STUDIES WITH CYCLOHEXIMIDE: TOXICITY TO WOOD-ROTTING FUNGI, AND UTILIZATION BY TISSUES AND ORGANIC COMPOUNDS

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John R. Vaughan
Major professor

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STUDIES WITH CYCLOHEXIMIDE: TOXICITY TO WOOD-ROTTING FUNGI, AND UTILIZATION BY TISSUES AND ORGANIC COMPOUNDS.

by

William Klemparens

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INTRODUCTION

Since 1948 when cycloheximide was found to be of potential value as a fungicide, practical experiments have shown it to be effective in many cases, both with and without phytotoxic effects. Rose mildew, Sphaerotheca pannosa (Wallroth) Leveille var. rosae Woronchine, was found to be controlled by a 2½ ppm. concentration, but with some injury to new leaves; bean mildew, Erysiphe polygoni, was controlled by a 10.0 ppm. with no phytotoxic effects. As a turf spray, the antibiotic apparently was very effective, and no phytotoxic effects were observed, from the use of applications as high as 1000 ppm. Cycloheximide at 10.0 ppm. indicated superior control of cucumber scab, Cladosporium cucumerinum, in one field trial. The antibiotic demonstrated further promise for control of cherry leaf spot, Higginsia hiemalis, under field conditions.

Inasmuch as the early experimental work demonstrated practical uses and possible applications, an attempt was made to determine the action of the antibiotic when in contact with organic tissues and compounds. The aims of the experimental work were partially to investigate the constancy of the fungicidal activity of the antibiotic under varied environmental conditions, and also a study of retent-

ion, absorption, recovery, or possible translocation in living and non-living tissues. It was also hoped that a correlation could be established between cytological aberrations produced in onion roots with the absorbed quantity of the antibiotic.

Fundamental knowledge of this type should be of vital interest to scientists dealing with antibiotics, and to those interested in fungicides.

LITERATURE REVIEW

Cycloheximide, an antibiotic produced by Streptomyces griseus, as a by-product in Streptomycin production (4,7,15,16), was initially suggested as a possible plant fungicide in 1948. In this first fungicidal experiment (3), powdery mildew, Erysiphe polygoni, on greenhouse beans, was found to be controlled effectively by cycloheximide concentrations between 5.0 and 10.0 ppm. Mention was also made of possible phytotoxic effects by higher concentrations.

In 1949, in Michigan, the effect of the antibiotic on seed germination in petri plates, and action and control on rose plants with mildew under greenhouse conditions was investigated (13). Radish seed inhibition ranged from 100% in those soaked in 100 ppm. cycloheximide to only a delay in germin-

ation at 1.0 and 5.0 ppm. The other seeds tested (pea, wheat, and bean) were inhibited less by the 100 ppm. concentration but displayed the same proportional change as the concertration was lowered. The rose mildew was controlled by 2½ ppm., but the increasing of the concentration to 5, 7½, and 10.0 ppm. caused injury to young and new leaves. Further investigations showed excellent inhibition in agar plates against Monilinia fructicola, Cladosporium cucumerinum and Fomes sp. Also, a recheck of the bean mildew trial (1948) resulted in control at 10.0 ppm., and no phytotoxic effects.

Another possible application for the antibiotic was described in 1949 by Vaughn and Hamner (12). As a spray for such turf diseases as "Dollar Spots*", "Brown Patch*," and "Melting Cut," the cycloheximide appeared to be at least the equal of the fungicides commonly used, and possibly superior - particularly for the "Melting Cut," caused by Helminthosporium sp.. However, the spray trials were limited by changing weather, and should be repeated for more definite results.

*"Dollar Spot" - caused by Sclerotinia homoeocarpa, and the "Large brown patch" caused by Pellicularia filamentosa = Corticium solani.

de Zeeuw and Vaughn, in 1950, reported the cycloheximide as the best apparent spray in a single field experiment with cucumber scab, Cladosporium cucumerinum (2). The antibiotic was used at 10.0 ppm. and compared with Crag 658, Tribasic copper sulfate, Dithane, and Zerlate.

As a postharvest spray for brown rot, Monilinia fructicola, in concentrations of 2.0, 5.0, and 10.0 ppm., four sprays of cycloheximide were compared with liquid lime sulfur and sulfur dusts by Peterson and Cation (8). After cold storage for two days, and common storage for four, the peaches sprayed with 2.0 ppm. cycloheximide showed 2% rotted, the 5.0 ppm. sprayed peaches 0% rotted, the 10.0 ppm. 1.0 % rotted, the lime sulfur sprayed and dusted peaches 3% rotted, and the unsprayed peaches 7% rotted. In another test, under conditions of heavy inoculum, a 20.0 ppm. cycloheximide spray was compared with wettable sulfur and to grower sprayed liquid lime sulfur. The cycloheximide sprayed peaches showed 84% rotted, the sulfur sprayed 67% rotted, and the grower sprayed 89% rotted.

In other trials with peaches, a 20.0 ppm. spray of cycloheximide was found to cause cracking in firm-ripe Halehavens, and possibly to

dissolve the skin coloring matter. The severity of these actions was less with lower concentrations. The same report indicated that cycloheximide may be a superior spray for cherry leaf spot, Higginsia hiemalis, in that 1.0 ppm. almost completely controlled heavily spotted Montmorency leaves, and these same trees held a high percentage of noticeably greener leaves for a longer time than the trees sprayed with "Tennessee 26", "Ferbam," "Crag Cherry Fungicide 341B," and "Nabam".

Gottlieb et al, in 1950 (5) in a report based on visual observation of growth in liquid media of six pathogenic fungi, all but one - Monilinia fructicola, were inhibited by a 100.0 ppm. cycloheximide concentration. The report also stated that in phytotoxicity tests with tomato, bean, peach, geranium, and strawberry; the strawberry alone was not injured by 100.0 and 1000.0 ppm. concentrations.

In Canada, eight fungi were tested for spore and mycelium inhibition, and for the effect of the antibiotic on pea seed germination by Wallen et al (14). Using the agar streak method of Waksman and Reilly, spore germination varied from complete in-

hibition in the case of Ascochyta pisi Lib. at 1.0 ppm. to slight inhibition at 50.0 ppm. with Penicillium sp.. However, using the filter paper disk method of Vincent and Vincent, which consisted of placing disks saturated with cycloheximide near the growing colony, no inhibition was found in concentrations up to 225 ppm. It was also shown that soaking infected pea seeds in varying concentrations of cycloheximide did not control the internal parasite A. pisi; but the pea seeds were injured as shown by reduced germination as the concentration increased.

The same workers also investigated the activity of the cycloheximide on germinating pea seeds. In the lower concentrations, an abnormal, discolored root, and a long shoot resulted, while in concentrations from 10.0 ppm. and up, there was little or no germination.

Recently Whiffen tested thirty-three plant pathogenic fungi in culture for effect from various concentrations of cycloheximide (17). If spores were produced, suspensions were streaked on agar plates; in the case of fungi not sporulating, a mycelial disk was used as inoculum. The concentration needed for complete inhibition after 48 and 72 hours was noted. The

antibiotic was found to cause complete inhibition for 72 hours in concentrations of less than 1 ppm. with Ustilago tritici, and ranged to 100.0 ppm. for 72 hour inhibition when used against Ramularia pastinaceae and Fusarium lycopersici.

MATERIALS AND METHODS

Since broad objectives were chosen with the hope that facts concerning absorption, retention, possible translocation or utilization, and/or recovery might be obtained, the experimental work was divided into six separate parts dealing with different phases or dissimilar organic compounds.

PART ONE

Biological Assay Technique

Inhibition of linear fungal growth on agar plates containing varying amounts of cycloheximide*, forms the basis for assaying unknown amounts of the antibiotic (see TABLE I).

* Crystalline, pure chemical used, unless otherwise stated.

Disks, five millimeters in diameter, of Poria microspora* taken from a six day old culture - were seeded in 2% malt extract agar (Difco, dessicated), and incubated at 30° C until the necessary measurements were made. The medium was made up in single lots, either 500ml. or 1000 ml., and the desired dilutions made by pipetting from a standard 1000.0 ppm. cycloheximide solution into smaller flasks. The smaller flasks, containing enough medium for four plates, were autoclaved, and the medium poured into previously labelled Petri dishes. Four replications were used in every case. The inoculum pieces cut with a curved, sharpened, brass tube were seeded - fungus side up - as near the center of the plate as possible. After incubation at 30° C for forty-eight hours, the first linear measurements were taken with the aid of a specially constructed box, illuminating the culture from beneath so as to define the periphery of the colony.

The growing colony was measured and the growth recorded (see TABLE I) at twenty-four hour intervals for six consecutive days.

* Poria microspora = Poria monticola, recently has become well-known as destructive to wood in service, (1).

PART TWO

Retention by Wood

The retention of cycloheximide by non-living tissues was investigated by using Basswood, Tilia glabra Vent. chips, and the bio-assay technique.

Forty Basswood chips (3/16 x 1 3/8 x 2 inches), were submerged in one liter of 200.0 ppm. cycloheximide for a period of three minutes. The solution remaining was retained for subsequent bio-assay (see TABLE III) and the chips dried to approximately the original moisture content. In repeated leaching trials, the chips were placed in one liter of distilled water and shaken vigorously for fifteen minutes. The chips were re-dried and the solution saved for bio-assay for cycloheximide content (see TABLE III). The control followed the procedure with chips not previously soaked in cycloheximide, but was determined only once as there was no evidence of either stimulation or inhibition in the assay plates.

Chromated zinc chloride (DuPont) leaching was also investigated. A biological assay table was set up (see TABLE II) and leaching trials with subsequent assays were attempted. The procedure was identical to that followed with cycloheximide except for the autoclaving. The chromated zinc

chloride precipitates under conditions of high heat so the chips were sterilized with propylene oxide (6), submerged in sterile distilled water, shaken vigorously as before, and under sterile conditions, the leaching water was added to warm, sterile medium for pouring into the assay plates.

The leaching trials with the chromated zinc chloride were discontinued, due to the resistance of the compound to leaching under these conditions.

PART THREE

Leutritz Technique

The Leutritz method of evaluating wood preservatives (10) was employed, using varied 100.0 ppm. treatments of cycloheximide and Ponderosa pine test blocks.

In this method, two feeder* chips, Pinus palustris, were placed ¼ inch apart on approximately two inches of soil in flat-topped jars. The jars were sterilized, and the feeder chips seeded with Poria microspora as an inoculum disk placed between the chips. After five weeks, when the chips

* Feeder chips of Pinus palustris served to "feed" inoculum to the test blocks.

were covered with mycelium which had also penetrated the soil, the previously weighed test blocks were placed $\frac{1}{4}$ inch apart upon the chips with a treated and an untreated test block in the same jar.

The treatment of the test blocks (approximately $\frac{1}{4}$ x 1 x 2 inches Pinus ponderosa) was as follows:

1. Cold soak in 100.0 ppm.* crude cycloheximide solution for twenty-four hours.

2. Cold soak in 100.0 ppm crystalline cycloheximide water solution for twenty-four hours.

3. Hot and cold bath** for one and one half hours in crude cycloheximide water solution.

4. Hot and cold bath in crystalline cycloheximide water solution for one and one-half hours.

5. Hot dip (three minutes) in 100.0 ppm. crude cycloheximide plus crankcase oil (at approximately 94°C.).

The jars were held for one hundred and twenty

* All treatments with 100.0 ppm.
** Hot and cold treatments averaging 95° C. and cooled in the same solution.

days in a humidity chamber which averaged 28° C and 87% relative humidity.* Loss in weight by the treated and untreated blocks is presented in TABLE IV.

PART FOUR

Synthetic vs Organic Medium

Two types of liquid medium were employed in this part of the experimental work.

Coon's synthetic broth (9) consisting of sucrose, 7.2 grams; dextrose, 3.6 grams; KNO_3, 2.02 grams; $MgSO_4$, 1.23 grams; KH_2PO_4, 2.72 grams; per 1000 cc. of distilled water, was used in comparison to 2% malt extract liquid medium. Four concentrations (0, 1, 10, and 20.0 ppm. cycloheximide) were used with each type of medium and four replicates containing 50 ml. of medium per flask.

Fusarium sp. was grown on a 2% malt extract agar plate and cut with the 5 mm. rod for seeding purposes. The seeded flasks were shaken continuously for four days, after which the mycelium was strained, dried and weighed (see TABLE V).

*Leutritz jars containing Quercus rubra blocks, and Fomes igniarius var. laevitagus as the wood-rotter were discarded. There was nearly a 100% failure of the fungus to spread to either the treated or the untreated blocks from the feeder chips.

The liquid media, and the mycelium, were retained for future antibiotic assay, as in TABLES VI and VII respectively.

PART FIVE

Onion Root Reaction

Onions which had been germinated for thirty-six hours in tap water, were transferred to flat dishes holding 100 ml. of distilled water or 100 ml. of 100.0 ppm. cycloheximide in distilled water.

Four onions in each dish were left for periods of 1,2,4 and 6 hours in the cycloheximide solution and in the distilled water checks. At each interval the onions were removed, and the solutions retained for biological assay. After a one minute wash in running water the onions were returned to 100 ml. of fresh distilled water for a twenty-four hour leaching period.

Growth patterns for comparison to TABLE I are found in TABLE VIII.

Although the roots were retained for assay, the absorption results were too indefinite to make this step of any value.

PART SIX

Cultural Inhibition

The inhibition in culture was determined with the method described in Part I (Biological assay technique).

The graphs represent a single repetition with the exception of Graph I constructed from the results of TABLE I, from which all unknown cycloheximide concentrations were estimated.

Graph III is based upon dry weight of Poria microspora growth in 50 ml. of 2% malt extract liquid media. Twelve flasks for each of five cycloheximide concentrations - 0.0, 0.01, 0.1, 1.0 and 10.0 ppm. were held for seven days at room temperature, after which three of each concentration were removed and the mycelial dry weights noted. Weight determinations were made for four successive days. Thus the graph is based upon three replications, while Graphs IV thru VII of Lentinus lepideus, Fomes igniarius var. laevigatus, Lenzites trabea, and Schizophyllum commune as the respective fungi, are with four replicates, and based upon linear measurements.

EXPERIMENTAL RESULTS

The master table (TABLE I) was established from the constant linear growth rates of Poria microspora on malt extract agar plates containing known cycloheximide concentrations. The numerous replicated trials showed the method to be adequately accurate to check the experimental work that was undertaken.

The choice of the fungus was fortunate as its high susceptibility to the antibiotic allowed accurate determinations of a small amount of cycloheximide. The susceptible fungus further allowed an almost unlimited range for detection merely by using dilutions for the higher concentrations. The fungus served admirably also in that its mycelial growth on agar plates is comparatively flat and quite consistently forms an almost perfect circle. The malt extract (Difco, dessicated) as a nutrient source was also found to yield results consistently within the ranges of the twenty-four hour periods, even when made up from two different lots. A burette technique for dilution into single plates under sterile conditions was found to be less accurate than making dilutions of a volume sufficient for the replication of four.

In periodical assays of the 1000 ppm stock

solution of cycloheximide, it was found that the antibiotic did not lose determinable amounts of fungicidal activity when stored in the refrigerator for periods up to three months.

TABLE I, and Graph I, indicate that the determination of a 0.01 ppm. concentration is below the scope of accuracy of the method due to overlapping of growth ranges. However, the 0.5, 1.0 and 2.0 ppm. cycloheximide growth ranges show clear cut, independent ranges at the twenty-four hour periods. This gave quite accurate determinations when used as the standard for comparison to growth rates in plates containing unknown cycloheximide concentrations.

The plates with 5.0 ppm. concentration showed no growth for the six day period, but did show slight growth after the sixth day and would recover if the inoculum was transferred to a plate containing no cycloheximide. The plates containing a 10.0 ppm. concentration, however, inhibited all growth and the inoculum, when transferred to a pure nutrient agar plate after the seventh day, would not recover.

It was noted that, except in 10.0 ppm. and up, growth on plates containing cycloheximide was preceded by a marked lighter colored diffusion zone.

TABLE I

Growth rates of Poria microspora in 2% malt extract agar plates containing cycloheximide.
Master table used for bio-assays

	Rate of growth in mm.*				
Day	2	3	4	5	6
0.0 ppm.	12.5	22.3	38.0	47.6	61.5
Range	9.5 to 14.5	17.2 to 25.5	25.2 to 39.3	37.7 to 49.3	54.5 to 63.2
0.01 ppm.	10.7	20.1	31.6	42.2	52.7
Range	8.2 to 12.2	18.5 to 22.7	24.2 to 35.2	36.7 to 45.0	49.7 to 56.7
0.5 ppm.	0	8.7	15.7	20.7	25.0
Range		8.2 to 12.0	11.2 to 17.5	17.7 to 24.5	23.5 to 31.7
1.0 ppm.	0	6.5	9.5	15.3	18.0
Range		5.2 to 7.2	9.2 to 12.5	10.2 to 17.0	15.7 to 22.0
2.0 ppm.	0	0	6.0	9.0	11.0
Range			5.1 to 7.2	8.5 to 9.5	9.7 to 12.7
5.0 ppm.	0	0	0	0	0
10.0 ppm. and up - no growth.					

* The daily measurements include the 5.0 mm. inoculum disk, and are based upon six trials with replicates of four.

The diameter of the zone varied inversely as the concentration of the antibiotic.

Table II, and Graph II demonstrate the growth rates made by Poria microspora in plates containing chromated zinc chloride. As previously mentioned, the chromated zinc chloride was to be used as one commonly employed water soluble wood preservative in contrast to the antibiotic, cycloheximide, as a wood preservative. Since attempts to analyze the original dipping solution of chromated zinc chloride after the three minute dip indicated there was still the original 5% solution present, and a trial calculated to yield leaching figures showed no leaching - even when concentrated ten times, this part of the experiment was discontinued. The table and graph do show a possible stimulation by very low concentrations of chromated zinc chloride, and a sharp, delimiting toxicity line as opposed to the gradual suppression with cycloheximide when slight dilutions are made.

Table II and Graph II are, however, based upon much larger concentration variations. In ppm. the 0.2% chromated zinc chloride would be 2000 ppm. and the 0.3% would be equivalent to 3000 ppm. The critical toxicity point for Poria microspora in culture is between 0.2% and 0.3% or 2000 and 3000 ppm. of

TABLE II

Growth rates of Poria microspora in 2% malt extract agar plates containing chromated zinc chloride.

	Rate of growth in mm.*				
Day	2	3	4	5	6
0.0%	12.5	22.3	38.0	47.6	61.5
Range	9.5 to 14.5	17.2 to 25.5	25.2 to 39.3	37.7 to 49.3	54.5 to 63.2
0.1%	13.5	26.0	38.9	48.2	60.2
Range	11.2 to 14.7	22.2 to 29.5	37.5 to 39.2	43.0 to 48.5	59.7 to 66.2
0.2%	6.5	14.6	21.7	30.5	37.7
Range	6.0 to 7.7	12.2 to 16.7	14.2 to 26.0	28.2 to 33.7	34.2 to 39.7
0.3%	0	0	0	0	0
Range					
0.4% and up - no growth					

* Each figure represents linear measurements of Poria microspora taken at twenty-four hour intervals, and are an average of twelve plates; e.g. they represent three trials with four replicates.

chromated zinc chloride, as opposed to 10.0 ppm. or less in the plates containing cycloheximide.

TABLE III, showing results of the assay for cycloheximide content in the solution having had the chips submerged in it for a three minute dip, indicates that the active antibiotic content was materially reduced - by mechanical adsorption by the wood, chemical alteration, or a combination of these factors. Two dilutions, made for 0.5 ppm and 1.0 ppm. growth rate comparisons to TABLE I, show that very little over 100.0 ppm. cycloheximide remains from the original 200.0 ppm concentration. The dilution made for a 1.0 ppm. reading fits the 0.5 ppm. dilution as found in TABLE I, and the 0.5 ppm. dilution of the dip solution falls just below the 0.01 ppm. range. The two, cross-checking each other, lead to the conclusion that 100.0 of the 200.0 ppm. cycloheximide was retained by the wooden chips.

Leaching trials I through V, in which the undiluted leaching water was used in the medium, indicate a leaching of between 1.0 and 2.0 ppm. for the first leach (1), slightly over 0.5 in (2), slightly less than 0.5 ppm. in (3), and progressively less in trials (4) and (5). The original solution, diluted for 1.0 ppm. reading, falls

TABLE III

Linear growth rates of Poria microspora on media containing cycloheximide leached from Tilia glabra chips.

	Rate of growth in mm.				
Day	2	3	4	5	6
Solution before dip	0	6.2	9.0	15.0	19.2
Solution after dip* 1)	8.2	12.5	21.0	25.5	32.5
2)	0.0	8.2	13.2	19.2	22.7
Leaching trials					
1	0	5.7	7.5	10.2	15.2
2	0	8.2	12.2	17.7	20.7
3	6.0	12.0	20.2	25.5	30.2
4	6.5	14.0	21.0	27.2	34.7
5	8.0	16.0	22.2	31.2	39.5

* Solution after dip: 1)- diluted for 0.5 ppm. reading. 2)- diluted for 1.0 ppm. The solution before the dip was diluted for a comparison with 1.0 ppm. in Table I, all others were assayed undiluted.

perfectly in that category in TABLE I.

The results of a modified Leutritz technique (10) experiment are shown in TABLE IV. Treatment (3), the 1½ hour hot and cold bath in water solution of the crude antibiotic, gave evidence of cycloheximide being an excellent water soluble wood preservative. The twenty-four hour cold soak solution prepared from the crystalline product, (Treatment 2), yielded results which indicated that the antibiotic was valueless as a preservative, as the treated blocks were more severely rotted than the untreated in both replicates. The twenty-four cold soak in crystalline prepared solution (Treatment 4) was also found to be an ineffective wood preservative. Although the treated blocks lost less weight than the untreated, the resulting figures were inconclusive. Treatment (5), an oil base cycloheximide solution, was entirely inconclusive, varying between the replicates.

All results may have been affected by the high humidity at which the jars were held, particularly as the antibiotic was used as a water soluble wood preservative. High humidity may lead to a higher loss in weight percentage when using the Leutritz technique (10, page 11).

Northern white cedar, Thuja occidentalis L.

TABLE IV

Percentage loss in weight of Quercus rubra blocks after being subjected to rotting conditions used in the Leutritz technique.

Treatment*		% loss in weight
(1) Cold soak in crude cycloheximide solution for 24 hours.	Treated	11.3
	Check	67.8
	Treated	4.1
	Check	67.6
(2) Cold soak in crystalline cycloheximide solution for 24 hours.	Treated	154.7
	Check	99.7
	Treated	70.5
	Check	48.1
(3) Hot and cold bath in crude cycloheximide solution for 1½ hours.	Treated	1.6
	Check	109.9
	Treated	1.9
	Check	72.9
(4) Hot and cold bath in crystalline cycloheximide for 1½ hours.	Treated	87.0
	Check	146.9
	Treated	2.1
	Check	9.9
(5) Hot dip (3 min.) in crude cycloheximide plus crankcase oil.	Treated	55.3
	Check	14.8
	Treated	109.7
	Check	118.6

* Test blocks removed after 120 days in a humidity chamber averaging 28° C. and 87% relative humidity. All treatments with 100 ppm. cycloheximide - Poria microspora as the test fungus.

fence posts were given treatments identical to those listed for the Leutritz trial, and were placed beneath greenhouse benches in four replications. After seven months, visual observations yield no indications of the superiority of one treatment over the others.

In TABLE V, in which increase in mycelial weight of Fusarium sp. in synthetic and organic liquid media is compared, the Coon's synthetic media is shown to be superior to the 2% malt extract when used with no cycloheximide concentration. Although there is a greater weight increase in the synthetic medium flasks containing 0.0 ppm. cycloheximide, the flasks of this same medium containing 10.0 ppm and 20.0 ppm. cycloheximide show considerably less mycelial growth than in the 2% malt extract with a 10 and 20 ppm. content.

The synthetic medium is evidently superior to the malt extract liquid medium when the antibiotic is not present, but inferior for producing mycelium when the antibiotic is added, i.e. the fungus is less inhibited in 2% malt extract medium containing cycloheximide than it is in the Coon's synthetic medium containing the same amount of cycloheximide.

The difficult analysis of TABLE VI, in which the antibiotic assays of the two media is presented,

TABLE V

Comparison of Fusarium sp. dry mycelial weight increase grown in two different media containing cycloheximide.

ppm.	Coon's Synthetic		2% Malt Extract	
	% Increase	Grams Increase	% Increase	Grams Increase
0.0	4200.0	0.0714	3411.7	0.0580
1.0	3929.4	0.0668	3652.9	0.0621
10.0	2405.8	0.0409	2694.1	0.0458
20.0	2017.6	0.0343	2576.4	0.0438

yields evidence of antibiotic presence, but also the presence of at lesst one possible metabolic product. The synthetic medium originally containing 1.0 ppm. cycloheximide, and the synthetic medium check, which were diluted one part of substrate with one part fresh 2% malt extract medium, showed no evidence of either cycloheximide or a metabolic product. Both fit well into the check range of the assay table (TABLE I). If the antibiotic were present in 100% active form, the original 1.0 ppm substrate assay should fit the 0.5 ppm. range in TABLE I. The malt extract check medium however, gives evidence of the presence of a toxic metabolic product when assayed in the same manner. Both the check and the original 1.0 ppm. medium gave considerable inhibition.

The check media for the 10.0 and 20.0 ppm. cycloheximide flasks, were diluted one part of substrate to nine parts of fresh and one part to nineteen parts of fresh medium respectively. The growth on assay plates shows definite stimulation of the linear growth rates when compared to the check growth in TABLE I. Stipulating that the stimulation is not only acting upon growth in the check media flasks but also upon the noticeably inhibited growth on the plates containing media from the 10.0 and 20.0 ppm cycloheximide flasks, TABLE VII is

TABLE VI

Growth rates of Poria microspora on 2% malt extract agar containing substrate from two different media which had supported fungal growth in cycloheximide concentrations.*

	Rate of growth in mm.				
	Coon's Synthetic				
Day	2	3	4	5	6
From flasks containing					
1.0 ppm.	11.0	22.7	33.3	43.2	48.5
0.0 ppm.	13.0	24.5	37.0	47.5	59.0
10.0 ppm.	7.5	16.5	23.5	29.5	35.0
0.0 ppm.	16.0	28.0	44.0	53.5	64.2
20.0 ppm.	5.5	11.5	19.0	24.7	29.5
0.0 ppm.	18.2	30.0	43.6	53.7	64.5
	Malt Extract				
1.0 ppm.	7.0	11.5	20.0	28.2	35.5
0.0 ppm.	7.2	12.0	21.2	32.0	40.0
10.0 ppm.	10.7	20.7	29.7	37.2	45.2
0.0 ppm.	17.0	28.2	41.7	54.2	67.0
20.0 ppm.	10.5	19.7	28.7	36.5	45.5
0.0 ppm.	17.0	30.2	41.7	54.7	65.7

* Media in which cultures had been grown was diluted, hoping to avoid action by possible metabolic waste products, i.e.; original 1 ppm. media diluted 1 to 1 to yield 0.5 ppm. cycloheximide if the original amount is available, and check media also diluted 1 to 1. 10 and 20 ppm. diluted (1 to 9 and 1 to 19) to yield 1 ppm. and comparable dilutions with the 0.0 media. Dilutions made with fresh 2% malt extract. Replicated four times in all cases.

TABLE VII

Revised liquid media assay showing growth rates after removal of the stimulation factor.

Coon's Synthetic					
10 ppm. (day)	2	3	4	5	6
% stimulation	28	25	16	12	4
Revised rate	5.4	12.4	19.7	26.0	33.6
20 ppm.					
% stimulation	45	34	15	14	5
Revised rate	3.0	7.6	16.1	21.2	27.0
2% Malt Extract					
10 ppm.					
% stimulation	36	26	10	14	9
Revised rate	6.6	15.3	26.7	32.0	41.3
20 ppm.					
% stimulation	36	35	10	15	7
Revised rate	6.6	12.8	25.8	31.0	42.3

presented with figures showing the percentage stimulation in the check plates and that percentage subtracted from the stimulated growth as found in TABLE VI. After the elimination of the stimulation factor, the assays, all of which were diluted for comparison at 1.0 ppm. concentration in TABLE VII are as follows: the 10.0 ppm. cycloheximide in Coon's synthetic medium assays at slightly less than 0.5 ppm. indicating that 50% of the cycloheximide was unavailable or altered; the 20.0 ppm. synthetic medium assays directly in the 0.5 ppm. range in TABLE I, indicating that 75% of the activity of the antibiotic was missing. The 10.0 ppm. cycloheximide in malt extract assay shows a concentration of the antibiotic slightly more than 0.01 ppm. cycloheximide, indicating that 0.1 ppm was present or approximately 99% was inactive. The 20.0 ppm. in malt extract assays at nearly the same level as the 10.0 ppm. flask. The difference between the theoretical 0.1 ppm. present in the supposed 10.0 ppm. malt extract medium and the 0.2 ppm. in the 20.0 ppm. cannot be determined by the method used. It is obvious that there is much higher retention of the antibiotic by the malt extract medium. The possibility that the antibiotic was previously utilized by the fungus is unlikely, since the

synthetic medium was the more suitable medium when no antibiotic was added.

Since the media retained or altered the antibiotic, or it was taken up by the fungus, an assay on the dried mycelium was made. The results in TABLE VIII indicate that the cycloheximide was not present, in detectable amounts, or was altered so as to eliminate any chance of anti-fungal action in the assay. Had the 0.1 grams of mycelium added to the 100 ml. of media been one thousandth part pure cycloheximide, it could have been assayed as an approximate 0.1 ppm. concentration. The growth rates all fall within the check range as found in TABLE I.

The results in TABLE X indicate that no antibiotic was taken up by the onion roots when they were immersed in the 100 ppm. cycloheximide for the 1,2,4 and 6 hour periods. However, the water in which the roots were leached for twenty-four hours and assayed undiluted, gave indications as follows: the one hour dip assayed very close to 0.5 ppm., two hour dip at 0.5 ppm., four hour dip at slightly over 1.0 ppm. concentration, and the six hour dip at 1.0 ppm. cycloheximide concentration.

The results indicate that very little, if any,

TABLE VIII

Assay of mycelium grown in two different media containing cycloheximide.*

Day	2	3	4	5	6
	Coon's Synthetic				
	Rate of growth in mm.				
From flask containing					
0.0 ppm.	13.5	24.7	35.5	48.5	62.5
1.0 ppm.	14.3	26.0	35.0	47.7	60.0
10.0 ppm.	11.0	21.0	28.7	38.7	52.7
20.0 ppm.	10.3	19.7	29.7	42.7	55.3
	Malt Extract				
0.0 ppm.	10.7	21.5	32.0	43.0	56.2
1.0 ppm.	10.0	19.2	29.7	42.0	56.0
10.0 ppm.	11.2	20.5	31.2	43.5	57.5
20.0 ppm.	13.2	26.0	36.2	46.7	59.7

* The washed mycelium from the Coon's synthetic broth and from the malt extract liquid media were collected after weighing, pulverized, and 0.1 gm. added to 100 ml. of 2% malt extract agar for the assay with Poria microspora.

of the antibiotic was taken up, but positive evidence of inhibitory material, possibly cycloheximide in the leaching water, is shown with the technique used.

When diluting one milliliter from the 100 ppm. original soaking solution to 99 of pure water, the possibility arises that ten, twenty, or more ppm. could have been taken up and that the 0.8 or 0.9 ppm. could not be differentiated from 1.0 ppm. by the bio-assay technique. In the assay of the leaching water, however, the undiluted use would allow assaying of the small amounts.

The indications are therefore, that the amount of antibiotic taken up in the original dip could not be detected by dilution from a concentration as high as 100 ppm., but that 0.5 ppm. was leached from the one hour and two hour dips, and 1.0 ppm. from the four and six hour dips. Unfortunately the lack of data as to the quantity absorbed allows no conclusions as to whether all or only a percentage of the antibiotic was removed in the subsequent leaching process.

The assay of the check plates gave no evidence of stimulation or inhibition from the presence of the onion roots for the 1,2,4 and 6 hour periods.

Graph III based upon the dry weight of mycelium

TABLE IX

Growth rates of Poria microspora on 2% malt extract agar plates containing cycloheximide solution which had been used as an onion root dip or as a leaching solution.

	100.0 ppm. dip*					Distilled water checks**					
Day	2	3	4	5	6	Day	2	3	4	5	6
Time						Time					
1 hr.	0	6.7	14.0	19.0	24.2	1 hr.	13.5	24.0	36.0	47.7	58.7
2 "	0	6.7	14.0	18.7	22.5	2 "	12.7	24.2	36.0	47.2	59.0
4 "	0	6.0	12.2	17.5	21.5	4 "	11.5	20.0	31.7	43.2	56.0
6 "	0	7.5	15.0	17.5	22.2	6 "	11.0	21.2	31.7	44.0	54.0
24 hour leach from trial:						24 hour leach from trial:					
1 hr.	5.5	12.0	18.0	21.5	28.7	1 hr.	11.5	23.0	33.0	44.2	55.7
2 "	0	8.7	16.0	21.5	28.0	2 "	12.7	23.2	33.2	45.0	56.2
4 "	0	0	8.7	12.7	16.0	4 "	10.7	20.7	30.5	41.0	54.0
6 "	0	5.5	12.7	17.5	21.5	6 "	12.0	21.2	31.2	41.5	55.0

* Diluted for 1.0 ppm. comparison in TABLE I.
** Incorporated into assay medium undiluted.

grown in liquid 2% malt extract media, shows a gradual suppression of total growth - grading through the suppression of a growth peak - to total inhibition in 1.0 and 10.0 ppm. cycloheximide. The graph also demonstrates that the antibiotic is more effective in the liquid media which contains no agar. Growth is not totally inhibited in agar plates containing the same, 1.0 ppm. concentration of the cycloheximide (see TABLE I and Graph I).

Graphs IV through VII show the inhibition by cycloheximide of four common wood-rotting fungi. All except Schizophyllum commune were totally inhibited by the 2 ppm. concentration.

DISCUSSION

The bio-assay technique developed for use in subsequent trials probing the action of the antibiotic, was found to be an accurate standard for determining very dilute, unknown cycloheximide concentrations by comparison of growth rates in agar plates.

The Leutritz technique showed that the crude cycloheximide was an effective wood preservative. However, despite the high retention by wood, and extremely high toxicity of the antibiotic to wood rotting fungi, crystalline cycloheximide treatments were not as conclusive as the treatments with the crude product. In a leaching comparison, chromated zinc chloride gave evidence of resistance to leaching, but needed two thousand times the concentration for the same cultural inhibition as cycloheximide.

Possible explanations for the inconclusive results are found in the very high humidity at which the jars were held, and in the fact that the extremely toxic substance leaches out in amounts sufficient to permeate the soil water with a lethal dosage of the antibiotic.

An explanation for the superiority of the crude product as a preservative should be sought. Also, since the leaching trial was undertaken using the crystalline antibiotic, there is a possibility that

the crude cycloheximide preparation would not yield the same leaching results.

The results also indicate that cycloheximide may be of value as a killing agent to be added to inorganic preservatives and water repellents.

The experimental results also showed that the cycloheximide was not as active as an inhibiting agent when in a highly organic media. Greater fungal growth was found in a synthetic broth containing only sucrose and dextrose as carbohydrate source, than in a 2% malt extract media. It was definite that one or more substances found in the malt extract - proteins, various carbohydrate and nitrogen sources, and enzymes or growth regulators - served to alter or retain the antibiotic. Agar also seemed to retain the cycloheximide, as a 10 ppm. concentration would inhibit all growth in a liquid medium, but would not totally inhibit in the same medium in agar plates. There was however no apparent recovery of the cycloheximide from the dried mycelium which had obviously absorbed and altered or closely retained the antibiotic.

The above facts indicate that further work could narrow the number of organic compounds showing high retention, and might yield more valid recovery data if a larger mycelial mass was assayed. A leaching study on growing mycelium should also be undertaken.

The lack of positive absorptive results when onion roots were dipped in 100.0 ppm. cycloheximide solutions for one, two, four and six hours suggests that more valid results might be gained by lowering the concentration of the original dipping solution. The lower initial concentration should allow an assay if only 10.0 or 20.0 ppm. cycloheximide are taken up by the growing tips. This could be aided further by the use of more onion roots per 100 ml. of solution to increase the proportional surface area. Valid results as to the amount of removed antibiotic activity compared with that leached from the roots would yield the quantity actually held by the roots.

The onion root absorption was undertaken, not only for purposes of determining action of the antibiotic when in contact with the growing tips of higher plants but with the hope that its absorptive power could be correlated with cytological aberrations found to be caused by the antibiotic. Wilson (18 1950) found that cytological recovery of the antibiotic was impossible from initial dippings in concentrations up to 200 ppm. cycloheximide. Also, the cytological results increase after the onion roots are removed from the cycloheximide solution and placed in the pure water.

As previously mentioned, a low concentration in the original onion root dip solution should yield a valid

assay as to the quantity of the antibiotic absorbed; this coupled with the assay on the leaching water could lead to correlating graphs constructed from cytological data and from the retention data.

Another line for possible continued study would be the investigation of the noticeably lighter colored zone produced by the fungi growing in the malt extract agar plates containing cycloheximide. There is a possibility that the zone represents removal or alteration of the antibiotic and is a diffusion gradient established by the fungus prior to its linear growth. Whatever the cause, it should be investigated for cycloheximide content.

CONCLUSIONS

It was found that with a biological assay using Poria microspora and 2% malt extract agar, it was possible to detect cycloheximide concentrations in amounts as low as 0.5 ppm.

The cycloheximide was shown to be forcibly absorbed by the wood used and could be leached out in quantities very near 1.0 ppm.. Despite the high retention and toxicity, the crystalline antibiotic was not an effective wood preservative in all trials attempted, however the crude preparation showed excellent preservative properties.

Cycloheximide was also shown to be somewhat less effective as an inhibiting agent when used in highly organic media. The assay of the medium in which the fungus was grown indicated that from 50% to approximately 99% of the antibiotic was removed or inactivated by the growing fungus. The synthetic medium containing cycloheximide suppressed fungal growth more than the 2% malt extract medium with the same antibiotic content. The negative results in assaying the dried mycelium is an indication that antibiotic activity of the cycloheximide was altered or destroyed.

The antibiotic also gave indications of being absorbed by growing onion roots. The amount absorbed was impossible to determine due to the high initial concentration, but the leaching data showed that 0.5 ppm. cycloheximide was leached from the one and two hour dipped roots, and 1.0 ppm. from the four and six hour dips. An assay of onion roots treated in this manner indicated that the amount of cycloheximide present was too small to be assayed, or that the antibiotic was in an altered, non-toxic form.

Substantiation of the data indicating that the antibiotic is considerably less active when in contact with organic matter, was found in the replicated liquid culture growth of Poria microspora. The fungus was totally inhibited by a 1.0 ppm. concentration in the

liquid culture, but not by this same concentration in agar plates.

Cycloheximide was shown to be highly toxic against four representative wood-rotting fungi, using linear measurements on agar plates as a basis for comparison. Three of the four were totally inhibited by a 2.0 ppm. concentration.

SUMMARY

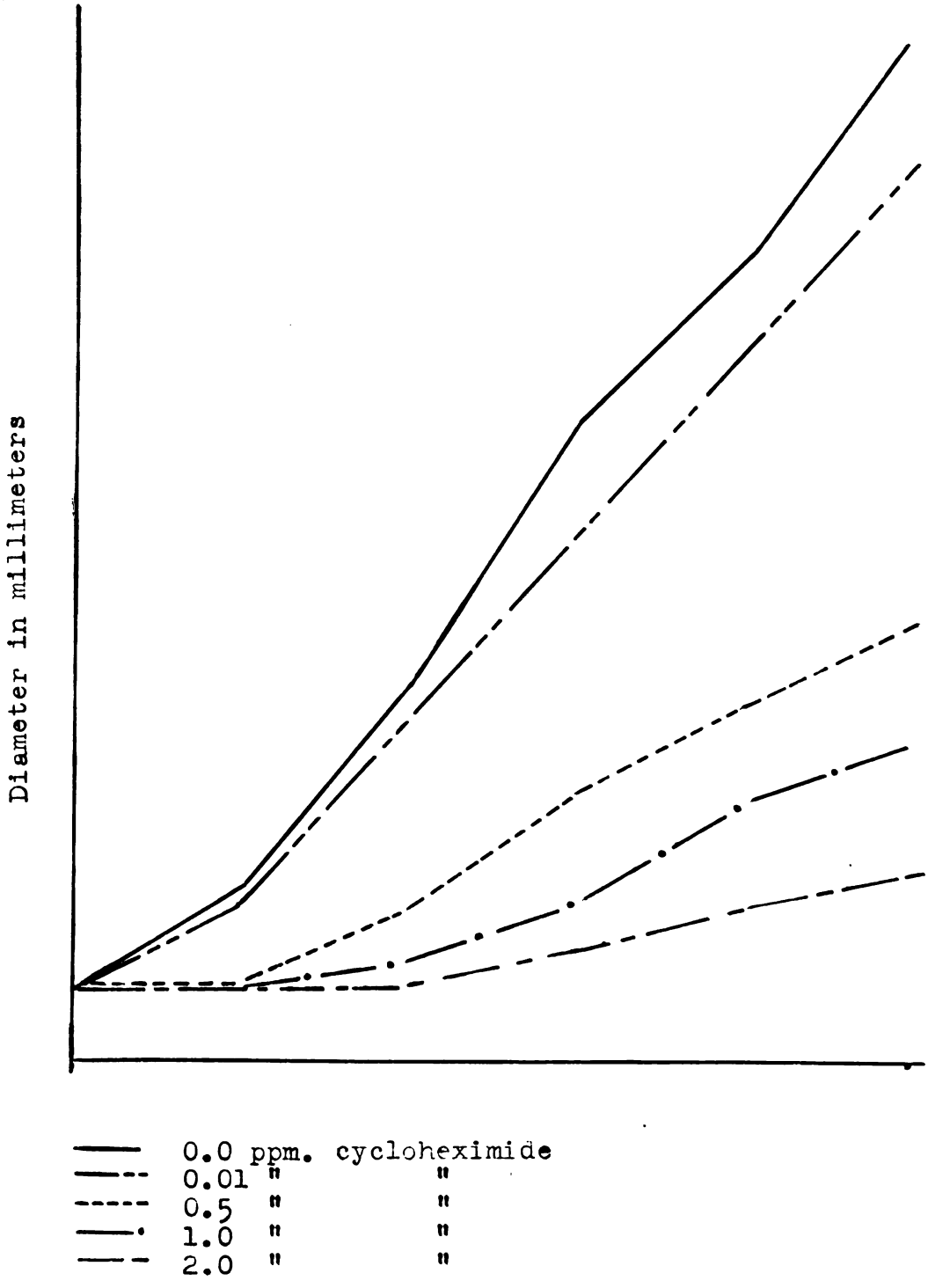
Pertinent facts brought out as a result of this work are as follows:

1. The antibiotic can be assayed biologically for concentrations as low as 0.5 ppm..
2. Cycloheximide was extremely toxic to wood-rotting fungi in very low concentrations, and was an effective wood preservative in two trials, but of doubtful value in others.
3. Cycloheximide is forcibly absorbed by wood tissue, and released in minute quantities on subsequent leaching in water.
4. The antibiotic is less effective as a fungal inhibitory agent when in highly organic media as compared to a synthetic medium.
5. Cycloheximide is absorbed by growing onion roots, and can be recovered by leaching these growing roots.

6. Recovery of the antibiotic from dried mycelium and dried onion roots was not possible under the conditions of the experiment.
7. Suggestions for further research are as follows:
    a. Study of possible absorption, translocation, and recovery in higher plants.
    b. Repetition of wood-rotting tests to prove or disprove that crude cycloheximide was superior to the crystalline product for wood preservation. A leaching study with the crude product would be valuable.
    c. Investigation of possible selective retention by organic compounds in nutrient media.
    d. Study of the mass effect upon retention by mycelium and higher plant tissue.
    e. Attempted recovery from larger masses of organic tissue.

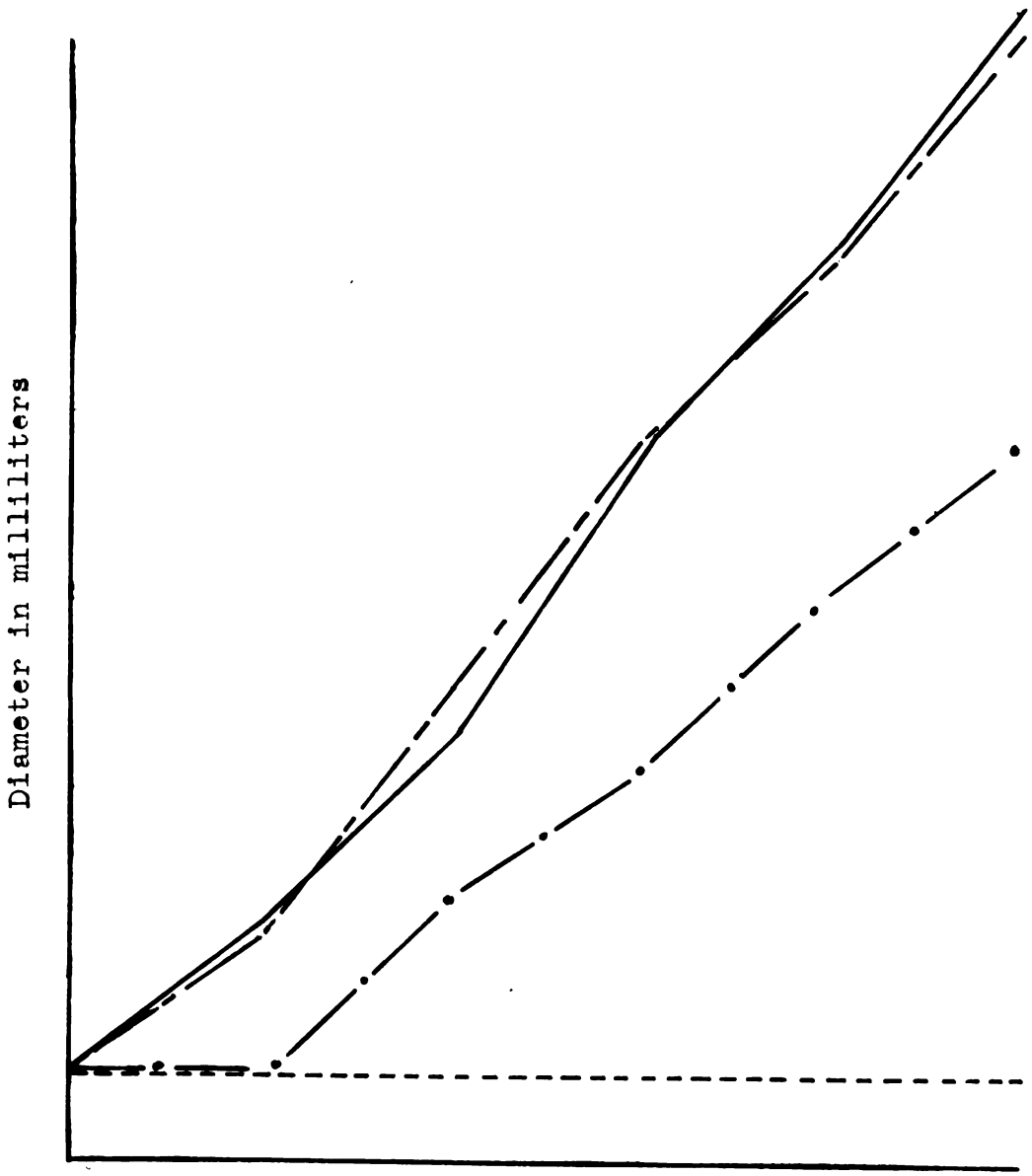
Graph I

Linear growth of Poria microspora in 2% malt extract agar plates containing cycloheximide.



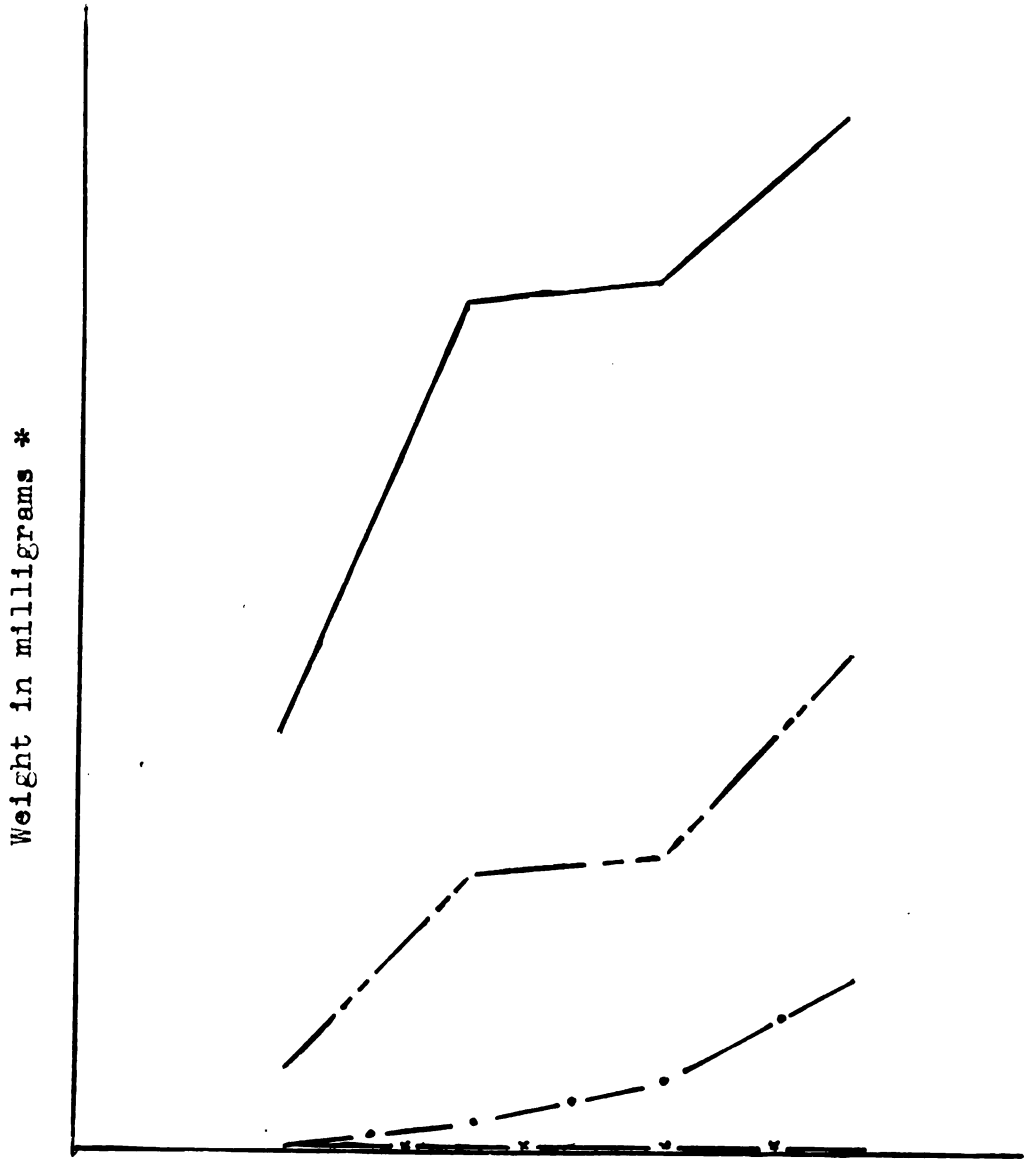
Graph II

Linear growth of Poria microspora in 2% malt extract agar plates containing chromated zinc chloride.



Graph III

Cultural inhibition of Poria microspora in 2% malt extract liquid media containing cycloheximide.

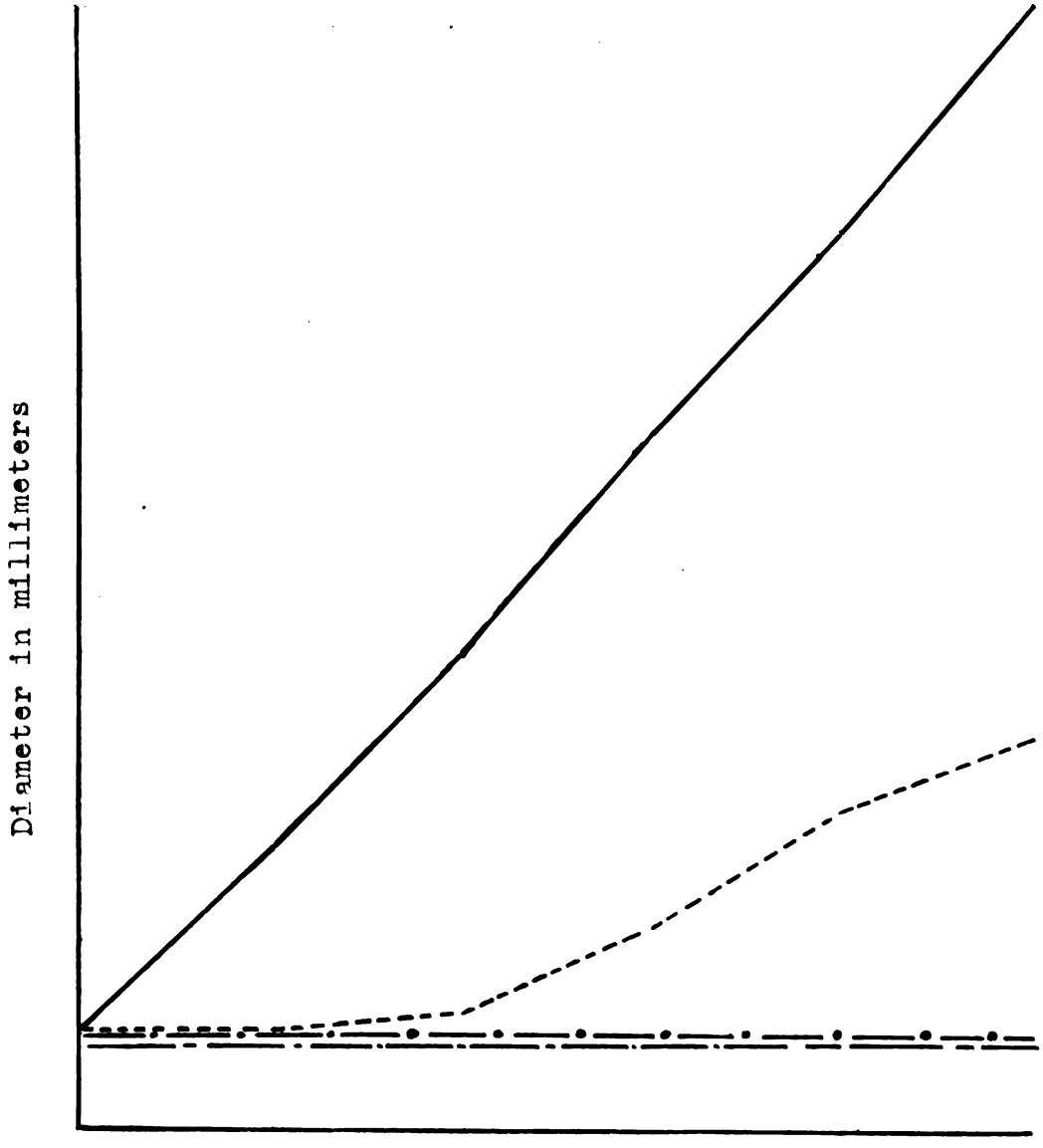


0.0 ppm. cycloheximide
0.01 " "
0.1 " "
1.0 and 10 ppm. cycloheximide

* Increased to mg. for graphing.

Graph IV

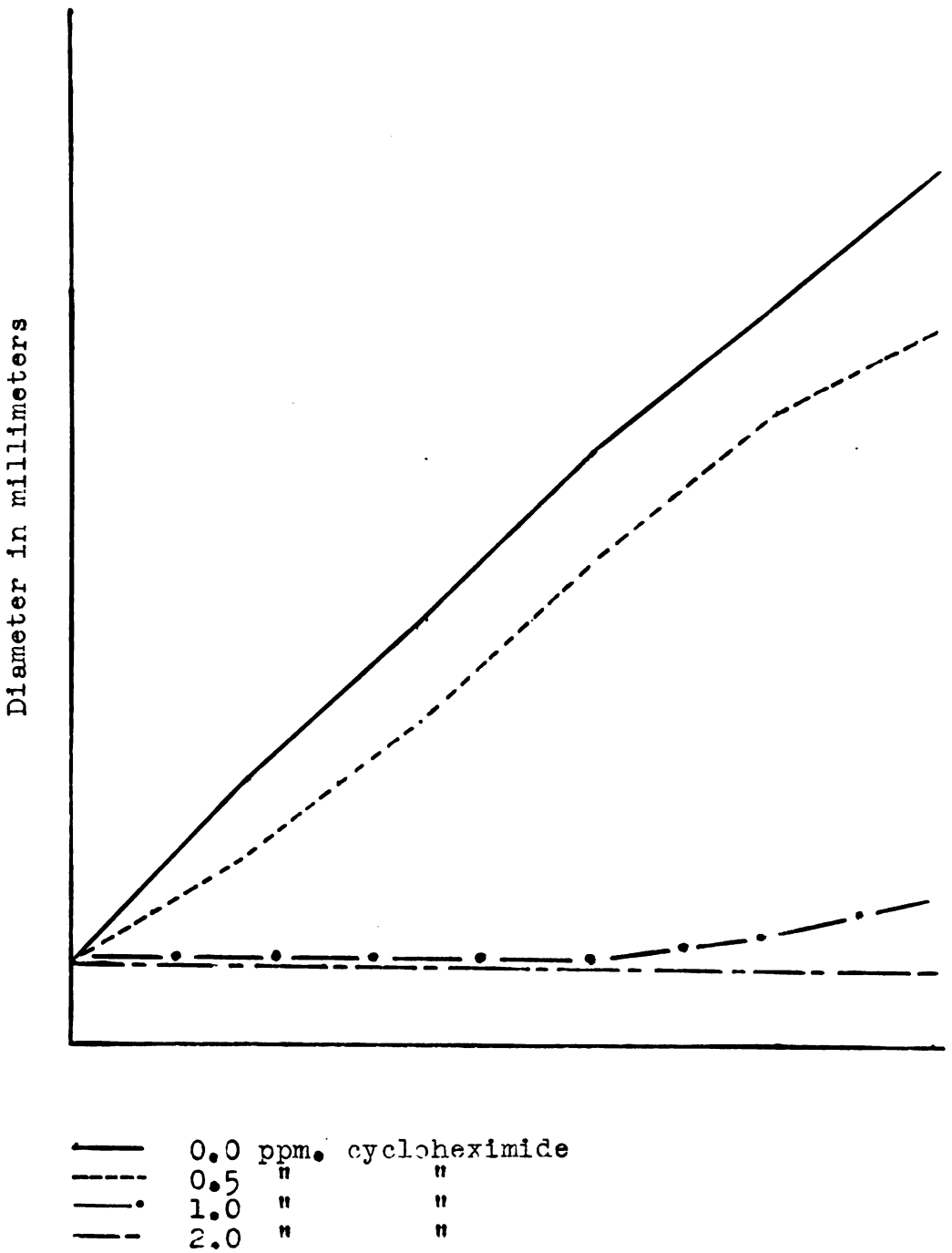
Linear growth of Lentinus lepideus in 2% malt extract agar plates containing cycloheximide.



0.0 ppm. cycloheximide
0.5 " "
1.0 " "
2.0 " "

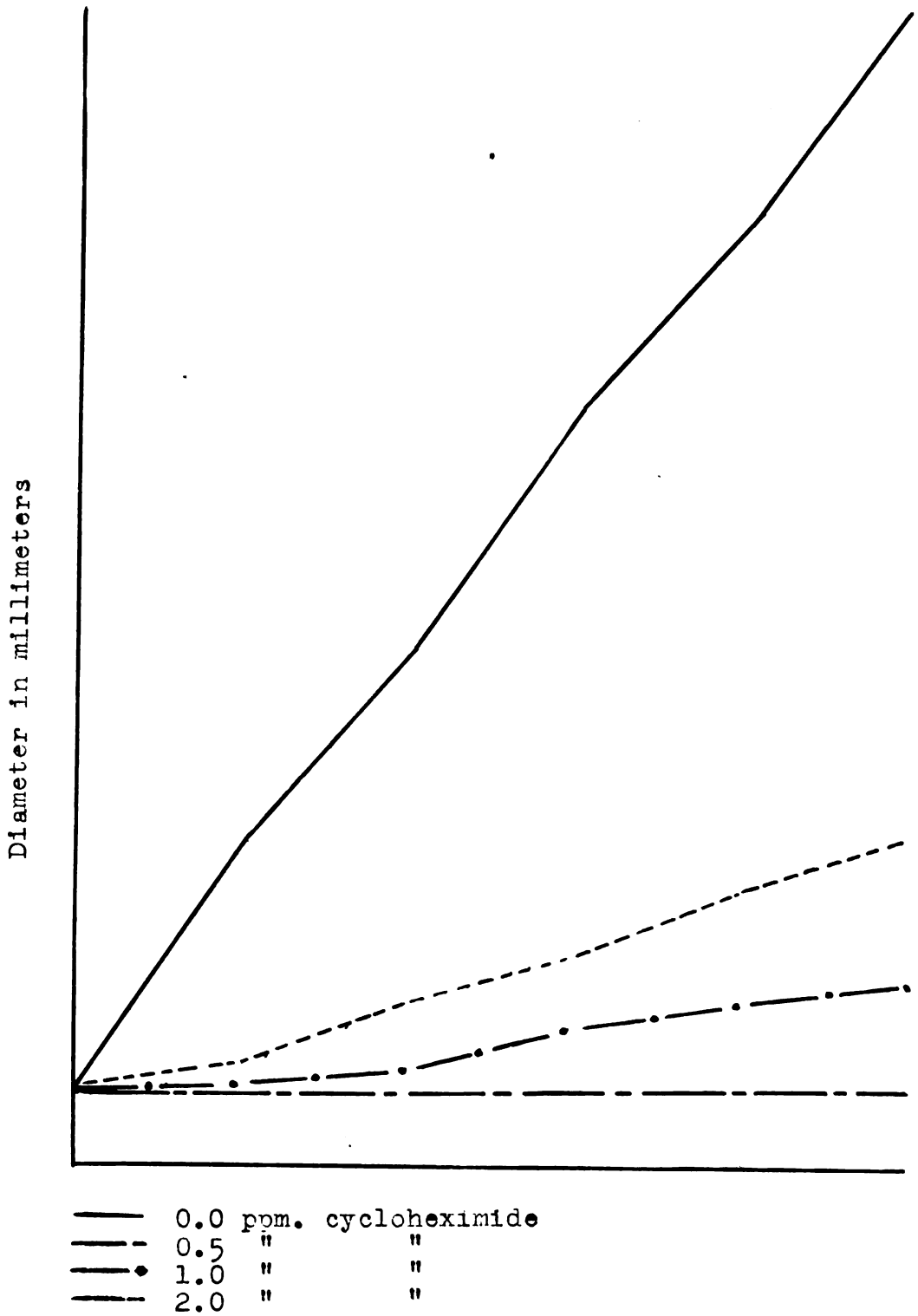
Graph V

Linear growth of Fomes igiarius var. laevigatus in 2% malt extract plates containing cycloheximide.



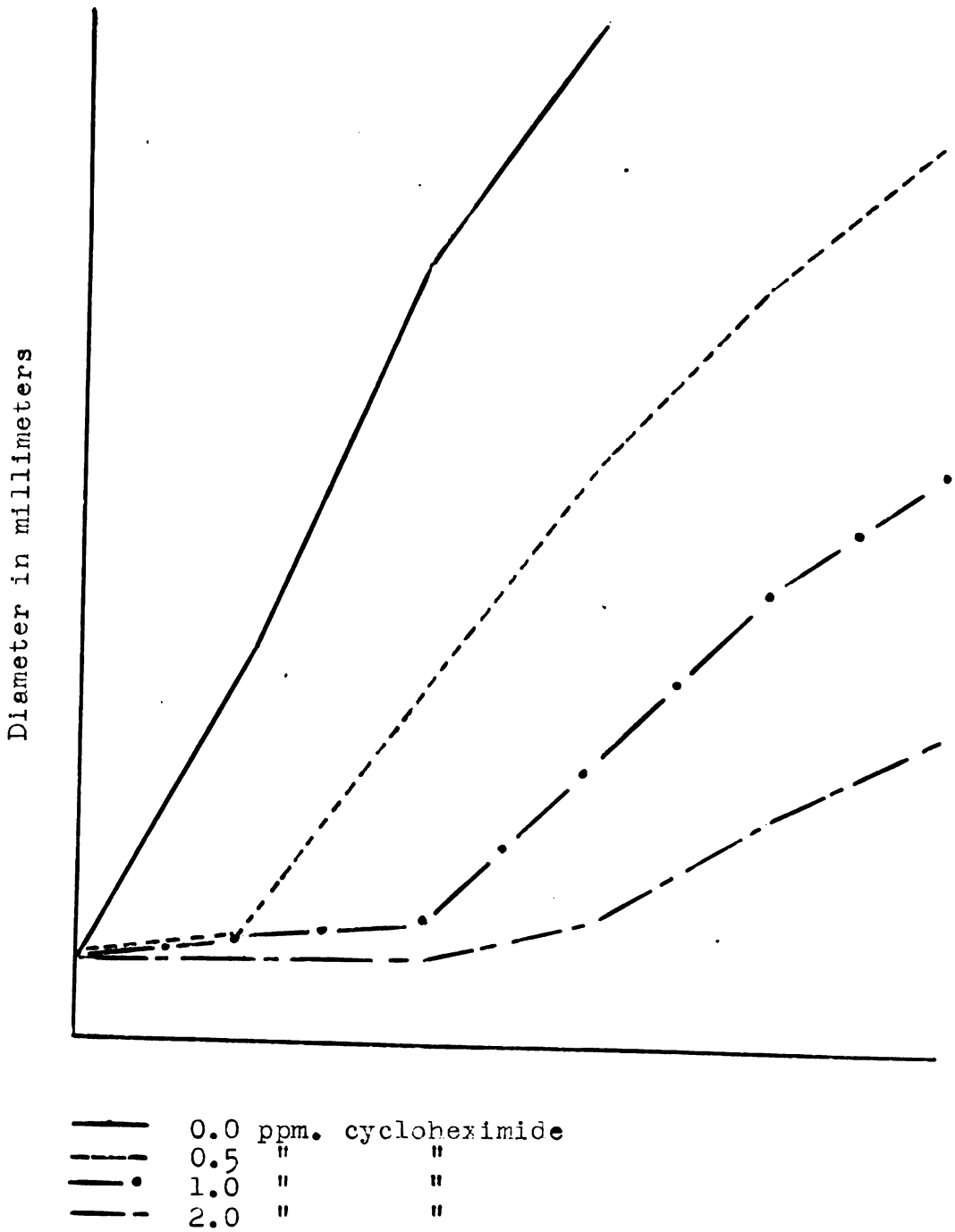
Graph VI

Cultural inhibition of Lenzites trabea in 2% malt extract agar plates containing cycloheximide.



Graph VII

Cultural inhibition of Schizophyllum commune in 2% malt extract agar plates containing cycloheximide.



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