IN VITRO STUDIES OF COLLETOTRICHUM PHOMOIDES UNDER THE INFLUENCE OF SODIUM PYRIDINETHIONE AND OTHER ANTIFUNGAL MATERIALS

By

Samuel Morris Ringel

AN ABSTRACT

Submitted to the School for Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

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Approved Everett C. Bender

The following antifungal test compounds were screened *in vitro* against *Colletotrichum phomoides* and *Helminthosporium sativum* employing Lilly and Barnett's glucose asparagine liquid medium: the sodium salt of 1-hydroxy-2(1H) pyridinethione, diaphine HCl, nystatin, compound M 4575, rimocidin sulfate, thiolutin, candicidin and endomycin.

Sodium 2-pyridinethione is moderately thermostable and breaks down slowly in aqueous solution at room temperature. Endomycin was found to be strongly adsorbed onto the surface of asbestos Seitz filter pads.

Sodium 2-pyridinethione is fungistatic at concentrations up to 0.12 µg/ml. The fungicidal nature of the compound is exhibited when the spores of *C. phomoides* remain in contact with the inhibitor for 24 hours at concentrations above 0.3 µg/ml. Exposures for 20 minutes at concentrations higher than 100 µg/ml. are also fungicidal.

Physiological studies revealed that reducing sugars such as D-glucose decreased the antifungal activity of sodium 2-pyridinethione. It is suggested that the inactivation is due to the formation of a complex involving glucose and 2-pyridinethione. This is supported by ultraviolet absorption spectra studies. The complex is thought to be a thio hemi-acetal. The role of glucose in plants is discussed with relation to 2-pyridinethione inactivation.

The mode of action of 2-pyridinethione was not determined. The inhibitor does not appear to act as a chelating agent for essential metals nor does it function as a substrate analogue for niacin. It is suggested that the sulfur portion of 2-pyridinethione is functional in causing inhibition.

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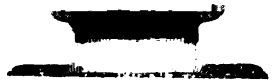

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CHAPTER I

INTRODUCTION

During the past decade, extensive efforts have been directed toward the development of antibacterial antibiotics for the control of human and animal diseases. Recently, this effort has extended into the field of phytopathology. With the exception of actidione (cycloheximide) which is commercially available for agricultural use against phytopathogenic fungi, all other antifungal antibiotics are still at best in the experimental stage. Nystatin (9, 11) shows excellent promise *in vivo* for the control of some of the systemic human mycoses such as histoplasmosis and moniliasis. Recently, a survey of the literature has been published concerning antibiotics (35) that may be of value in controlling plant diseases. Although chemosynthetic agents are the mainstay of the protective and erradicant spray programs, new synthetic materials are also constantly sought after from the viewpoints of cheapness, ease of application, low mammalian toxicity and stability (when desired).

In plant pathology, the biological evaluation of an antimicrobial compound is based on *in vitro* as well as *in vivo* studies. Fortunately, such a joint program was made available by the Horace H. Rackham Research Endowment grant

in the Department of Botany and Plant Pathology. This thesis is concerned exclusively with the in vitro phase of the physiological studies and trial application of antifungal materials for the control of plant diseases.

At this point it seems appropriate to clarify some of the terms that are used in this study specifically with regard to the effects exhibited by materials antagonistic to fungi. McCallan and Wellman (38) stated that in the field of phytopathology "A fungicide may be defined as an agent that kills or inhibits the development of fungus spores or mycelium." They further stated that in the restricted sense, however, fungicidal refers to the property of killing fungi and fungistatic, the property of inhibiting. In this study, the following terms were used in the restricted sense:

> Fungistatic: the growth-preventing or restricting effect on fungus spores or mycelium by an agent, so long as it is in contact with the spores or the mycelium.
>
> Fungicidal action: the killing effect of an agent on fungus spores or mycelium after contact for a limited time, making certain that the agent is completely removed from the organism following the contact time.
>
> Inhibition: see fungistatic.
>
> Antifungal: the antagonistic effect of an agent on fungus spores or mycelium but not specifying whether the effect is due to fungistatic or fungicidal action or a combination of both.

The objectives of this study in vitro were to evaluate:

a. The minimal inhibition concentrations (MIC) of certain test antifungal compounds against selected test phytopathogenic fungi in liquid medium.

b. The stability of certain test antifungal compounds.

c. The effect of four different carbohydrates and two nitrogen sources on the activities of rimocidin and pyridinethione when using Colletotrichum phomoides C80A as the test organism.

Due to the favorable screening results obtained with sodium pyridinethione and the fact that virtually no work had previously been done with this compound, it was deemed advisable to investigate pyridinethione further, again using Colletotrichum phomoides C80A as the test organism, with respect to:

d. Mode of action.

e. Fungistatic-fungicidal properties.

f. The effect upon the growth curve.

g. The inactivation of sodium pyridinethione by D-glucose and supported by spectrophotometric studies.

Literature Review

The basic moiety of pyridinethione is the heterocyclic nitrogen portion called pyridine. The first reported antimicrobial action by an analogue of pyridine was made by Browning et al. (10) in 1923. Binz and Räth (6) in 1928 reported pyridine arsonic acid to be effective against micrococci and streptococci in mice. Wooley and his coworkers (59) in 1938 found pyridine-3-sulfonic acid caused nicotinic acid deficiencies in dogs. McIlwain (39) shortly thereafter reported that pyridine-3-sulfonic acid inhibited

the growth of certain bacteria. The antibacterial and antifungal action of pyridine analogues of thiamine have received some attention (47, 60, 62). Shaw *et al.* in 1950 (52) synthesized a cyclic thiohydroxamic acid from 2-bromopyridine, N-oxide and reported a high *in vitro* activity against a variety of microorganisms. Pansy *et al.* (42) found this compound, 1-hydroxy-2(1H) pyridinethione, to be extremely effective in controlling fungi *in vitro* at concentrations of 0.04 - 1.0 μg/ml. Allison and Barnes in 1956 (4) reported 2-pyridinethione to be moderately effective *in vivo* against some phytopathogenic fungi and also gave an *in vitro* ED_{50} value of 0.03 - 0.4 ppm against conidia of *Monilinia fructicola*. Sander and Allison in 1956 (50) showed that 1-hydroxy-2(1H) pyridinethione is translocated down in cucumber seedlings but suggested that the material is inactivated in the young shoots and cotyledons. Soo-Hoo and Grunberg (53) conducted studies with various salts of 3-pyridinethiol with good results against dermatophytic as well as phytopathogenic fungi *in vitro*.

With regard to other antifungal materials used in this study, Hazen and Brown in 1951 (27) reported the isolation of a new antifungal antibiotic which they named fungicidin (now called nystatin). This material is effective *in vitro* against medical and plant pathogenic fungi at concentrations up to 10 μg/ml. Nystatin is reported to be quite effective *in vivo* against such systemic mycoses as histoplasmosis and

moniliasis (11, 9). Compound M 4575, an antibiotic, (55) has an *in vitro* minimal inhibition concentration of 6 - 12 µg/ml. Rimocidin, first described by Davisson *et al.* (15) in 1951, is an antibiotic inhibiting many of the human pathogenic fungi *in vitro* at concentrations of one to five µg/ml. Grosso (24) in 1954 tried rimocidin as a greenhouse spray for the control of tobacco blue mold. Thiolutin has been tried for the control of mycotic infections of man as well as *in vivo* screening against phytopathogenic bacteria and fungi (13, 40, 22); so far, the results have not been too promising. Candicidin, an antibiotic discovered by Lechevalier and his co-workers (34) is reported to have an activity spectrum against yeast and yeast-like organisms but is only poorly active against the filamentous fungi and exhibits no activity against bacteria. The authors reported this compound to be very soluble in water but it is thermolabile and showed a marked loss of potency after being kept in an aqueous solution for 24 hours at room temperature. Alcorn and Ark (2) reported good results using candicidin as a protectant spray as well as having some value as a protectant dip for peaches (3). Gottlieb *et al.* (23) in 1951 described endomycin as a broad spectrum antifungal antibiotic which was also effective against some gram positive and gram negative bacteria *in vitro*. Endomycin was

reported to inhibit Colletotrichum phomoides at 40 µg/ml. in broth and in addition, this antibiotic was also thermostable. Klomparens and Vaughn (33) found that endomycin showed increased antifungal activity when Fusarium lycopersici was grown in liquid medium containing maltose as the carbon source.

Many microbial inhibiting agents act by virtue of being antimetabolites and several excellent reviews have been published on this matter (58, 61). Sulfanilamide acts as a competitor for para-aminobenzoic acid which was found to be an essential metabolite for many microorganisms (26, 36). Zentmeyer (64) in 1944 suggested that 8-hydroxyquinoline, an excellent fungistatic agent, functions by chelating zinc and thus depriving such fungi as Fusarium oxysporum var. lycopersici of this essential metal. Reversal of inhibition was accomplished by adding excess zinc to the medium. Pyridine is the fundamental moiety of nicotinic acid and nicotinic acid amide which in turn are found in coenzyme I or II (diphosphopyridine nucleotide or triphosphopyridine nucleotide). Coenzymes I and II are required by many of the dehydrogenases. Various substrate analogues of nicotinic acid have been thus found to act as inhibitors, some examples being pyridine-3-sulfonic acid (20, 59), halogen substituted nicotinic acids (30, 31) and 2-sulfanilamido-5-nitropyridine (14). Isonicotinic acid hydrazide (isoniazid) is bacteriostatic

for the tubercle bacillus *in vivo*. The mode of action appears to be that of a competitive inhibitor for nicotinic acid (56) as well as an antimetabolite against pyridoxal synthesis (7, 63).

From the preceding paragraph, it can be shown that by modifying the substrate in some way (be it *in vitro* or *in vivo*), the activity of an antimicrobial agent can sometimes be altered. Thus, Horimoto and his co-workers (29) in 1954 observed that the various B vitamins, nicotinic acid, DPN and some of the sugars are effective in reducing the bacteriostatic action of isonicotinic acid hydrazide on tubercle bacilli. Di Raimondo (16) in 1953 also reported that the vitamin B complex group reduced the activities of the antibiotics terramycin and tyrothricin against *Micrococcus pyogenes* var. *aureus*. According to Weinberg (57), the inhibition of *Mycobacterium avium* by the antibiotics aureomycin and terramycin is significantly increased by the addition of phosphate to the medium.

CHAPTER II

MATERIALS AND METHODS

A. Source and Maintenance of Materials for Study

1. Antifungal Compounds

The following is a list of the compounds employed as antifungal agents together with the methods for preparing the stock solutions:

Sodium pyridinethione[1] (Squibb): very soluble in water.

Diaphine HCl (Pfizer): very soluble in water.

Nystatin (Squibb): soluble in acid methanol.

M 4575 (Squibb): raise to pH 11 with NaOH, solution then occurs; back titrate immediately with HCl.

Rimocidin sulfate (Pfizer); soluble in water, stock solutions of one mg/ml. will be slightly cloudy.

Thiolutin (Pfizer): sparingly water soluble, 50 µg/ml., solubility can be increased to 150 µg/ml. by using 70 percent methanol.

Candicidin A (Dept. of Microbiology, Rutgers University): very soluble in water.

Endomycin (Upjohn): soluble in water containing a little $NaHCO_3$.

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[1] 1-hydroxy-2(1H) pyridinethione (MC 3277), so designated by E. R. Squibb and Sons. When a further supply of this material became necessary, lot no. Py-354-75c was provided which turned out to be slightly more active than the previous material used.

With the exceptions of diaphine and pyridinethione, the hitherto listed compounds are antibiotic in source. All test materials were maintained in a dry state at 5° C. until removed for use.

2. Test Organisms

The following fungi were employed as test organisms:

Colletotrichum phomoides (Sacc.) Chester[1], obtained from Indiana tomato fruits in the summer of 1953 and designated as isolates C80A and 10I.

Helminthosporium sativum Pam., King and Bakke[2], isolated from barley in the "Thumb" area, Michigan, and designated as isolate 925.

Helminthosporium carbonum Ullstrup[2].

Ustilago hordei (Pers.) K. and S.[2], isolate obtained from Madison, Wisconsin and designated as isolate R6.

Puccinia sorghi Schw.[2]

Colletotrichum lagenarium (Pers.) E. and H.[3]

C. lagenarium was maintained on V-8 agar slants in order to induce ample sporulation. The uredospores of P. sorghi were stored at 5° C. All other organisms were routinely kept on potato dextrose agar slants with the exception of U. hordei which required a three percent glucose supplement.

Cultures obtained from the following faculty members of the Dept. of Botany and Plant Pathology at Michigan State University, East Lansing, Michigan:

[1]Dr. E. S. Beneke

[2]Dr. R. L. Kiesling

[3]Dr. D. J. de Zeeuw

In order to minimize the possibility of biochemical variation, sub-culturing was kept to a minimum by allowing no more than five successive transfers from the originally supplied cultures.

B. Techniques Employed

1. Medium Used

For the purposes of assay testing and physiological studies, Lilly and Barnett's (36) synthetic glucose asparagine medium was used. This medium, compounded on the liter basis, contained D-glucose, 10 g.; L-asparagine, 2 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; KH_2PO_4, 1.0 g.; Fe, 0.2 mg.; Zn, 0.2 mg.; Mn, 0.1 mg.; thiamine HCl[1], 100 µg. and biotin[1], 5 µg. The pH was adjusted to 6.0 with a Beckman glass electrode pH meter. The medium was dispensed in various ways, as described later, and autoclaved at 240° F. for 15 minutes. The medium was removed from the autoclave as soon as possible to offset caramelization. All materials used in the formulation of the medium were of reagent grade purity.

2. Glassware Cleaning Procedure

All glassware employed in this study was previously cleaned in a synthetic detergent solution followed by a tap

[1]Kindly furnished by Hoffmann-La Roche Inc., Nutley, New Jersey.

water wash and a distilled water rinse. Pipettes were cleaned by soaking in chromic acid followed by seven rinses for each in tap and distilled water.

3. Screening of Antifungal Materials

In all cases, solutions of the test antifungal compounds were freshly prepared prior to use. The compounds were sterilized in the following manner: one series via Seitz filtration and another series via autoclaving directly with the medium, thus affording information regarding thermostability. Lilly and Barnett's glucose asparagine liquid medium was used exclusively, intentionally omitting solidifying agents such as agar which may tie up some of the test compound by adsorption. For example, when nystatin was screened, the minimal inhibition concentration against C. phomoides C80A on agar was 100 µg/ml. whereas in liquid, it was 10-50 µg/ml.

Spore inocula were used in the assays by preparing aqueous spore suspensions from two-week old culture slants. The spore suspensions were adjusted, using a Levy Hemacytometer, so that a concentration of 400-500 spores per ml. of medium resulted when one ml. was used as inoculum for 20 ml. of medium.

Aseptic techniques were maintained throughout the procedure. One ml. of inoculum was pipetted into a sterile

Petri plate followed by the medium and then by the Seitz sterilized test compound (in the heat sterilized series, the test compound had already been added to the medium prior to autoclaving). The test compounds were serially diluted so as to give the desired concentration series for the assay. The plates were gently swirled after the addition of each constituent to insure adequate mixing. All assay plates and controls were run in duplicate, incubated at ca. 25° C. for five days and the results recorded as plus or minus growth.

4. "Shelf Life" Stability Determinations of Antifungal Materials

In those instances where the test compounds showed good promise (minimal inhibition concentrations of 10 µg/ml. or less), further studies were conducted to determine the length of time that potency is retained in aqueous solutions at 10 µg/ml. of materials at 5° C. and at room temperature. This is termed the "shelf life" of the compound. In addition, sodium pyridinethione was maintained at one µg/ml. under the same temperatures. Activity was determined by the Petri plate assay method but in one instance where sodium pyridinethione was kept at one µg/ml., the evaluation was determined by weighing the recovered dry mycelium. This procedure is discussed in the section dealing with

techniques employed in the physiological studies on C. phomoides C80A (page 19).

5. Spore Germination Tests

Spore germination tests were set up for a 24-hour period employing the glass slide technique with the test tube dilution method as described by the American Phytopathological Society, Committee on Standardization of Fungicidal Tests (5). Distilled water was used as the suspending medium for the spores.

6. Physiological Studies on Colletotrichum phomoides C80A

a. Varying the medium with respect to the carbon source. Four different carbohydrates were investigated separately in conjunction with varying concentrations of the test antifungal compound. The basal medium employed was Lilly and Barnett's synthetic glucose asparagine, complete with the exception of the carbohydrate. The standard medium contained glucose equivalent to four g. carbon per liter; hence the following sugars were used in the quantities specified so as to give four g. carbon per liter: D-glucose, 10 g.; sucrose, 9.5 g.; D-xylose, 10 g. and alpha-lactose.H_2O, 10 g. The medium was compounded on the liter basis but made up to 960 ml. volume. This was done so that the subsequent addition of one ml. inoculum and one ml. of the antifungal

material to 48 ml. of medium would yield 50 ml. at the proper concentration of nutrients. Forty eight ml. of medium was dispensed into 250 ml. Erlenmeyer flasks which were then plugged with cotton (non-absorbent) and autoclaved at 240° F. for 15 minutes. The contents were removed from the autoclave as soon as possible to help offset caramelization.

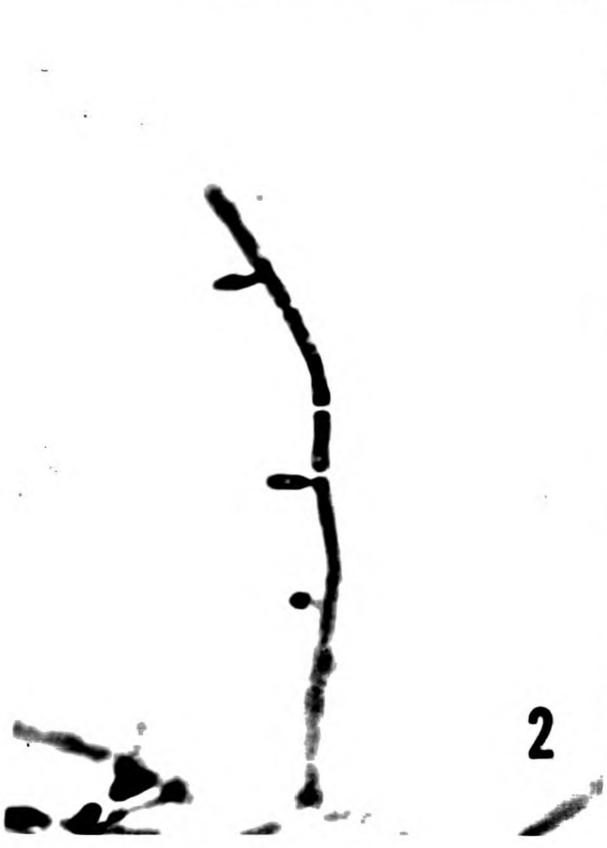
One ml. of spore inoculum containing 150,000 spores (equivalent to 3,000 spores per ml. of medium) was aseptically added to each flask followed by one ml. of a freshly prepared, unless otherwise specified, Seitz filtered solution of the antifungal material at that concentration designed to yield the desired final concentration in μg/ml. The flasks were gently swirled after the addition of each constituent. All variations along with the controls were run in triplicate.

b. Varying the medium with respect to the nitrogen source. Ammonium nitrogen as well as nitrate nitrogen sources were investigated separately in conjunction with varying concentrations of the antifungal material. The standard Lilly and Barnett medium used was complete with the exception of the nitrogen source (asparagine). The standard medium contains asparagine equivalent to 0.424 g. N/liter; hence the following nitrogen compounds were used in the quantities specified so as to give the same amount of nitrogen (0.424

g./liter); sodium nitrate, 2.57 g.; and ammonium chloride, 1.62 g. The methods employed were the same as those previously described for the carbohydrate studies.

c. Preparation of inoculum. In the beginning, spore inocula for the physiological studies were routinely obtained from sporulating PDA slant cultures. Because of some unexplained factor or combination of factors, cultures of C. phomoides C80A, incubated two weeks or longer at 25° C., produced decreasingly smaller crops of conidia or often none at all, although prolific quantities of acervulus-like bodies were apparent. This situation was detrimental in work requiring specific spore concentrations in the inocula. Unsuccessful attempts were made to induce sporulation on agar slants by the use of such media as, Sabouraud, corn meal and yeast extract incubated over a temperature range of 16 to 30° C. When the physiological studies were first undertaken, it was noted that cultures incubated on the Kershaw rotary shaker produced a turbidity in the liquid medium. This was found to be due to prolific sporulation. These conidia were morphologically similar to those produced on semi-solid media. Acervuli do not form in the shake cultures, except on the aerial mycelial ring above the splash line. The typical appearance of the organism in shake culture is pellet-like. Plate I shows that the conidia are borne directly on the hyphae or on short projecting conidiophores.

PLATE I



Colletotrichum phomoides C80A conidial formation.

Liquid shake culture, incubated four days at ca. 25° C.

Fig. 1. A portion of a pellet showing some conidia. Ca. x 525.

Figs. 2 and 3. Enlargement showing conidia borne on short conidiophores or directly on the hyphae. Ca. x 1,000.

Some species of the genus Fusarium also produce abundant quantities of conidia in liquid shake cultures. Utilizing this information, it was possible to set up a simple procedure to harvest the spores of C. phomoides C80A, wash and use them as inoculum. The following procedure was devised:

(1) Fifty ml. of the standard glucose asparagine liquid medium was inoculated with a one ml. aqueous suspension of mycelial fragments obtained from a PDA slant culture. The flasks were incubated on a Kershaw rotary shaker at 140 rpm for four days at ca. 25° C. at the end of which time, large quantities of conidia were produced imparting a milky appearance to the medium.

(2) Aspetically, a portion of the culture supernatant, containing the suspended conidia, was decanted into a cotton plugged, sterilized 30 ml. pour-out-lip, round-bottomed centrifuge tube.

(3) The material was centrifuged in an "International Centrifuge, Size 1, Model SBV" for one minute at an average Relative Centrifugal Force of 95 gravities. This forced down any pellets still present while the spores remained in suspension.

(4) Aseptically, the spore suspension was decanted into another sterilized, cork-plugged, 50 ml. tapered-end centrifuge tube and recentrifuged for five minutes at 300 gravities. This served to centrifuge the spores down. The clear supernatant was rejected.

(5) The spore mass was washed twice with 10 ml. each of sterile distilled water, and each time step (4) was repeated. The spores were finally resuspended in 10 ml. sterile distilled water.

(6) The spore count was determined with a Levy Hemacytometer and subsequently diluted to the desired concentration.

Cultures inoculated with spores obtained in the above procedure gave results comparable with those obtained with inoculum secured from agar slants. Refrigerating the spore suspension at 5° C. for two days prior to use yielded likewise comparable results. Using spore inoculum prepared in this manner has the following advantages over inoculum obtained from agar slant cultures:

(1) Approximately 150-200 million spores can be recovered compared to 8-10 million spores from an exceptionally heavy sporulating slant culture.

(2) The obtained spore inoculum is free from hyphal and acervulal debris which can act as focal growth points.

(3) The possibility of residual nutrient carryover is eliminated.

d. Incubation conditions. In all instances involving the carbon and nitrogen source variations, the flasks were prepared in two series, one to be incubated in a

stationary position and the other to be agitated on a Kershaw rotary shaker at 140 rpm. Since the results of a stationary culture series did not differ from those of the shake cultures, the former were omitted. Because of mechanical difficulties involving the rotary shakers, some experiments have been run only in the stationary culture series. Occasional difficulties were encountered in trying to control the incubation temperature. This influenced the rate of growth of the organism under study and consequently made comparisons between experiments difficult. After the installation of constant temperature devices by the Department of Botany and Plant Pathology, the temperature was held fairly uniformly at ca. 25° C. with the exception of a few weeks during the summer of 1955. The incubation period for shake cultures was six days and for stationary cultures, nine days.

e. Harvesting and data presentation. At harvest time, the mycelium from each flask was filtered via suction on a Buchner funnel through a nylon filter. The mycelium was then washed three times with distilled water, placed in a numbered, tared aluminum weighing cup and dried overnight at 60° C. The cups with the dried mycelia were then kept in a dessicator until weighed. The pH of the filtrate was determined at harvest time. Each value reported, unless

otherwise indicated, is the average of three flasks and expressed as mg. dry weight.

f. Size of inoculum. As previously stated, the standard spore concentration employed for the physiological studies is 3,000 spores per ml. of medium. In one experiment, the spore load was unintentionally adjusted to 130 spores/ml. medium instead of the standard concentration. At this low spore load and at varying concentrations of sodium pyridinethione, no agreement between replicate flasks of shake cultures was possible with the exception of the control flasks. All replicate treatments did agree however in the stationary incubated cultures. Table I shows the individual readings within given treatments for shake and stationary cultures at low spore loads as compared to the individual readings obtained for shake cultures under the same conditions but with the normal spore load inoculation of 3,000 spores per ml. of medium. At the end of six days, the shake culture flasks containing the low inoculum all exhibited irregular sized pellets and often just one large mycelial clump per flask, with the exception of the control flasks which had good pellet growth of uniform small size. The series inoculated with the normal spore load, for the most part, appeared homogeneous, macroscopically, and irregular mycelial clumps were observed only when the organism

TABLE I

EFFECT OF SPORE CONCENTRATION ON AGREEMENT BETWEEN REPLICATE FLASKS TREATED WITH SODIUM PYRIDINETHIONE*

Na Pyridinethione µg/ml.		Concentration of Inoculum		
		130 Spores/ml. Medium		3,000 Spores/ml. Medium
		Shake Cult.	Stat. Cult.	Shake Cult.
		mg.	mg.	mg.
0.00	rep 1	205	190	146
	rep 2	192	196	154
	rep 3	194	183	146
0.04	rep 1	188	190	220
	rep 2	118	184	215
	rep 3	207	168	202
0.08	rep 1	88	145	195
	rep 2	145	145	192
	rep 3	202	148	---
0.12	rep 1	109	79	46
	rep 2	139	79	69
	rep 3	188	71	35
0.32	rep 1	no growth	4	no growth
	rep 2	" "	2	" "
	rep 3	" "	2	" "

*C. phomoides C80A cultured in glucose asparagine medium. Incubated for six days at ca. 25° C. and expressed as mg. dry mycelium per fifty ml. medium.

was greatly retarded at the higher concentrations of sodium pyridinethione. This appearance of irregular mycelial clumps at the higher concentrations agree with the results of Oster and Golden (41) who stated that the greatest variations were exhibited at either very low or very high concentrations of test antifungal substances. Duckworth and Harris (18) found that spores of Penicillium chrysogenum at one million spores per ml. of medium gave the best non-anastamosed hyphal growth in submerged cultures. In addition, Foster (19) stated that the uneven large clump-like colonies in submerged cultures are mostly due to an inadequate amount of viable inoculum. In the previously described observations, an inoculum size of 130 spores per ml. of medium apparently was adequate for the control flasks in submerged culture since a good uniform growth was obtained. Under the influence of even minute concentrations of sodium pyridinethione (0.02 µg/ml.), it was to be expected that some of the spores would be inhibited. The remaining number of spores, which were able to germinate, probably was at a sub-optimal level and thus caused the irregular mass like growths. At the higher spore loads of 3,000 per ml., the number of spores unaffected by the inhibitor was perhaps still large enough to induce the uniform pellet like growth and hence, good reproducibility between replicate flasks.

g. Concentration range of antifungal materials used in study. A preliminary investigation showed the working range of the antifungal materials employed in the physiological studies to be:

Rimocidin: one to ten µg/ml.

Sodium pyridinethione: 0.02 to 0.32 µg/ml.

7. Additional Techniques Related to the Sodium Pyridinethione Study

Additional methods and techniques pertaining to the physiological study with sodium pyridinethione will be discussed in Chapter III, entitled RESULTS AND OBSERVATIONS.

CHAPTER III

RESULTS AND OBSERVATIONS

A. Screening of Antifungal Materials

The results presented in Table II indicated that sodium pyridinethione, rimocidin and endomycin were most effective against the organisms tested. It was due to these results that sodium pyridinethione and rimocidin were selected for some of the physiological studies. Endomycin, although showing good potential, was not included in the physiological studies because it was screened some time after the project was in progress.

Seitz filtered endomycin preparations showed no activity against the test organisms at concentrations up to 200 µg/ml. It was noted that the normal yellow color of the endomycin stock solution was colorless after passing through the Seitz filter. The antibiotic had apparently been adsorbed onto the asbestos filter pad of the Seitz apparatus which in turn was colored yellow. A duplicate experiment yielded the same results. Because endomycin has been reported to be thermostable (23), it was sterilized by autoclaving directly with the medium. Plates II, III and IV show the difference between the two treatments of endomycin.

TABLE II

ACTIVITIES OF ANTIFUNGAL MATERIALS MIC[1] IN μG/ML.
GLUCOSE ASPARAGINE LIQUID MEDIUM[2]

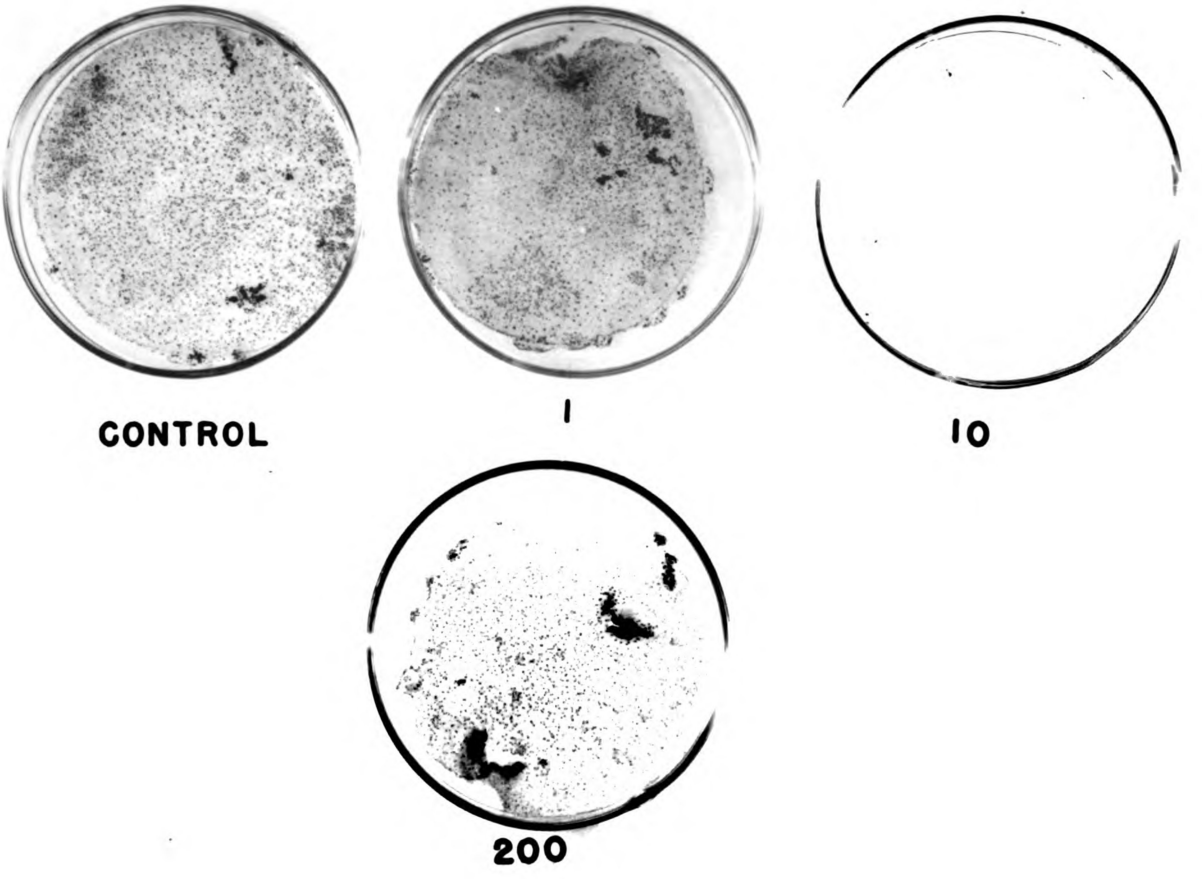
Material	C. phomoides C80A	C. phomoides 101	H. sativum 925
Na pyridinethione	< 1	< 1	< 0.1
Diaphine HCl	-	100-200	50
Nystatin	> 10	50	> 50
M 4575	> 200	> 50	> 200
Rimocidin SO_4	< 10	< 10	< 10
Thiolutin	> 15	> 15	> 15
Candicidin A	-	200	200
Endomycin[3]	< 10	< 10	< 10

[1]Minimal inhibition concentration.

[2]Incubated for four days at ca. 25° C.

[3]Sterilized via autoclaving directly with the medium; all other materials Seitz-filter sterilized.

PLATE II

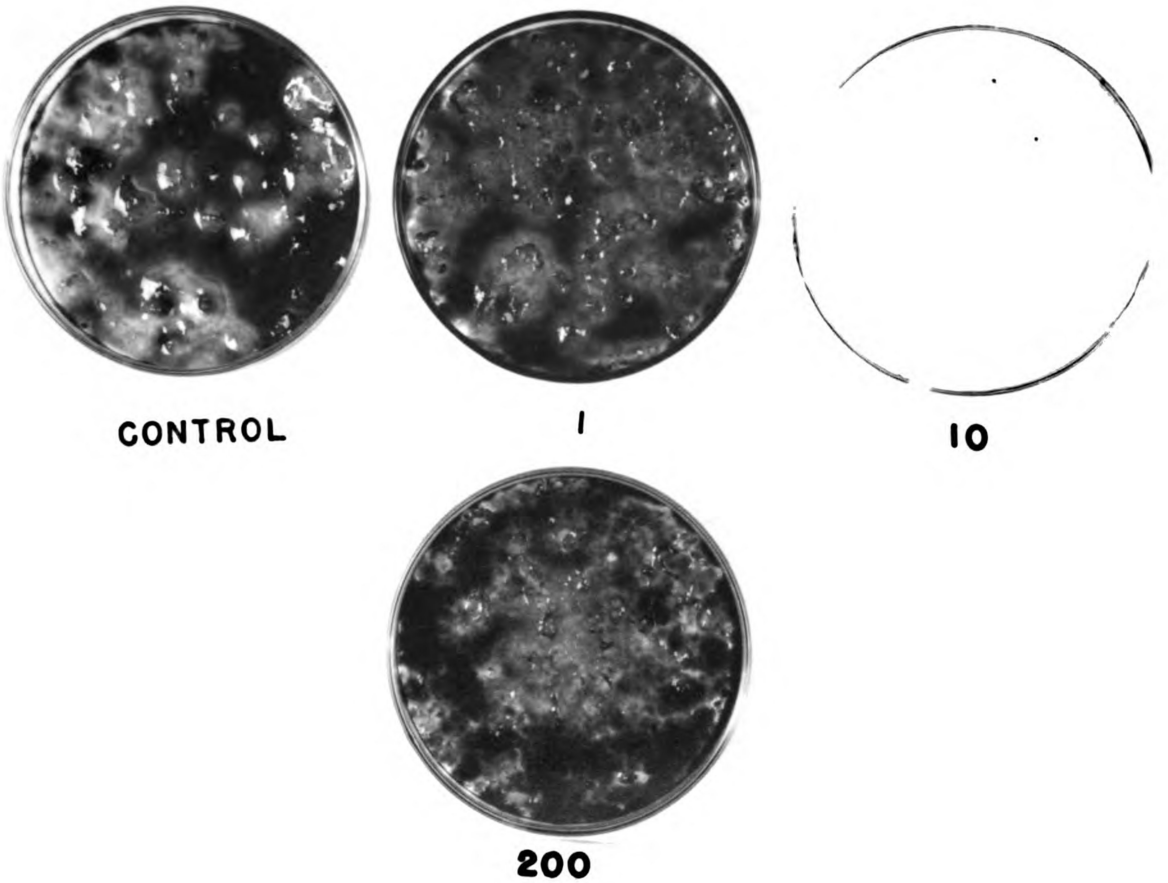


Concentration series of endomycin in µg/ml. against Colletotrichum phomoides C80A in liquid medium. Incubated for 35 days at ca. 25° C.

Upper: Control plate and plates containing autoclaved endomycin.

Lower: Seitz-filtered endomycin.

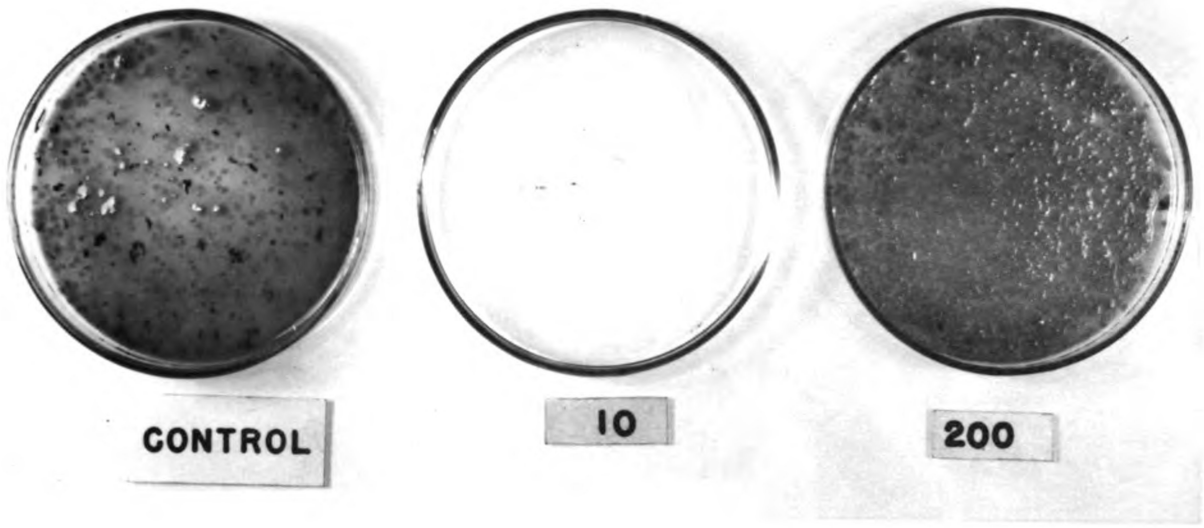
PLATE III



Concentration series of endomycin in µg/ml. against Helminthosporium sativum 925 in liquid medium. Incubated for 35 days at ca. 25° C.

Upper: Control plate and plates containing autoclaved endomycin.

Lower: Seitz filtered endomycin.



Concentration series of endomycin in µg/ml. against Ustilago hordei R6 in liquid medium. Incubated for 35 days at ca. 25° C.

Left to right: Control, autoclaved and Seitz-filtered endomycin.

B. Stability Tests

Since the antifungal compounds sodium pyridinethione, rimocidin and endomycin exhibited relatively high *in vitro* activities, they were further evaluated with regard to various stability aspects. Although diaphine did not show very good screening results, it was included in these tests by way of comparison.

1. Thermostability

Losses in potency were observed when the compounds sodium pyridinethione, rimocidin, diaphine and endomycin were subjected to autoclaving with the medium for 15 minutes at 240° F. Diaphine exhibited the greatest loss due to heat inactivation whereas the other compounds remained fairly stable. In the case of sodium pyridinethione, the medium darkened somewhat upon autoclaving. The results are shown in Table III.

2. "Shelf Life"

a. Sodium pyridinethione and rimocidin via assay plate test. Using *H. sativum* 925 and *C. phomoides* C80A as the test organisms, these compounds when stored at 10 µg/ml. in glucose asparagine medium retained their activity for:

TABLE III

THERMOSTABILITY OF ANTIFUNGAL MATERIALS MIC IN μG/ML. GLUCOSE ASPARAGINE LIQUID MEDIUM*

Compound	Method of Sterilization			
	Seitz Filtration		Autoclaving With the Medium	
	C. phomoides C80A	H. sativum 925	C. phomoides C80A	H. sativum 925
Na Pyridinethione	< 1	< 0.1	10	5-10
Rimocidin	< 10	< 10	< 10	< 10
Diaphine	100-200	50	200	200
Endomycin	< 10**	< 10**	10	10

*Cultures incubated for four days at ca. 25° C.

**Non-sterilized (unheated).

TABLE IV

ANTIFUNGAL ACTIVITY OF STORED SODIUM PYRIDINETHIONE TESTED AT 0.12 μG/ML. GLUCOSE ASPARAGINE LIQUID MEDIUM AGAINST C. PHOMOIDES C80A AND REPORTED AS MG. DRY MYCELIUM PRODUCED IN SIX DAYS AT CA. 25° C.

Na Pyridinethione	Tested		
	Immediately	After 25 Days	After 5 1/2 Months
Freshly prepared	50	--	--
Stored at 5° C.	--	45	133
Stored at room temperature	--	40	203

C. phomoides C80A in the control yields 200 mg. dry mycelium.

sodium pyridinethione: six months at 5° C.
six months at room temperature.

rimocidin: 2 1/2 months at 5° C.
1 1/2 months at room temperature.

b. A more critical determination of the "shelf life" of sodium pyridinethione. Since most of the physiological work was to be concerned with this compound, it was deemed advisable to determine whether this compound underwent degradation during an incubation period at those concentrations 0.02 to 0.32 µg/ml., used in the physiological study. Aqueous, Seitz filtered solutions of sodium pyridinethione at a concentration of 10 µg/ml. were maintained at both 5° C. and at room temperature for varying periods of time. At appropriate intervals, portions were removed, diluted to a final concentration of 0.1 µg/ml. medium and shake flasks were inoculated with spore suspensions of C. phomoides C80A, incubated on Kershaw rotary shakers and the mycelium processed according to the method described in Chapter II. Thus, at the end of a 25-day storage period, the material kept at either room temperature or at 5° C. exhibited no loss of potency. On the other hand, at the end of 5 1/2 months, there was virtually an 100 percent loss in potency at both room temperature and 5° C. These results are presented in Table IV. Since the incubation periods do not exceed nine days in the physiological studies, no problem was presented with respect to the self-degradation of sodium

pyridinethione on the basis of this information. On the contrary, sodium pyridinethione can be considered a fairly stable antifungal material.

c. Endomycin. Six concentrations ranging from 0.1 to 200 µg/ml. were tested against three organisms. Non-sterilized (unheated) endomycin, together with a combination of 20 units penicillin and 40 units streptomycin per ml. of medium to keep down bacterial contamination, retained full potency even after an incubation period of 35 days. The autoclaved endomycin lost a little of its potency during heating since some growth was apparent at the end of 35 days (Tables V and VI). The "shelf life" of endomycin is good, as can be seen in Plates II, III and IV.

3. The Effects of Different Sterilization Treatments on the Potencies of Sodium Pyridinethione and Rimocidin

Because of bacterial contamination which would otherwise be present, the antifungal materials had to be sterilized prior to being brought into contact with the test organism(s). The selected method used during the physiological studies was Seitz filtration since it was shown that autoclaving directly with the medium altered the activity level of the antifungal materials. In order to determine whether any material was lost in the process of Seitz filtration, by adsorption,

TABLE V

ANTIFUNGAL ACTIVITY OF AUTOCLAVED ENDOMYCIN AT VARYING CONCENTRATIONS IN GLUCOSE ASPARAGINE LIQUID MEDIUM CULTURES INCUBATED AT CA. 25° C.

Conc. of Endomycin in µg/ml.	C. phomoides C80A		H. sativum 925		U. hordei R6	
	4 da.	35 da.	4 da.	35 da.	4 da.	35 da.
0.0	++	+++	++	+++	+	+++
0.1	++	+++	++	+++	+	+++
1.0	++	++	++	+++	+	+++
10.0	-	+	-	+++	-	-

TABLE VI

ANTIFUNGAL ACTIVITY OF NON-STERILIZED (UNHEATED) ENDOMYCIN AT VARYING CONCENTRATIONS IN GLUCOSE ASPARAGINE LIQUID MEDIUM CULTURES INCUBATED AT CA. 25° C.

Conc. of Endomycin in µg/ml.	C. phomoides C80A		H. sativum 925	
	4 da.	35 da.	4 da.	35 da.
0.0	++	+++	++	+++
0.1	++	+++	++	+++
1.0	++	++	++	++
10.0	-	-	-	-

it was deemed advisable to compare the activities of materials sterilized by Seitz filtration to those sterilized via sintered glass filtration. An ultra-fine (bacterial) sintered glass funnel was employed. As indicated in Graph I, some activity is lost when sodium pyridinethione is Seitz-filtered and that there is a complete loss upon autoclaving (compare this to the results obtained in the section on thermostability). In the case of rimocidin however, a negligible amount of activity is lost upon Seitz filtration and it is only moderately inactivated by autoclaving.

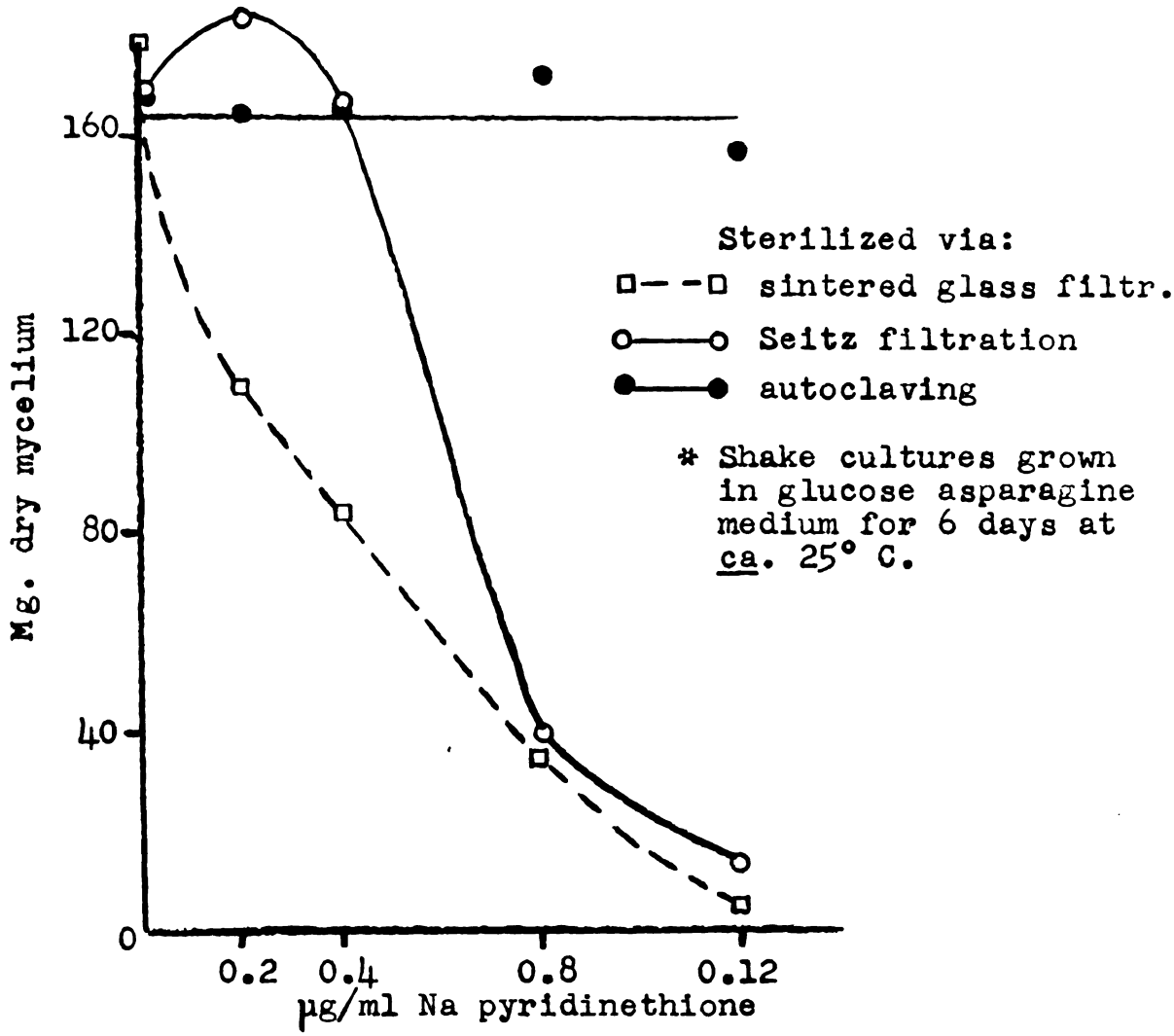
C. Physiological Studies with Rimocidin

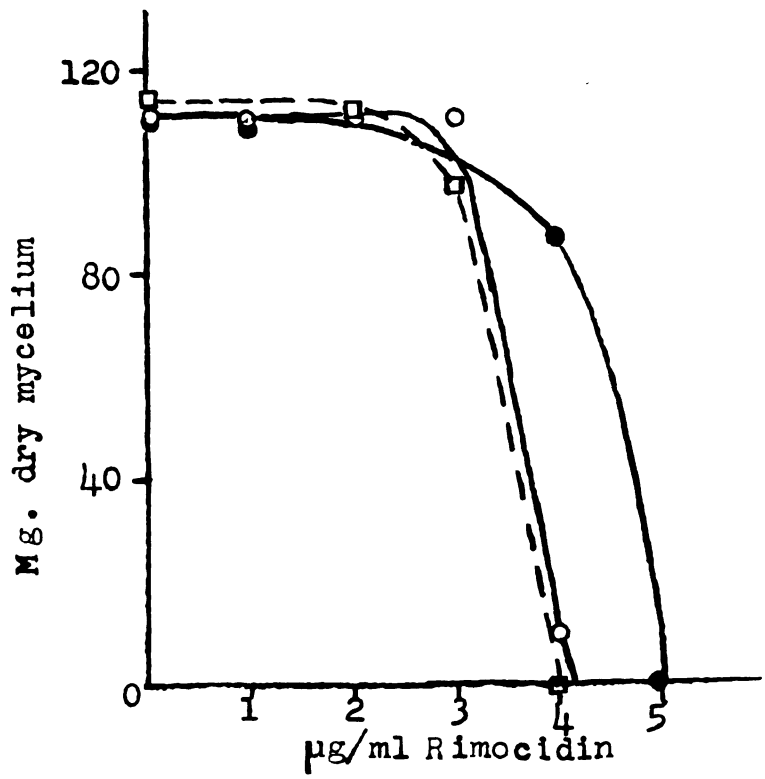
1. The Effects of Different Sugars on Antifungal Activity

The results whereby varying concentrations of the antibiotic rimocidin were used in conjunction with four different sugars are presented in Graph II. The antibiotic activity rate was markedly increased when D-glucose or sucrose was used in comparison to D-xylose or alpha-lactose. The differences in incubation times were due to temperature difficulties. The glucose and sucrose phases were conducted at the same time under temperatures of 28°-30° C. and therefore were harvested at the end of four days which was the original schedule. In the case of the xylose and lactose run, also conducted simultaneously, the temperature in the incubation

GRAPH I

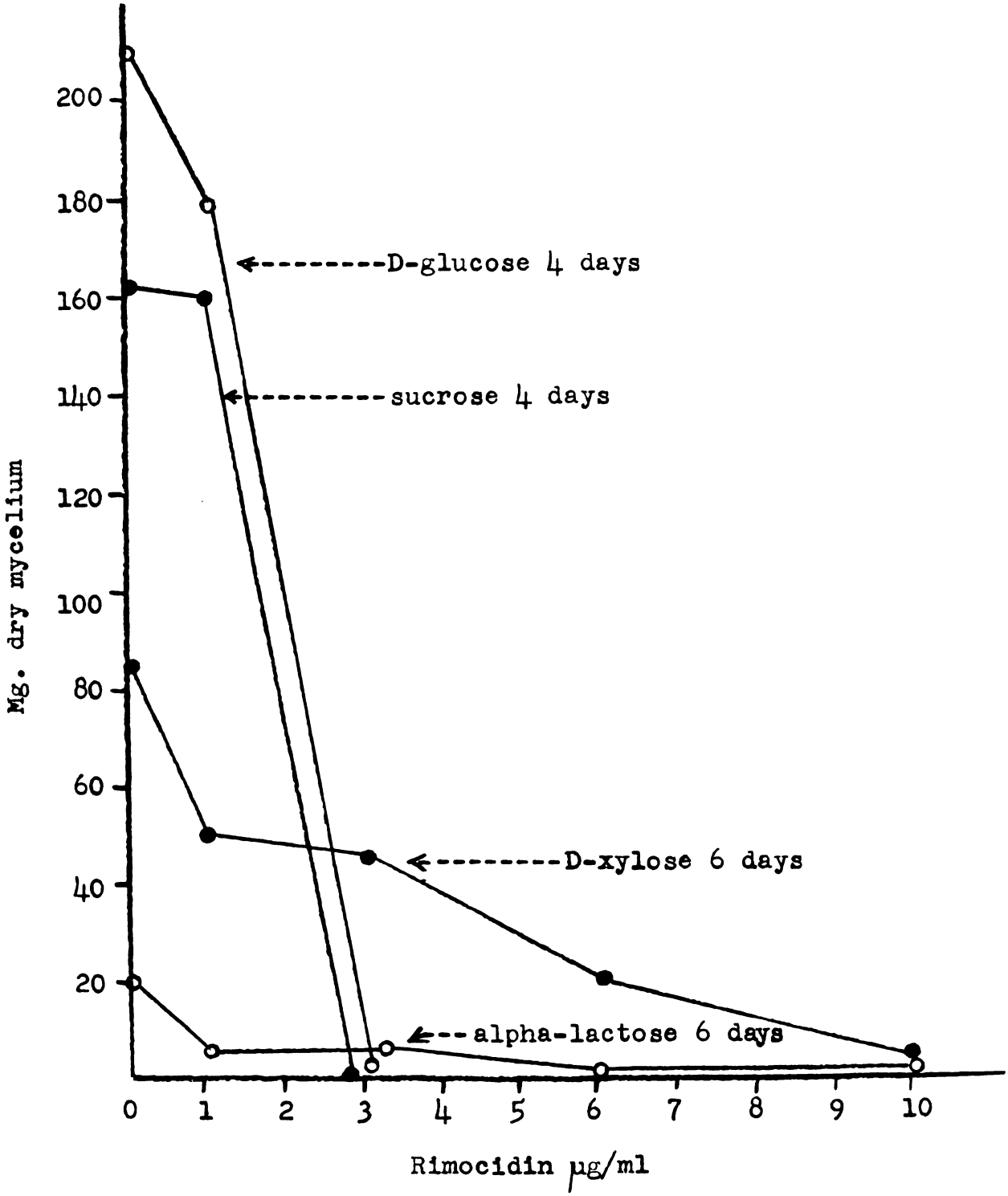
THE AMOUNT OF GROWTH OF C. PHOMOIDES C80A IN THE PRESENCE OF NA PYRIDINETHIONE OR RIMOCIDIN WHICH HAVE BEEN SUBJECTED TO DIFFERENT STERILIZATION TREATMENTS*





GRAPH II

RESPONSE OF C. PHOMOIDES C80A TO INCREASING CONCENTRATIONS OF RIMOCIDIN IN CONJUNCTION WITH DIFFERENT SUGARS SHAKE CULTURES[1]



[1]Inoculum obtained from PDA slant cultures.
D-glucose and sucrose incubated at 28-30° C.
D-xylose and alpha-lactose incubated at temperatures ranging up to 33° C.

room rose to 33° C. At this high temperature, the organism was retarded and hence required a longer incubation period. The harvest time of six days was arbitrarily set in the latter case.

2. The Protective Effect of D-Glucose and Sucrose on Rimocidin

Shake flask cultures of C. phomoides C80A involving a sucrose and a D-glucose series at 6 and 10 µg/ml. of rimocidin each were maintained over a 38-day period. From the data presented in Text Figure 1, it is seen that when the organism was incubated in a glucose medium, growth was evident in nine days when the concentration of rimocidin was six µg/ml. and at 15 days when at 10 µg/ml. In the sucrose medium, highly retarded growth was observed at the end of 27 days at six µg/ml. and total inhibition was still maintained at the 10 µg/ml. level at the end of 38 days at which time the experiment was terminated.

PROTECTIVE EFFECTS OF TWO SUGARS ON THE STABILITY OF RIMOCIDIN TESTED AGAINST C. PHOMOIDES C80A[1]

Rimocidin in µg/ml.	Sugar	Incubation in Days				
		9	15	22	27	38
6	D-glucose	growth				
10	D-glucose	-	growth			
6	Sucrose	-	-	-	growth	
10	Sucrose	-	-	-	-	-

[1]Recorded as the incubation time required for the appearance of growth.

Text Figure 1

D. Antimicrobial Properties of Sodium Pyridinethione

1. Phenol Coefficient Against *Micrococcus pyogenes* var. *aureus*

The phenol coefficient for an aqueous solution of sodium pyridinethione was evaluated against *Micrococcus pyogenes* var. *aureus* in conformance with the U. S. Food and Drug Administration procedure (49). In addition, a Shippen's modification was run whereby the test compound was removed from the bacteria via dilution. This was done to determine whether the inhibition was due to bacteriostatic or bacteriocidal properties of the sodium pyridinethione. The phenol coefficient of sodium pyridinethione for *Micrococcus pyogenes* var. *aureus* was found to be 43 but the Shippen's modification showed the compound to be bacteriostatic, since growth ensued after removal of the test compound.

2. Spore Germination Tests*

Spore germination tests were set up for a 24-hour period employing the glass slide technique (5) in which the test tube dilution method was used with distilled water as the suspending medium. *Colletotrichum phomoides* C80A, *Colletotrichum lagenarium*, *Helminthosporium carbonum* and *Puccinia sorghi* were used as the test organisms because of the difference of cell size and wall thickness between species.

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*The spore germination studies were conducted jointly with Mr. Clare B. Kenaga of the Dept. of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan.

Dosage response curves produced by sodium pyridinethione against three organisms are presented in Graph III. The dosage response curves for C. phomoides C80A and C. lagenarium were closely parallel to one another, as might be expected with two similar organisms.

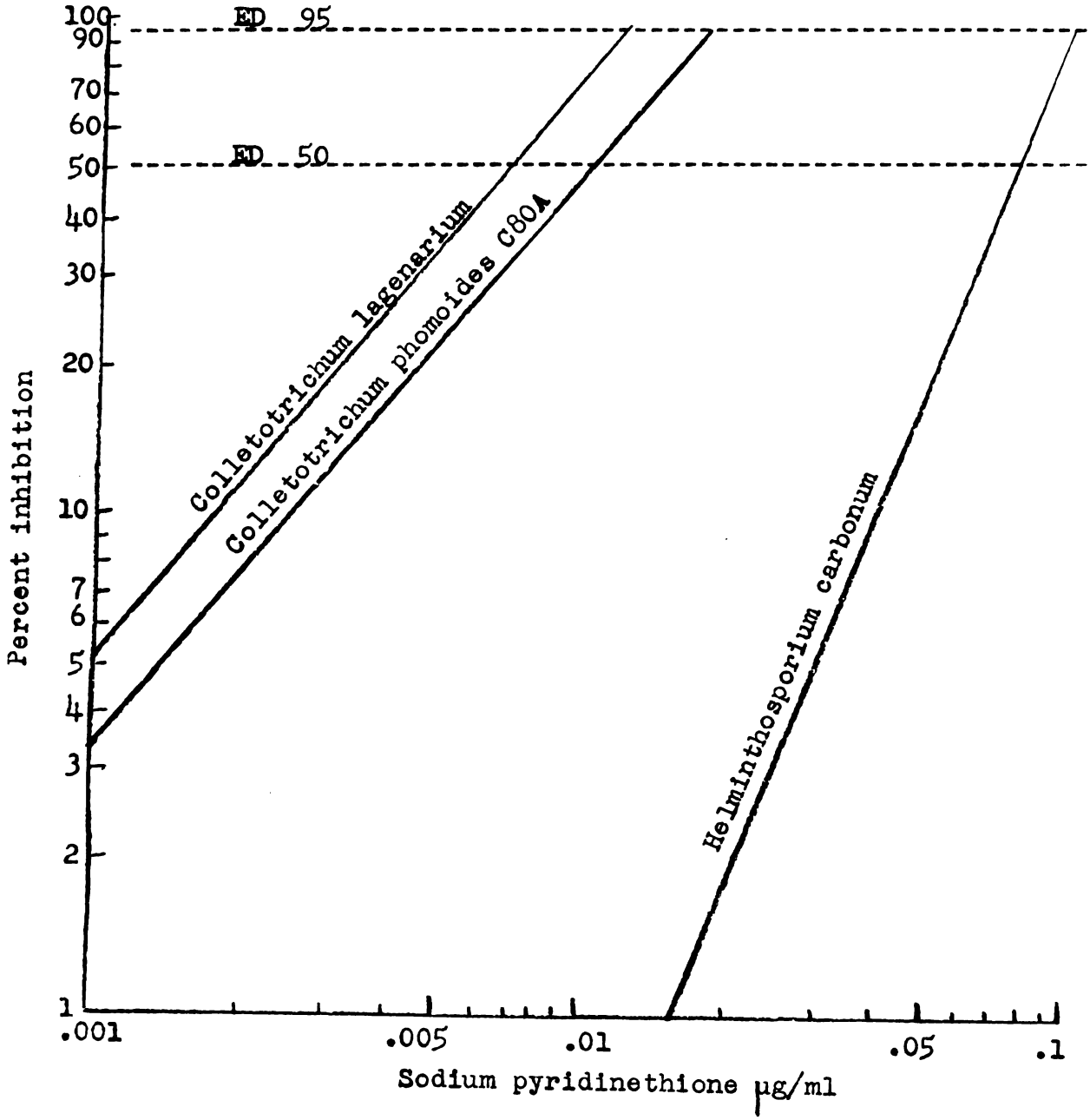
Plates V, VI and VII show the effect of sodium pyridinethione on the spores of C. phomoides C80A, H. carbonum and P. sorghi. Increasing sub-lethal concentrations of the test material induced marked changes in germ tube formation. This was especially evident in the case of H. carbonum, where 0.05 µg/ml. of the inhibitor caused vesicle formation at the end of the germ tube. The response of C. phomoides C80A was quite dramatic, since as little as 0.005 µg/ml. of sodium sodium pyridinethione induced retardation in germ tube development and total inhibition occurred at 0.05 µg/ml. To sum up, the concentrations of sodium pyridinethione in µg/ml required to completely inhibit spore germination for 24 hours in distilled water at ca. 25° C. were as follows: C. phomoides C80A, 0.05; C. lagenarium, 0.05; H. carbonum, 0.10 and Puccinia sorghi, 0.05.

3. Fungicidal Property

The preceding spore germination tests yielded an index of inhibitory activity on the part of sodium pyridinethione but the results are limited in value. Although inhibition

GRAPH III

DOSAGE RESPONSE CURVES FOR SODIUM PYRIDINETHIONE
BASED ON SPORE GERMINATION



Logarithmic: three by three inch cycles

PLATE V

Spore germination test. The effect of sodium pyridinethione on spores of Colletotrichum phomoides C80A. Twenty-four hour incubation at 25° C.

Fig. 1. Control. Ca. x 675.

Fig. 2. 0.005 µg/ml. sodium pyridinethione. Ca. x 675.

Fig. 3. 0.01 µg/ml. " " "

Fig. 4. 0.05 µg/ml. " " "

PLATE V

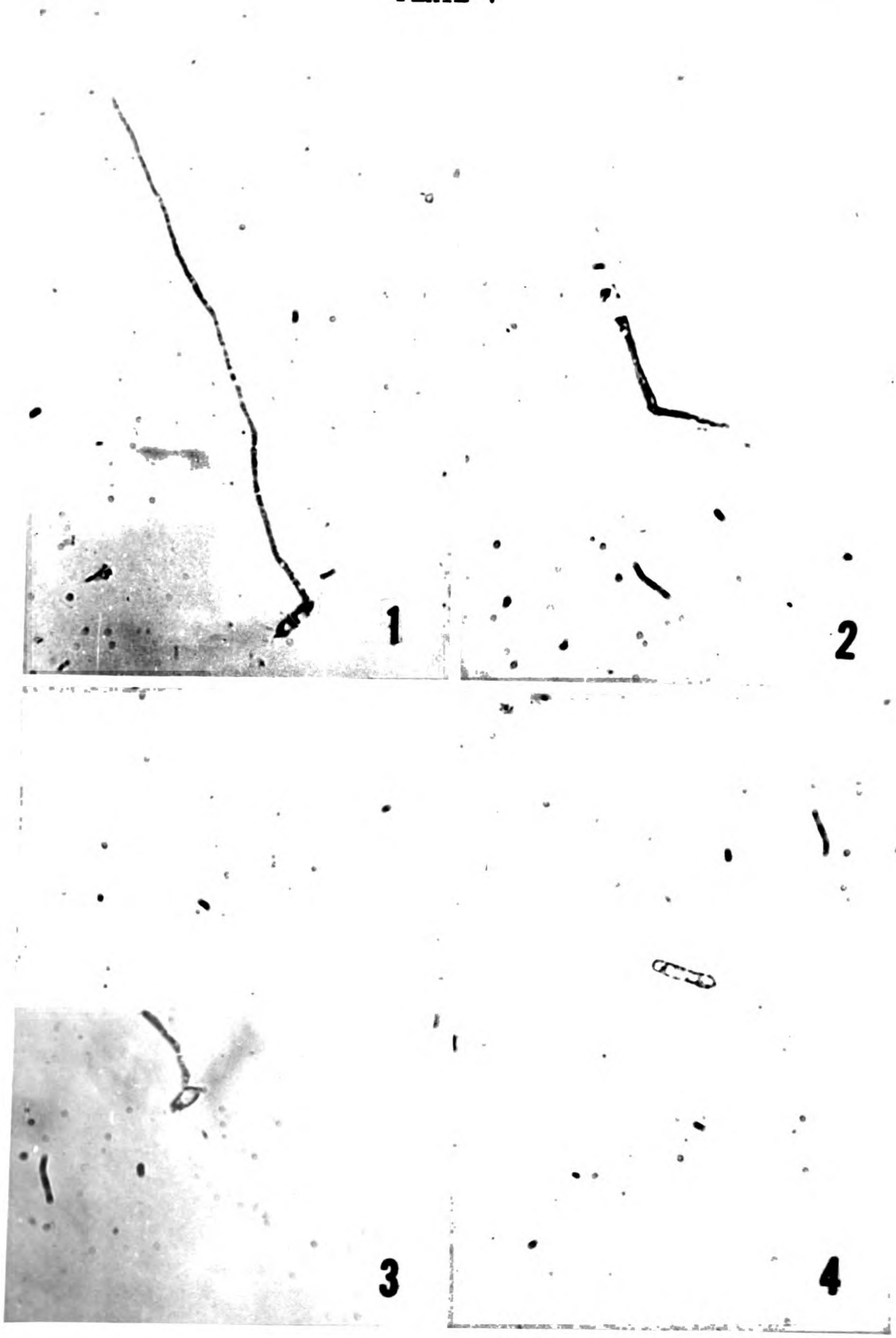


PLATE VI

Spore germination test. The effect of sodium pyridinethione on spores of Helminthosporium carbonum. Twenty-four hour incubation at 25° C.

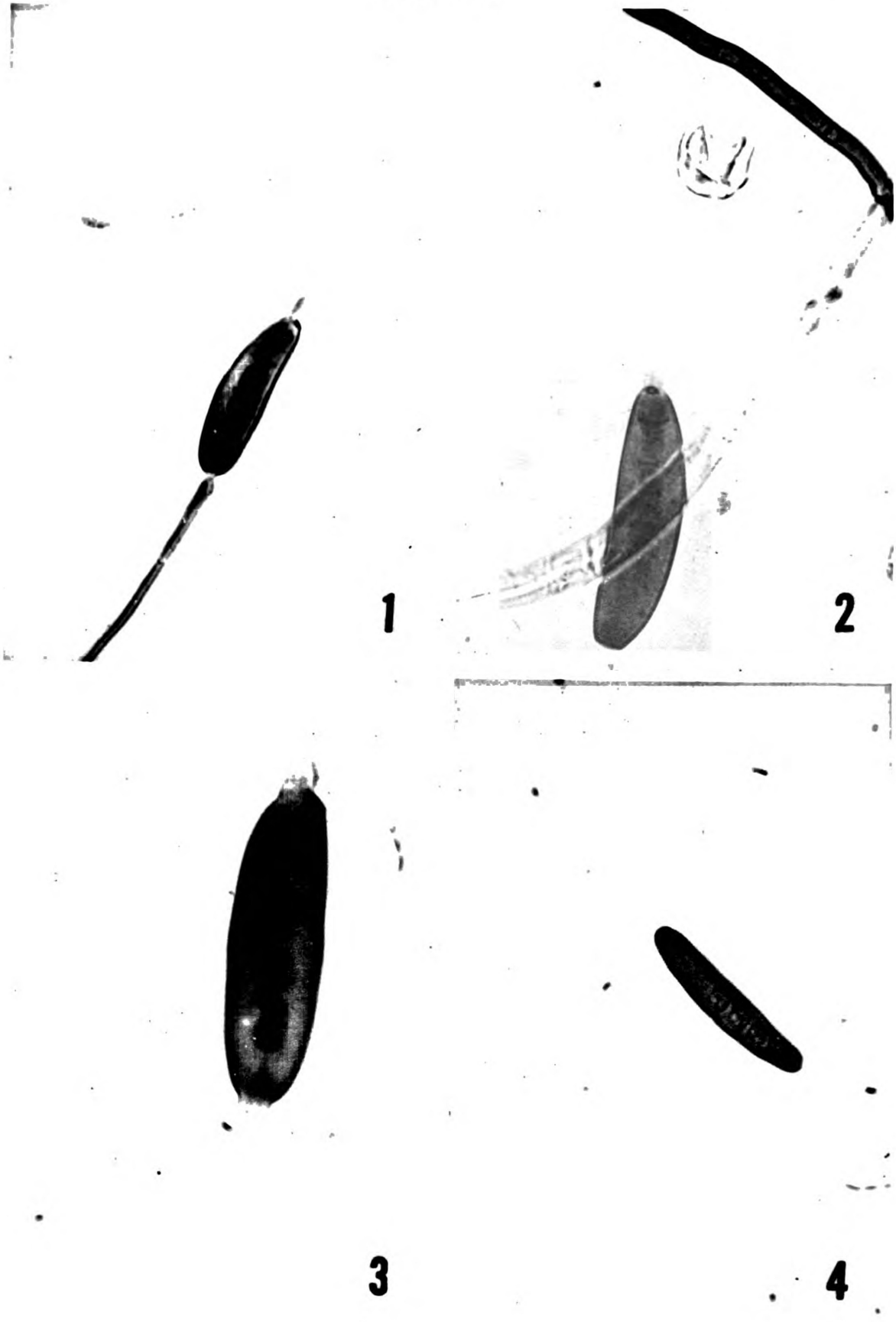
Fig. 1. Control. Ca. x 525.

Fig. 2. 0.05 µg/ml. sodium pyridinethione. Ca. x 1,000.

Fig. 3. 0.1 µg/ml. " " "

Fig. 4. 1.0 µg/ml. " " Ca. x 525.

PLATE VI



1

2

3

4

,000.

25.

PLATE VII

Spore germination test. The effect of sodium pyridinethione on uredospores of Puccinia sorghi. Twenty-four hour incubation at 25° C.

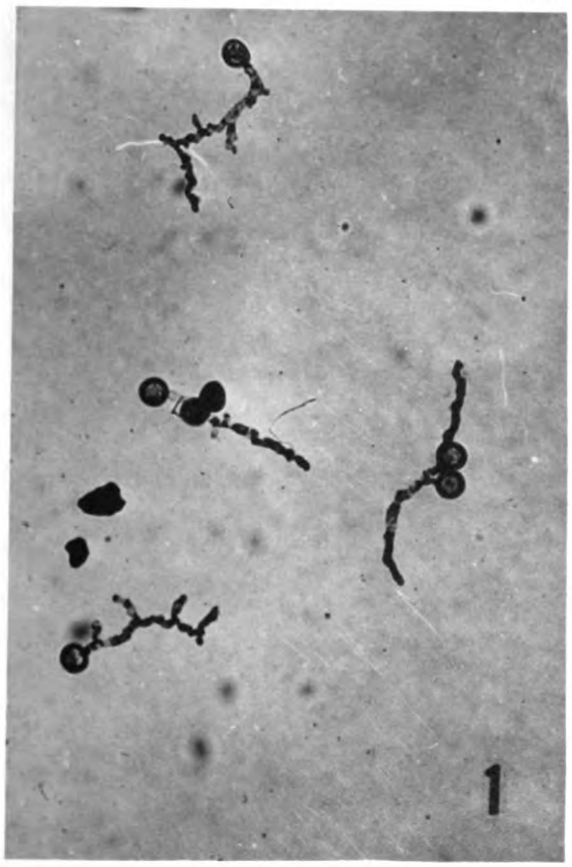
Fig. 1. Control. Ca. x 150.

Fig. 2. 0.01 µg/ml. sodium pyridinethione. Ca. x 150.

Fig. 3. Control. Ca. x 640.

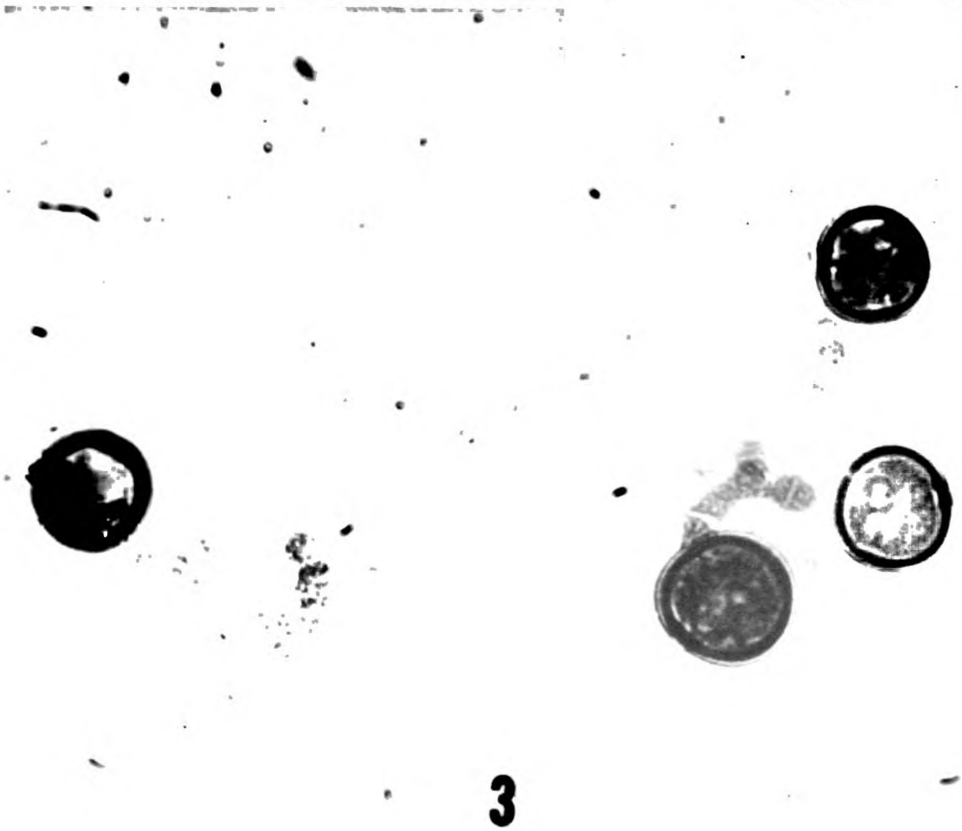
Fig. 4. 0.01 µg/ml. sodium pyridinethione. Ca. x 640.

PLATE VII



1

2



3

4

occurred at very low concentrations, it was not possible to deduce whether the action was fungistatic or whether the spores were actually killed. Therefore, in order to test the fungicidal capacity of sodium pyridinethione, an experiment was designed whereby the spores of C. phomoides C80A were allowed to remain in contact with varying amounts of the test compound for two periods of time after which, the viability was determined. The following procedure was employed:

a. Seitz filtered aqueous dilution series of sodium pyridinethione were prepared ranging in concentration from 0.1-1,000 µg/ml.

b. Washed spores of C. phomoides C80A were standardized so that there would be 300,000 spores per ml. of test compound (this is 100 times greater than the concentration of spores used as inoculum in the physiological studies).

c. After exposure times of 20 minutes and 24 hours at 26° C., three ml. portions of the sodium pyridinethione and spore mixtures were aseptically removed and centrifuged at 300X gravity in an International Centrifuge, Size 1, Model SBV for ten minutes. The supernatant was discarded and the remaining spores washed three times with five ml. portions of sterile distilled water and centrifuged each time. The final centrifuged spore mass was suspended in three ml. sterile distilled water, mixed and one drop deposited on a Petri plate containing the standard glucose asparagine agar medium.

McCallan and Wellman (38) used a similar technique, the major difference being that they employed a 20-second, centrifuged wash period. Since the conidia of C. phomoides are small in mass, longer centrifuge times were used in order to bring down the spores. The results as tabulated in Text Figure 2 indicate that after a 20 minute exposure, fungicidal

action occurred at greater than 100 but less than 1,000 µg/ml. On the other hand, after a 24-hour exposure, the spores of C. phomoides C80A were virtually all killed at the lowest concentration tested namely 0.1 µg/ml. and 100 percent kill took place between 1.0 and 10.0 µg/ml. Plate VIII shows the comparison of growth between the controls and the spores treated at sub-lethal concentrations. These results confirm the observations made in shake cultures from which only a few fragmented spores were recoverable at the 0.32 µg/ml concentration level after a two-day period. In this experiment, all the spores were killed upon a 20 minute exposure to concentrations of sodium pyridinethione greater than 100 µg/ml. These spores were still intact however, when examined microscopically. On the other hand, at the end of the 24 hour exposure time, spores in contact with as little as 0.1 µg/ml. of sodium pyridinethione were seen microscopically to be in a state of disintegration. At concentrations above 10 µg/ml., no spores were visible although much debris was seen microscopically.

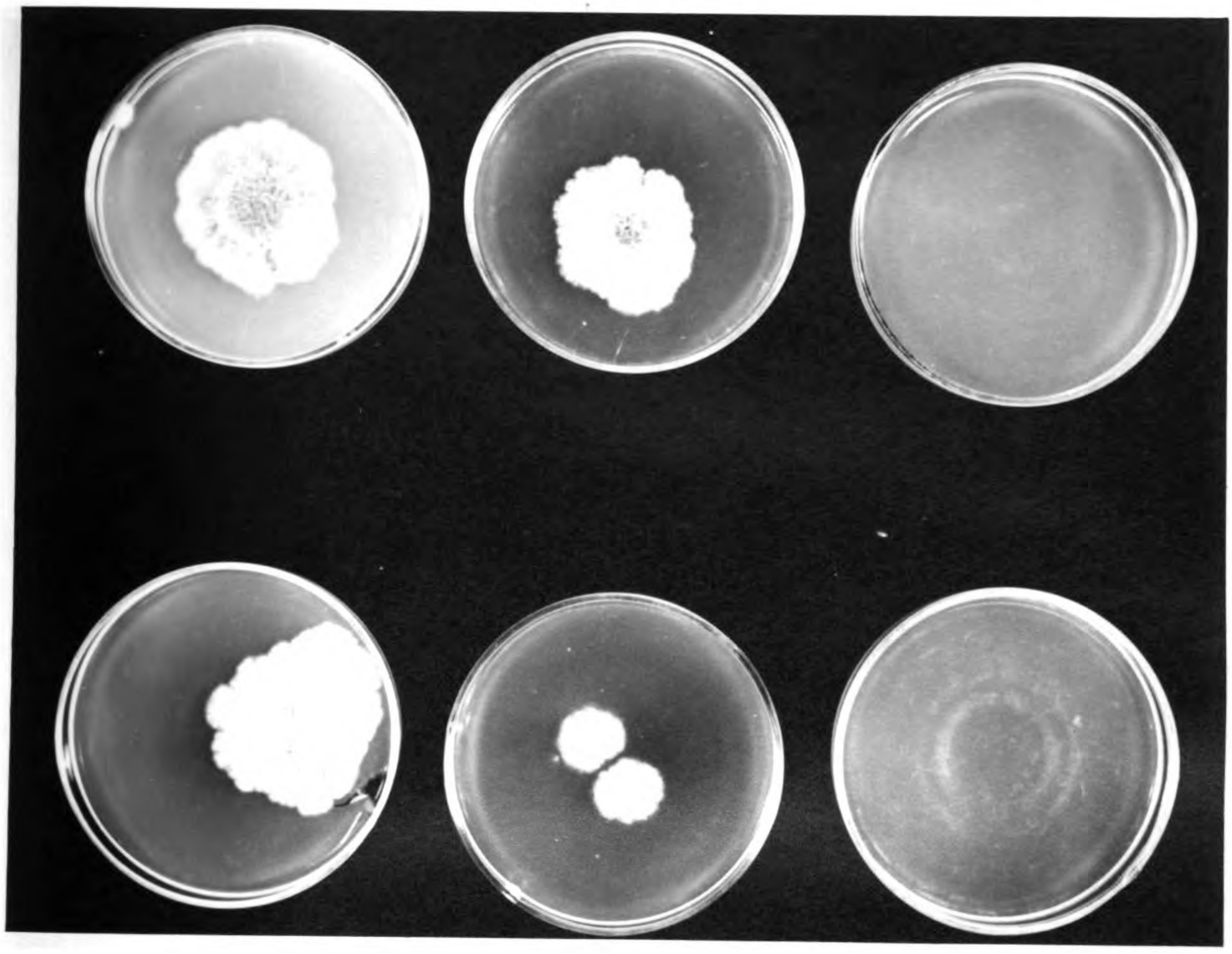
FUNGICIDAL PROPERTIES OF SODIUM PYRIDINETHIONE ON THE SPORES OF C. PHOMOIDES C80A[1]

Inhibitor Removed After Contact Time For	Sodium Pyridinethione µg/ml					
	0	0.1	1.0	10	100	1,000
20 minutes	+++	+++	+++	+++	+++	-
24 hours	+++	+	+	-	-	-

[1]Inhibitor removed by washing the spores three times with distilled water. One drop of washed spore suspension was placed on glucose asparagine agar and incubated for five days at 26° C. Growth recorded as +.

Text Figure 2

PLATE VIII



Fungicidal test for sodium pyridinethione.

The inhibitor was removed after finite contact times and the treated spores of C. phomoides C80A plated out on glucose asparagine agar for five days at 26° C.

Top row: 20 minute exposure

left to right: control, 100 µg/ml. and 1,000 µg/ml.

Bottom row: 24 hour exposure

left to right: control, 1.0 µg/ml. and 10 µg/ml. At 1.0 µg/ml., only a few spores germinated, to yield 2 small colonies.

E. Physiological Studies with Sodium Pyridinethione

1. The Effect of Varying the Carbohydrates

Four different carbon sources were investigated separately in conjunction with varying concentrations of the test compound to determine how nutritive environmental conditions might influence antifungal activity towards a phytopathogenic fungus such as C. phomoides C80A. These results are presented in Table VII. In addition, the data have been plotted with the weight of the dry mycelium against the concentration of sodium pyridinethione. Graph IV shows that sucrose was the most effective sugar in enhancing the antifungal property of the test compound. At 0.02 µg/ml., growth was inhibited 82 percent and total inhibition took place at slightly over 0.03 µg/ml. This corresponds quite well with the spore germination tests wherein the spores of C. phomoides C80A were totally inhibited at 0.05 µg/ml. of an aqueous solution of sodium pyridinethione. C. phomoides C80A does not utilize alpha-lactose to any extent[1] and was not markedly affected by increasing concentrations of sodium pyridinethione. The general shapes of the curves for both D-glucose and D-xylose are similar at the lower portion with respect to the slopes. A sharp drop in growth is evident up to 0.12 µg/ml. and thereafter, a more gradual decrease

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[1]This agrees with the results obtained by Lilly and Barnett (37).

TABLE VII

RESPONSE OF C. PHOMOIDES C80A TO INCREASING CONCENTRATIONS OF SODIUM PYRIDINETHIONE IN CONJUNCTION WITH DIFFERENT SUGARS SHAKE CULTURES INCUBATED FOR SIX DAYS[1]

Na Pyridinethione in µg/ml.	D-glucose		Sucrose		D-Xylose[2]		∝-Lactose[2]	
	mg.[3]	pH[4]	mg.	pH	mg.	pH	mg.	pH
0.00	200	7.8	145	7.6	220	7.4	39	8.5
0.02	199	7.7	27	6.1	-	-	-	-
0.04	212	7.6	+1	6.1	170	7.0	10	7.7
0.08	193	7.4	0	6.1	105	6.8	3	7.0
0.12	50	6.1	0	6.1	55	6.5	+1	6.0
0.32	0	6.1	0	6.1	9	5.6	0	6.0

[1] D-glucose and sucrose incubated at 22-28° C. D-xylose and alpha-lactose incubated at ca. 25° C.

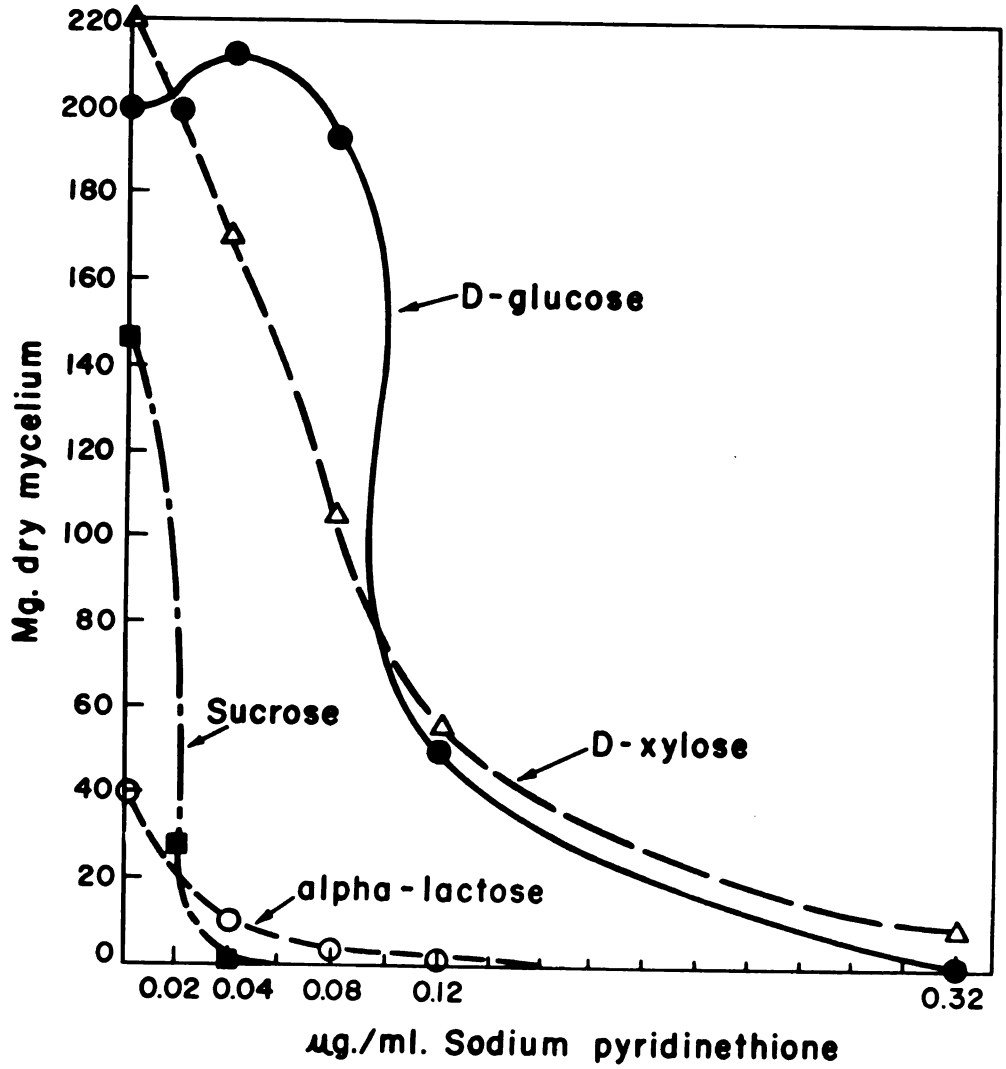
[2] Spore inoculum obtained from PDA slant cultures.

[3] Weight of dry mycelium.

[4] pH of filtrate at the time of mycelial recovery.

GRAPH IV

RESPONSE OF C. PHOMOIDES C80A TO INCREASING CONCENTRATIONS OF SODIUM PYRIDINETHIONE IN CONJUNCTION WITH DIFFERENT SUGARS SHAKE CULTURES INCUBATED FOR SIX DAYS[1]



[1] D-glucose and sucrose incubated at 22-28° C.
D-xylose and alpha-lactose incubated at ca. 25° C.
spore inoculum obtained from PDA slant cultures.

occurred until at 0.32 µg/ml., the organism was totally inhibited. In the glucose series, the cultures in the control flasks and those containing 0.02 µg/ml. sodium pyridinethione showed signs of autolysis. This was evidenced by a darkening of the medium and the mycelial pellets. The latter also appeared to be partially fragmented. Autolysis of C. phomoides C80A is accompanied by an increase in pH which is evident in Table VII and is more noticeable in Table XIII. No autolysis was observed in the other flasks in the glucose series containing concentrations of sodium pyridinethione greater than 0.02 µg/ml. Thus, the apparent stimulation in the glucose curve in Graph IV is erroneous.

2. The Effect of Sugar Concentration on the Antifungal Property of Sodium Pyridinethione

Medium constituents in some way influence the antifungal activity of sodium pyridinethione. This is based on the observation that in the presence of the Lilly and Barnett basal medium to which sucrose has been added as the carbon source, the test compound was very effective causing complete inhibition at slightly over 0.04 µg/ml. This is the same MIC obtained in germination tests of spores of C. phomoides C80A, with the washed spores, sodium pyridinethione and distilled water as the only materials present.

The sugars D-glucose, D-xylose and alpha-lactose, on the other hand apparently had some effect on the activity of the compound since 0.32 µg/ml. was required to induce total inhibition. Further investigations were carried out by adding increasing concentrations of either D-glucose or sucrose to the medium containing a standard concentration of sodium pyridinethione. This was done to see whether an increased concentration of these two sugars may have any additional effect on the antifungal activity of sodium pyridinethione.

The standard Lilly and Barnett glucose asparagine medium contains 10 g. glucose/liter which is equivalent to 4 g. available carbon. The concentrations of D-glucose and of sucrose used in this experiment were equal to 1/2, one, two and three times the amount of available carbon found in the standard medium. Employing routine procedures, the inoculated flasks were incubated in a stationary position for nine days at 24° C. and the mycelial dry weights evaluated. Percent inhibition by sodium pyridinethione was calculated the following way:

$$1 - \frac{A}{B} \times 100 = \text{percent inhibition}$$

where A is the dry mycelial wt. with Na pyridinethione
and B is " " " " without "

In order better to evaluate the sugar to inhibitor relationship, molar ratios were employed. The empirical formula of the sodium salt of 1-hydroxy-2(1H) pyridinethione

is C_5H_4NNaOS (54) and hence the molecular weight (MW), excluding degree of purity, is 149. Disregarding the salt formation by sodium, the MW is 127, thus involving a factor of 1.2 in determining molar concentrations of pyridinethione when the sodium salt is used. Since the variation of sugar concentration is based on the standard concentration of four g. of carbon per liter, the amount of sodium pyridinethione to be used was chosen by the inspection of Graph IV. Desiring a concentration that falls in a good working range, the following amounts of sodium pyridinethione[1] were chosen:

For glucose: 0.12 µg/ml. medium, equivalent to 0.1 µg/ml. medium (8×10^{-7} M) pyridinethione.

For sucrose: 0.02 µg/ml. medium, equivalent to 0.017 µg/ml. medium (1.34×10^{-7} M) pyridinethione.

The results as tabulated (Tables VIII and IX) show that increasing the concentration of D-glucose while maintaining the same concentration of the test compound served to inactivate progressively the antifungal capacity of the sodium pyridinethione. On the other hand, increasing the concentration of sucrose had no effect on either diminishing or increasing the antifungal properties of sodium pyridinethione. Not related to the pyridinethione study but interesting nevertheless, was the observation that in the control flasks, C. phomoides C80A grew best when the sugar concentrations were between one and two percent (D-glucose 11.2 $\times 10^{-2}$ M and sucrose 5.6×10^{-2} M). At sugar concentrations

[1]Lot no. Py-354-75c, for explanation see page 8.

TABLE VIII

THE INFLUENCE OF D-GLUCOSE CONCENTRATION ON THE ANTIFUNGAL ACTIVITY OF PYRIDINETHIONE[1]

Glucose Conc. x 10^{-2} M	Minus Pyridinethione		With Pyridinethione (8×10^{-7} M)		I/Glucose[3] Molar Ratio	Percent Inhibition
	Dry Mycelium mg.	pH[2]	Dry Mycelium mg.	pH[2]		
2.8	97	7.7	< 1	5.5	2.86×10^{-5}	+99
5.6[4]	191	6.9	45	4.8	1.44×10^{-5}	76
11.2	329	5.5	197	5.1	7.14×10^{-6}	40
16.8	368	5.3	274	5.3	4.76×10^{-6}	26

[1] Tested against C. phomoides C80A, stationary cultures incubated 9 days at 24° C.

[2] pH of filtrate at the time of mycelial recovery.

[3] I symbolizes the inhibitor pyridinethione.

[4] Equivalent to four g. of carbon per liter (the standard conc. used in Lilly and Barnett's synthetic glucose asparagine medium).

TABLE IX

THE INFLUENCE OF SUCROSE CONCENTRATION ON THE ANTIFUNGAL ACTIVITY OF PYRIDINETHIONE[1]

Sucrose Conc. x 10^{-2} M	Minus Pyridinethione		With Pyridinethione (1.34×10^{-7} M)		I/Sucrose[3] Molar Ratio	Percent Inhibition
	Dry Mycelium mg.	pH[2]	Dry Mycelium mg.	pH[2]		
1.4	106	8.0	32	6.1	9.6×10^{-6}	70
2.8[4]	220	7.3	32	5.8	4.8×10^{-6}	85
5.6	278	6.0	81	6.0	2.4×10^{-6}	71
8.3	291	5.2	88	5.8	1.6×10^{-6}	70

[1] Tested against C. phomoides C80A, stationary cultures incub. 9 days at 24° C.

[2] pH of filtrate at the time of mycelial recovery.

[3] I symbolizes the inhibitor pyridinethione.

[4] Equivalent to four g. of carbon per liter (the standard conc. used in Lilly and Barnett's synthetic glucose asparagine medium).

of two percent and greater, the fungus produced acid(s) in contrast to the more alkaline conditions normally observed.

3. Decreased Antifungal Activity in the Presence of Acetaldehyde

On the basis of the preceding results showing inactivation of sodium pyridinethione in the presence of glucose but not in the presence of sucrose, it was thought that the reducing properties of glucose might be responsible for the decreased antifungal effect. With the exception of sucrose, the sugars that were examined (D-glucose, D-xylose and alpha-lactose) are all reducing sugars. In other words, they possess a free carbonyl or aldehyde group. If this in some way inactivates pyridinethione, it should be possible to add a substance such as acetaldehyde to a medium containing sucrose and sodium pyridinethione and cause an inactivation of the inhibitor. A series of experiments was set up whereby twice the concentration of sodium pyridinethione normally required to induce total inhibition was added to flasks containing the basal medium and sucrose. To a portion of these flasks, acetaldehyde was introduced at the same molar concentration as D-glucose (5.6×10^{-2} M).

The results indicate that acetaldehyde does inactivate sodium pyridinethione to some extent. While it is not as active as D-glucose the data presented in Table X show that,

TABLE X

SODIUM PYRIDINETHIONE INACTIVATION BY ACETALDEHYDE
C. PHOMOIDES C80A STATIONARY CULTURES
INCUBATED AT CA. 25° C.

Treatment		Amount of Growth In				
Sugar	Combined With	2 days	5 days	10 days	14 days	16 days
Sucrose	———	+	++	+++	+++	+++
	CH_3CHO	+	++	+++	+++	+++
	Na pyr.	-	-	-	-	-
	Na pyr. + CH_3CHO	-	±	+	++	+++
D-Glucose	———	+	++	+++	+++	+++
	CH_3CHO	+	++	+++	+++	+++
	Na pyr.	-	±	++	+++	+++
	Na pyr. + CH_3CHO	-	+	++	+++	+++

Concentration of constituents:
sucrose: 2.8×10^{-2} M (one percent)
D-glucose: 5.6×10^{-2} M (one percent)
acetaldehyde: 5.6×10^{-2} M
sodium pyridinethione: 0.08 µg/ml.

in the medium containing an acetaldehyde-sucrose mixture, the antifungal activity of sodium pyridinethione was diminished, whereas a high level of activity was maintained in the sucrose medium alone. Thus, acetaldehyde is also capable of reacting in some way with pyridinethione to inactivate its antifungal mechanism. Apparently, this is due to the aldehyde group of the compound, since in this respect, it is the same as the reducing sugars.

These results also showed that, in the presence of D-glucose, C. phomoides C80A was able to overcome the inhibitory effects of sodium pyridinethione. Therefore, the compound is fungistatic and not fungicidal at least under the conditions tested.

4. The Effect of Time on Mixtures of D-Glucose and Sodium Pyridinethione

It is evident from the preceding observations that the antifungal capacity of sodium pyridinethione was reduced when the material was in contact with reducing sugars, specifically D-glucose, or acetaldehyde. It was not known however, whether the fungus itself played any part in this inactivation (or reversal of activity) or if the inactivation of pyridinethione was due solely to exogenous interactions with the medium constituents. In order to shed light on this problem, a series of experiments was designed to determine

the ultraviolet absorption spectra for mixtures of sodium pyridinethione and D-glucose. In another series, various contact times were allowed, between sodium pyridinethione and certain medium constituents. For the assay of antifungal potency that remained after the various contact periods, the test organism C. phomoides C80A was used.

a. Spectrophotometric determination. Since most materials exhibit definite absorption spectra patterns, a resulting chemical reaction between two materials may often be observed spectrophotometrically by a comparison of the ultraviolet absorption spectra of the reactants and their mixture. Sodium pyridinethione and D-glucose were thus prepared in the same fashion employed in the microbiological studies. The ultraviolet absorption spectra were then determined at varying time intervals for the components as well as for the mixture in an effort to determine any change(s).

The materials and techniques employed were as follows:

(1) Sodium pyridinethione[1] at a concentration of 4 µg/ml. in distilled water (the maximum concentration of 0.32 µg/ml. as used in the physiological studies was too low to be determined spectrophotometrically) was Seitz filtered in order to duplicate the routine procedure. Graph V [A] shows a slightly different pattern for Seitz filtered material as compared to sodium pyridinethione which was not Seitz filtered. The latter absorption spectrum curve more closely duplicates the results obtained by the Squibb Laboratories (54). The Seitz filtered material

[1]Lot no. Py-354-75c, for explanation see page 8 .

behaved as if some acid was present (48) but the pH of the two preparations were about the same since the pH of the distilled water used was 5.7, the pH of sodium pyridinethione (4 µg/ml.) was 6.1 and that of the Seitz filtered sodium pyridinethione (4 µg/ml.) was 6.6

(2) D-glucose at a concentration of nine mg/ml. in distilled water and autoclaved at 240° F. for 15 minutes.

(3) Mixtures of (1) and (2) at double strength of ingredients so as to give the same final concentration as the ingredients alone.

(4) The ultraviolet absorption spectra were determined with the aid of a Beckman Quartz Spectrophotometer, Model DU, employing quartz cuvettes which were calibrated for correction.

(5) The solutions of D-glucose, sodium pyridinethione and the mixtures of the two were stored at 26° C. for varying periods of time before analysis.

(6) The ultraviolet absorption spectra curves were plotted from the data obtained as, optical density (OD) against the wavelength λ (in mµ).

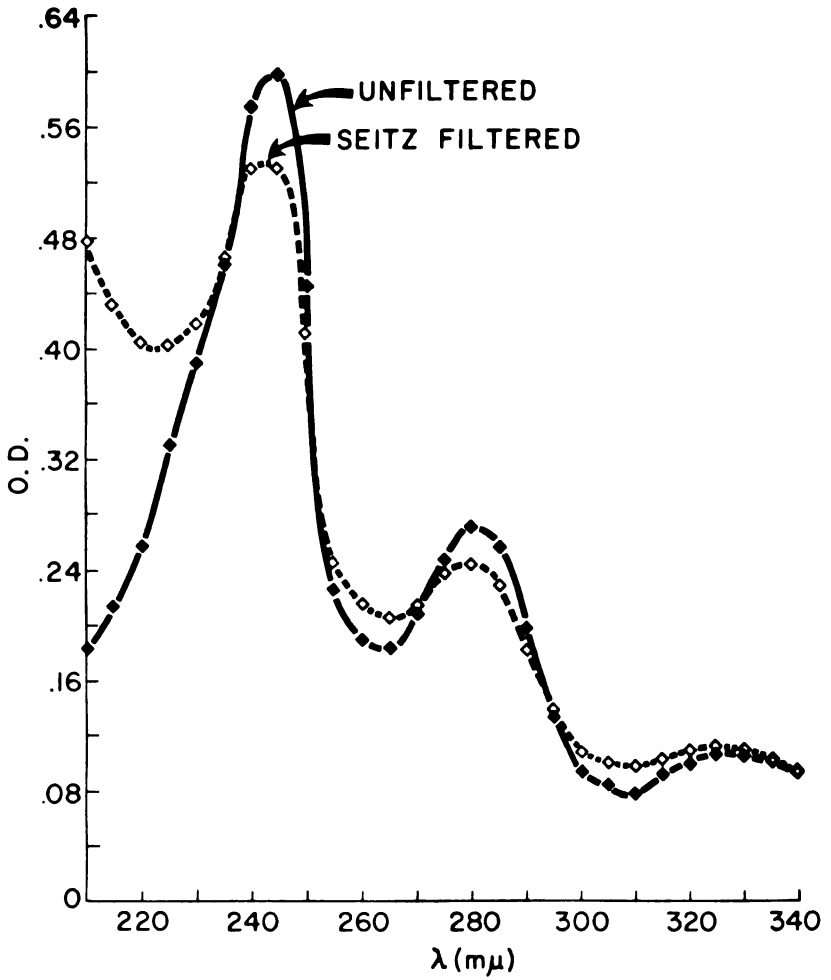
(7) The criterion for determining whether any reaction took place was by comparing the observed ultraviolet absorption spectra of the mixture to the one calculated by adding the spectra of D-glucose and sodium pyridinethione together. If no reaction occurred, then the curves for the observed and the calculated mixtures should be the same.

The results as shown in Graphs V and VI indicated that a reaction occurred in a mixture of sodium pyridinethione and D-glucose. In addition, the reaction was dependent on time. In the instance where the mixture was prepared just at the time of taking the readings, no reaction took place since the observed and the calculated mixture curves

GRAPH V

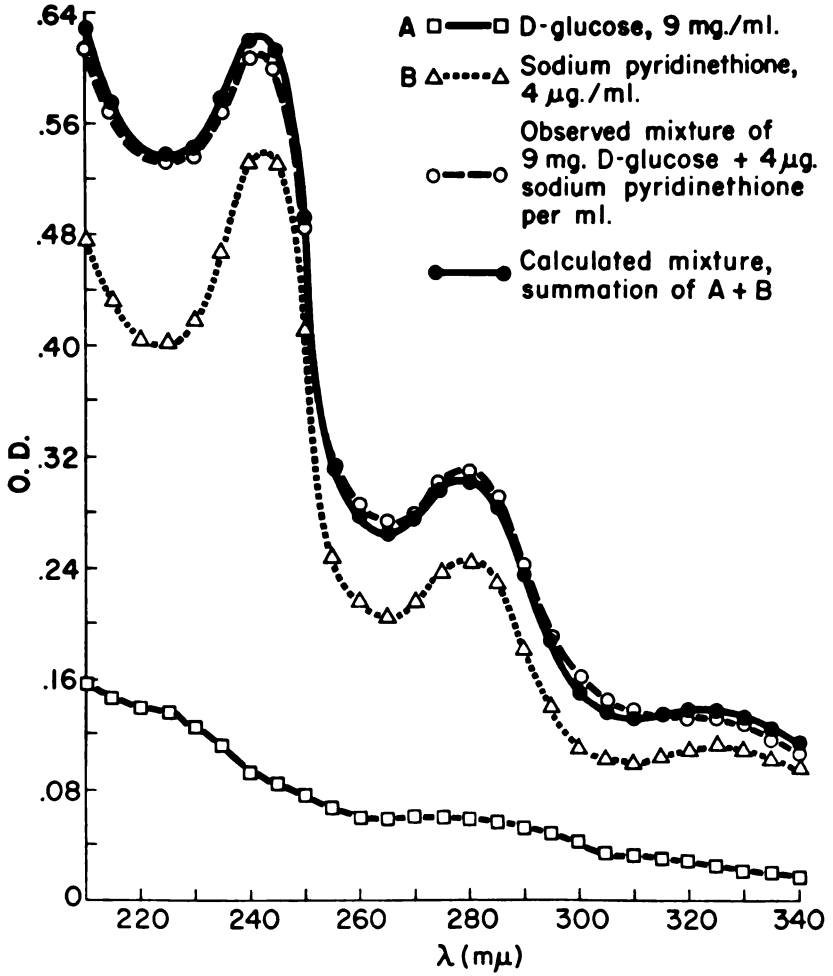
[A]

ULTRAVIOLET ABSORPTION SPECTRA
1-HYDROXY-2(1H)PYRIDINETHIONE, SODIUM SALT
4 μG/ML. DISTILLED WATER KEPT AT 26° C. FOR 12 HOURS



[B]

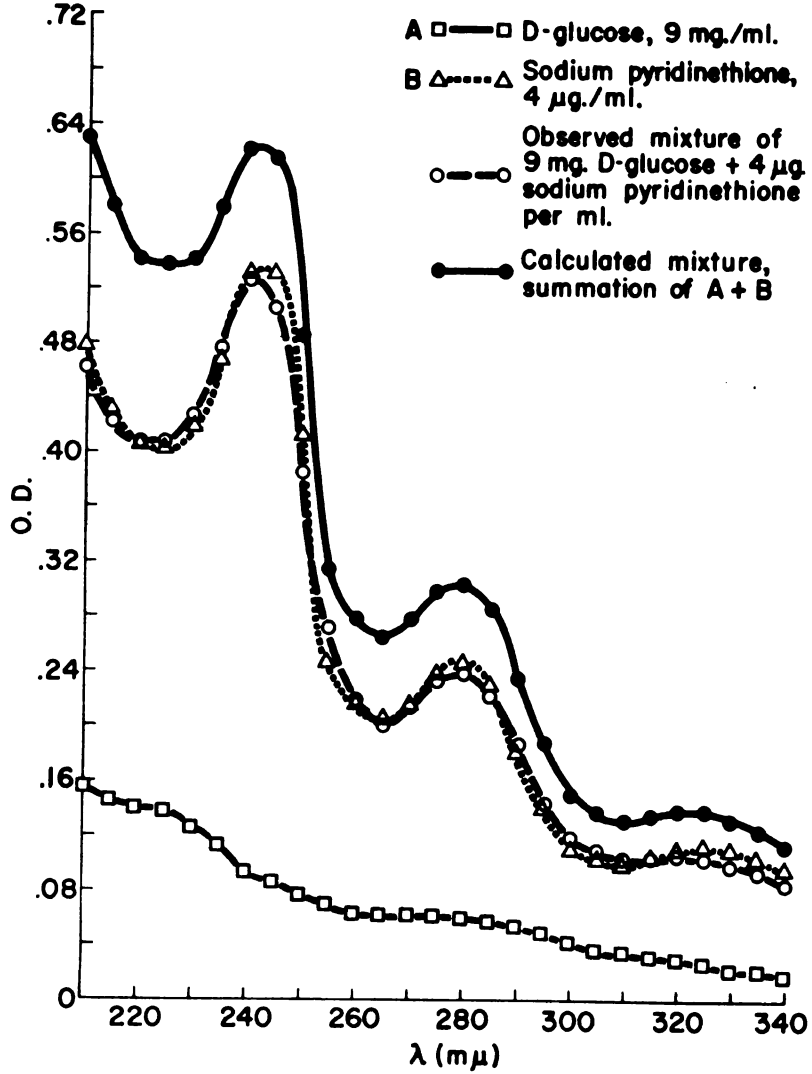
ULTRAVIOLET ABSORPTION SPECTRA
MIXTURE PREPARED IMMEDIATELY BEFORE READING
INGREDIENTS KEPT AT 26° C. FOR 12 HOURS



GRAPH VI

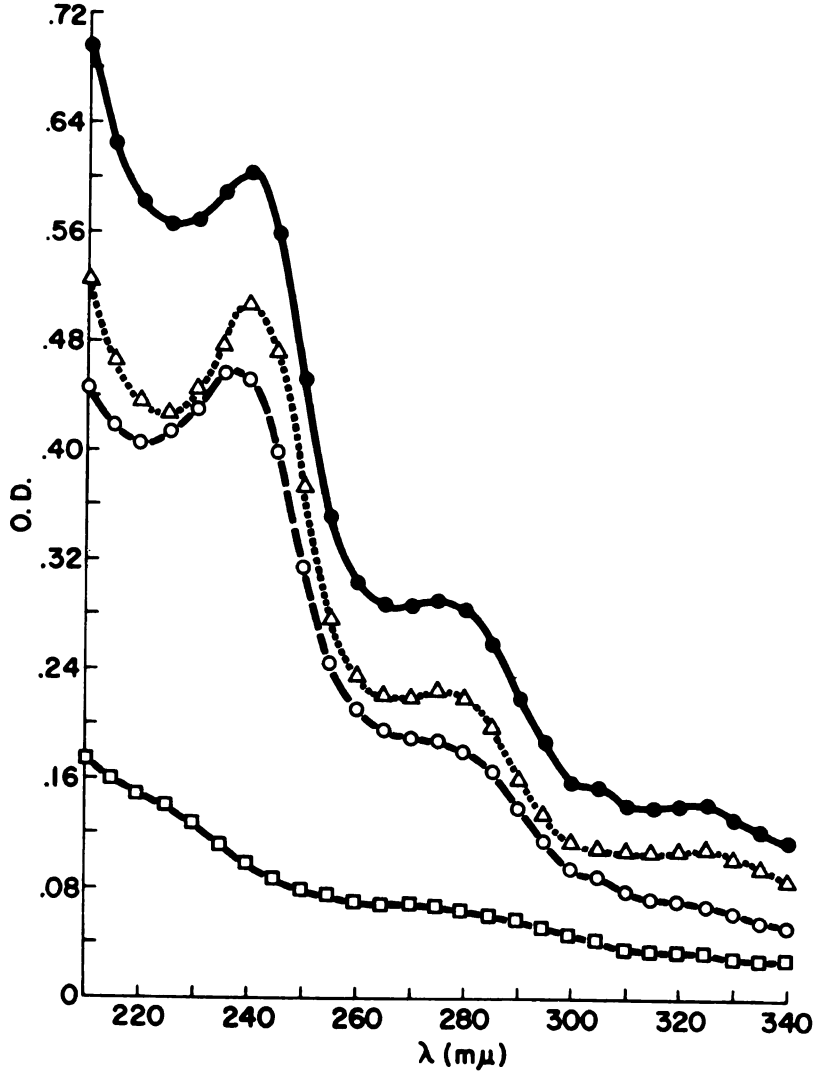
[A]

ULTRAVIOLET ABSORPTION SPECTRA
12 HOUR MIXTURE
INGREDIENTS KEPT AT 26° C. FOR 12 HOURS



[B]

ULTRAVIOLET ABSORPTION SPECTRA
EIGHT DAY MIXTURE
INGREDIENTS KEPT AT 26° C. FOR EIGHT DAYS



were the same (Graph V [B]). On the other hand, when sodium pyridinethione and D-glucose were in contact with one another for a 12-hour period at 26° C., the observed and the calculated mixture curves were not the same (Graph VI [A]). This observed mixture curve was almost exactly the same as the curve for sodium pyridinethione, indicating that the mixture curve was predominantly influenced by the pyridine moiety. An observed eight-day mixture curve (Graph VI [B]) was not significantly different from the 12-hour mixture except that the extinction coefficient has been somewhat further reduced. In addition, it was observed that some change occurred in the pyridinethione solution at the end of eight days since its ultraviolet absorption spectrum curve became somewhat less pronounced when compared to the 12-hour sample of sodium pyridinethione.

b. Bioassay determination. This method, involving the use of C. phomoides C80A, was used to assay the antifungal potency remaining after various contact periods between sodium pyridinethione and certain medium constituents. The standard Lilly and Barnett synthetic glucose asparagine medium was used containing one percent D-glucose (5.6×10^{-2} M and equivalent to four g. C per liter). By basal medium, it is understood that all the constituents were present with the exception of D-glucose. The complete medium indicates the basal plus D-glucose. Four series were set up as follows:

(1) Complete medium (control)

(2) Complete medium plus sodium pyridinethione

(3) Basal medium plus sodium pyridinethione. The D-glucose is added at inoculation time.

(4) Basal medium. Glucose and sodium pyridinethione mixture is added at inoculation time.

Each series was prepared in sufficient replication, each replicate stored in 300 ml. soft glass media bottles with screw caps to offset evaporation, so that three replicates of each series would be inoculated with C. phomoides C80A at the end of the following contact periods: 0, one week, two weeks, three weeks and four weeks.

Inocula were prepared in the routine manner employed for the other physiological studies and concentrations of 3,000 spores/ml. medium were used. All medium constituents were autoclaved at 240° F. for 15 minutes except sodium pyridinethione, which was sterilized via Seitz filtration. Sodium pyridinethione[1] was employed at a concentration of 0.32 µg/ml. medium. All the series, prior to and after inoculation, were maintained at 26° C. The inoculated bottles were incubated for nine days in a stationary position and the results evaluated on the basis of the dry mycelial weights obtained in the routine manner.

The results as presented in Table XI indicate that when sodium pyridinethione was added to the complete glucose asparagine medium, the antifungal activity diminished with

[1]Lot no. Py-354-75c, for explanation see page 8.

TABLE XI

INACTIVATING THE ANTIFUNGAL PROPERTY OF SODIUM PYRIDINETHIONE ON PROLONGED CONTACT WITH D-GLUCOSE TESTED AGAINST *C. PHOMOIDES* C80A[1]

Contact Time Prior To Inoculation (in weeks)	Mg. Dry Mycelium			
	Complete Medium		Basal Medium	
	Control	With Na Pyridinethione[2]	With Na Pyridinethione[2] D-Glucose Added at Time of Inoculation	Mixture of D-Glucose and Na Pyridinethione[2] Added at Inoc. Time
0	183	< 1	0	0
1	185	49	0	0
2	183	98	0	0
3	172	160	0	0
4	176	179	0	0

[1]Stationary cultures incubated for nine days at 26° C.

[2]0.32 µg/ml.

increasing contact time. At the end of four weeks, the compound was completely inactivated. Evaluating the data in another way, the percent inhibition by sodium pyridinethione is presented in Table XII according to the method used on page 51, whereby:

$$1 - \frac{A}{B} \times 100 = \text{percent inhibition}$$

A is the dry mycelial weight with sodium pyridinethione
B " " " " " without " "

When sodium pyridinethione was present in the complete medium at a concentration of 0.32 µg/ml., there was a 26 percent breakdown at the end of one week.

Increased contact time of a mixture containing D-glucose and sodium pyridinethione showed no breakdown according to the bioassay test, even though the ultraviolet absorption spectra analysis indicated that some sort of reaction did occur. Likewise, there was no indication of breakdown when the antifungal material was in contact with the basal medium alone. Hence, on the basis of the bioassay results, the following are suggested:

(1) that the fungus C. phomoides C80A takes no part in the inactivation of sodium pyridinethione, and

(2) that the inactivation of sodium pyridinethione takes place in the presence of aldehyde containing materials such as glucose and also requires some other factor or factors as are present in the basal medium.

TABLE XII

EXTENT OF INACTIVATION WHEN SODIUM PYRIDINETHIONE IS IN CONTACT WITH THE COMPLETE MEDIUM[1]

Contact Time (in weeks)	Percent Inhibition[2]	Percent Inactivation of Na Pyridinethione
0	+99	-
1	73	26
2	46	54
3	7	93
4	0	100

[1] Lilly and Barnett synthetic glucose asparagine medium.

[2] Based on the data in Table XI.

Concentration of D-glucose is 5.6×10^{-2} M.
" " Na pyridinethione is 2.1×10^{-6} M.
I/glucose molar ratio is 3.8×10^{-5}.

5. The Effect of Varying the Nitrogen Source

Ammonium nitrogen as well as nitrate nitrogen sources were investigated separately in conjunction with varying concentrations of the antifungal material. In one series, ammonium chloride was used for the nitrogen source while in another series, sodium nitrate was employed. The results were compared to those obtained with the standard medium containing L-asparagine. The concentration of L-asparagine in the Lilly and Barnett standard medium is equivalent to 0.424 g. nitrogen per liter. Therefore, the concentrations of ammonium chloride and of sodium nitrate were adjusted to contain the same amount of equivalent nitrogen.

Text Figure 3 and Graph VII show that inorganic nitrogen as nitrate or ammonium had no significant effect on the antifungal activity of sodium pyridinethione when compared to one another. However, total inhibition was attained at 0.12 µg/ml. in either case, as compared to 0.32 µg/ml. for asparagine.

C. phomoides C80A does not grow as well in an inorganic nitrogen medium as compared to a medium containing an organic nitrogen source such as asparagine. The organism grew rather poorly when ammonium chloride was used. This was jointly evidenced by a marked drop in pH to 2.6.

RESPONSE OF C. PHOMOIDES C80A TO INCREASING CONCENTRATIONS OF SODIUM PYRIDINETHIONE IN CONJUNCTION WITH DIFFERENT NITROGEN SOURCES
SHAKE CULTURES INCUBATED AT CA. 25° C. FOR SIX DAYS

Na Pyridinethione	Weight of Dry Mycelium					
	$NaNO_3$		NH_4Cl		L-Asparagine	
µg/ml.	mg.	pH[1]	mg.	pH	mg.	pH
0.00	154	6.2	135	2.6	190	7.9
0.04	153	6.8	126	2.6	200	7.7
0.08	11	6.1	35	3.4	193	7.6
0.12	0	6.1	0	6.0	40	6.1
0.32	0	6.1	0	6.0	0	6.1

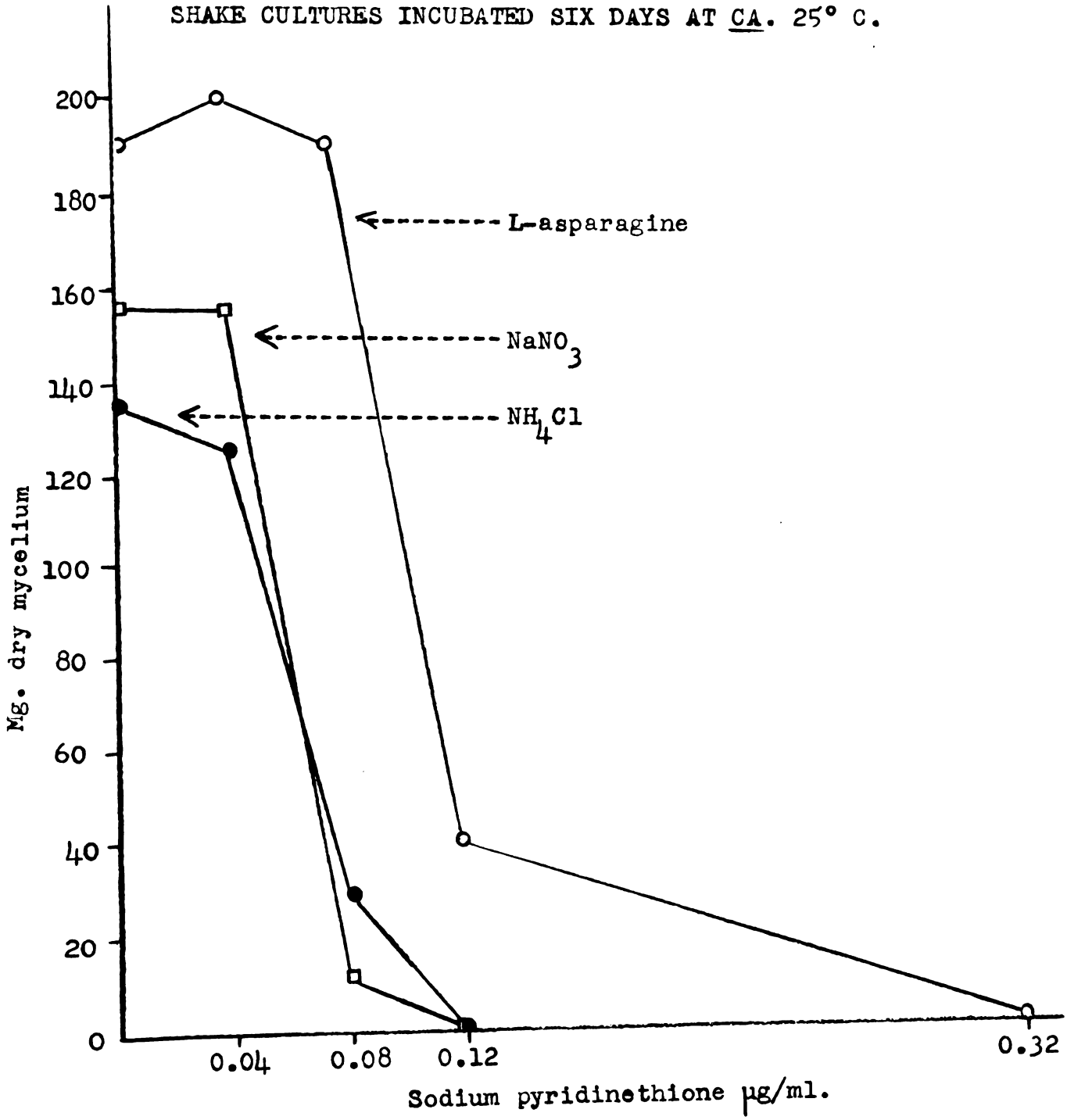
[1]pH of filtrate at the time of mycelial recovery

Text Figure 3

Apparently the fungus preferentially absorbed the ammonium ion while the chloride anion remained in the medium, the latter perhaps combining with H ions to form HCl thus accounting for the marked decrease in pH. This appeared to be substantiated because, as the organism was progressively inhibited by increasing concentrations of sodium pyridinethione, the pH increased until at total inhibition the pH of the medium was the same as the initial pH prior to incubation.

GRAPH VII

RESPONSE OF C. PHOMOIDES C80A TO INCREASING CONCENTRATIONS OF SODIUM PYRIDINETHIONE IN CONJUNCTION WITH DIFFERENT NITROGEN SOURCES
SHAKE CULTURES INCUBATED SIX DAYS AT CA. 25° C.



6. Growth Curves of C. phomoides C80A in Response to Varying Concentrations of Sodium Pyridinethione

It was considered advisable to determine what effects if any, increasing concentrations of sodium pyridinethione would have when added to the standard glucose asparagine medium at the time of inoculation with spores of C. phomoides C80A.

Low concentrations of the inhibitor added to the medium at the time of inoculation induced a lag in the growth curve as indicated from the results in Table XIII and Graph VIII. At the end of four days, inhibition was still virtually complete at the 0.12 µg/ml. level whereas the retardation was progressively lessened at decreasing concentrations of the antifungal material. The control and treatments at 0.02 and 0.04 µg/ml. showed very similar growth peaks at the end of six days and thereafter decreased to the same level at 8 1/2 days, the latter indicative of senescence. The retardation period at the 0.12 µg/ml. level was prolonged beyond four days and thereafter growth was very rapid until, at the end of 8 1/2 days, it was equivalent to the six day peaks for the other treatments. There was a gradual increase in the pH of the medium to 7.5 at which time, the fungus reached its maximum rate of growth. During autolysis, the pH increased to 8.0 - 8.2.

TABLE XIII

GROWTH PATTERNS OF C. PHOMOIDES C80A SUBJECTED TO DIFFERENT LEVELS OF SODIUM PYRIDINETHIONE AT THE TIME OF INOCULATION[1]

Harvest Time		μg/ml Sodium Pyridinethione			
		0.00	0.02	0.04	0.12
2 days	mg.[2]	10	2	+1	0
	pH[3]	6.1	6.0	6.0	6.0
4 days	mg.	187	115	33	+1
	pH	6.2	6.2	6.2	6.0
6 days	mg.	204	190	208	76
	pH	7.4	7.6	7.3	6.1
8 1/2 days	mg.	165	162	162	208
	pH	8.2	8.0	8.0	7.5

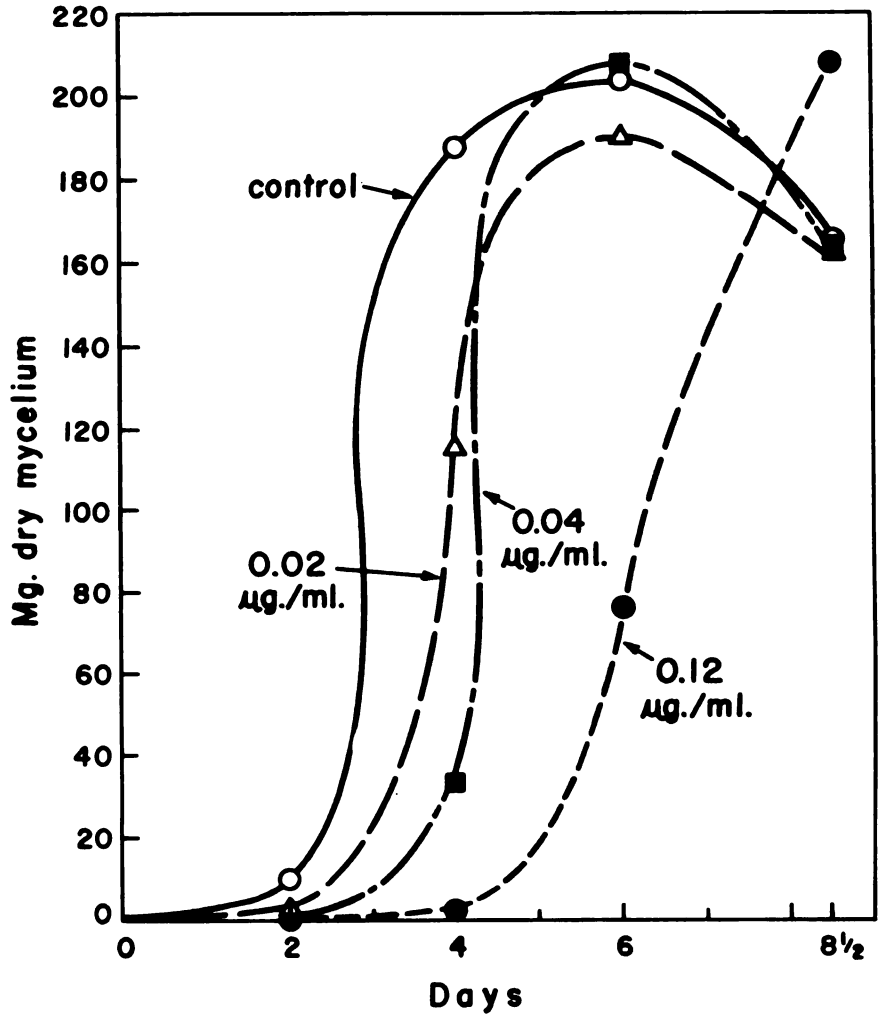
[1]Glucose asparagine medium shake cultures, incubated at 24-27° C.

[2]Dry mycelial weight.

[3]pH of filtrate.

GRAPH VIII

GROWTH CURVES OF C. PHOMOIDES C80A SUBJECTED TO DIFFERENT LEVELS OF SODIUM PYRIDINETHIONE AT THE TIME OF INOCULATION[1]



[1] Glucose asparagine medium shake cultures, incubated at 24-27° C.

7. The Effect of Adding Sodium Pyridinethione During Various Phases of the Growth Curve of C. phomoides C80A

In order to determine at what phase of its growth curve C. phomoides C80A was most susceptible to the antifungal action of sodium pyridinethione, three series of flasks containing the standard glucose asparagine medium were prepared. The antifungal material was added at the following times:

Series (1) at inoculation time

Series (2) 24 hours after inoculation

Series (3) 36 hours after inoculation.

The concentration of sodium pyridinethione used was 0.12 µg/ml. medium.

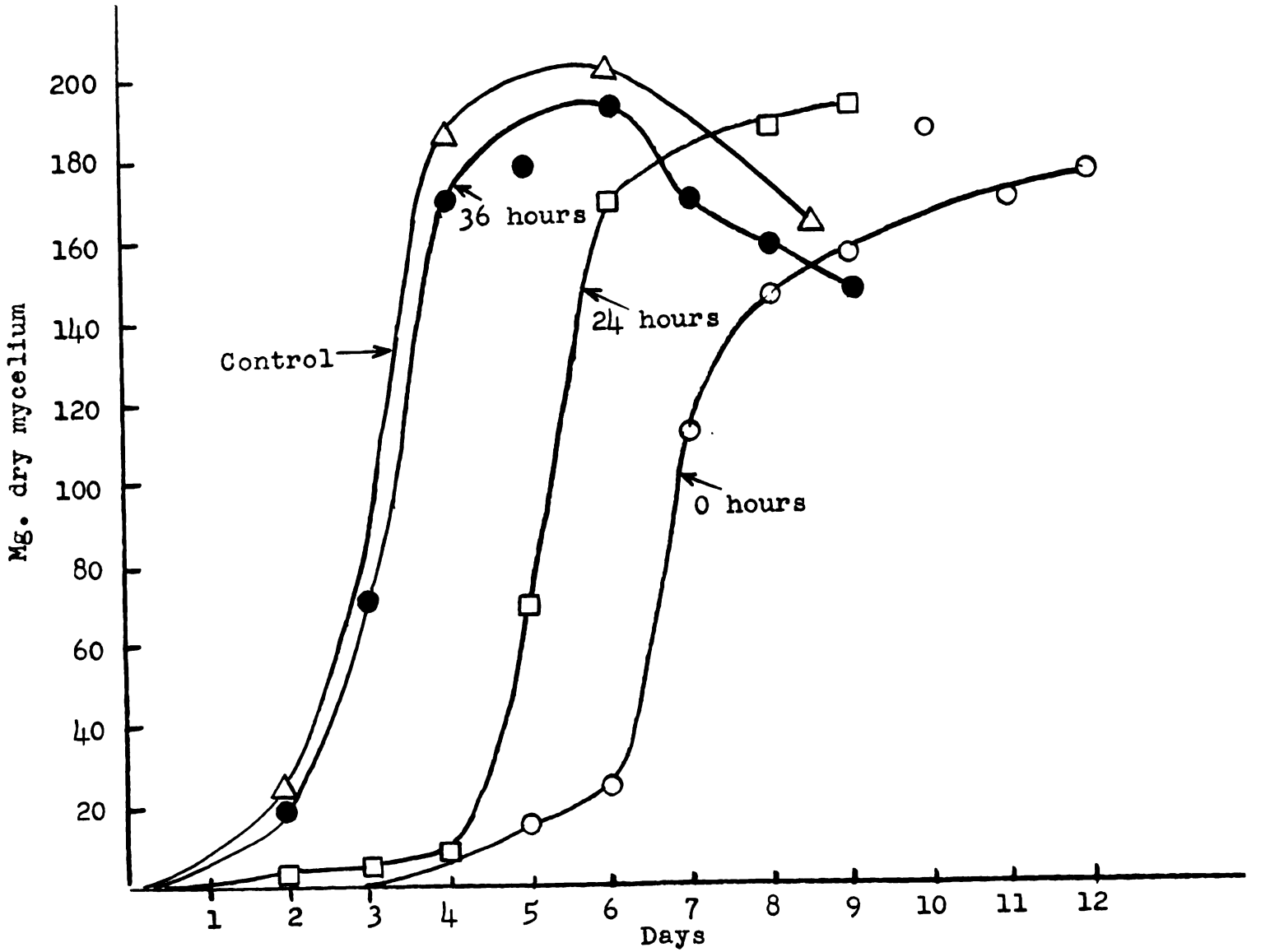
On the basis of the results which have been plotted as growth curves in Graph IX, the fungistatic action of sodium pyridinethione was diminished when the material was added to flasks containing spore inocula which had been allowed to germinate. Adding the inhibitor to 36-hour old cultures of C. phomoides C80A had no effect on the organism, since its growth curve was virtually the same as that for the control cultures.

8. Mode of Action

If its mechanism of action is known, an inhibitory agent may find wider use, not only from the practical standpoint

GRAPH IX

GROWTH CURVES OF C. PHOMOIDES C80A IN RESPONSE TO 0.12 µG/ML. SODIUM PYRIDINETHIONE ADDED AT DIFFERENT TIMES[1]



[1]Glucose asparagine medium shake cultures, incubated at ca. 25° C.

but more specifically from the standpoint of basic physiological research, especially if it acts as an antimetabolite.

Sodium pyridinethione while fungicidal at higher concentrations, exerts a fungistatic effect at the low concentrations (0.02 - 0.32 µg/ml.) used in this study. In one of the few papers published to date dealing with this new compound, Donovick et al. in 1952 (17) reported that the pyridinethione as well as the 8-hydroxyquinoline activity against Mycobacterium tuberculosis var. bovis BCG was reversed by histidine. Since 8-hydroxyquinoline was known to be an excellent chelating agent (64), these workers suggested the possibility that iron chelation by these two compounds was reversibly offset by the competitive action of histidine for the iron in the medium. It was, therefore, considered advisable to pursue further this concept in the current investigation. In addition, the possibility that the mode of action of pyridinethione might be due to the competition for a substrate analogue such as niacin was also investigated. This latter suggested role for the activity of pyridinethione was based on the fact that such known pyridine compounds as pyridine-3-sulfonic acid (39) and isoniazid (56) do act by virtue of their competitive and, therefore, reversible inhibition for niacin and niacin-containing compounds of metabolic importance.

a. As a chelating agent. First in order to duplicate the findings of Donovick and his co-workers (17) under conditions applicable to this investigation, varied concentrations of histidine were added to the sucrose asparagine medium containing sodium pyridinethione. In addition, a comparison was made employing the same medium that was used by the above investigators, a modified Kirchner's medium, except that Tween 80 was substituted for Triton A-20. The next step in this investigation was to increase the trace metals concentrations of the sucrose asparagine medium in the presence of 0.32 µg/ml. sodium pyridinethione and the test fungus C. phomoides C80A to see whether inhibition could be reversed. The complete sucrose asparagine medium contained the standard concentrations of the following metals: 0.2 mg. Fe, 0.2 mg. Zn and 0.1 mg. Mn. The basal medium was complete with the exception of the metal under investigation which was added at one, ten and 100 fold concentrations. Moreover, all the metals in the medium were simultaneously increased according to the previously listed concentrations. The shake cultures were incubated for six days at ca. 25° C.

Table XIV is a compilation of the results indicating that histidine inactivated the inhibitory properties of pyridinethione when tested against C. phomoides C80A in a sucrose asparagine as well as a modified Kirchner's

TABLE XIV

INACTIVATION OF SODIUM PYRIDINETHIONE BY HISTIDINE
TESTED AGAINST C. PHOMOIDES C80A

Histidine Concentration		I/Histidine* Molar Ratio (Na pyr. 8×10^{-7} M)	Sucr. Aspar. Medium		Kirchner's Medium	
Molar	mg/ml.		Percent Inhibition	Percent Reversal of Inhibitor	Percent Inhibition	Percent Reversal of Inhibitor
4×10^{-2}	6.0	0.00002	16	84	7	93
8×10^{-3}	1.2	0.0001	64	36	73	27
1.6×10^{-7}	0.00024	0.5	100	0	100	0

*I symbolizes the inhibitor pyridinethione.

medium. Donovick (17) reported an 83 percent reversal of the inhibitor pyridinethione when using M. tuberculosis var. bovis BCG. This corresponds to the results obtained by this author, since the same histidine to pyridinethione ratio, an 84 percent reversal was obtained in the sucrose asparagine medium and a 93 percent reversal in the modified Kirchner's medium. It is to be noted here that, on a molar basis, an histidine to pyridinethione ratio of 10,000:1 was necessary for an inhibition reversal of 36 percent when tested against C. phomoides C80A in a sucrose asparagine medium.

No reversal of inhibition was noted when as much as one hundred times the standard concentrations of zinc, iron and manganese were added to the glucose asparagine medium containing 0.32 µg/ml. sodium pyridinethione and inoculated with the spores of C. phomoides C80A.

These results suggest that the inhibitory action of pyridinethione is not due to the inactivation of metals by chelation. With the exception of iron, the excessive amounts of the metals used neither enhanced nor decreased the amount of growth in the control cultures. In the case of iron, at concentrations of 20 µg/ml., a slight toxic effect was evidenced by somewhat decreased mycelial weights.

b. As a competitor for niacin or DPN (diphosphopyridine nucleotide). Niacin[1], nicotinamide[1] and DPN were separately added to flasks containing glucose asparagine medium and 0.32 μg/ml. (2.15×10^{-6} M) sodium pyridinethione. Spores of the organisms C. phomoides C80A and H. carbonum were tested separately. Since DPN is thermolabile, it was sterilized via Seitz filtration and added to the autoclaved medium at the time of incorporating the inhibitor and the spore inocula. The concentrations of niacin and nicotinamide employed were, on a molar basis, as much as 100 times greater than that of the inhibitor. DPN was used at the same molar concentration as the inhibitor.

The results were all negative, since neither niacin, nicotinamide nor DPN reversed the inhibitory action of pyridinethione. This indicates that pyridinethione does not act as an antimetabolite for niacin or DPN.

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[1]Kindly furnished by Hoffmann-La Roche, Inc., Nutley, New Jersey.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Screening of Antifungal Compounds *In Vitro*

Of the six antibiotic and two synthetic materials screened for antifungal activity against two isolates of *Colletotrichum phomoides* (C80A and 10I) and *Helminthosporium sativum*, the compounds sodium pyridinethione, rimocidin and encomycin exhibited the best activities with minimal inhibition concentrations of less than 10 μg/ml.

On the basis of this investigation, candicidin A showed poor antifungal activity against the test organisms; in addition, the material was degraded rapidly. While this information corresponds to that obtained by Lechevalier (34), Alcorn and Ark (2, 3) indicated candicidin to be a good protectant dip and spray as well as a fairly stable compound. This apparent conflict of results may be due to the different sources of the materials. In this investigation, the material was obtained directly from the laboratory wherein the compound was first described (34) and perhaps from the first batch produced. The candicidin which Alcorn and Ark used was supplied by a pharmaceutical house and presumably could have been a more active preparation.

Aside from the preliminary screening conducted at the outset of this investigation, no attention was paid to nystatin from the phytopathological viewpoint since three other materials exhibited higher *in vitro* activities. Currently however, nystatin shows excellent promise of being the first successful antifungal antibiotic in the field of medicine. Hazen and Brown (27), the discoverers of this antibiotic, report that it is not inactivated by either blood or horse serum. Campbell *et al.* (11) demonstrated good mammalian tolerance to high dosages in addition to its effect against histoplasnosis, and Brown *et al.* (9) showed nystatin to be quite effective *in vivo* for the control of moniliasis.

This investigation confirmed the report made by Gottlieb *et al.* (23) that endomycin is thermostable. The antibiotic however is strongly adsorbed. The implication of this latter observation could be its impracticability as a soil drench for the control of such diseases as damping off. However, since endomycin is active at fairly low concentrations and does not deteriorate either upon autoclaving or prolonged contact with the medium used, it should merit further investigation.

The stability of sodium pyridinethione was investigated. Glucose asparagine medium containing 10 µg/ml. sodium pyridinethione was still capable of completely inhibiting the test fungi after a storage period of six months at room

temperature. This however did not indicate the amount of degradation that took place, since if even 75 percent loss occurred, there would still be 2.5 µg/ml. sodium pyridinethione present in the medium which would be more than sufficient to inhibit C. phomoides C80A whose MIC is 0.32 µg/ml. in glucose asparagine medium. Hence, when an aqueous solution of the inhibitor was stored at 10 µg/ml. and subsequently tested at 0.1 µg/ml., it was found that full potency was retained for 25 days at room temperature. On the other hand, the sodium pyridinethione was completely inactivated after storage at room temperature for 5 1/2 months. Sixty-six percent of the activity was lost when the inhibitor was kept at 5° C. for the same period of time. Thus with respect to time, aqueous solutions of sodium pyridinethione were slowly inactivated. This supports the earlier data obtained by the Squibb Institute for Medical Research wherein physical techniques were employed in the stability tests of this compound (54).

Physiological Studies with Rimocidin

Rimocidin is quite effective against C. phomoides C80A in the presence of sucrose or glucose as compared to xylose or lactose. The observation that controls grew much better when cultured with glucose or sucrose than when cultured with xylose or lactose indicates a higher metabolic

rate for the organism in the presence of the former sugars. This seems to correlate well with the antifungal activity. Highly active tissues such as meristematic growing points in plants are usually more susceptible to phytotoxic effects when sprayed with antifungal compounds such as rimocidin (45).

When flasks containing glucose asparagine medium and rimocidin at a concentration of 10 µg/ml. were inoculated with a spore suspension of C. phomoides C80A, a fungistatic effect was observed for a period of 15 days after which time, growth became apparent. If sucrose was substituted, all other conditions being equal, the fungistatic effect was prolonged considerably, in this case for 38 days, at which time the experiment was terminated. A possible explanation for this might be attributed to the reducing property of glucose which may serve to break down the rimocidin over a period of time thus allowing the spores which were held in check, to germinate. Sucrose on the other hand, apparently does not contribute to the inactivation of rimocidin.

Antimicrobial Properties of Sodium Pyridinethione

According to the results based on the Shippen's modification of the phenol coefficient test, sodium pyridinethione appears to be bacteriostatic. The results of the spore germination tests indicate that 0.05 µg/ml. causes 100 percent inhibition for the spores of C. phomoides C80A at the end

of 24 hours. This, however, does not tell us whether the compound is fungistatic or fungicidal.

At very low concentrations, in the presence of glucose asparagine medium, sodium pyridinethione is fungistatic, since increasing the concentration from 0.02 - 0.12 µg/ml. merely induced greater lag periods in the growth curves of C. phomoides C80A. Hartelius (26) obtained similar results for Aspergillus niger using increasing concentrations of sulfanilamide. The fungicidal nature of sodium pyridinethione became apparent when its concentration was increased beyond 0.3 µg/ml. and the spores of C. phomoides C80A remained in contact with the inhibitor for 24 hours. Short time exposures for 20 minutes at concentrations of over 100 µg/ml. were also fungicidal.

Physiological Studies with Sodium Pyridinethione

Sodium pyridinethione was most effective against C. phomoides C80A in the presence of sucrose; total inhibition occurred at 0.04 µg/ml. Sucrose does not hinder the efficiency of sodium pyridinethione since the spore germination test for this fungus in distilled water showed total inhibition to be at 0.05 µg/ml. The sugars D-glucose, D-xylose and alpha-lactose, on the other hand, apparently had some effect on the activity of the compound. In the presence of these sugars, 0.32 µg/ml. was required to induce total inhibition

(46). At this point, an explanation is in order to account for the differences obtained when the fungus was grown in the presence of xylose (compare Graph II to Graph IV). In the rimocidin series, the xylose controls grew poorly, whereas in the pyridinethione series, the xylose controls grew very well. Early stationary culture work confirmed the observations made by Lilly and Barnett (37). that Colletotrichum phomoides grows poorly on xylose at first but after a lag period, is capable of utilizing this sugar as well as glucose. Since the xylose run in the rimocidin series was made at uncontrollably high temperatures, the non-utilization phase was probably extended, so that even at the end of the six-day incubation period, growth was still poor. In the case of the sodium pyridinethione series however, the lag phase for xylose was considerably shortened since the 25° C. incubation temperature was more favorable for the organism.

The sugar studies done on rimocidin, indicated that fast growing vigorous mycelium is rendered more susceptible to the antifungal action of rimocidin. There seems to be no such relationship between growth rate and susceptibility to sodium pyridinethione. The difference of antifungal activity when tested with different sugars in this latter case might be explained in another way.

Because the carbohydrates employed, with the exception of sucrose, are reducing sugars, it was suspected that their

free carbonyl groups may have something to do with inactivating, at least in part, the antifungal properties of sodium pyridinethione. This is supported by the results with acetaldehyde which, when added to the medium containing sucrose and pyridinethione also diminished the antifungal activity. In addition, increasing the concentration of glucose inactivated the inhibitor progressively, whereas the same level of activity was maintained at various sucrose concentrations. The inactivation of pyridinethione in a glucose asparagine medium is also dependent on time. The presence of glucose alone is not sufficient to completely inactivate the inhibitor but requires some additional constituent(s) of the medium. According to Agren (1), the presence of phosphate accelerated a complex formation involving glucose and cysteine. The medium used in this study also contained phosphate in the form of KH_2PO_4. It is therefore suggested that the constituents of the medium be investigated separately by addition to a glucose pyridinethione mixture and the results determined via bioassay.

The suggestion is hereby made that a definite chemical reaction occurs between pyridinethione and glucose. This is substantiated by the ultraviolet absorption spectra curves of glucose and pyridinethione mixtures. Should spectrophotometric studies be conducted with the complete medium containing pyridinethione, it could be expected that much more pronounced results will be obtained.

Some speculation as to the chemical nature of this glucose (or other carbonyl containing compound) and pyridinethione interaction seems to be in order at this point. The reader is referred to Plate IX for the proposed chemical scheme concerning the inactivation of pyridinethione by glucose.

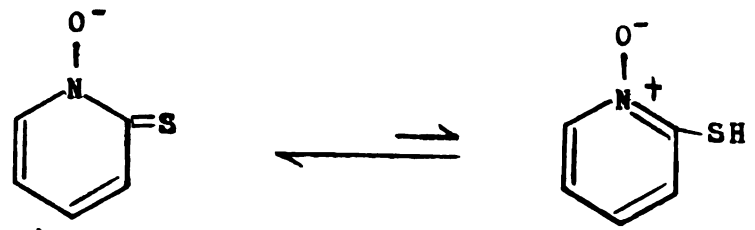
The Squibb research group (54) state that pyridinethione exists in equilibrium with its tautomeric form pyridinethiol. Employing the nitroprusside test (25), this author was unable to detect the presence of sulfhydryl groups in fresh aqueous solutions of either pyridinethione or its sodium salt. Accordingly, Brewster (8) stated that equilibrium often favors the aldo or keto form rather than the enol form of a tautomeric mixture. Thus, Plate IX (A) shows the structural formulas of both pyridinethione* and pyridinethiol* with the tautomeric equilibrium far to the left in favor of pyridinethione. Plate IX (B) shows that a possible reaction between glucose and pyridinethiol is the formation of a thio hemi-acetal. A literature review indicated that such a reaction is chemically feasible. Holleman, in his organic chemistry text (28), stated that alpha-picoline (2 methyl pyridine) undergoes a condensation with acetaldehyde to form a hemi-acetal. Cavallini (12) supported Schubert's concept (51) that thio hemi-acetals are formed between thiols (glutathione, thioglycollic acid)

*See bibliographical reference (54).

PLATE IX

PROPOSED SCHEME FOR THE INACTIVATION
OF PYRIDINETHIONE BY GLUCOSE

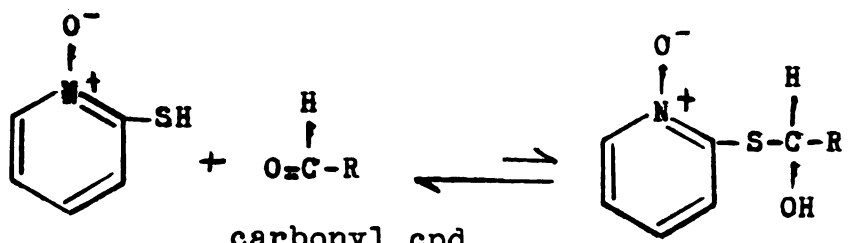
(A)



1-hydroxy-2(1H)-pyridinethione
(keto form)

2-pyridinethiol-1-oxide
(enol form)

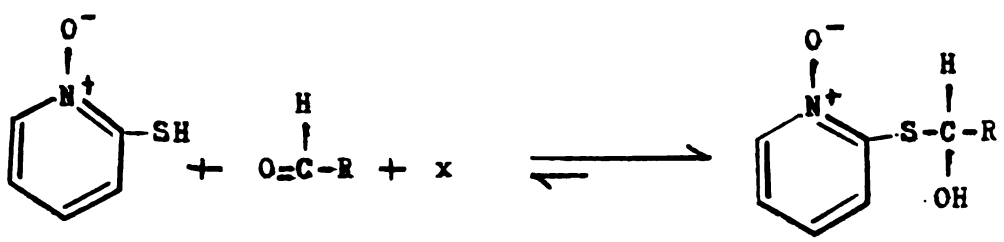
(B)



carbonyl cpd
(viz. glucose)

thio
hemi-acetal

(C)



and acetaldehyde, pyruvate and glucose. In their studies on glyoxalase activity, thio hemi-acetal formation was supported by Jowett and Quastel (32), Platt and Schroeder (43) and Racker (44). While the ultraviolet absorption spectra curves help support the possibility of a glucose-pyridinethiol interaction, bioassay results indicate some additional factor(s), as yet undetermined by this investigator, is required to shift the equilibrium of the reaction to the right, so that most of the pyridinethiol becomes tied up in the thio hemiacetal complex. This is expressed in Plate IX (C).

The overall reaction whereby the antifungal activity of pyridinethione becomes inactivated, is a slow process. A possible explanation might be attributed to the relationship of the tautomeric equilibrium between pyridinethione and pyridinethiol. Since it has already been suggested that very little pyridinethiol is present at any one time, the pyridinethione is only gradually depleted to maintain the tautomeric equilibrium as the pyridinethiol becomes tied up with the sugar.

Providing that a radioactively tagged preparation of pyridinethione can be obtained, it is suggested that further substantiation of the pyridinethiol-glucose complex be investigated via autoradiography. In short, this involves comparing the R_f values of glucose and the glucose-pyridinethiol

complex by paper chromatography. If the mixture spot contains glucose but has a non-glucose R_f value and also exhibits radioactivity upon exposure to an X-ray plate, the implications suggest a union of glucose and pyridinethiol.

The explanations given for the inactivation of pyridinethione have been made on the basis of indirect association. Since the training of this investigator has been in the field of microbiology, he feels that elucidation concerning the verification and elaboration of the thio hemi-acetal formation between glucose and pyridinethiol belongs to the realm of the chemist.

Mode of Action of Pyridinethione

The author does not at this time know why histidine is able to inactivate the antifungal effects of pyridinethione. Histidine does not appear to act as a competitor with the inhibitor for iron since excess iron does not reverse the inhibition by pyridinethione. The results indicate therefore that pyridinethione does not act as a chelating agent to deprive the fungus of essential metals. In addition, it does not appear to be a substrate competitor for niacin or DPN. The mode of action has not yet been determined by this investigator but he believes that the sulfur containing portion of the pyridinethione is important in the mechanism of inhibition. This assumption

is based on the previous suggestion of a thio hemi-acetal formation between the carbonyl group of glucose and the sulfhydryl group of pyridinethiol which is concomitant with inactivation.

Other Significant Conclusions

For the purpose of replication between laboratories, a description of the test medium and the concentration of its constituents can be quite important in reporting activity levels of an antimicrobial agent. The results with sodium pyridinethione can be cited as an example. In sucrose asparagine medium (the sucrose concentration is immaterial), the MIC for C. phomoides C80A is 0.04 µg/ml. If in place of sucrose, one percent glucose is used, the MIC increases to 0.32 µg/ml. Hence eight times more sodium pyridinethione was required in the latter test. Moreover, if a higher glucose concentration, say two percent, were used in the same basal medium, a much greater concentration of the inhibitor would have been required.

Quite often antifungal test compounds require much greater concentrations to control phytopathogens in vivo as compared to the in vitro control of the same organisms (45). These larger levels required in vivo may be due to factors or combination of factors which serve to decrease the efficiency of the compound. Some of these factors

may be lack of penetration, sensitivity to sunlight (ultraviolet light), thermolability, self degradation in solution and chemical inactivation by other sprays or plant exudates. Since pyridinethione is inactivated by reducing sugars, it might be conceivable that if used as a protectant systemic spray, the antifungal agent may become inactivated, at least in part, by the glucose present in the plant sap. Thus, Sander and Allison (50) recently reported that 2-pyridinethiol-1-oxide while translocated downward in cucumber seedlings, nevertheless was found to be inactivated when extracts of the cotyledons were assayed. This investigator suggests that the inactivation might have been due to the large concentrations of reducing sugars found in the cotyledons of such plants as cucumber at the time of germination.

The manner in which carbohydrates influence activity appears to differ between antimicrobial materials. In the case of pyridinethione, reducing sugars serve to inactivate the compound by combining with it. When the metabolic rate of a fungus is enhanced by certain sugars, its susceptibility to rimocidin is increased. On the other hand, this correlation seems to be reversed in the case of sulfonamides, since Gerundo (21) reported that bacterial inhibition occurred if no sugars were added to the medium or if the sugar was not one usually fermented by the bacterial organism.

CHAPTER V

SUMMARY

1. Of eight antifungal test compounds screened *in vitro* against *Colletotrichum phomoides* (C80A and 10I) and *Helminthosporium sativum*, the following compounds exhibited minimal inhibition concentrations of less than 10 µg/ml.: sodium pyridinethione, rimocidin and endomycin.

2. Sodium pyridinethione is moderately thermostable and breaks down only slowly in aqueous solution at room temperature.

3. Endomycin was found to be strongly adsorbed onto the surface of asbestos Seitz filter pads.

4. Using *Colletotrichum phomoides* C80A as the test organism, the *in vitro* antifungal effect of rimocidin was markedly increased in the presence of D-glucose or sucrose as compared to D-xylose or alpha-lactose. Rimocidin at a level of 10 µg/ml. in the presence of glucose asparagine medium maintained fungistatic activity for a period of 15 days. On the other hand, when sucrose was substituted, the fungistatic effect was increased to over 38 days.

5. The concentrations of sodium pyridinethione in µg/ml. required to completely inhibit spore germination for 24 hours in distilled water at *ca.* 25° C. were as follows:

Colletotrichum phomoides C80A, 0.05; Colletotrichum lagenarium, 0.05; Helminthosporium carbonum, 0.10 and Puccinia sorghi, 0.05. At 0.05 µg/ml. sodium pyridinethione, abnormal vesicle formation was noted at the end of the germ tubes of H. carbonum. As little as 0.005 µg/ml. was needed to induce retardation in germ tube development of C. phomoides C80A.

6. Sodium pyridinethione is fungistatic at concentrations up to 0.12 µg/ml. The fungicidal nature of the compound is exhibited when the spores of C. phomoides C80A remain in contact with the inhibitor for 24 hours at concentrations above 0.3 µg/ml. Short time exposures for 20 minutes at concentrations higher than 100 µg/ml. are also fungicidal.

7. Increasing concentrations of sodium pyridinethione at fungistatic levels progressively retard the growth curves of C. phomoides C80A.

8. In the presence of sucrose asparagine medium, sodium pyridinethione totally inhibited C. phomoides C80A at 0.04 µg/ml. When the reducing sugars D-glucose, D-xylose or alpha-lactose were substituted, 0.32 µg/ml. of the inhibitor was required to produce the same effects.

9. Asparagine medium containing increasing concentrations of D-glucose progressively inactivated sodium pyridinethione. This inactivation is also dependent on time. Sucrose had no effect on the antifungal inactivation of the compound.

10. It is suggested that glucose inactivates pyridinethione by the formation of a thio hemi-acetal complex. Other carbonyl containing chemicals such as acetaldehyde also inactivate pyridinethione. The postulation of an interaction between glucose and the inhibitor is supported by ultraviolet absorption spectra data. The role of glucose in plants is discussed with relation to pyridinethione inactivation.

11. The mode of action of sodium pyridinethione remains unknown to this investigator. The inhibitor does not appear to act as a chelating agent for essential metals nor does it function as a substrate analogue for niacin. It is suggested that the sulfur portion of pyridinethione is functional in causing inhibition.

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