paper. Some chemicals used as preserving agents readily emulsify in an agar-salt dispersion medium, but others such as coal-tar creosote form rather unstable emulsions. Dean and Downs, in 1918, proposed the use of gum arabic as a protective colloid for creosote emulsions (3). However, in 1918 Humphery and Fleming reported that gum arabic materially reduced the toxicity of the creosote(7). As was stated previously, Schmitz advocated the inadequate method of merely shaking by hand the preservative agar-salt mixture. The new procedure involves the preparation of a finely dispersed emulsion of poison in agar-salt to form what is known as the master solution. The master solution is pipetted into the cups containing the pure nutrient medium in proportional quantities to give the desired concentrations of preservative in the final mixture. It has been found that the master solution

mixed homogeniously with the pure nutrient-gel melt with a minimum of shaking or stirring. The poisoned emulsions were prepared by swirling pure molten agar-malt with the chemical toxicant in the Waring Blender, an electrical agitator which operates on the same principle as the ordinary malted milk mixer.

In addition, the idea of having new growth attack the poisoned gels instead of using the conventional 14 day growth is in itself a drastic alteration of the old test method.

There were also other minor differences in procedure which will be taken up individually in the detailed account which follows.

Procedure Followed in Using the New Method

Preparation of the Gels

1. The agar-nutrient was made according to the formula prescribed by Sehnitz et al. The water was first heated to 90° and the agar-agar mixed in until a clear sol resulted. Then the malt was added, and the mixture cooled to 60° at which temperature the melt was poured.
2. The molten gel was measured out in a graduated cylinder, 40 cc. being poured into each cup and 360 cc. being poured into the bottom of the pan around the cups. The amount of 100 cc. was transferred to a 250 cc. Erlenmeyer flask, aluminum foil being used to cover the top of the flask.
3. The tops were placed over the pan bottoms and the holes in the top covered with short strips of masking tape.

Sterilization

1. The testing pans and the master solution flasks were subjected to 15 lbs. steam pressure for 15 minutes in the steam autoclave. The sterilization was generally performed within a day after pouring in order to avoid attack from molds. If equipment and gels could not be sterilized immediately, they were conveniently stored in a refrigerator to prevent mold growths.

Introduction of Preservative

1. If poisoning was done immediately after sterilization, the gels were kept in the molten state by temporary storage in a heating oven until the master solution could be prepared. If there was a time lapse between the sterilization and poisoning operations so that the gels set, they were returned to the molten state by heating in the oven.
2. The Waring Blender was either sterilized with the gels in the autoclave or was sterilized by washing with ether and flaming.
3. The molten gel designated for the master solution was poured into the Waring Blender together with a weighed quantity of preservative. The mixture was swirled for two minutes. The hot emulsion was then pipetted through the hole nearest the periphery of the cover with a delivery pipette, the top being rotated slightly after each measuring out operation so that the hole was aligned in turn with the center of each cup as the master solution was introduced. Master solution was not put into one of the 7 cups in order to provide for a control. It was convenient to regulate the addition of the master solution so that samples were arranged in decreasing concentration.

4. After the master solution had been added, the contents of each cup (except the control cup) was stirred with a hooked glass stirring rod. The cover was rotated with each stirring operation. Samples were stirred from the most dilute to the most concentrated to eliminate the necessity of wiping the stirring rod after each operation. The rod was sterilized before use by dipping in alcohol and flaming. After the stirring was finished, the strip of masking tape was replaced over the orifice.
5. The molten gels were allowed to set and were then placed in the constant-temperature box (28°) until planted.

Innoculation

1. A handy tool for cutting innocula from the fungus source cultures proved to be a cork borer of an appropriate size. The innocula were transferred by using a homemade instrument fashioned from a piece of common bailing wire. A length of wire about 8 inches was obtained and the ends flattened by hammering on an iron anvil. Each end could then be easily bent to the desired angle. The transferring instrument and the cork borer were sterilized before use by dipping in alcohol and flaming. A round inoculum was cut from the source culture and then transferred through the center hole of the cover to the pure agar-nutrient medium in the center of the test pan. The masking tape used to cover the hole was folded back in this operation and later replaced after the plug had been put into the pan. In order to cut down the possibility for mold contamination during the operations of poisoning and innoculating, doors and windows were

closed and wet towels were hung around the work table. These precautions served to minimize air movements and settle any spore-carrying dust which might be stirred up.

Incubation

1. After innoculation, test pans were incubated in a constant-temperature box at 20°. It was found that an incubation period of from three to four weeks was required before growth reached the poisoned samples. In these experiments, no time limit was fixed for standard observation due to the fact that some fungus innocula took longer than others to begin growth on the pure medium after planting, and because the rate of growth was not constant. The latter observation contradicted the findings of Falck in 1907 (4).

C. Observations and Remarks on the Test Cultures

Drying Effects

After a period of time in the constant-temperature box, some samples appeared to undergo syneresis to some extent. This effect was reduced considerably by putting an open beaker of water in the box with the gels.

Contamination by Molds

Mold contamination was prevalent to a greater extent in the new test cultures than in the Erlenmeyer flask cultures used in the old agar test. This was presumably due to mold spores passing through the gap between the cover and the bottom. Contamination of the cultures did not occur at all

during the winter months, but in the fall and spring some of the gels were affected. One possible way to rectify this flaw in the process might be to spread a temperature-resistant grease along the rim of the bottom pan before sterilising. Another suggestion could be to fasten a strip of sponge rubber along the rim.

III. EXPERIMENTAL RESULTS

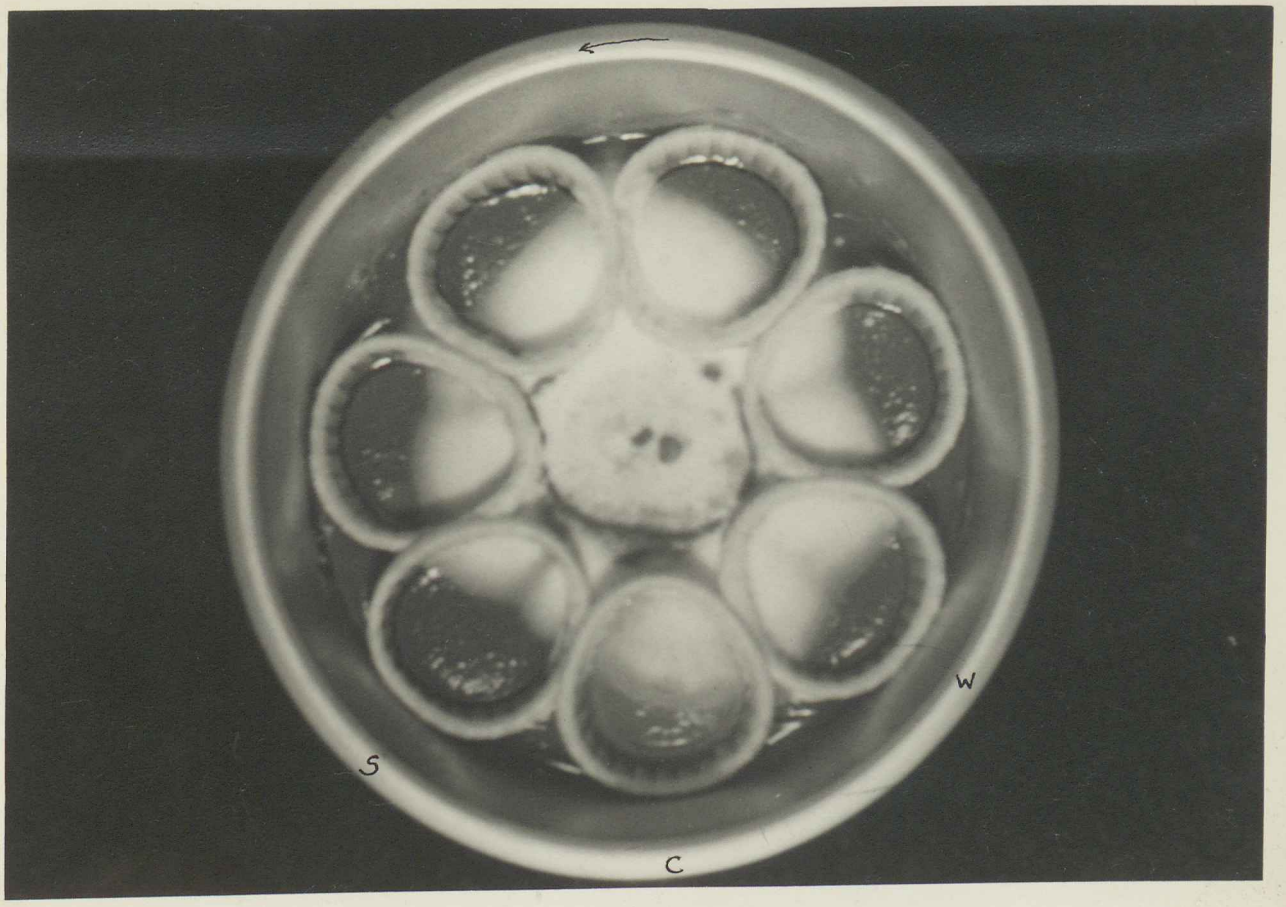
The Use of Octyl Alcohol as a Preservative

Concentration ranges of normal octyl alcohol up to .875 % were tested for toxicity against Lentinus lepideus. In all cases, a thriving growth was observed over the poisoned samples. Using the conventional agar-malt method and employing Erlenmeyer flasks as culture receptacles, a co-worker reported the inhibition point for the same chemical on the same fungus as being approximately .05% (6).

The Use of Low-Residue Coal-Tar Creosote as a Preservative

Concentration ranges of from .02% to .194% creosote were tested for toxicity against Lentinus lepideus. Likewise, in all cases, the fungus growth was heavy on the poisoned samples. By the standard agar test method, using the same creosote, the growth was inhibited at approximately a .05% creosote concentration. (6)

Lentinus Lepideus Growing Over Creosoted Agar-Malt Samples



Data

Preservative: Low-Residue Coal-Tar Creosote

Growth Time: 21 days

Strongest Concentration: .194%

Weakest Concentration: .037%

Key

S: Strongest Concentration

W: Weakest Concentration

C: Control

(The arrow indicates the direction of increasing conc.)

IV. DISCUSSION OF RESULTS

The fact that the experimental results of this new modified test differed so greatly from results obtained by the conventional agar method led this investigator to two alternative explanations. Either the thriving growth of *lentinus lepideus* which was observed on the poisoned agar-malt media derived its nutrition from the unpoisoned nutrient-agar and from the paper cups, or wood preservatives have a much lower toxic effect upon the attack of independently-nourished new fungus growth than they have upon 14 day old growth which is virtually required to either make use of the nutrient from the poison gel or die.

Considering how much higher the concentrations of preservatives tolerated, as observed by the new method, are than those observed by the old method, the argument for the former alternative seems strongly valid. However, why would the fungus growth choose to pass over such an unfavorable poisonous area when it would not span the low metal partitions of the ice cube trays? Then too, on many of the new experimental test cultures, the saprophyte flourished so well over the poisoned samples that even little knobby growths, which were probably precursors of fruiting bodies, were in evidence. It seems highly improbable that the *lentinus* mycelium would produce such structures if it were not deriving food material from the underlying region. Furthermore, it was observed that once the fungus had grown over the paper cups and down into the gels, that the rate of growth over the gel was much faster than over the surface of the paper cup. Why would the growth progress more rapidly over an unfavorable medium than over the paper?

Conclusive evidence that hyphae actually penetrate the cup gels was obtained by cross-sectional observations under the microscope. This facts weakens considerably the first explanation, although it might be feasible that the rhizoidal hyphae do not absorb materials from the poisoned gels or that hydrolytic enzyme action is stopped by the poison but the growth flourishes because it is still extracting food material from the unpoisoned gel in the center of the test pan. Nevertheless, observations of the growth habits lead one to accept the second proposal.

A simple explanation might be that the toxic effect of wood preservatives is not as great against the attack of thriving new growth as it is against an older growth due to the fact that the older growth is more susceptible to the poison. A more mechanistic explanation offered is that the poison absorbed by a fungus mycelium is dispersed throughout the entire organism. In the case in question, several sections of a large mycelium are absorbing poison into the hyphae. In this way, the concentration of poison in any one hypha of the overall growth is not as great as in the case where the entire mycelium is forced to live off the same poisoned nutrient. Hence, the reason why concentrations of the same preservatives support growth by the new method but inhibit growth by the standard method. This theory is also justified by the findings of Robards who reported a far lower concentration of toxic compound required when a plug of actively growing mycelium was placed on poisoned agar than when a poisoned agar "sausage" was placed on an actively growing mycelium.(11)

V. SUMMARY

The modified agar test method offers the following advantages over the standard agar dish test:

1. The fungus attack is much less susceptible to poisoning due to the fact that it is a part of a large growth deriving its nutrition from a pure agar-malt medium. Hence, the method offers a much more rigorous test of the chemical preservative.
2. The test pans contain 7 individual test cups, thus making the equipment more compact and the procedure more convenient.

Among the disadvantages are:

1. Only inhibition points and not killing points can be determined by the new method.
2. At the present stage of development the testing cultures are more easily contaminated.
3. The time required for a test run is almost two weeks longer.

It is felt that the advantages of the modified test may greatly overbalance the disadvantages. It is hoped that a more extensive investigation will be made to more fully determine its capabilities as a method for testing the effect of chemical preservatives upon the attack of wood fungi.

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