STUDIES ON THE FUNGICIDAL ACTIVITIES AND PHYTOTOXIC PROPERTIES OF SEVERAL ANTIFUNGAL COMPOUNDS

By

Clare Burton Kenaga

A THESIS

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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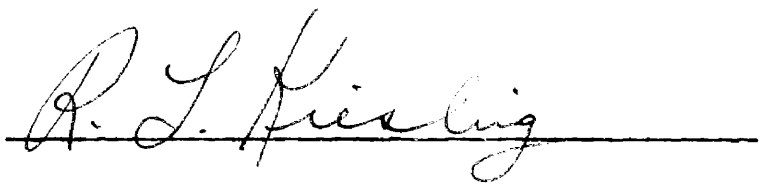
Approved 

ABSTRACT

Acti-dione, Panogen 15, hexachlorobenzene and the Omadine salts of copper, zinc, manganese and sodium when applied as foliar sprays controlled Phoma lingam on cabbage, Colletotrichum lindemuthianum on bean and Puccinia sorghi on corn. The best overall control was obtained with sodium Omadine, copper Omadine and Panogen 15.

Concentrations of sodium Omadine necessary to produce total inhibition of the spores of Colletotrichum lagenarium, C. phomoides and Helminthosporium carbonum in distilled water were 0.05, 0.05 and 0.10 ppm respectively. Vesicles or swellings were occasionally produced at the tips of the germ tubes of H. carbonum at concentrations of 0.05 ppm. The lethal threshold for growth of Lemna minor under the influence of sodium Omadine was between 0.5 and 0.1 ppm. Concentrations as low as 0.005 ppm produced a marked decrease in growth.

Sodium Omadine does not act as a systemic fungicide, and up to 50 ppm of the compound is inactivated immediately upon contact with extracts from radish and tomato.

The ultraviolet absorption spectra of guttation water of cucumber seedlings grown at temperatures of 68° to 70° F in sand treated with sodium Omadine indicated a lower amount of inorganic substances were probably present than from control plants. At higher temperatures these differences were not present, and the absorption spectra curves for control plants from both high and low ranges of temperatures were entirely different.

The hypothesis was made that limited amounts of -SH groups were present in aqueous solutions of sodium Omadine, and that therefore several chemical reactions characteristic of the mercaptans were possible. Circumstantial evidence was presented as the basis for the hypothesis, and evidence is presented to show that some of the reactions characteristic of mercaptans do take place.

Evidence was presented to the effect that in a solution containing water-soluble metal salts there is a replacement of the sodium ion attached to the Omadine molecule by the metal element in solution.

A second hypothesis was made that the Omadine salts act as chelators in which the preformed metal chelates, possessing a fat-water solubility balance, permeate the cellular membranes and preform a toxic reaction within the cell. Evidence is given in support of this hypothesis.

A third hypothesis was made that once the Omadine molecule enters the cell it undergoes reduction, leaving the pyridine ring which is the toxic property. Circumstantial evidence is presented in support of this hypothesis.

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CHAPTER I

INTRODUCTION

A study of the influence of several antibiotic and synthetic organic materials upon certain plant pathogens and their hosts was initiated in 1954. These compounds were screened against several organisms causing foliar diseases and formed the basis for further selection of materials employed in physiological studies of host reaction, possible systemic action, inactivation and mode of action.

The derivatives of 1-hydroxy-2(1H) pyridinethione (Omadine) were selected from a group of artifungal materials for physiological studies on the basis of the screening trials since they exhibited low phytotoxicity and excellent antifungal properties. The sodium salt of 1-hydroxy-2(1H) pyridinethione was selected for most of these studies since it was water soluble. These compounds represent a new group of antifungal materials with which little work had previously been done.

LITERATURE REVIEW

The antibiotic thiolutin was screened for chemotherapeutic activity in 1952 by Gopalkrishnan and Jump (17). They found thiolutin to be more active *in vivo* than *in vitro*, in the control of Fusarium wilt of tomato, although higher concentrations of this antibiotic produced a stunting accompanied by marked epinasty of the leaves. Nickell and Finlay (43) found that 1 ppm thiolutin produced some stimulation of growth of

Lemna minor. Although many accounts of this antibiotic have appeared in the literature concerning its antibacterial and antifungal activity of human and plant pathogens, thiolutin has met with very limited success (17,67,8,41,22).

Rimocidin, an antibiotic, was first described by Davisson et al. (11) in 1951 who found it inhibited many of the human pathogenic fungi in vitro at low concentrations. Malcolmson and Bonde (36) found that rimocidin sulphate alone and together with Agrimycin gave good control of the bacterial rots, Erwinia atroseptica and Pseudomonas fluorescens, on potato seed pieces. Vaartaja (67) reported that rimocidin at a wide range of concentrations controlled Rhizoctonia solani and Pythium debaryanum on pine and birch seed in Petri dishes without marked phytotoxicity.

Antibiotic XG (later called fungistatin XG) was found by Hobby et al. (23) in 1949 to be effective against the growth of many human pathogenic fungi in vitro, while Wallen and Skolko (71) in 1950 found it to be effective as a seed treatment for the control of Ascochyta pisi on peas.

Hazen and Brown in 1951 (21) reported that fungicidin (later called nystatin) was effective in vitro against human and plant pathogens at concentrations of 10 ppm and lower.

Acti-dione (cycloheximide) was isolated from Streptomyces griseus by Whiffen et al. in 1946 (74). Considerable material exists in the literature concerning this antibiotic. Whiffen in 1948 (73) found it highly active in vitro against human pathogenic fungi. Felber and Hamner in 1948 (15) found that this antibiotic controlled mildew on bean.

Wallen et al. (72) reported it had a high phytotoxicity against pea seed. Gottlieb et al. (18) reported that Acti-dione when applied as foliar sprays was very phytotoxic. Cation (7) and Hamilton and Szkolnik (20) reported control of cherry leaf spot with foliar sprays of 1 to 2 ppm. Vaughn (68) found this antibiotic effective against turf diseases.

Hexachlorobenzene (perchlorobenzene) was found effective in controlling dwarf bunt as well as common bunt of wheat by Siang and Holton (58) in 1953, who attributed the control to inhibitory action of vapor on spore germination. Purdy in 1956 (51) found that in five Pacific Northwest states, hexachlorobenzene was the most effective chemical for wheat smut control.

Martin (38) described Panogen (methylmercuric dicyandiamide) as a fungicide for use on seed of cereals, flax, sorghums, cotton and sugar beet. He stated further that it was used in Sweden since 1938 and in North America since 1949 as a seed disinfectant. Leukel (34) reported Panogen to be effective in controlling bunt on wheat, loose and covered smut on oats and stripe disease on barley.

The prototype for a new class of compounds possessing antifungal activity, a cyclic thiohydroxamic acid, was synthesized by Shaw et al. in 1950 (57). This compound exists in two tautomeric forms and as such may be chemically described as 1-hydroxy-2(1H) pyridinethione, or 2-pyridinethiol, 1-oxide (62,45). Pansy et al. in 1953 (50) conducted screening studies against numerous microorganisms in vitro by this compound and found it to exhibit antifungal activity at concentrations as low as 0.04 ppm. Norman (44) reported that low concentrations of

this compound and several of its analogs inhibited root elongation and dry matter increase in cucumber and barley, but foliar applications at comparable or higher concentrations did not produce noticeable responses in top growth or development. However, Kenaga and Kiesling (29) found that repeated application of the sodium derivative as foliar sprays and root drenches caused chlorosis and stunting. Allison and Barnes in 1956 (1) evaluated 2-pyridinethiol, 1-oxide and several derivatives and found them to be promising as foliage and soil fungicides, and also claimed that the copper and zinc derivatives had a high residual activity on tomato foliage. Couch and Cole (9) reported that the disulfide provided a limited amount of control on rust of Merion bluegrass, and in 1957 (10) these authors reported that it provided significant reduction of melting-out of Kentucky bluegrass. Disulfide, manganese and thio-urea derivatives of 2-pyridinethiol, 1-oxide were found effective in reducing the disease incidence of Alternaria blight when tested in mist stream propagation of carnations in the greenhouse in 1956 by Skiver (59). Sander and Allison (56) reported that 2-pyridinethiol, 1-oxide was absorbed through the leaves of cucumber seedlings and translocated downward, and suggested that the chemical was inactivated in extracts of young shoots and cotyledons. Ringel and Beneke (54) showed that the sodium derivative was most effective in inhibiting the growth of Colletotrichum phomoides in liquid culture when in the presence of sucrose, and that the reducing sugars D-glucose, D-xylose and alpha-lactose reduced the inhibitory effect of the compound. Ringel (53) suggested that the sodium derivative was not a competitor for the

substrate analogues niacin, nicotinamide and diphosphopyridine nucleotide. This writer also reported that histidine caused a 36 percent inactivation of the sodium derivative when present at a molar ratio of 10,000 to 1 respectively, and that an excess of zinc, iron and manganese failed to reverse the inactivation. He therefore, concluded that the inhibitory action of this compound was not due to chelation of metals. One of the antifungal compounds reported in the literature and closely related to 2-pyridinethiol, 1-oxide was the compound 3-pyridinethiol. The disulfide and metal derivatives of this latter compound were studied for antifungal activity by Soo-Hoo and Grunberg in 1950 (61), who found them to possess a high antifungal activity.

CHAPTER II

MATERIALS AND METHODS

Materials Employed

Compounds: Their Source and Maintenance

The following is a list of antifungal materials employed in this study and their source:

Thiolutin, diaphine HCl, rimocidin sulfate and fungistatin XG: furnished by Charles Pfizer and Company, Brooklyn, New York.

Panogen 15, (2.2% methyl mercury dicyandiamide in liquid carrier) and hexachlorobenzene, (40% furnished by Panogen, Inc., Ringwood, Illinois.

Acti-dione (cycloheximide): furnished by the Upjohn Company, Kalamazoo, Michigan.

Nystatin (fungicidin): furnished by the Squibb Institute for Medical Research, New Brunswick, New Jersey.

Sodium, zinc, manganese and copper Omadine, (salts of 1-hydroxy-2 (1H) pyridinethione); and Omadine disulfide, (2,2'-dithiodipyridine-1,1'-dioxide): the sodium salt was originally furnished by the Squibb Institute for Medical Research, New Brunswick, New Jersey, now a division of Olin Mathieson Chemical Company, Baltimore, Maryland, who furnished succeeding lots of this and remaining Omadine compounds.

Acti-dione, thiolutin, fungistatin XG, nystatin and rimocidin sulfate were antibiotic materials. Diaphine HCl, Panogen 15 and the Omadine compounds were synthetic organic compounds.

Diaphine HCl, rimocidin sulfate, Panogen 15, Acti-dione and disulfide and sodium Omadine were soluble in water at the concentrations used, and fresh stock solutions of these materials were prepared before use. The remaining compounds were not soluble at this stock

concentration, and an emulsifying agent, Tergitol No. 7 (38), was employed to keep them in suspension. All of the test materials were maintained in a dry state at room temperature until used. Throughout this study each concentration of a test compound in solution or otherwise is given as concentration of active ingredients.

Fungal Organisms and Their Culture

The fungal organisms employed in this study together with the common names of diseases they produce are given in Table I.

C. lindemuthianum was maintained on bean-pod agar slants. To obtain abundant sporulation, flasks containing sterilized barley were inoculated with a spore suspension and allowed to grow for four days after which spores were collected by rinsing with sterile water and straining through cheesecloth.

C. lagenarium was maintained on V-8 agar to induce abundant sporulation. Spores of P. sorghi and P. graminis tritici were collected via cyclone spore-collector from the diseased leaves of their respective host plants and stored under refrigeration. The remaining fungi were maintained on potato dextrose agar.

Host Plants

The following varieties of economic plants obtained from Ferry-Morse Seed Co., Detroit, Michigan, were used in this study:

Golden Cross Bantam corn
National Pickling cucumber
Michelite bean
Marglobe tomato[1]

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[1] Marglobe variety of tomato was used in initial screening trials, but was replaced with Bonny Best tomato in all later runs and physiological studies.

TABLE I. Test organisms and common names of diseases they produce.

Organism	Disease
Alternaria solani (Ell. and G. Martin) L. R. Jones and Grout[4]	Early Blight of Tomato
Colletotrichum lagenarium (Pers.) E. and H.[3]	Cucumber Anthracnose
C. lindemuthianum (Sacc. and Magn.) Briosi and Cav. alpha strain[1]	Bean Anthracnose
C. phomoides (Sacc.) Chester[2]	Tomato Anthracnose
Glomerella cingulata (Stan.) Spauld. and Schrenk[4]	Bitter Rot of Apple
Helminthosporium sativum (Pam.) King and Bakke[4]	Spot Blotch of Barley
H. carbonum Ullstrup[4]	Leaf Spot of Corn
Phoma lingam (Tode and Fr.) Desm.[4]	Blackleg of Crucifers
Puccinia graminis tritici Eriks. and E. Hemm.[4]	Stem Rust of Wheat
P. sorghi Schw.[4]	Corn Rust

Fungal organisms in the form of cultures or spores were obtained from the following faculty members of the Department of Botany and Plant Pathology at Michigan State University, East Lansing, Michigan:

[1]Dr. A. Andersen
[2]Dr. E. S. Beneke
[3]Dr. D. J. de Zeeuw
[4]Dr. R. L. Kiesling.

Bonny Best tomato
Scarlet Globe radish
Mandarin Chinese cabbage
Marion Market cabbage

Methods Employed

Studies of the Various Test Compounds

1. In vivo screening of test compounds

Spores of test organisms were suspended in distilled water, sprayed by atomizer onto the foliage of their respective hosts, and allowed to dry for 45 minutes. Test compounds were then applied by atomizer to rows of inoculated plants until the point of run-off (40). Cardboard strips set upright between rows protected plants on either side from drift or accidental spray contamination. The plants were then kept in a moist chamber for three days and removed to a bench. Readings were taken after lesions appeared on the untreated inoculated checks and were based on a numerical gradation of 1 to 10; 1 indicating no visible symptoms, and 10 indicating death of the plant. At least seven replications were used in each experiment.

Cabbage plants 3-4 weeks old were used in the initial trials involving P. lingam. Since it was necessary to remove the bloom from this type of cabbage by rubbing the leaves before inoculation, subsequent trials were run using Chinese cabbage which was susceptible to this test organism and free from bloom. Beans in the first to third trifoliate-leaf stage were used in the C. lindemuthianum trials; and corn in the 4-5 leaf stage, in the P. sorghi trials.

A. solani and C. phomoides on tomato proved unsuccessful as test organisms due to failure to obtain constant and uniform infection.

Kunkel's method (31) of increasing sporulation in culture by scraping the surface of the culture and using fluorescent light was employed (52). Spore production was greatly increased, but infection either because of the strain or because of the conditions, was not materially increased. Marglobe variety of tomato was replaced with a more susceptible variety, Bonny Best (40), with little or no increase of infection. Therefore, these fungal organisms were dropped from the screening program.

In initial trials all compounds were applied at 200 ppm, with the exception of Acti-dione and Panogen 15. These latter compounds served as checks, and concentrations of 3 ppm and 4.4 ppm respectively were employed unless otherwise stated.

2. Phytotoxicity

Phytotoxicity tests were run for all compounds tested at 200 ppm with the exception of Acti-dione and Panogen 15, see above. Diaphine HCl was tested further at 100, 50, and 25 ppm. Compounds were applied as foliar sprays.

3. Experiments on the systemic activity of several antifungal compounds in the control of C. lindemuthianum on bean

a. Via detached leaf culture

This study was designed to evaluate the materials under test for systemic action (25,37,76). Acti-dione, Panogen 15, hexachlorobenzene and the four Omadine salts were tested. Mature and near mature leaflets were cut from bean plants, immediately dipped into a spore suspension of C. lindemuthianum, and allowed to dry for a period of five minutes. They were then placed petiole

down in glass jars (1½ inches in diameter by 3 inches tall) containing a 10 percent sucrose solution to which one of the antifungal compounds had been added. Acti-dione was tested at 1 ppm, Panogen 15 at 0.11 ppm and the other compounds at 5 ppm. Eight replications of each treatment was run. Control runs of inoculated and uninoculated leaves were placed in a 10 percent sucrose solution with eosine. The jars were covered with Petri dish tops and placed under continuous light. Readings were taken after 5 days and based on numerical gradation of 1 to 10 as previously defined.

b. Via hydroponics

Test compounds were added to Hoagland's nutrient solution (16, Table B, p. 119) as follows: Acti-dione at 3 ppm, Panogen 15 at 0.11 ppm, hexachlorobenzene at 5 ppm and the four Omadine salts at 5 ppm respectively. An untreated nutrient check was also run. Pint jars covered with brown wrapping paper and dipped in paraffin served as containers. Bean seedlings germinated in paper toweling were placed in the jars and supported by 1/4 inch wire mesh screen cut into squares and fitted over the jar tops. Each test compound was replicated five times. Distilled water was added as needed to maintain the solution level and prevent concentration of salts. After four days the plants were inoculated with a spore suspension of C. lindemuthianum and left in the inoculation chamber for two days, then removed to a greenhouse bench. Readings were taken as previously described.

Studies of Sodium Omadine

1. Spore germination tests[1]

The glass slide technique with the test tube dilution method was employed as described by the American Phytopathological Society, Committee on Standardization of Fungicidal Tests (2,3). Distilled water was used as the suspending medium for the spores. C. phomoides, C. lagenarium, H. carbonum and P. sorghi were used as the test organisms and provided a wide range of difference of cell size and wall thickness.

2. Stability of sodium Omadine on bean foliage

Sodium Omadine at 200 ppm was sprayed on 12 bean plants to test its stability on foliage without washing. Sprayed plants were held for two hours, two days and four days at 85° to 90° F daytime temperature. Leaf punches (33) from treated and untreated plants were bioassayed together with filter-paper discs which were run as bioassay controls.[2]

3. Growth response of Lemna minor L. to sodium Omadine (27)

Microorganism-free stock cultures of L. minor were obtained as follows: roots were removed and rosettes pulled apart into separate fronds by use of fine forceps. The fronds were wrapped in several folds of cheesecloth and weighed down in a large beaker, then washed with running tap water for 12 hours. After a momentary dipping in mild sodium hypochlorite solution, followed by rinsing in sterile distilled

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[1] The spore germination studies were conducted jointly with Dr. Samuel M. Ringel, former Graduate Research Assistant of the Botany and Plant Pathology Department, Michigan State University, East Lansing, Michigan.

[2] This procedure is discussed under section entitled "Filter-paper disc bioassay technique," page 24.

water, each frond was placed in a separate bottle containing sterile liquid medium (43, Table A, p. 119). The bottles were laid at an angle under continuous light in an air conditioned culture room with controlled temperature of 22° C.

Forty-eight ml of liquid medium were poured in culture bottles and sterilized via autoclave for 20 minutes at 15 pounds pressure. Two ml of sterile distilled water were added to each of seven bottles which served as checks. Four concentrations of sodium Omadine were sterilized via sintered-glass filter, and two ml of each concentration were added aseptically to each of seven bottles. The final concentrations were 5, 1, 0.5, and 0.1 ppm. Rosettes of L. minor were transferred immediately to each bottle. Experimental transfers for each experiment were accomplished by placing microorganism-free rosettes of four fronds in individual bottles. All transfers for a given experiment came from one stock flask. Wet weight determinations were made after a growth period of four weeks. A second experiment was run using concentrations of 0.1, 0.05, 0.01, and 0.005 ppm sodium Omadine. A six-week growth response study was also made with sodium Omadine at 0.05 ppm. Controls and treatments were harvested every week for six weeks. Seven replications were used.

4. Translocation studies of sodium Omadine

a. Via hydroponics

Bean plants in the fifth trifoliate-leaf stage were placed in Hoagland's nutrient solution (16, Table B, p. 119) containing 5 ppm and 25 ppm sodium Omadine. The plants were placed under continuous light for four days at 78° F, after which they were

removed, washed and the secondary roots and leaves cut from the stem. Nine root and stem sections 1/8 inch in length were cut from each of the plants as follows: the first at root tip, the next two at 1/2 inch intervals up the root, and the last six at 1 inch intervals. The sections were placed on bioassay plates with the cut side down. The plates were incubated for 48 hours and examined for antifungal activity by the presence of zones of inhibition.

b. Via bioassay of guttation and stem exudate from plants grown in treated sand

Tomato, corn and cucumber seedlings respectively were grown in sand in six-inch pot saucers. The seedlings were allowed to wilt slightly, then one drench application of sodium Omadine at 50 ppm was applied until an excess of liquid was in evidence at the surface of the sand. Care was taken not to splash any compound on the seedlings. Distilled water drench was applied to saucers of control plants. Bell-jars were placed over the pot saucers, and water of guttation was collected with a syringe and blunt needle. Collections were made each evening for three successive evenings following treatment. Each collection was bulked as to treatment and control. The cotyledons were cut from the stems of cucumber seedlings four days after treatment. The sap which was exuded at the cut surface of each stem was collected 12 hours after cutting. The water of guttation and stem exudate was immediately examined for biological activity by means of bioassay. Sodium Omadine at

concentrations of 1, 5 and 25 ppm in distilled water and in guttation water were used as bioassay checks.

To determine the extent of the activity present in the sand of the treated pot saucers, the tomato seedlings were removed from their saucers but the bell-jars were left covering the saucers. No water was added to these saucers. After sixteen days the moisture from the treated pot saucers was examined by bioassay to determine the presence of biological activity.

c. Via bioassay of split-stem sections of tomato seedlings grown in treated sand

Tomato seedlings were grown in treated sand in pot saucers as above. After three days several plants from both control and treated groups were cut off at the soil line, the leaves removed, and the stems split lengthwise and plated cut-side down on bioassay plates.

d. Via bioassay of expressed juice from tomato and cucumber seedlings grown in treated sand

Cucumber and tomato seedlings were grown in sand to which sodium Omadine was applied as a drench. The aerial portions of the plants were removed, washed and blotted dry three days following treatment. Stems and leaves of both tomato and cucumber seedlings were ground in a mortar and the juice expressed through cheesecloth. Water-drenched plants served as controls. Each sample was examined for biological activity by means of bioassay.

e. Via bioassay of expressed juice from foliar treated plants

Foliar applications of 100 and 200 ppm sodium Omadine respectively were sprayed on two lots of 3-inch high tomato seedlings grown in sand. The sand was flushed from the roots of the plants 12 hours after treatment, and the plants were washed in running tap water followed by a distilled water rinse and then blotted dry. The plants were then separated into roots, stems and leaves. Each of these separations was ground in a mortar and the juice expressed through cheesecloth. Each lot of juice was immediately bioassayed against G. cingulata, with juice expressed from untreated plants serving as controls. The leaves of two lots of radish seedlings were sprayed with 100 ppm and 200 ppm sodium Omadine respectively, and a set of untreated seedlings served as controls. This experiment was performed in the same manner as the experiment just described, with the exception that juice was expressed from roots, hypocotyls and leaves.

5. Physiological studies on the influence of sodium Omadine on host plants

a. Ultraviolet spectrophotometric determination

Cucumber seedlings grown in sand in pot saucers were treated with a root drench of 50 ppm sodium Omadine. Water of guttation from treated plants was collected after each of four succeeding 12-hour periods. Samples from the untreated control plants were collected after the first and the last periods only. All samples were stored under refrigeration. The samples were diluted 1 to 4 with

glass-distilled water and the ultraviolet absorption spectra for each sample determined by use of a Beckman Model DU spectrophotometer. A glass-distilled water solution of sodium Omadine at 4 ppm was prepared and the ultraviolet absorption spectra obtained.

Three other experiments were run similarly, except that water of guttation was collected from both treated and untreated seedlings at the end of each 12-hour period for seven periods, encompassing 84 hours from the time of original drench application.

b. Free and combined amino acid studies of treated plants

Foliage of Chinese cabbage, tomato, cucumber and bean plants was sprayed with one application of sodium Omadine at 200 ppm. After 20 hours the foliage from treated and untreated check plants was removed, washed, and the free amino acid samples extracted and examined by means of paper chromatography for quantitative differences.[1] Combined amino acid samples were extracted from tomato and cucumber plants and similarly examined. Cucumber and bean seedlings were grown in pot saucers and treated with one drench application of sodium Omadine at 100 ppm. After three days the foliage was harvested and free amino acid samples extracted and examined as above.

c. Reducing sugar studies of treated plants

Somogyi's test[2] for reducing sugars was run on cucumber,

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[1] Paper chromatography: procedures and techniques, section 2, page 25.

[2] Test for reducing sugars, section 3, page 27.

tomato, bean and Chinese cabbage, all of which had been treated with one foliar spray of sodium Omadine at 200 ppm. The foliage was removed for sample extraction 24 hours after treatment. These same types of plants were also grown in six-inch pot saucers in sand to which one application of sodium Omadine at 50 ppm had been added. The foliage was removed for sample extraction after four days.

Concentrations of D-glucose at 0.05 to 1 percent were examined by this method and plotted on logarithmic scale graph paper. The sample readings of reducing sugars were thus measured in equivalent amounts of D-glucose.

6. Inactivation of sodium Omadine

a. Guttation water

Guttation water from corn and cucumber seedlings was examined to determine if any sodium Omadine inactivation properties were present. One part sodium Omadine was added to four parts of both boiled and unboiled guttation water from corn and cucumber respectively. The final concentrations of the compound were 1, 5, 12.5, 25, and 50 ppm in each trial. The solutions were allowed to stand 0 and 12 hours, then bioassayed against G. cingulata. Distilled water concentrations of sodium Omadine were used as checks.

b. Expressed juice of plant parts

The leaves and hypocotyls of three-week old radish seedlings were harvested separately, washed, blotted dry, ground in a mortar

and the juices of each expressed through cheesecloth. Each lot of juice was separated into 2 ml samples and placed in capped vials. One-half ml amounts of sodium Omadine at five concentrations were pipetted into the samples of leaf and hypocotyl juice respectively. The final solutions of juice contained 5, 10, 25, 50 and 100 ppm sodium Omadine. These vials of treated juice were allowed to stand for 12 hours at room temperature and then bioassayed. Samples of leaf and hypocotyl juice containing concentrations of 10 and 50 ppm of the inhibitor as well as distilled water checks were bioassayed immediately after preparation.

The leaves and stems of three-week old tomato seedlings were harvested separately, washed, blotted dry, ground in a mortar and the juices expressed through cheesecloth. The juices were separated into eight 2 ml samples and prepared and bioassayed similarly to the previously discussed experiment on radish juice.

Radish leaves and hypocotyls were harvested, washed, blotted dry and ground separately in a mortar. The juice was expressed through cheesecloth and divided into samples of 0.9 mm each. Four samples each from leaves and hypocotyls were boiled for 5 minutes in a water bath, removed and allowed to cool. One-tenth ml of sodium Omadine at two concentrations was added to each of the boiled and unboiled samples so that the final concentrations were 50 ppm and 100 ppm. Distilled water was added to other samples as controls. After 2 hours the samples were bioassayed, and the unboiled samples were examined for differences in free amino acids by means

of chromatography[1] and reducing sugars via Somogyi's method.[2] Leaves from cucumber and tomato seedlings were harvested and treated as just described for radish.

c. D-Glucose

An aqueous solution of sodium Omadine at 100 µg/ml and D-glucose at 225 mg/ml was prepared. The solution was bioassayed after stand- at room temperature for 10 days. Separate solutions of sodium Omadine and D-glucose were used as bioassay controls.

Studies Concerning Mode of Action of the Omadine Compounds

1. Purification and investigation of chemical composition

a. Studies of technical grade sodium Omadine by paper chromatography

Four-tenths ml samples of sodium Omadine at 100 µg/ml in glass-distilled water were chromatogramed. Glass-distilled water saturated n-butanol was employed as the solvent. The solvent had a pH of 6.7 and the sodium Omadine a pH of 6.9. Descending chromatography with a development time of 15 hours was employed. The resulting spots on developed chromatograms, visible under fluorescent light[3] were outlined in pencil. Discs 11 mm in diameter were cut from the center of the outlined spots, and bioassayed.

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[1] Paper chromatography: procedures and techniques, section 2, page 25.

[2] Test for reducing sugars, section 3, page 27.

[3] Sodium Omadine was detected on paper chromatograms as a "quench" spot under fluorescent light at quantities of 10 µg or greater.

Autobiograms of the above chromatograms were made as follows: the edges of two 18 inch square glass panes were covered with Scotch masking tape. One-quarter inch wide cardboard strips were inserted under the masking tape along the edges of the glass on one side of each pane. Potato dextrose agar seeded with spores of G. cingulata was poured onto the surface of one of the glass plates. The chromatogram strips were placed on the surface of the solidified agar. The second glass pane was positioned over the first, and the outline of the chromatogram strips and the pencil outlines of the "quench" spots on the chromatograms were outlined on the surface of the upper glass pane. The chromatogram strips were removed after 5 minutes and the upper glass pane sealed to the first with masking tape. The assay plate was allowed to stand for 36 hours at room temperature. The outlines of the zones of inhibition were marked on the surface of the upper glass pane and also on the respective chromatograms.

b. Purification

All lots of the Omadine compounds were supplied as technical grade compounds. Attempts to obtain chemically pure compounds from the manufacturer were unsuccessful. Communications from Squibb Institute for Medical Research (62) and Olin Mathieson Chemical Corporation (45) stated that the sodium salt was insoluble in the common organic solvents, and that it could contain as much as two moles of water of crystallization depending upon its method of

preparation. However, if a solvent were found in which sodium Omadine were soluble at room temperature or slightly higher, purification of the inhibitor could be readily accomplished by recrystallization. Therefore, over thirty organic solvents were used in an attempt to find one in which sodium Omadine was soluble. None was found; however, it was noted that in several solvents there was a color change of the solvent while the sodium Omadine appeared to remain unchanged. It was hypothesized that impurities were soluble and could therefore be removed by several successive exposures to fresh solvent followed by filtration. Iso-propyl alcohol, tertiary-amyl alcohol, and secondary-butyl alcohol were selected as solvents. Technical grade sodium Omadine Id. JYP-8Cr-1134 was exposed to four washings of each of these solvents respectively. After the final filtration, the filtrates were dried for 12 hours under vacuum. Melting points of the technical grade compound and the compound as extracted by each of the three solvents were determined. The ultraviolet absorption spectra for 10 µg/ml of the compound from each extraction method was determined and compared to that presented in a brochure by the Squibb Institute for Medical Research (62). The extracted compounds were also bioassayed and chromatogramed, and compared to technical grade sodium Omadine.

c. Chemical tests

The sodium azide:iodine reaction (5), the sodium nitroprusside test (66), the platinic iodide test (75), and the bromine

reaction (64) were used to determine the presence of -SS and -SH groups of technical grade and "purified" sodium Omadine in paper chromatograms. Omadine disulfide, methionine and cysteine were run as checks for all chemical tests employed.

2. Location of the action of the Omadine compounds

Spores of G. cingulata were suspended in sterile glass-distilled water, centrifuged and resuspended in sterile glass-distilled water five successive times. Washed spores were standardized so that there would be 600,000 spores per ml. Seven 2 ml samples of the spore suspension were pipetted into separate test tubes. Disulfide, sodium, ferric and copper Omadine solutions, as well as sodium Omadine:cupric chloride, and cupric chloride solutions were prepared at 2 ppm in sterile glass-distilled water. Two ml samples of each of the above solutions were added to the 2 ml samples of spore suspensions. The final spore suspension was 300,000 spores per ml, and the final concentrations of metal salts and Omadine compounds were all 1 ppm. A sterile glass-distilled water check was also used. The spore suspensions were allowed to stand for 12 hours and 24 hours, after which they were centrifuged and resuspended in 4 ml sterile glass-distilled water five successive times. One drop of the final spore suspensions was deposited on a Petri plate containing potato dextrose agar. After three days the plates were examined for growth.

In a second experiment, spores were washed as before and standardized so that there were 1,000,000 spores/ml. The final spore suspension in

the treatments was 500,000 spores/ml. Ferric chloride, zinc chloride, manganous sulfate and cupric chloride were added respectively to a sterile glass-distilled water solution of sodium Omadine. The final solutions contained 2 ppm of the metal salt plus 2 ppm sodium Omadine. Check solutions of sodium Omadine and each of the four metal salts respectively were prepared at 2 ppm. A glass-distilled water check was also included. Drops of the final spore suspensions were placed on Petri plates containing potato dextrose agar as before. The remaining spore suspensions were centrifuged and the spores resuspended in 4 ml of 2 percent sucrose. One drop of the spore suspension in sucrose was deposited on microscope slides which were inverted and suspended in a moist chamber for 24 hours, after which time a spore germination count was made.

Additional techniques related to this study

1. Filter-paper disc bioassay technique

The filter-paper technique proved excellent for assay of water soluble inhibitors, but was of little value for assay of insoluble compounds (30). Many modifications of this technique have been published (19,32,35,65,70). The method employed throughout this study is as follows: potato dextrose agar was melted and allowed to cool to approximately 42° C, after which it was seeded with spores of G. cingulata and poured into Petri dishes. Each Petri dish contained about 10 ml of media spread thinly over the bottom of the dish. Discs 11 mm in diameter were cut from large sheets of Whatman filter paper No. 1 by means of a cork

borer. The discs were saturated with samples to be examined and the excess removed by momentary blotting on clean filter paper by means of forceps. After two days zones of inhibition (clear zones) were measured in mm from the edges of the discs to the rim of growth.

2. Paper chromatography: procedures and techniques

a. Extraction and preparation of samples

Free amino acids were extracted by a modification of the method of Dent et al. (13). Composite samples of fresh leaves were pulverized in a blender with sufficient 95 percent ethanol so that the final concentration of ethanol was approximately 80 percent. The mash was evaporated to dryness in a 60° C oven, ground in a mortar, passed through a fine wire mesh screen, placed in a capped bottle and stored at room temperature. Two ml of 80 percent ethanol were added to every 10 mgm of dried sample. This was placed in a refrigerator for 24 hours, and shaken at intervals. The insoluble material was removed by filtration and washed with 80 percent ethanol (0.5 ml per 10 mgm original dried material). The ethanol extracts were bulked and 3 volumes of chloroform added to each volume of ethanol extract. After shaking, the resulting aqueous layer was removed and stored under refrigeration (4). The extraction with ethanol diminished interference by substances such as inorganic ions and certain proteins (26), while chloroform extraction further removed many fatty materials. The chloroform remaining in the aqueous layer aided in preserving the amino acids and sugars present (63).

Combined amino acids were extracted by the method employed by Hrushovetz (26). The residue was treated with the ethanol and chloroform extraction method as employed for free amino acids. The resulting aqueous layer containing the amino acid hydrolysates was exposed to 6N NH_4OH vapors under a bell jar, until pH 7 was reached.

The removal of inorganic ions and fatty substances via these methods was excellent. When placed in a Reco Electric Desalter, Model R1500, samples repeatedly drew less than 0.2 amperes at 30 volts. This is as good or better ion extraction than could be obtained by use of such an electrolytic desalting apparatus alone.

b. Application of samples

Samples were applied to Whatman No. 1 filter paper strips and sheets via a multiple spotting apparatus designed and built by the writer (28, pp. 115-17). Sample volumes of 0.1-0.2 ml of free amino acids and 0.05-0.1 ml of combined amino acids were spotted.

c. Further techniques

The one-dimensional descending technique (5) proved expedient, with n-butanol : acetic acid : water = 250:60:250 v/v/v, the solvent. The chromatograms were developed by spraying with 0.25 percent w/v ninhydrin in acetone. To fully develop the color, the sprayed chromatograms were dried in a hood then placed in a moist atmosphere at 90° C for 10 minutes.

Semi-quantitative determinations of samples from treated and untreated plants were made by use of a Photovolt Densitometer Model 425 (5).

3. Test for reducing sugars

The samples were prepared using the methods of extraction employed in preparation of samples for paper chromatography. Two ml of Somogyi's reagent (60) were added to 2 ml of the sample and the mixture placed in a boiling water bath for 10 minutes. After cooling, 2 ml of Nelson's reagent (42) were added and the resultant mixture shaken and allowed to stand for 20 minutes. The sample was then brought up to 100 ml with glass-distilled water and read in a Klett-Summerson Photoelectric Colorimeter, Model 800-3, using a blue filter No. 42.

4. Precipitate test for aqueous solutions of sodium Omadine

In a brochure from the Squibb Institute for Medical Research (62), it was stated that the water-insoluble Omadine salts such as the copper or the zinc salt may be prepared by the treatment of an aqueous solution of the sodium salt with an aqueous solution of a water-soluble metal salt. Since the resulting reaction caused a precipitate to form, it was reasoned that this could be employed as a test for sodium Omadine. It was found that the resulting water-insoluble salts if present at concentrations of 100 µg/ml or above readily formed a visible precipitate. If present at lower concentrations, the precipitate settled out upon standing, and as low as 10 µg were detected by filtration since the precipitate imparted a slight discoloration to the filter paper.

No other Omadine derivatives reacted with these water-soluble metal salts. This test was employed for aqueous solutions of sodium Omadine.

CHAPTER III

RESULTS

Studies of the Various Test Compounds

1. In vivo screening of test compounds

Thiolutin, diaphine HCl, rimocidin sulfate, fungistatin XG and nystatin failed to control P. lingam on cabbage, and A. solani and C. phomoides on tomato (55). Sodium Omadine, Panogen 15 and Acti-dione gave excellent control of all three diseases at concentrations of 200, 4.4 and 3 ppm respectively. In the P. lingam trials (Table II), hexachlorobenzene, Panogen 15 and zinc, manganese, copper and sodium Omadine provided a high degree of control at the two levels of concentration employed (Plates I-II). In experiments 1 and 4 the Omadine treatments and Panogen 15 gave significantly better control in relation to Acti-dione. Hexachlorobenzene gave significantly better control than Acti-dione in experiments 1, 2 and 3. Panogen 15, and copper and sodium Omadine gave better control than hexachlorobenzene on Chinese cabbage in experiments 3 and 4.

Highly significant differences in control were obtained in experiments 5, 6 and 7 between treated and untreated beans inoculated with C. lindemuthianum, except with the Acti-dione treatment in experiment 7 (Table III). Panogen 15 and sodium, copper and zinc Omadine treatments provided highly significant control in comparison to Acti-dione in

TABLE II. Influence of various antifungal materials on disease incidence of *Phoma lingam*.

Experiment No.	Concentration of active ingredients	Treatment Means				
		Cabbage		Chinese Cabbage		
		1	2	3	4	10
Check		5.86	8.00	8.00	7.36	7.6
Acti-dione	3 ppm	4.00	4.86	5.50	4.38	---
Hexachlorobenzene	200 ppm	3.00	3.14	3.88	3.75	3.4
	100 ppm	----	----	----	----	3.9
Panogen 15*	4.4 ppm	2.71	2.86	3.13	2.75	3.1
	2.2 ppm	----	----	----	----	4.5
Zinc Omadine	200 ppm	2.71	2.43	2.50	3.38	3.1
	100 ppm	----	----	----	----	4.6
Manganese Omadine	200 ppm	2.57	2.29	2.75	3.00	3.3
	100 ppm	----	----	----	----	4.7
Copper Omadine	200 ppm	2.43	2.57	3.25	2.63	2.7
	100 ppm	----	----	----	----	3.5
Sodium Omadine	200 ppm	2.29	2.43	2.63	2.63	2.9
	100 ppm	----	----	----	----	3.5
L.S.D. .05		0.74	0.72	0.39	0.74	0.55
.01		0.99	0.96	0.53	0.98	0.73

*Methyl mercury dicyandiamide at 4.4 ppm is roughly equal to 3 ppm of mercury.

PLATE I.

Spray trials for control of Phoma lingam on Chinese cabbage (experiment 10).

Left: untreated check.

Center: treated with hexachlorobenzene at 200 ppm.

Right: treated with hexachlorobenzene at 100 ppm.

Left: untreated check.

Center: treated with Panogen 15 at 4.4 ppm.

Right: treated with Panogen 15 at 2.2 ppm.

Left: untreated check.

Center: treated with zinc Omadine at 200 ppm.

Right: treated with zinc Omadine at 100 ppm.


CK
Hex 200
CK
Pan 200
Pan 100
CK
Zn Pan 200
Zn Pan 100

PLATE II.

Spray trials for control of *Phoma lingam* on Chinese cabbage (experiment 10).

Left: untreated check.

Center: treated with manganese Omadine at 200 ppm.

Right: treated with manganese Omadine at 100 ppm.

Left: untreated check.

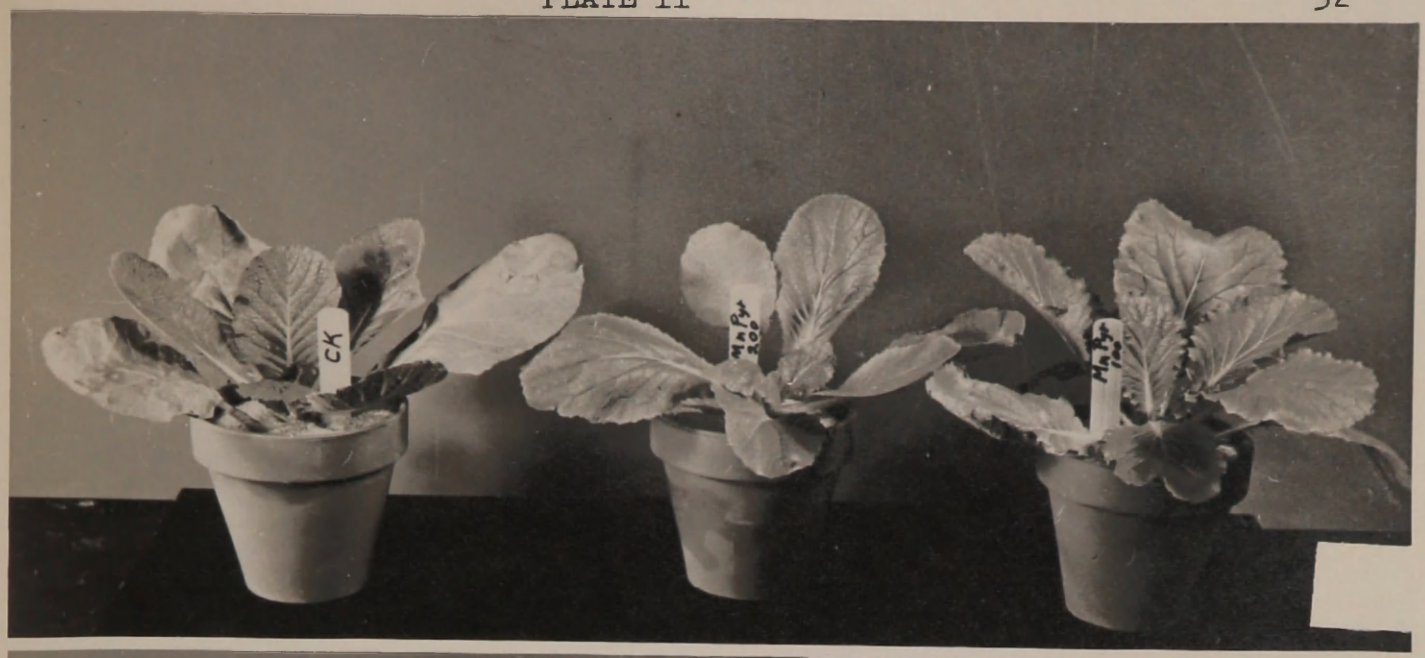
Center: treated with copper Omadine at 200 ppm.

Right: treated with copper Omadine at 100 ppm.

Left: untreated check.

Center: treated with sodium Omadine at 200 ppm.

Right: treated with sodium Omadine at 100 ppm.


CK
Mn Pyr 300
Mn Pyr 100


CK
Cu Pyr 300
Cu Pyr 100


CK
Mn Pyr 300
Mn Pyr 100

TABLE III. Influence of various antifungal materials on disease incidence of Colletotrichum lindemuthianum on beans.

Experiment No.	Concentration of active ingredients	Treatment Means			
		5	6	7	11
Check		8.25	7.00	9.00	6.5
Acti-dione	3 ppm	2.88	4.86	8.00	---
Hexachlorobenzene	200 ppm	4.25	4.14	4.29	3.1
	100 ppm	----	----	----	3.3
Panogen 15	4.4 ppm	2.50	3.14	1.57	1.3
	2.2 ppm	----	----	----	1.4
Zinc Omadine	200 ppm	2.25	1.86	4.14	3.5
	100 ppm	----	----	----	5.5
Manganese Omadine	200 ppm	3.13	4.14	4.29	5.5
	100 ppm	----	----	----	5.9
Copper Omadine	200 ppm	2.00	1.57	4.29	5.4
	100 ppm	----	----	----	5.6
Sodium Omadine	200 ppm	2.00	1.86	3.71	4.0
	100 ppm	----	----	----	4.9
L.S.D. .05		1.46	1.00	2.08	0.51
.01		1.94	1.34	2.76	0.67

experiments 6 and 7 (Plates III-VI). Copper, sodium and zinc Omadine gave better control than hexachlorobenzene in experiment 5. Experiments 5 and 6 (Table III) were completed during the months of January and February during which time the greenhouse maintained an even temperature of about 22° C. Experiment 7 was completed during late March; and 11, in April when the greenhouse temperatures rose appreciably during the daylight hours (Table III). Control of C. lindemuthianum was reduced at the higher temperatures during experiments 7 and 11 (Table III) for all treatments except Panogen 15. Panogen 15 gave significantly better control than other treatments in experiment 7 and highly significant control in relation to other treatments in experiment 11.

Results in the first P. sorghi trial (Table IV, exp. 8) were erratic with hexachlorobenzene and zinc Omadine giving no control in this experiment, but all other treatments gave good control. In the second trial (Table IV, exp. 9), the treatment means for Acti-dione, hexachlorobenzene, Panogen 15 and zinc, manganese, copper and sodium Omadine showed a high degree of significance over the inoculated untreated checks. All of the other compounds in this experiment were better than Acti-dione.

2. Phytotoxicity

Thiolutin at 200 ppm when applied as foliar sprays produced epinasty and bronzing of the leaves of tomato plants, but only a slight mottling on bean plants (55). Foliar sprays of fungistatin XG caused epinasty of tomato, leaf crinkle on cabbage, slight leaf puckering on beans, and chlorotic spots on cucumber leaves (55). Foliar sprays of diaphine HCl

PLATE III.

Spray trials for control of *Colletotrichum lindemuthianum* on bean (experiment 7).

Left: untreated check.

Right: treated with Acti-dione at 3 ppm.

Left: untreated check.

Right: treated with hexachlorobenzene at 200 ppm.


Control
Act.

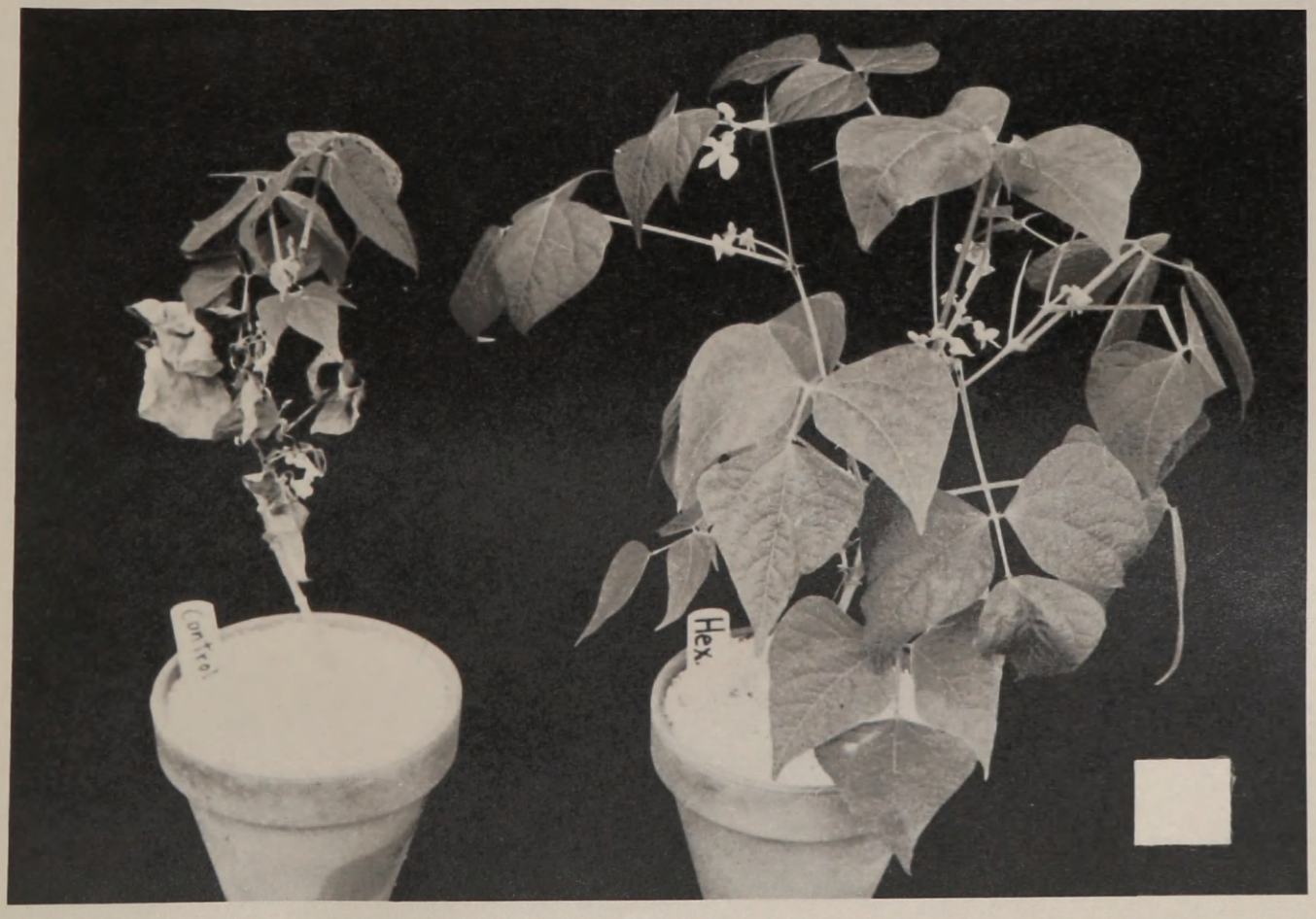

Control
Hex.

PLATE IV.

Spray trials for control of Colletotrichum lindemuthianum on bean (experiment 7).

Left: untreated check.

Right: treated with Panogen 15 at 4.4 ppm.

Left: untreated check.

Right: treated with zinc Omadine at 200 ppm.


Control
Pan.

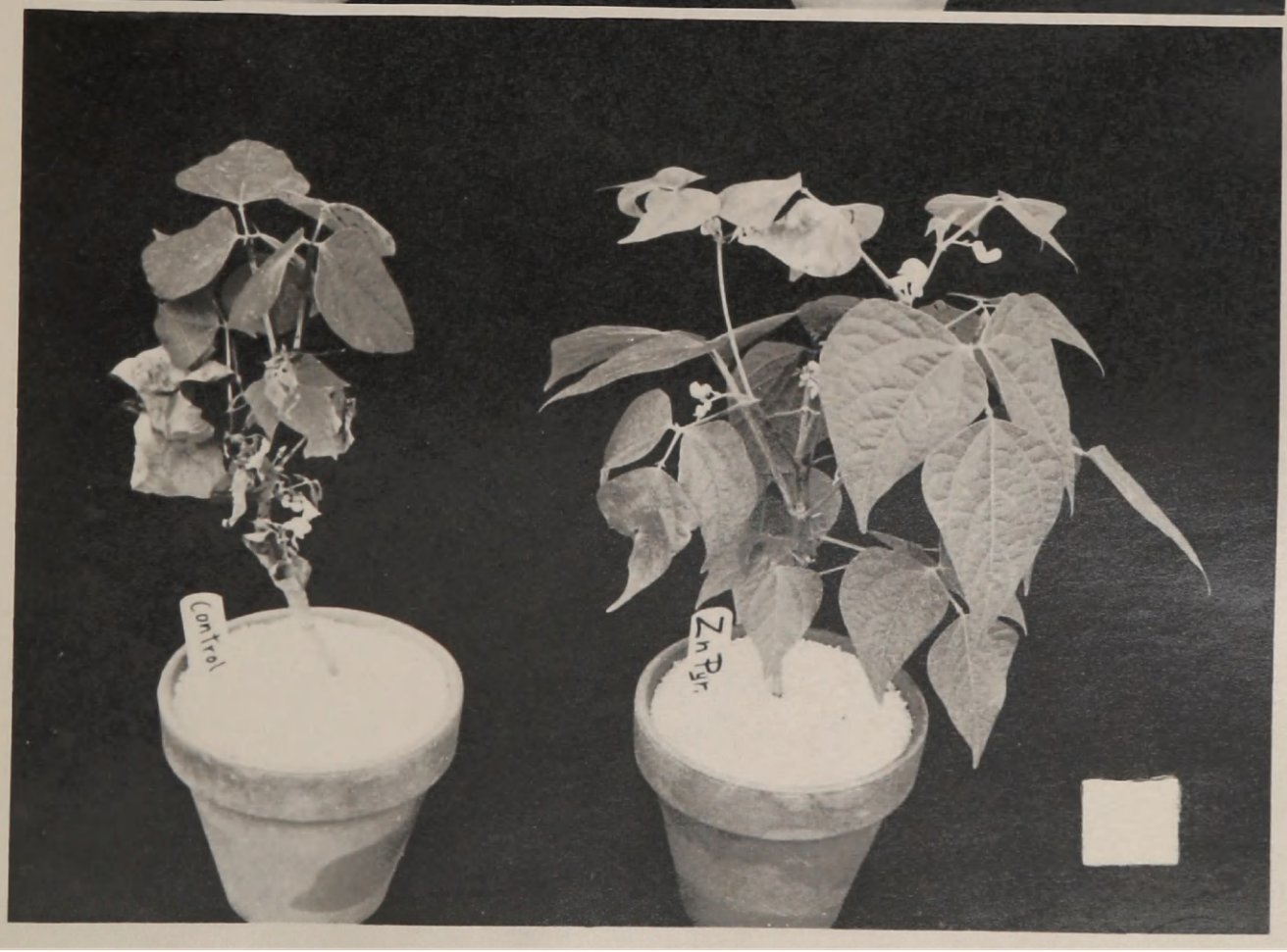

Control
Zn Pyr.

PLATE V

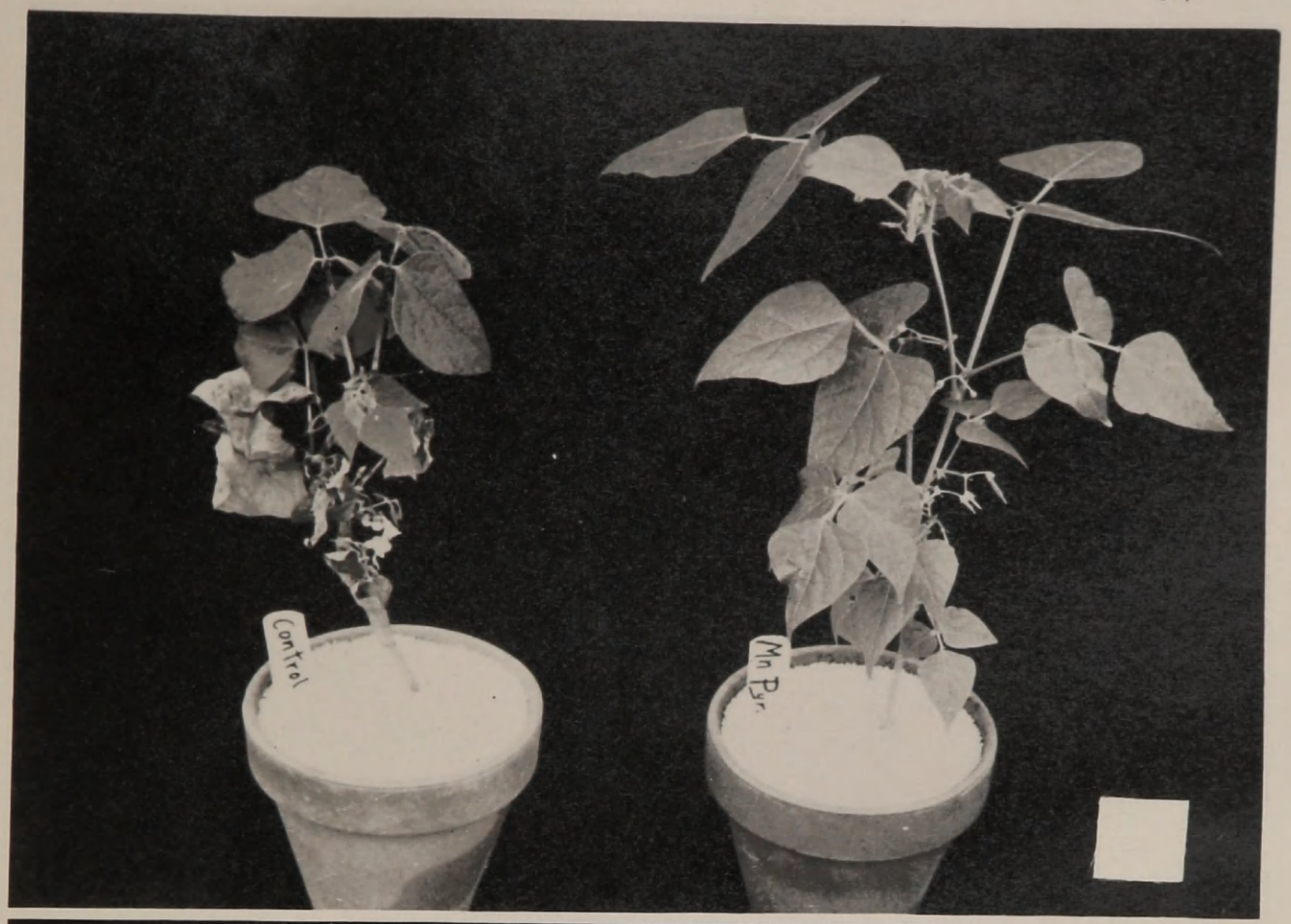
Spray trials for control of Colletotrichum lindemuthianum on bean (experiment 7).

Left: untreated check.

Right: treated with manganese Omadine at 200 ppm.

Left: untreated check.

Right: treated with copper Omadine at 200 ppm.


Control
Mn Pyr.


Control
Cu Pyr.

PLATE VI.

Spray trials for control of Colletotrichum lindemuthianum on bean (experiment 7).

Left: untreated check.

Right: treated with sodium Omadine at 200 ppm.

Reading from left to right: untreated check, treated with Acti-dione at 3 ppm, Panogen 15 at 4.4 ppm and hexachlorobenzene at 200 ppm.


Control
Na Pr.


Pan.
Hex

TABLE IV. Influence of various antifungal materials on disease incidence of Puccinia sorghi on corn.

Experiment No.	Concentration of active ingredients	Treatment Means 8	9
Check		5.00	3.50
Acti-dione	3 ppm	2.71	2.70
Hexachlorobenzene	200 ppm	4.43	1.90
Panogen 15	4.4 ppm	2.57	1.80
Zinc Omadine	200 ppm	4.29	2.00
Manganese Omadine	200 ppm	2.86	1.80
Copper Omadine	200 ppm	2.43	1.60
Sodium Omadine	200 ppm	2.00	1.90
L.S.D.	.05		
	.01		

produced a toxic reaction on bean, cucumber (Plate VII), tomato and cabbage, but not on corn. This toxic reaction generally consisted of chlorotic spots or chlorotic leaf margins on leaves which were immature at time of application. Diaphine HCl produced chlorotic rings on cabbage which were similar in appearance to certain virus symptoms. In most cases, however, the host plant recovered, and any new growth that appeared was either free of injury or only slightly injured. At lower concentrations of 100, 50 and 25 ppm, diaphine HCl still produced marked chlorosis on cucumber and beans, but a marked reduction in phytotoxicity was exhibited at lower concentrations on cabbage and tomato (55). Rimocidin sulfate applied as foliar sprays at 200 ppm caused slight puckering on bean and cucumber leaves, but the damage was not severe (55). Acti-dione when applied as a foliar spray at 3 and 5 ppm caused necrotic spots on the leaves of tomato, cucumber, bean and cabbage plants; however, phytotoxicity has been reported with this material (18). Foliar sprays of Panogen 15 and Nystatin were not phytotoxic at 4.4 and 200 ppm respectively, but hexachlorobenzene at 200 ppm caused necrotic spots on foliage. At 200 and 100 ppm, copper Omadine foliar sprays were more phytotoxic than zinc, manganese and sodium Omadine. Single foliage applications of copper Omadine at 200 and 100 ppm on Chinese cabbage caused appreciable plant stunting, while zinc, manganese and sodium Omadine at 200 ppm produced only slight stunting and leaf puckering (Plate VII). Single foliage applications of the four Omadine salts at 200 ppm were not phytotoxic to corn and beans. Wherever phytotoxic reactions were caused by foliar sprays of Omadine salts, the plants recovered quickly with new growth appearing normal.

PLATE VII.

Phytotoxicity caused by one foliar spray of diaphine HCl at 200 ppm on cucumber.

Left: untreated check.

Right: treated plant showing chlorosis.

Phytotoxicity caused by one foliar spray of diaphine HCl and sodium Omadine at 200 ppm on bean.

Left: untreated check.

Center: sodium Omadine treated plant showing puckering.

Right: diaphine HCl treated plant showing chlorosis.


56
57

Seedlings grown in hydroponic solutions containing Omadine salts at 5 ppm exhibited root stunting and little secondary root formation. Root stunting was severe in 0.11 ppm Panogen 15, but less severe in 5 ppm hexachlorobenzene, while the plants in 3 ppm Acti-dione solution died within three days. After 8 days, the young leaves of plants in the hexachlorobenzene solutions showed necrotic brown spots. Tomato and cucumber seedlings grown in sand were harvested several days after application of 50 ppm sodium Omadine as a root drench. The roots were stunted, discolored and generally severely injured (Plate X). Roots severely injured were not recovering from this injury, but new roots were being regenerated up the stem at or above the soil line.

3. Experiments on the systemic activity of several antifungal compounds in the control of C. lindemuthianum on bean

In the detached leaf culture experiment, C. lindemuthianum inoculated bean leaflets were placed in jars with the petioles extending into a nutrient solution containing one of the antifungal compounds. Hexachlorobenzene, Panogen 15 and zinc, copper and sodium Omadine showed a high degree of significance over inoculated checks, while manganese Omadine was significantly better (Table V). Fungal and bacterial contaminates were a problem in this experiment, and the Acti-dione treatment was discarded because of excessive contamination. Leaflets in control jars containing eosine showed that the dye was translocated throughout the xylem system of the blade inside of 24 hours. It was therefore assumed that the antifungal compounds were also translocated throughout the xylem system in this period of time.

TABLE V. Influence of various antifungal materials via systemic action on disease incidence of Colletotrichum lindemuthianum on bean leaflets in detached leaf culture.

		Concentration of active ingredients	Treatment Means
Check			8.63
Hexachlorobenzene		5 ppm	5.13
Panogen 15		0.11 ppm	3.88
Zinc Omadine		5 ppm	4.50
Manganese Omadine		5 ppm	6.63
Copper Omadine		5 ppm	5.75
Sodium Omadine		5 ppm	5.75
L.S.D.	.05		1.63
	.01		2.18

In the hydroponic experiment in which beans were grown in solutions containing test compounds and then inoculated, the disease incidence was erratic. However, sufficient infection occurred to determine that foliage infection by C. lindemuthianum was not controlled by any systemic activity of Panogen 15, Acti-dione, hexachlorobenzene, or the Omadine salts.

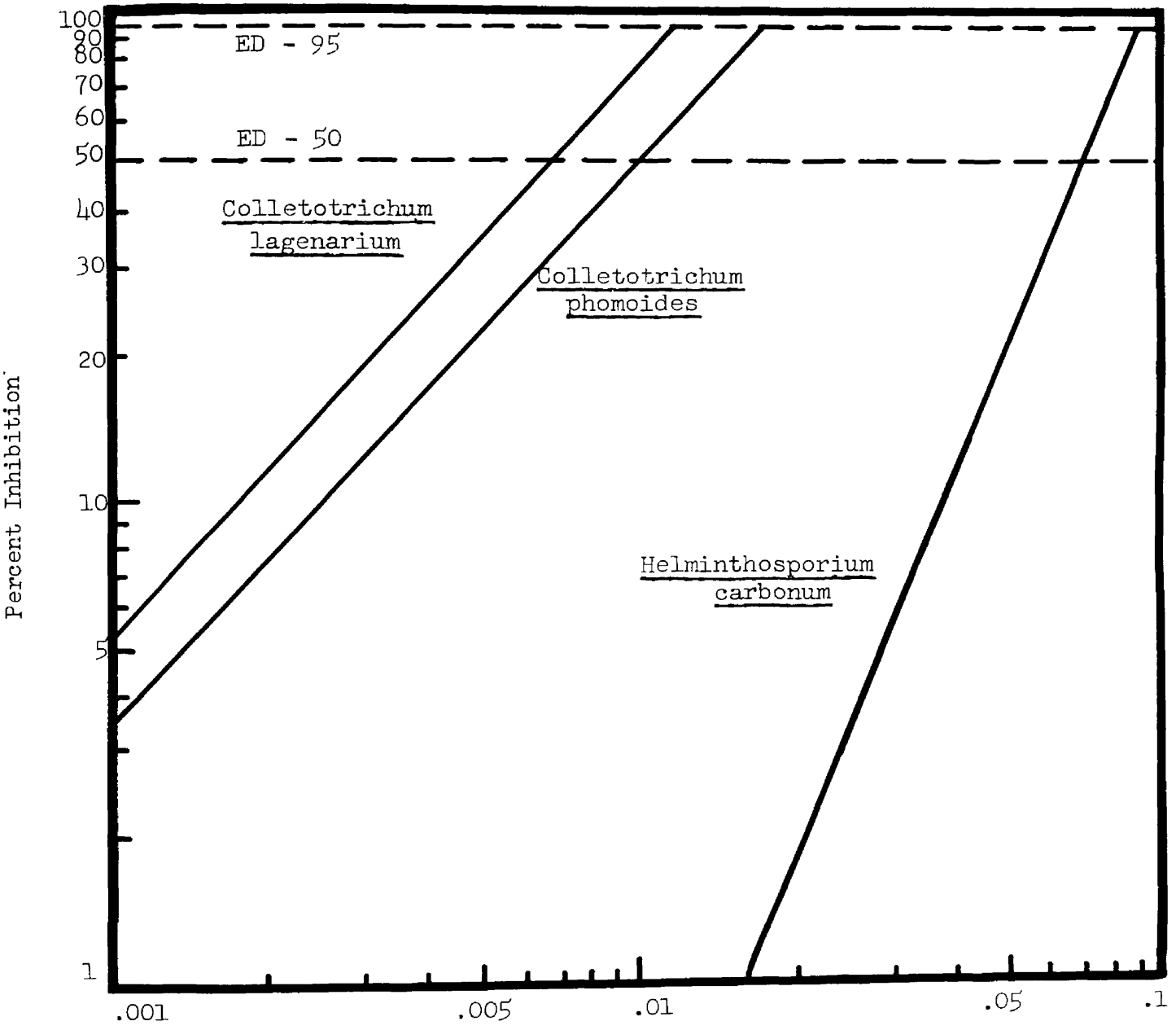
Studies of Sodium Omadine

1. Spore germination tests

Dosage response (DR) curves for C. lagenarium, C. phomoides and H. carbonum under the influence of sodium Omadine are presented in Graph I.

GRAPH I

Dosage response curves of the spores of three fungal organisms under influence of sodium Omadine at several concentrations.



Logarithmic: three by three inch cycles

The three DR curves are linear in nature, with those for the Colletotrichum species closely parallel, being similar in slope and position. The ED 50[1] values for C. lagenarium, C. phomoides and H. carbonum were 0.007, 0.011 and 0.08 ppm sodium Omadine respectively; and total inhibition occurred at 0.05, 0.05 and 0.10 ppm respectively.

Deformities of germ tubes below ED 50 values were noted in several cases. The most noticeable of these was a swelling or vesicle occasionally produced at the tip of germ tubes of H. carbonum (Plate VIII) at 0.05 ppm of the compound.

2. Stability of sodium Omadine on bean foliage

Leaf discs, punched from bean leaves 2 hours after being sprayed with sodium Omadine at 200 ppm, developed an inhibition zone 2.55 mm wide when bioassayed. Leaf discs removed 48 hours after spraying developed inhibition zones 1.4 mm wide and those removed 96 hours after spraying showed an inhibition zone 0.275 mm wide.

3. Growth response of L. minor to sodium Omadine

Data from the two experiments using sodium Omadine at concentrations of 5 to 0.1 ppm and 0.1 to 0.005 ppm sodium Omadine are presented in Graph II. These data represent a growth period of four weeks and are presented as percent change from control on a wet weight basis. The -94 percent as given in Graph II for the concentration 0.1 ppm is an average of two experiments at this concentration.[2] The lethal threshold

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[1] The effective dose for 50 percent spore inhibition is hereinafter referred to as ED 50, often termed the lethal dose (LD) by some authors (14).

[2] The values for the sodium Omadine concentration 0.1 ppm in the two experiments were -93.5 percent and -94.2 percent respectively.

PLATE VIII.

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

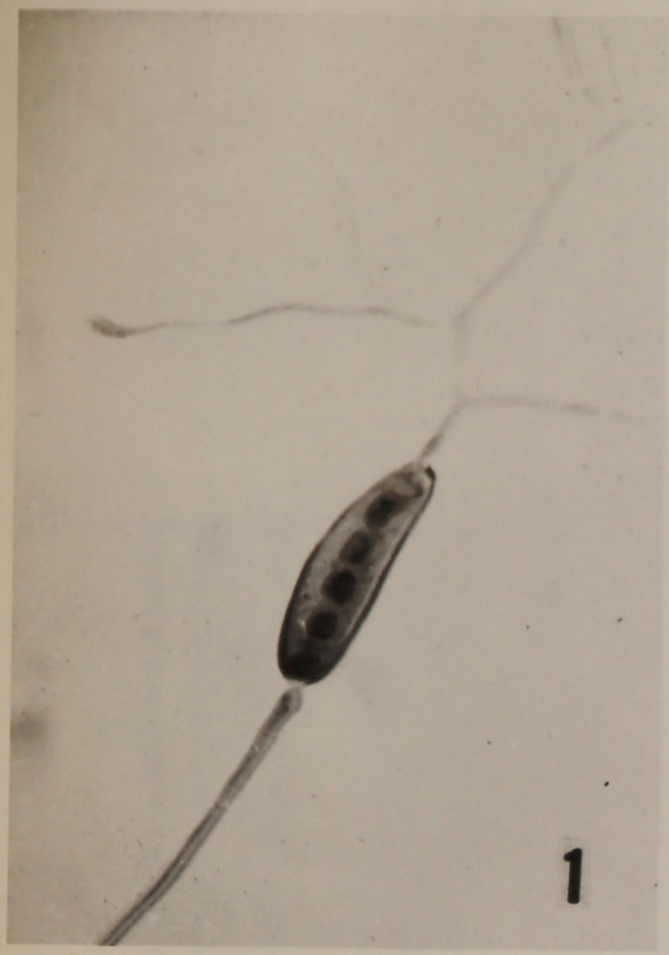
1. Control (magnified 525 diameters).

2. 0.05 ppm sodium Omadine (magnified 1,000 diameters). Note the swelling or vesicle at the end of the germ tube.

3. 0.1 ppm sodium Omadine (magnified 1,000 diameters).

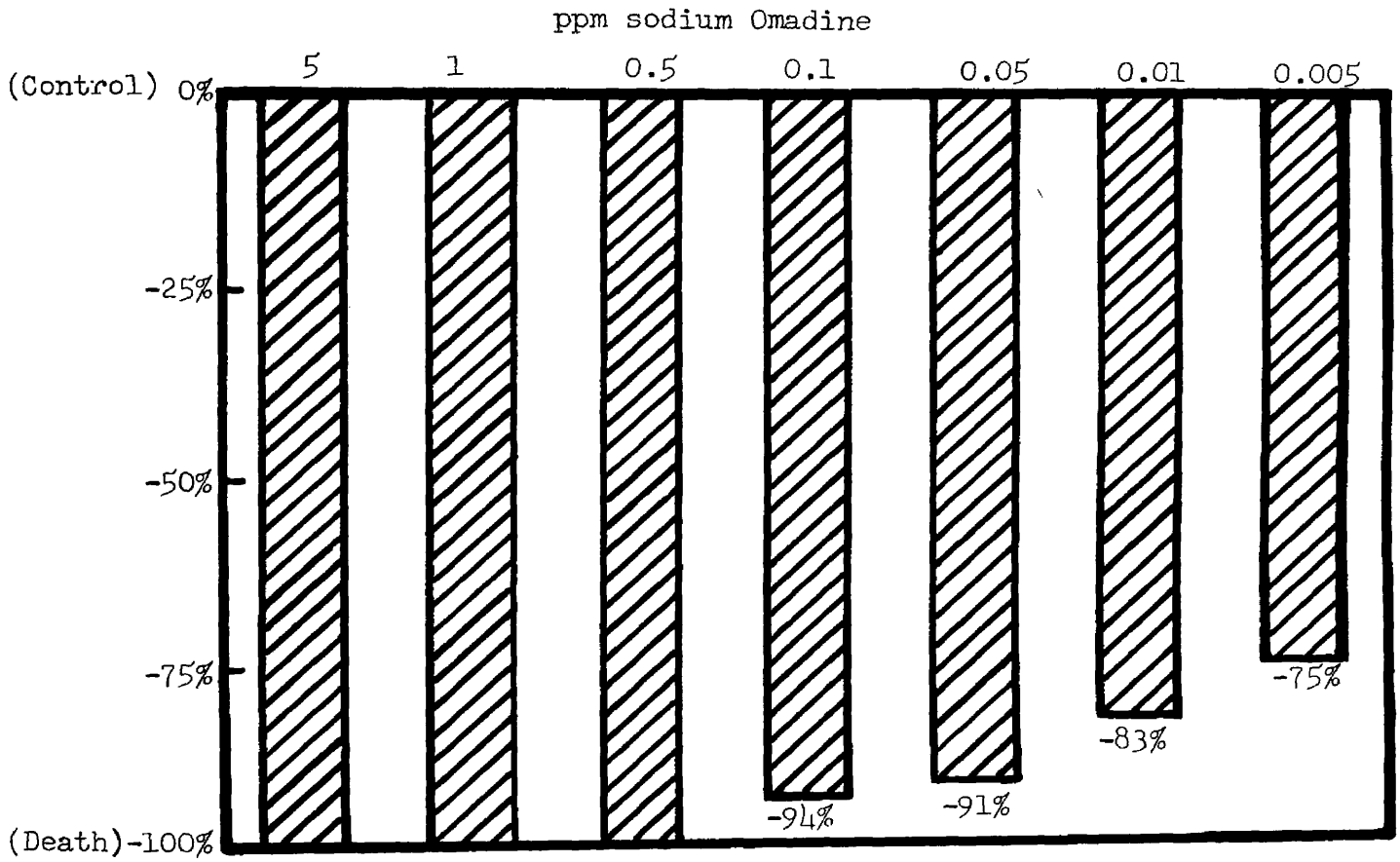
4. 1.0 ppm sodium Omadine (magnified 525 diameters).

PLATE VIII



GRAPH II

Influence of sodium Omadine at seven concentrations on the growth of Lemna minor given in percent change from control on wet weight basis (after 4 weeks)



for growth of L. minor under these conditions was between 0.5 and 0.1 ppm sodium Omadine. Of significance is the fact that 0.005 ppm of the compound caused a marked retardation in growth. At all sub-lethal concentrations there was a considerable decrease in frond size as compared to the controls. A comparison between untreated checks and 0.05 ppm sodium Omadine at the end of four weeks growth is seen in Plate IX.

The influence of 0.05 ppm sodium Omadine upon L. minor over a six week growth period is very striking (Graph III). The lag period of growth in the treatments has been considerably extended. The plants appeared to have recovered from the influence of the treatments at the end of the fifth week. Frond color and size appeared more normal at the end of the sixth week.

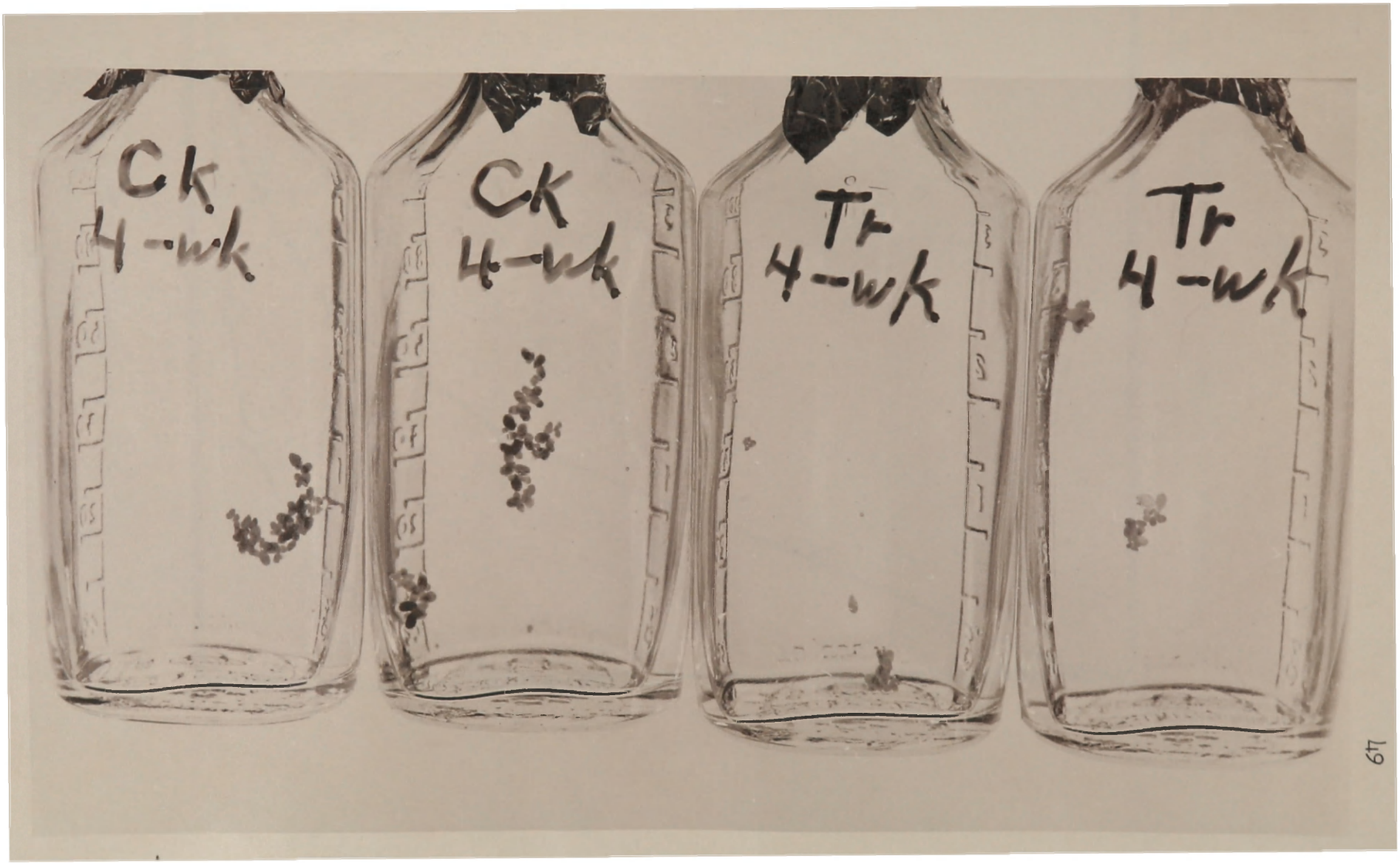
4. Translocation studies of sodium Omadine

Bean plants placed in Hoagland's solution containing 5 ppm and 25 ppm sodium Omadine were removed after four days and stem and root sections cut out and bioassayed. A measurable amount of activity was found in stem sections two inches from the root tip in those plants grown in solutions containing 25 ppm, but only 0.5 inches from the root tip in solutions 5 ppm. The roots extended approximately 1.5 inches into the solutions. However, it should be noted that these roots were slightly discolored and less turgid than roots of the control plants, indicating injury and perhaps death of the roots.

The results of bioassay experiments on guttation water from corn, cucumber and tomato seedlings grown in treated sand, indicate that the compound was not present in the active state in the guttation water of

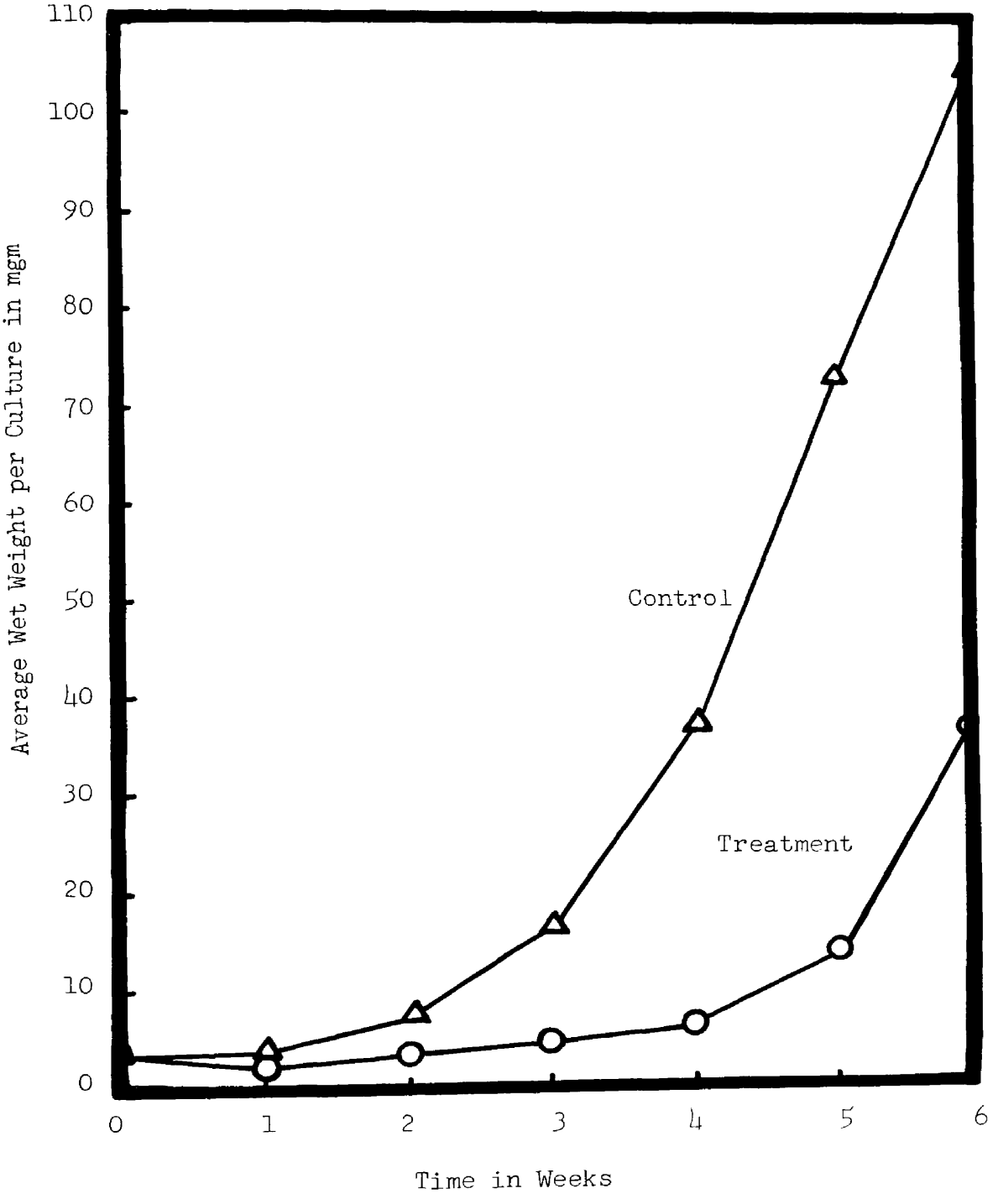
PLATE IX.

Growth of Lemna minor after a four-week period. Untreated controls on the left and 0.05 ppm sodium Omadine treatment on the right.



GRAPH III

Influence of 0.05 ppm sodium Omadine on the growth of Lemna minor.



these plants at concentrations above 1 ppm.[1] Due to the lack of biological activity, results of these experiments are not presented in tabular form. A bioassay of the sap from the cut ends of cucumber seedlings was made simultaneously. No activity was demonstrated in the sap from treated plants. However, it was noted that considerable bacterial growth was present in the bioassay discs containing exudate from untreated plants, while the discs containing exudate from treated plants were free from bacterial growth. No activity was demonstrated in the split-stem bioassay of tomato seedlings grown in treated sand. Data from bioassay experiments on expressed sap from cucumber, tomato and bean seedlings grown in treated and non-treated sand indicated that the compound was not present in the stems or leaves in the active state at concentrations above 1 ppm.

Table VI presents the results of the bioassay of sand moisture 16 days after application of 50 ppm sodium Omadine to sand in which tomato seedlings were grown. When compared to the bioassay of aqueous solutions of sodium Omadine (Table VII), the sand moisture demonstrated a biological activity equal to 25 ppm sodium Omadine.

The juice expressed from tomato seedlings which had been sprayed 12 hours previously with 100 and 200 ppm sodium Omadine respectively was bioassayed against G. cingulata (Table VIII). The results indicate the presence of 1-2 ppm of the active compound in the leaves and stems, but not in the roots of tomato seedlings. Table IX summarizes results of

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[1] The limitations of the bioassay technique were such that quantities of the inhibitor were undetectable below 1 ppm.

TABLE VI. Bioassay of moisture from sodium Omadine treated and non-treated sand in which tomato seedlings were grown: sixteen days after drench treatment.

	Inhibition zone in mm from edge of discs Average of 4 bioassay discs	
	Moisture from sodium Omadine treated sand	Moisture from non-treated sand
Plate 1	11.25	0
Plate 2	10.75	0
Plate 3	10.50	0
Over-all average	10.83	0

Table VII. Bioassay of distilled water dilutions of sodium Omadine.

	Inhibition zone in mm from edge of discs Average of 8 bioassay discs					
Concentrations:	5 ppm	10 ppm	25 ppm	50 ppm	75 ppm	100 ppm
	6.33	8.12	10.75	13.75	16.75	18.67

TABLE VIII. Bioassay experiments on juice expressed from roots, stems and leaves of tomato seedlings sprayed with sodium Omadine.

	Inhibition zone in mm from edge of discs Average of 24 bioassay discs		
	Roots	Stems	Leaves
Foliage spray 200 ppm sodium Omadine	0	4.21	3.33
Foliage spray 100 ppm sodium Omadine	0	3.21	2.46
Untreated Control	0	0	2.42

TABLE IX. Bioassay experiments on juice expressed from roots, hypocotyls and leaves of radish seedlings sprayed with sodium Omadine.

	Inhibition zone in mm from edge of discs Average of 36 bioassay discs		
	Roots	Hypocotyls	Leaves
Foliage spray 200 ppm sodium Omadine	1.78	2.86	1.53
Foliage spray 100 ppm sodium Omadine	t*	1.85	0.90
Untreated Control	0.42	1.75	1.50

* t = slight clearing, but not measurable.

bioassays of juice expressed from the roots, hypocotyls and leaves of radish seedlings sprayed with sodium Omadine 12 hours previous to bioassaying. The results indicate the presence of approximately 1 ppm of the active compound in the roots and hypocotyls when the foliage had been sprayed with 200 ppm of the compound. No activity was observed from the 100 ppm treated plants. No activity was found in the leaves from the 200 ppm treated plants.

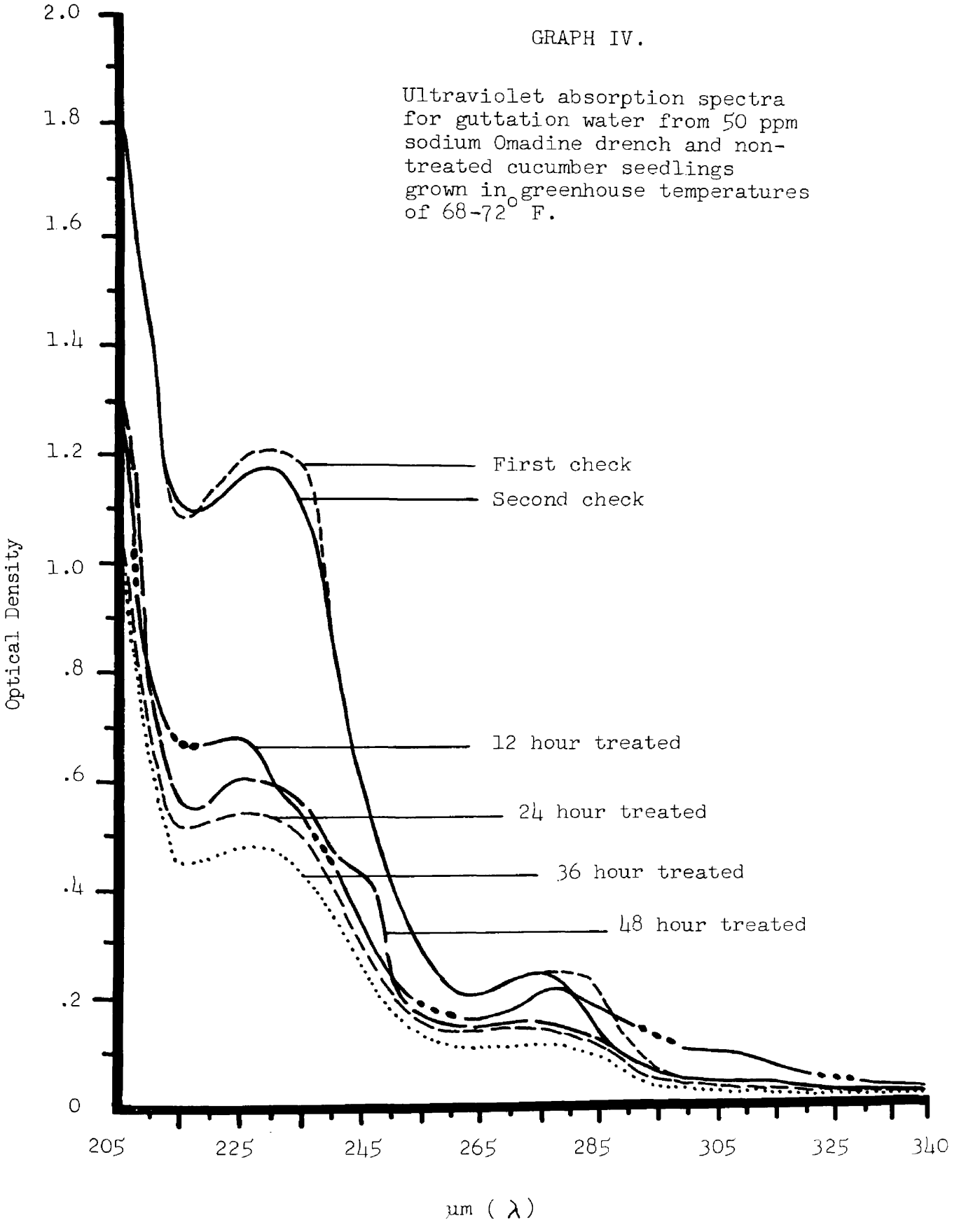
5. Physiological studies on the influence of sodium Omadine on host plants

a. Ultraviolet spectrophotometric determination

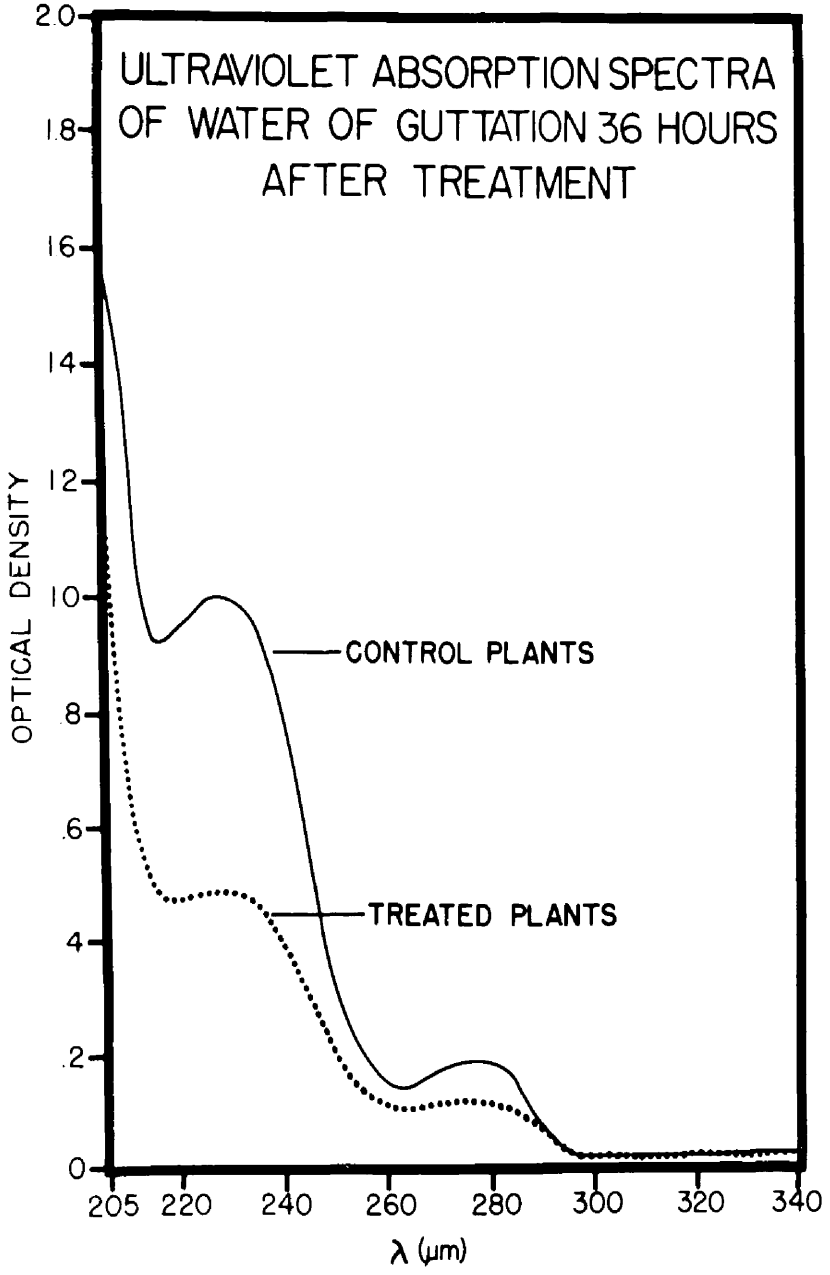
The ultraviolet absorption spectra of water of guttation from cucumber seedlings grown in treated and untreated sand are presented in Graphs IV and V. The absorption spectra for the two samples from treated seedlings are more variable than the checks, and their curves are considerably below those of the checks (Graph IV). Three later experiments were completed in which water of guttation from treated and untreated cucumber seedlings was collected at 12-hour intervals. The absorption spectra curves for the samples from treated plants 12 to 48 hours after treatment were considerably below those of the untreated control plants. However, 60 to 84 hours after treatment the absorption spectra curves approached those from the untreated control plants (Graph V). Ultraviolet spectrophotometric determinations of various aqueous dilutions of guttation water from treated plants and control plants proved that the major differences between guttation samples from treated plants versus

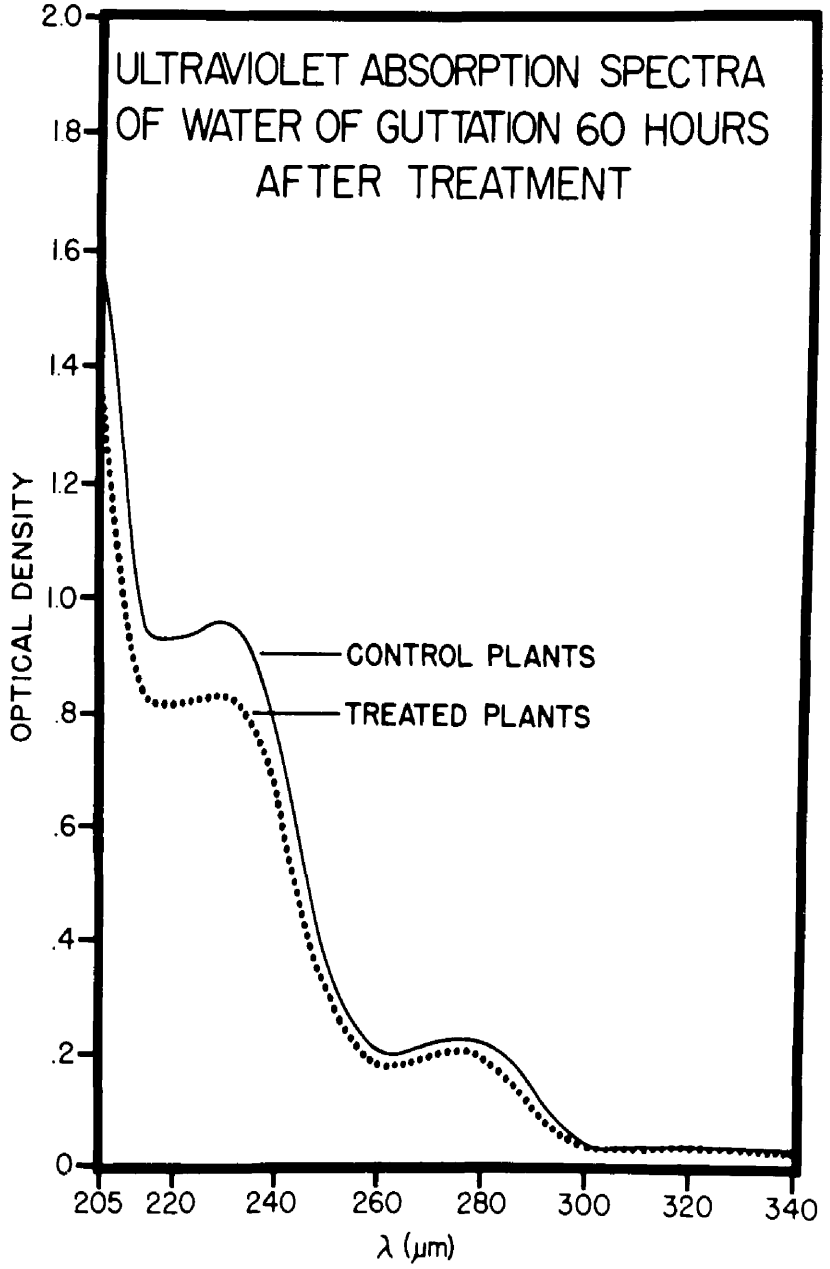
GRAPH IV.

Ultraviolet absorption spectra for guttation water from 50 ppm sodium Omadine drench and non-treated cucumber seedlings grown in greenhouse temperatures of 68-72° F.



Guttation water collected from cucumber seedlings grown in greenhouse temperatures of 68-70° F.





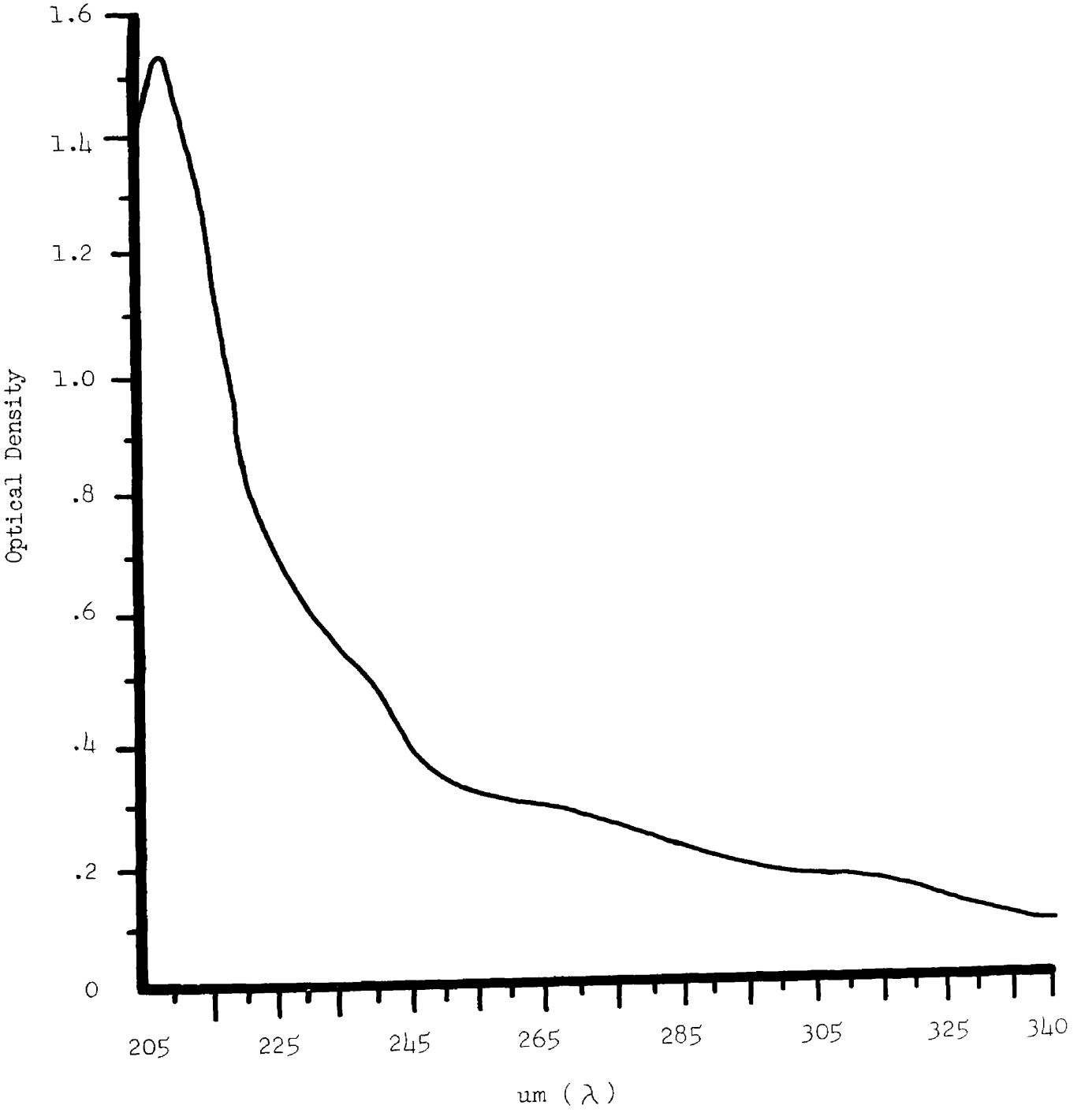
untreated control plants were differences in concentration. Substances in the guttation water from treated plants were not present in as great a concentration as in the untreated control plants.

Guttation samples from treated and control plants were analyzed for amino acids and reducing sugars. Ninhydrin failed to give a color reaction to a spot on filter paper to which a one ml sample of guttation water had been concentrated. Further tests showed that no amino acids were found in guttation water from both treated and control plants. Somogyi's test for reducing sugars in guttation water proved negative.

An interesting temperature phenomenon was observed in these studies. The plants in the first experiments were grown in the greenhouse in temperatures of 68° to 70° F. Several later experiments were run when greenhouse temperatures ranged from 76° F at night to 90° F in the day. When the ultraviolet absorption spectra was determined for samples of guttation from plants grown in higher temperatures, it was found that the absorption spectra curves for the treated and control plants were identical. Furthermore, the guttation samples were examined undiluted, and the character and slope of these latter curves (Graph VI) were entirely different from the absorption spectra curves of guttation water from plants grown in lower temperatures. The nature of this difference due to temperature was not examined. The quantity of guttation water produced by the seedlings at these higher temperatures was many

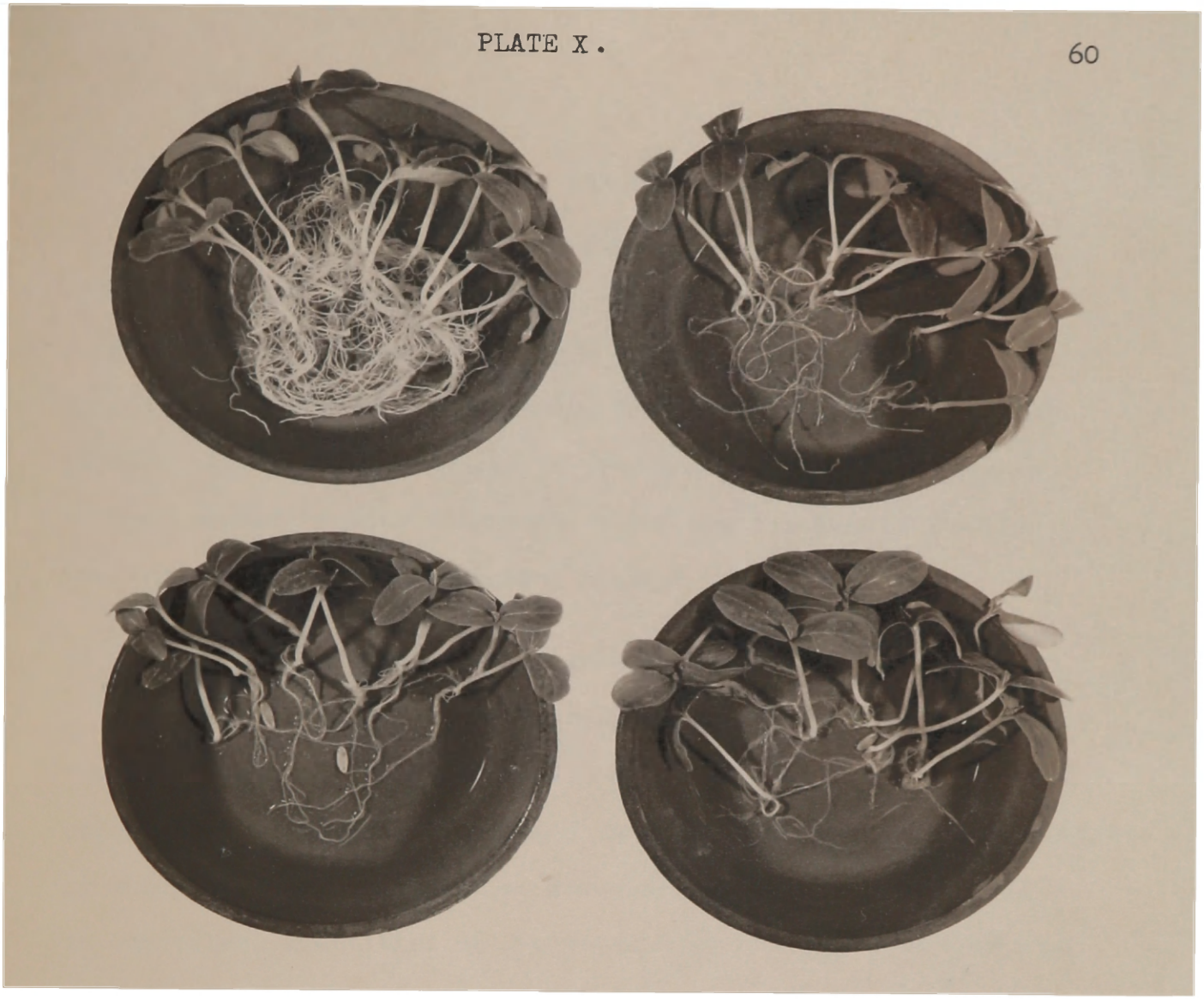
GRAPH VI

Ultraviolet absorption spectra for undiluted guttation water from 50 ppm sodium Omadine, and water drench treated cucumber seedlings grown in greenhouse temperatures of 76-90° C.



times that produced at lower temperatures. It was also found that addition of fertilizer to the sand slightly altered the slope and character of the curve, but this alteration, due to a different level of fertility, was negligible.

It was noticed that less water of guttation was collected from treated seedlings than the untreated controls. Therefore, pot saucers containing an equal number of cucumber seedlings were treated with a water drench and a drench application of sodium Omadine at concentrations of 50, 100 and 200 ppm respectively. The following volumes of water of guttation were collected 24 hours later: 0.8 ml from the water check, and 0.5 ml, 0.25 ml and 0.15 ml respectively from the treatments. The volumes of guttation water collected were inversely proportional to dosage of sodium Omadine. Pot saucers each containing eleven cucumber seedlings were treated 4 days apart with two drench applications of distilled water and sodium Omadine at 50, 100 and 200 ppm respectively. The pot saucers were placed in the greenhouse at temperatures of 55° to 65° F. Four days after the second treatment, the sand was flushed from the roots of the seedlings. The roots of the plants of all treatments (Plate X) were yellowed and stunted, with the 200 ppm treatment showing the greatest damage. Twisting and curling of cotyledons of seedlings from the 200 ppm treatment were also noted.



Cucumber seedlings grown in pot saucers in sand to which two drench treatments 4 days apart of sodium Omadine were applied. Photographed 8 days after the first treatment.

Upper left: water-drench control.

Upper right: 50 ppm sodium Omadine.

Lower left: 100 ppm sodium Omadine.

Lower right: 200 ppm sodium Omadine.

b. Free and combined amino acid studies of treated plants

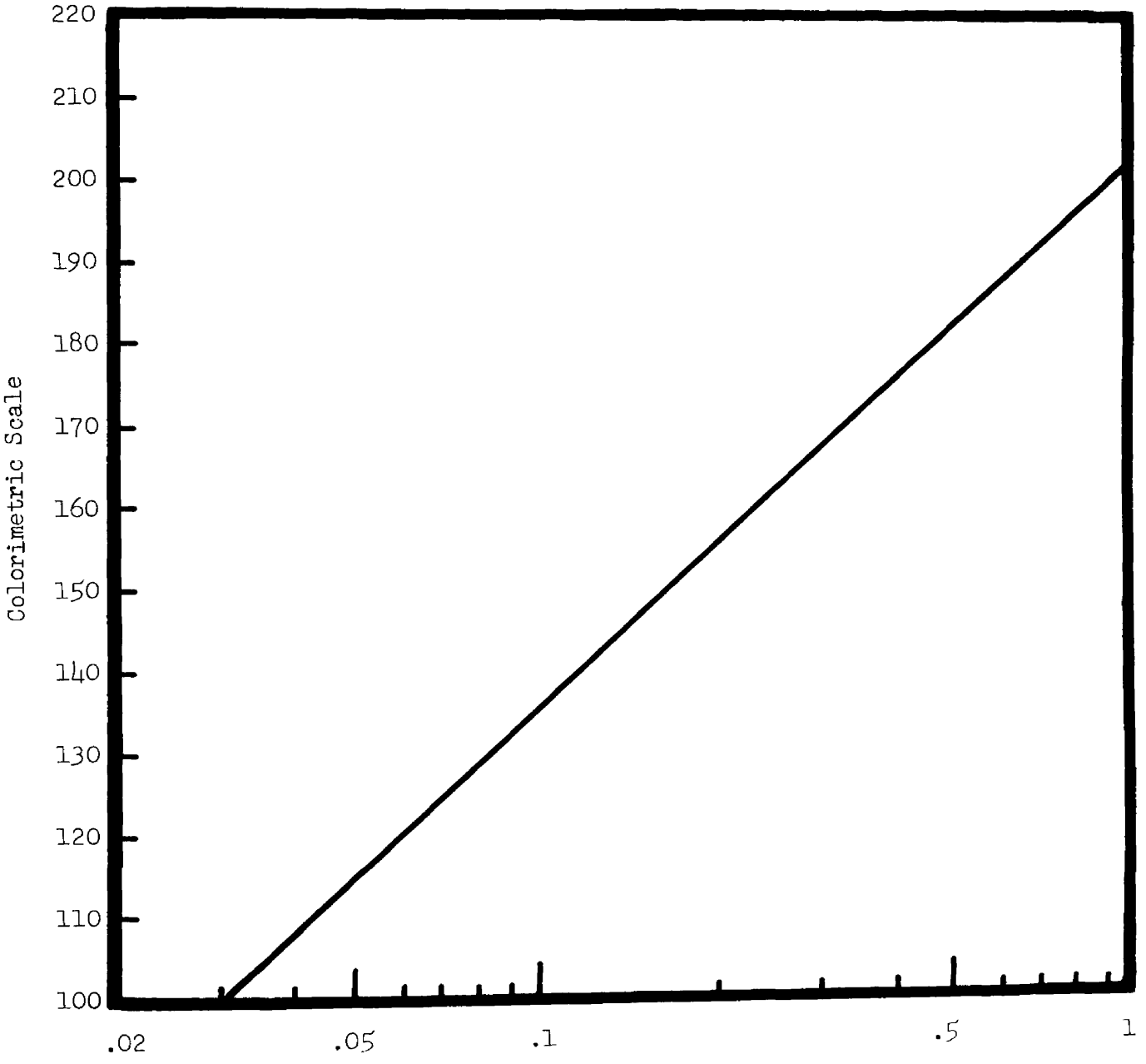
Semi-quantitative determinations of free and combined amino acids of treated plants were made via paper chromatography. No differences in amino acids were observed between plants treated with foliar applications of sodium Omadine and untreated control plants. Also, no differences in free amino acids were observed between plants treated with root drench applications of sodium Omadine and water drench control plants.

c. Reducing sugar studies of treated plants

Data from various concentrations of D-glucose were determined via the Somogyi method and read in a Klett-Summerson photoelectric colorimeter. These data, plotted on Graph VII, served as a standard by which unknown quantities of reducing sugars could be estimated in equivalent quantities of D-glucose. The colorimetric readings of extracts of control and treated plants as determined via the Somogyi method could not be directly evaluated, since the color change was a logarithmic function of the quantity of reducing sugars present in the extracts. By converting the colorimetric readings to equivalent amounts of D-glucose, quantitative differences between the reducing sugars of the treated plant extracts and control plant extracts were expressed in percent change from controls (Table X). The amounts of D-glucose equivalent to reducing sugars in the extracts from the foliar treated cucumber, bean, Chinese cabbage and tomato plants were all below those of the untreated control

GRAPH VII

Concentrations of 0.05 - 1% D-glucose as determined via the Somogyi method and read in a Klett-Summerson Photoelectric Colorimeter.



Semi-logarithmic: 3 cycles x 10 divisions

TABLE X. Reducing sugar determinations of extracts from control and sodium Omadine treated plants as ascertained via the Somogyi method. Foliar spray treated plants harvested 24 hours after 200 ppm sodium Omadine treatment. Root drench treated plants harvested 4 days after application of 50 ppm sodium Omadine. Determinations are based on an average of eight plants per treatment.

Plants	Colorimetric Readings of Untreated Control Plants	Equivalent Amount of D-glucose Expressed in Percent of Solution w/v	Colorimetric Readings of Treated Plants	Equivalent Amount of D-Glucose Expressed in Percent of Solution w/v	Percent Change from Control of Treated Plants Based on Equivalent Amounts of D-glucose
	Foliar Application				
Cucumber	187	0.520	173	0.380	-27
Bean	150	0.170	130	0.085	-50
Chinese cabbage	186	0.512	160	0.272	-53
Tomato	117	0.054	109	0.042	-22
	Root Drench Application				
Cucumber	174	0.390	192	0.730	+87
Bean	147	0.150	155	0.200	+33
Chinese cabbage	180	0.460	187	0.520	+13
Tomato	105	0.036	122	0.064	+78

plants. The reverse of this was observed for the root-drench treated plants. The leaves of cucumber, bean, Chinese cabbage and tomato plants treated with one foliar application of 200 ppm sodium Omadine from which extracts were made after 24 hours, contained 27, 50, 53 and 22 percent less equivalent amounts of D-glucose respectively than the controls. The leaves of the same variety of plants treated with one drench application of 50 ppm sodium Omadine from which extracts were made after 4 days contained 87, 33, 13, and 78 percent more equivalent amounts of D-glucose respectively than the controls.

6. Inactivation of sodium Omadine

Boiled and unboiled guttation water from corn and cucumber seedlings when bioassayed at 0 and 12 hours showed no inactivation of sodium Omadine.

The activity of sodium Omadine was reduced when combined with juice extracted from untreated radish and tomato seedlings. Concentrations of 5 to 100 ppm sodium Omadine in sap expressed from radish leaves and hypocotyls were bioassayed immediately and after incubating for 12 hours. All concentrations in leaf sap below 10 ppm at 0 and 12 hours showed total inactivation (Table XI). The activity of the 100 ppm solutions were greatly reduced at the end of the 13-hour period. The juice from the hypocotyl did not inactivate the compound as completely as that from the leaf. In similar experiments with sap from tomato leaf and stem tissues (Table XII) biological activity was reduced. However,

TABLE XI. Bioassay experiments on sodium Omadine treated extract expressed from radish seedlings.

	Inhibition zone in mm from edge of discs Average of 12 bioassay discs							
	Plated Immediately			Plated after 12 hours				
	Check	10 ppm	50 ppm	5 ppm	10 ppm	25 ppm	50 ppm	100 ppm
Sap from leaves	1.63	1.42	1.42	1.75	1.73	1.54	1.63	5.00
Sap from hypocotyl	1.00	1.75	3.33	1.25	2.25	1.33	1.58	4.08

TABLE XII. Bioassay experiments on sodium Omadine treated extract expressed from tomato seedlings.

	Inhibition zone in mm from edge of discs Average of 12 bioassay discs							
	Plated Immediately			Plated after 12 hours				
	Check	10 ppm	50 ppm	5 ppm	10 ppm	25 ppm	50 ppm	100 ppm
Sap from leaves	5.50	5.58	6.25	5.42	5.83	6.58	6.17	5.67
Sap from stem	3.75	4.83	5.75	3.67	3.75	4.75	4.75	5.08

the inactivation was not as complete as the inactivation by the sap from radish. Antifungal activity of sodium Omadine at 50 and 100 ppm in boiled extracts from radish leaves and hypocotyls and tomato leaves was not reduced. The data for this experiment are now shown. There were no differences of reducing sugars or free amino acids between distilled

water:expressed juice and 100 ppm sodium Omadine:expressed juice from radish, cucumber and tomato leaves after a two hour incubation period.

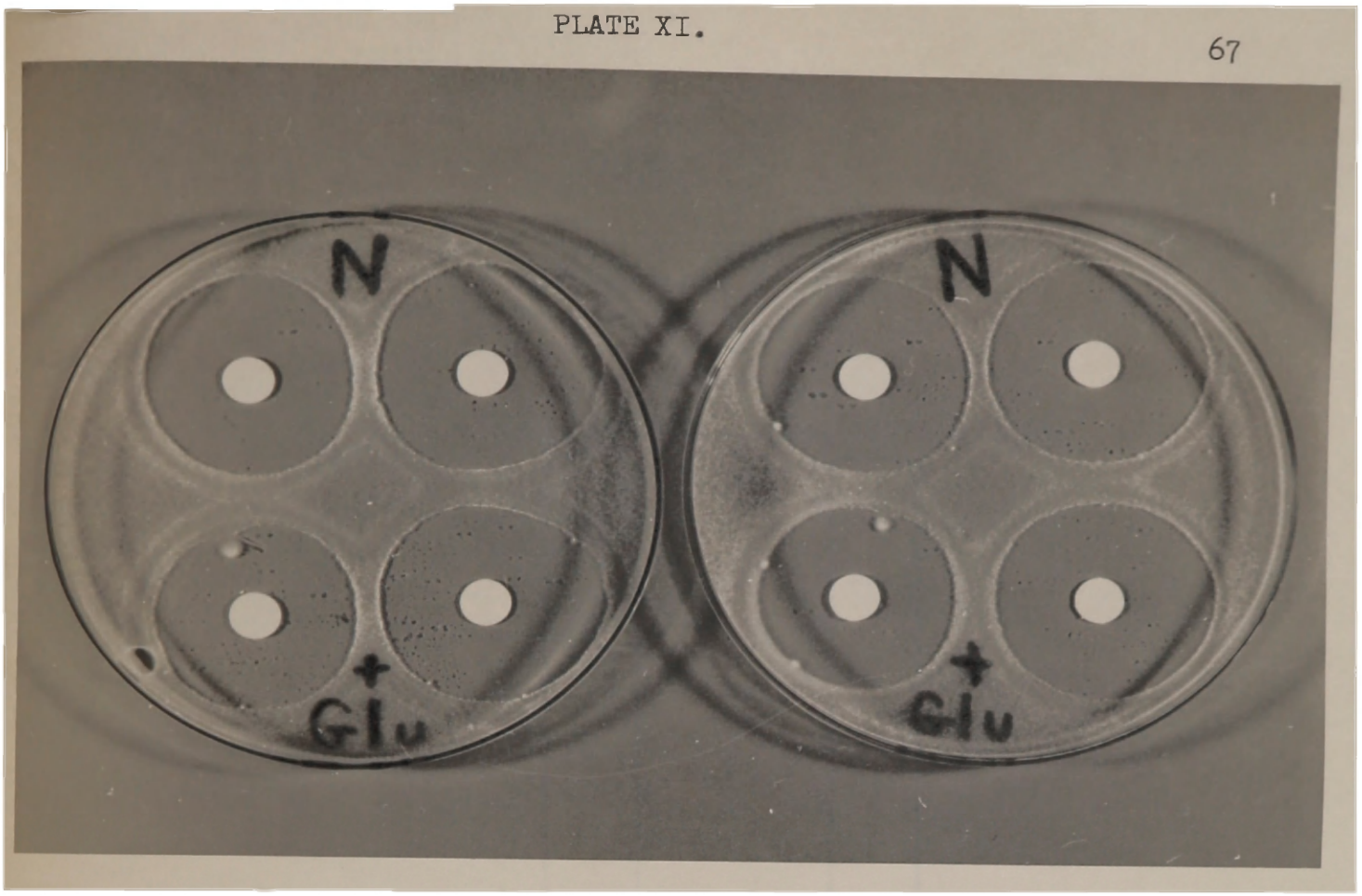
An aqueous solution of sodium Omadine at 100 µg/ml and D-glucose at 225 mg/ml when bioassayed against G. cingulata after an incubation period of 10 days, gave an inhibition zone the same size as the 100 µg/ml sodium Omadine control (Plate XI). The D-glucose control solution gave a 2 mm inhibition zone. No reduction in inhibitory properties of sodium Omadine by the D-glucose was observed.

Studies Concerning Mode of Action of the Omadine Compounds

1. Purification and investigation of chemical composition

a. Studies of technical grade sodium Omadine by paper chromatography

Chromatograms of technical grade sodium Omadine, lot number Id. JYP-8Cr-1134 yielded three inhibitory areas when bioassayed (Figure I). On fifteen replications the first area, or spot, had an R_f value of 0.099; the second, 0.687; and the third, 0.887. The first spot had an elongated "tail" below it which disappeared when 15 and 20 µg samples were chromatogramed. Discs from the center of these three spots, including one from the tail of the first spot, gave the following comparative inhibition zones when bioassayed: the first spot produced the greatest zone of inhibition; the tail from the first spot produced the second largest; the second and third spots were about equal in inhibition and slightly less than the tail from the first spot (Plate XII). The autobiograph of the technical grade compound, Figure II, shows



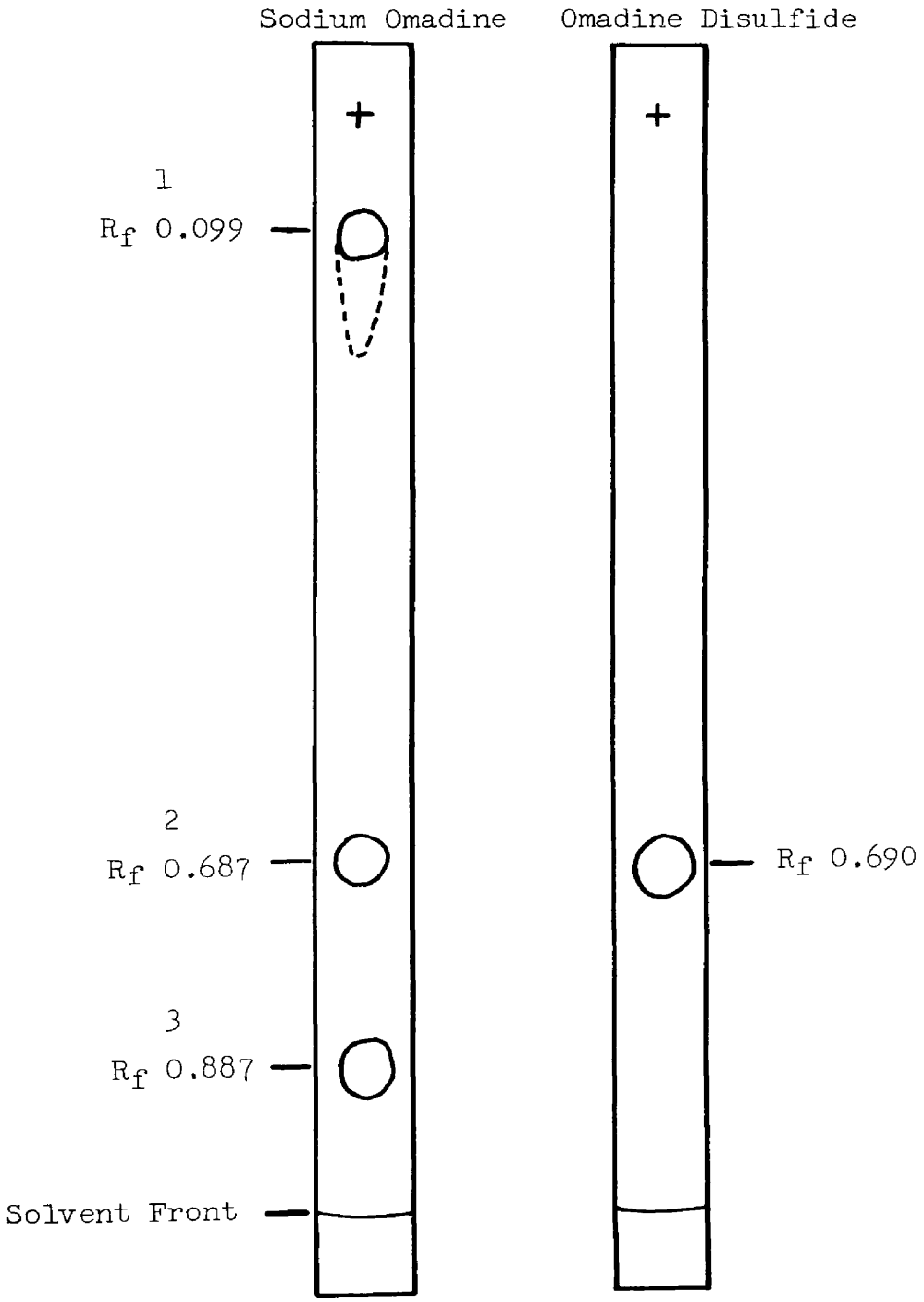
Bioassay plates of sodium Omadine at 100 µg/ml alone and in combination with D-glucose at 225 mg/ml. Solutions bioassayed after standing at room temperature for 10 days.

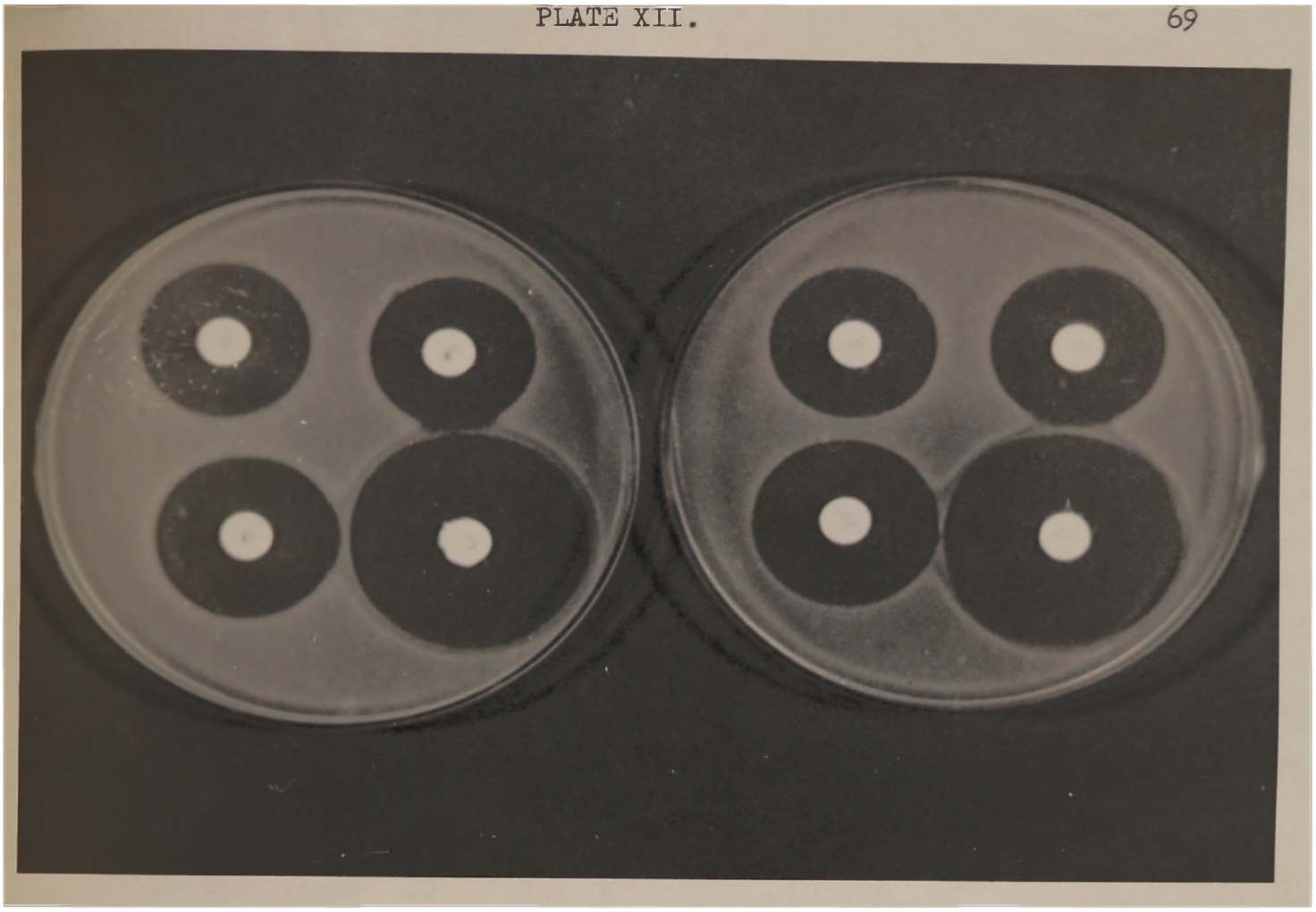
Upper four bioassay discs: sodium Omadine.

Lower four bioassay discs: sodium Omadine plus D-glucose.

FIGURE I

Chromatograms of 40 µg of sodium and disulfide Omadine showing quench-spots as observed under fluorescent light





Bioassay from fluorescent "quench" spots of chromatograms of technical grade sodium Omadine, developed in water-saturated n-butanol. The discs in the two bioassay plates are from corresponding spots from two different chromatograms.

Upper row left: third "quench" spot.

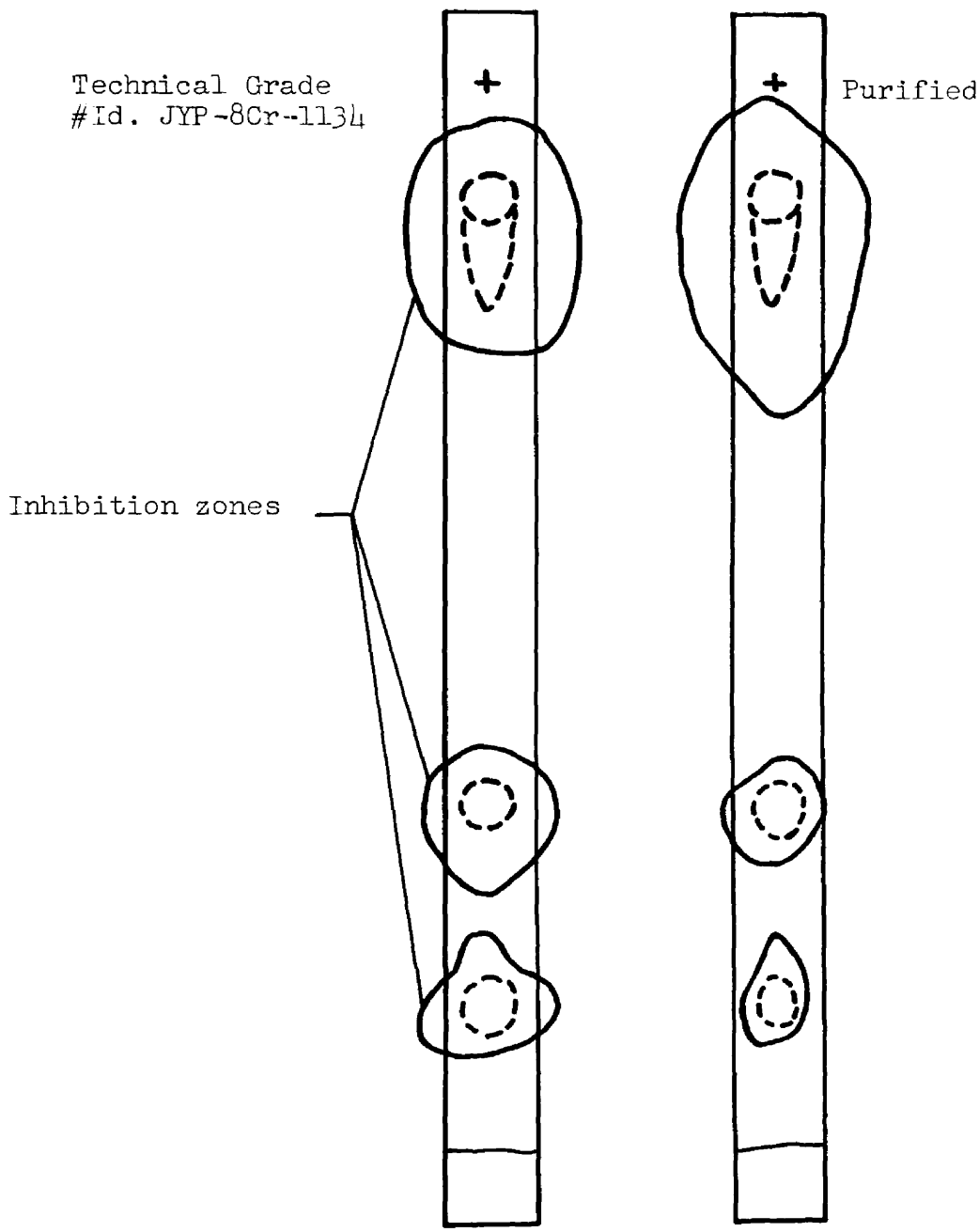
Upper row right: second "quench" spot.

Lower row left: tail from first "quench" spot.

Lower row right: first "quench" spot.

FIGURE II

Autobiographs of 40 µg of technical grade and purified sodium Omadine.



inhibition zones around these active spots and gives a clearer picture as to relative quantities of inhibitor present.

The first spot was eleuted with glass distilled water and rechromatogramed. Three active spots with the same R_f values as before were obtained. The second spot when treated similarly yielded mainly the second spot and a smaller third spot, and a very small quantity of the first spot. The third spot when rechromatogramed yielded only the third spot. Further aqueous eleutions from all three spots were given the precipitate test for the presence of sodium Omadine, to which the first spot gave a positive reaction, and the second and third a negative reaction.

Omadine disulfide when chromatogramed gave a biologically active "quench" spot which had the same R_f value as the second spot from chromatograms of sodium Omadine (Figure I). Eleutions of the second spot were co-chromatogramed[1] with Omadine disulfide and resulted in a large active spot in the number two position, and slight spots in the first and third positions. When disulfide and sodium Omadine were co-chromatogramed, spots one and three were normal, but the second spot was very large. This is further proof that Omadine disulfide and the substance causing the second spot on chromatograms of sodium Omadine are identical, both being the Omadine disulfide derivative.

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[1] The eleuted material and the solution of Omadine disulfide were applied to the same spot and chromatogramed together.

b. Purification

The melting point (MP) of technical grade sodium Omadine, Id. JYP-8Cr-1134, was 214-218° C.[1] The MP for sodium Omadine fractions "purified" in tertiary-amyl, secondary-butyl and iso-propyl alcohol were 250-253° C, 251-254° C, and 270-274° C respectively. Bioassay experiments of 4 ppm of "purified" fractions in tertiary-amyl alcohol gave an average inhibition zone of 9.7 mm, while average inhibition zones from secondary-butyl alcohol and iso-propyl alcohol were 9.85 mm and 8.88 mm respectively. Technical grade sodium Omadine at 4 ppm gave average inhibition zones of 8.25 mm. The "purified" fraction from iso-propyl alcohol was discarded since it showed the least amount in gain of biological activity, and since its MP was higher than the MP of the chemically pure compound (45).

The ultraviolet absorption spectra for 10 µg/ml of sodium Omadine "purified" in tertiary-amyl and secondary-butyl alcohol and technical grade sodium Omadine respectively were determined (Graph VIII). Absorption spectra curves from sodium Omadine "purified" from the alcohols showed it to be at higher concentrations than the technical grade sodium Omadine. The absorption spectra curves for sodium Omadine fractions "purified" from the alcohols show excellent similarities to the ultraviolet absorption spectra curve for chemically pure sodium Omadine as presented in a brochure from the Squibb Institute for Medical Research (62). The absorption

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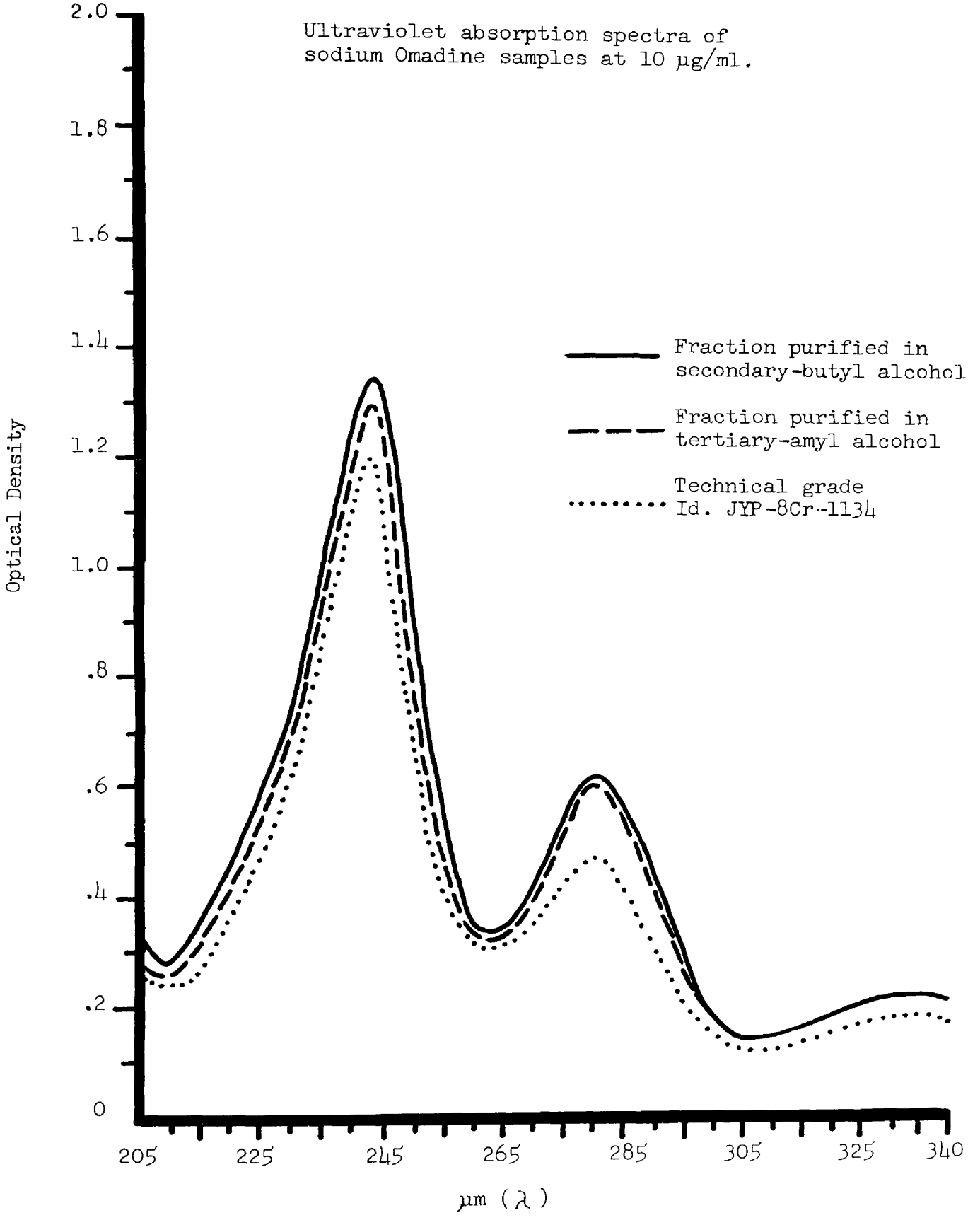
[1] The chemically pure compound was reported to have a MP of 252-254° C (45).

GRAPH VIII


Ultraviolet absorption spectra of
sodium Omadine samples at 10 µg/ml.
Optical Density
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
205
225
245
265
285
305
325
340
µm (λ)
Fraction purified in secondary-butyl alcohol
Fraction purified in tertiary-amyl alcohol
Technical grade Id. JYP-8Cr-1134

spectra curve for the fraction "purified" in secondary-butyl alcohol was slightly higher than that for tertiary-amyl alcohol, and therefore, the former was selected for all further use whenever a "purified" sample of sodium Omadine was required.

Chromatograms of the "purified" sodium Omadine gave the same three biologically active spots as the technical grade. However, autobiographs for chromatograms from the "purified" compound yielded greater activity in the first spot and less activity in spots two and three when compared to autobiographs for chromatograms from the technical compound (Figure II).

c. Chemical tests

Chemical tests for the presence of -SS and -SH groups in chromatograms of technical grade and "purified" sodium Omadine did not indicate the presence of either sulfur group. Methionine and cysteine gave positive reactions in each of the four tests, however, Omadine disulfide, whose chemical structure is known to contain an -SS group (45,62) did not give a positive reaction.

2. Location of the action of the Omadine compounds

Plates XIII through XVI show the growth of spores of G. cingulata which had been washed, brought in contact with solutions of several Omadine derivatives and combinations of sodium Omadine plus water-soluble metal salts and appropriate controls, rewashed, and tested for viability on culture plates. Plates XIII and XIV show the growth of spores exposed

to the following treatments: water check; sodium, copper, ferric and disulfide Omadine derivatives; sodium Omadine plus cupric chloride; and cupric chloride check. All treatments were present at 1 ppm. Results of growth of spores subjected to a 12 hour treatment is presented in Plate XIII. Good growth was present from spores from the water check and sodium Omadine treatment; spores from the cupric chloride and ferric and disulfide Omadine treatments gave moderate growth; the copper Omadine treatment resulted in less growth; and the sodium Omadine:cupric chloride treatment greatly reduced the viability of the spores. Growth of spores subjected to a 24 hour treatment (Plate XIV) corroborates and amplifies these results. Good growth is noted from spores in the water check and somewhat less in the sodium Omadine treatment; fair growth from the Omadine disulfide treatment; less growth from the cupric chloride treatment; poor growth from the copper and ferric Omadine treatments; and greatly reduced growth from the sodium Omadine:cupric chloride treatment.

Growth of spores after 24 hour exposures to each of cupric chloride, zinc chloride, manganous sulfate and ferric chloride alone and in combination with sodium Omadine; and sodium Omadine and water checks is presented in Plates XV and XVI. Good growth was present from spores in the water check and sodium Omadine treatment. Less growth was present in all other treatments. The sodium Omadine:cupric chloride treatment produced the greatest reduction in spore viability with very little growth in evidence from this treatment. Cupric chloride alone reduced spore viability more than did the sodium Omadine, but not nearly as

much as did the two compounds together. The combination of zinc chloride and sodium Omadine resulted in less dense growth than either the zinc chloride or sodium Omadine alone. The ferric chloride:sodium Omadine treatment allowed much less growth than was allowed by either of the two compounds in separate treatments. The manganous sulfate:sodium Omadine treatment resulted in a less dense growth than either of the two compounds in separate treatments.

After 24 hours in 2 percent sucrose, the percent inhibition of spores from the preceding experiment was determined (Table XIII). These data show that the sodium Omadine treatment reduced spore

TABLE XIII. Percent inhibition* of spores of Glomerella cingulata after 24 hours in 2 percent sucrose, after prior 24 hour exposure to treatments.

Compounds at 1 ppm	Percent inhibition of single compound	Percent inhibition of compound plus sodium Omadine at 1 ppm
Sodium Omadine	18.5	----
Cupric chloride	52.0	89.0
Zinc chloride	29.5	44.5
Ferric chloride	25.5	68.5
Manganous sulfate	15.0	42.5

*These data have been corrected to percentages of inhibition of the viable spores as determined from a distilled-water check.

germination by 18.5 percent. When cupric chloride, zinc chloride, ferric chloride or manganous sulfate was added to the sodium Omadine, a greater percentage of the spores were inhibited, especially noticeable was the reduction caused by cupric and ferric chloride. However, since each of the four metal salts caused a reduction in spore germination, in evaluating the reduction of spore germination by a combination of sodium Omadine and one of the metal salts, the inhibition caused by sodium Omadine and that by the metal salt alone must be taken into consideration. The 89 percent inhibition resulting from the sodium Omadine: cupric chloride treatment is greater than the additive effect of the two compounds alone, which would be 70.5 percent. The 44.5 percent inhibition of the sodium Omadine:zinc chloride treatment is in all respects equal to the additive effect, 48 percent, of the two compounds alone. The 68.5 percent inhibition of the sodium Omadine:ferric chloride treatment is greater than 44 percent, which would be the additive effect of the compounds alone. The 42 percent inhibition of the sodium Omadine: manganous sulfate treatment is also greater than 33.5 percent, the additive effect of the two compounds alone. To summarize, sodium Omadine alone did not cause a great amount of spore inhibition, but in combination with cupric chloride resulted in a multiple effect causing almost 90 percent inhibition. A striking multiple effect is also noted by ferric chloride:sodium Omadine, and to a lesser degree by manganous sulfate:sodium Omadine.

PLATE XIII.

Growth of spores of *Glomerella cingulata* which had been washed, brought in contact with various compounds for 12 hours, rewashed, and a drop of spore suspension from each treatment placed on potato dextrose agar plates for four days. Each compound in the treatments was present at 1 ppm.

Left: distilled water control.

Upper row from left to right: sodium Omadine, sodium Omadine and cupric chloride, cupric chloride.

Lower row from left to right: copper Omadine, ferric Omadine, Omadine disulfide.

PLATE XIII.

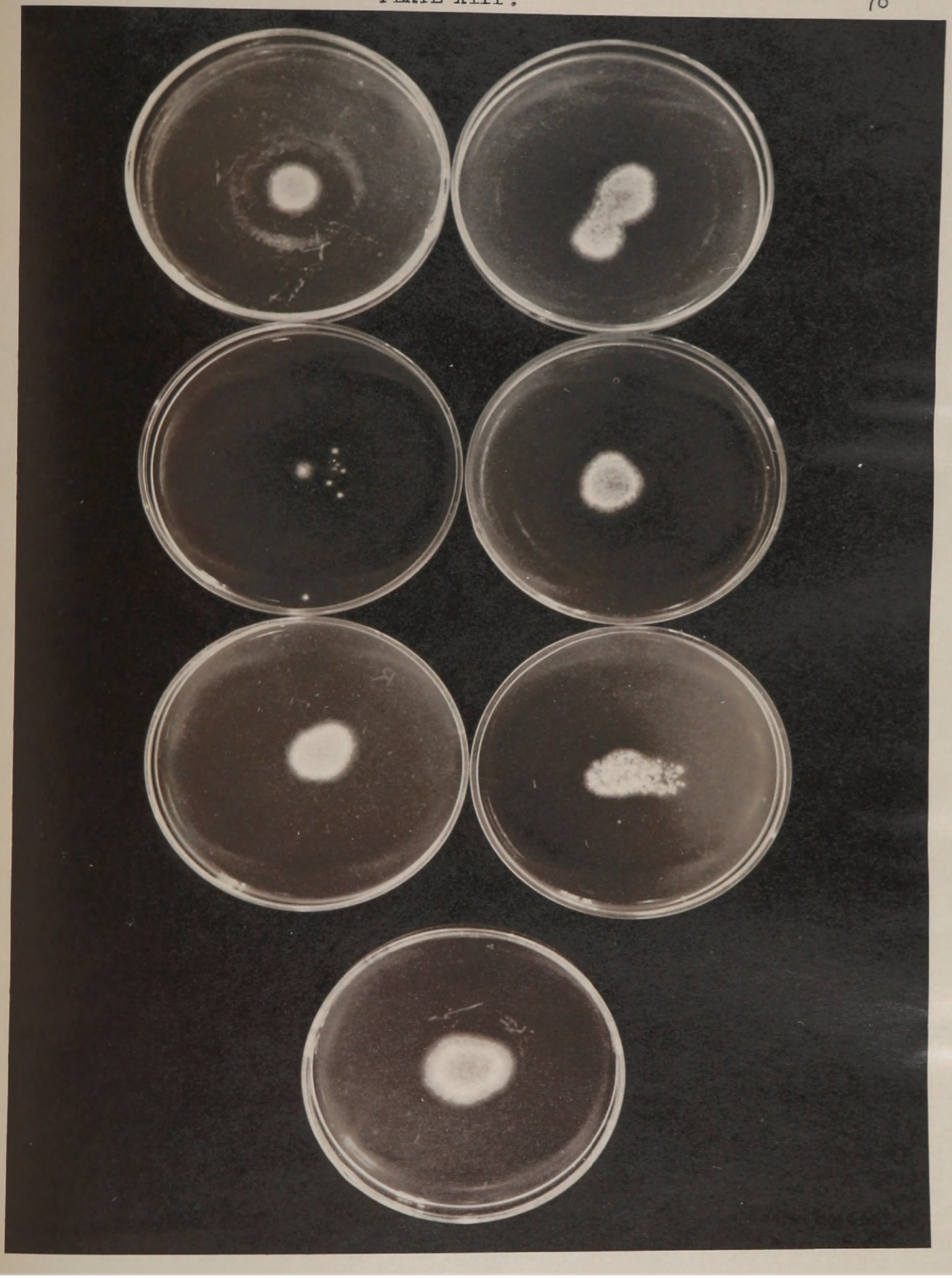


PLATE XIV.

Growth of spores of Glomerella cingulata which had been washed, brought in contact with various compounds for 24 hours, rewashed, and a drop of spore suspension from each treatment placed on potato dextrose agar plates for four days. Each compound in the treatments was present at 1 ppm.

Left: distilled water control.

Upper row from left to right: sodium Omadine, sodium Omadine and cupric chloride, cupric chloride

Lower row from left to right: copper Omadine, ferric Omadine, Omadine disulfide.

PLATE XIV.

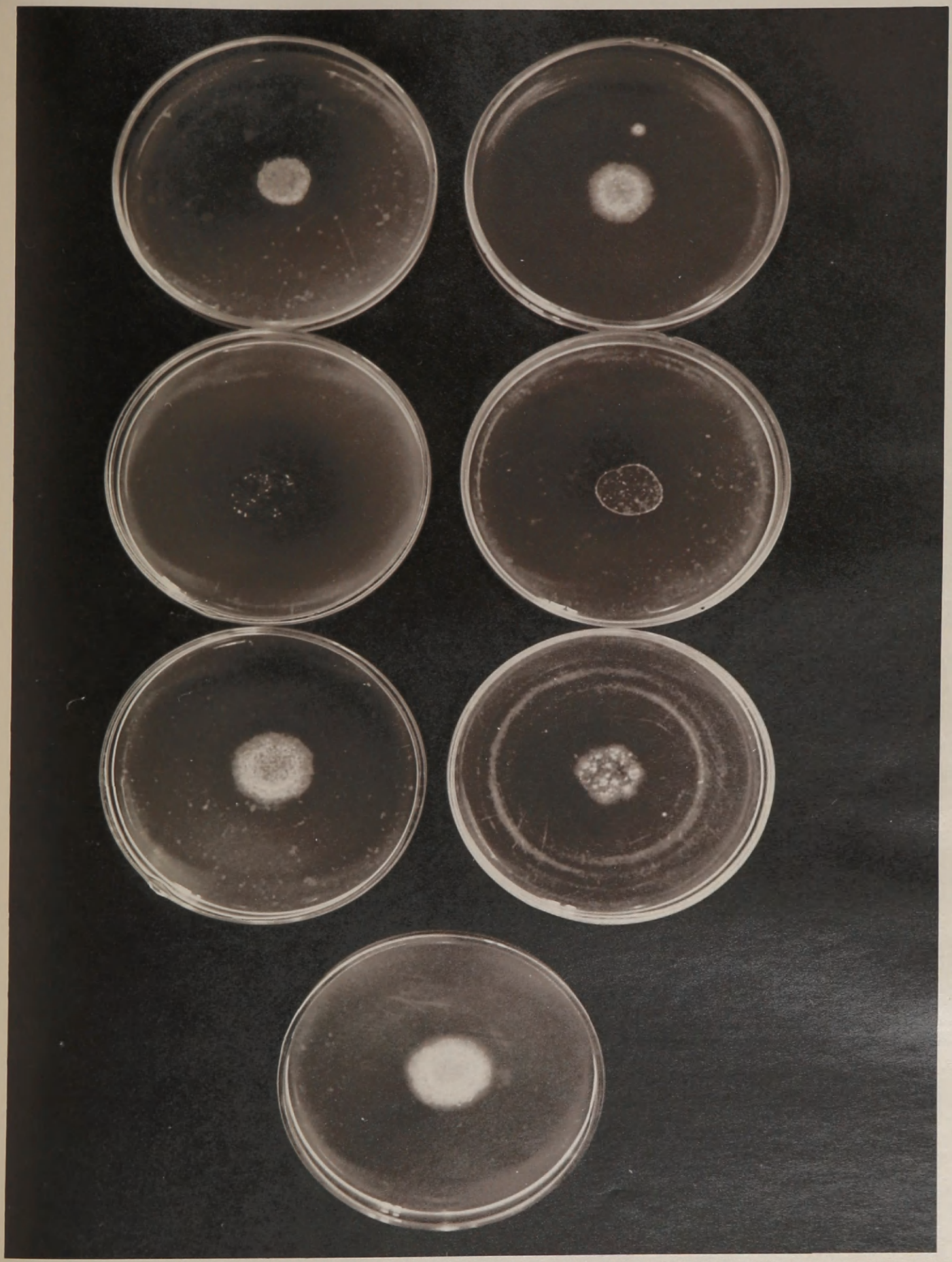


PLATE XV.

Growth of spores of Glomerella cingulata which had been washed, brought in contact with various compounds for 24 hours, rewashed, and a drop of spore suspension from each treatment placed on potato dextrose agar plates for four days. Each compound in the treatments was present at 1 ppm.

Upper row left to right: distilled water control, sodium Omadine and cupric chloride, sodium Omadine and zinc chloride.

Lower row left to right: sodium Omadine, cupric chloride, zinc chloride.

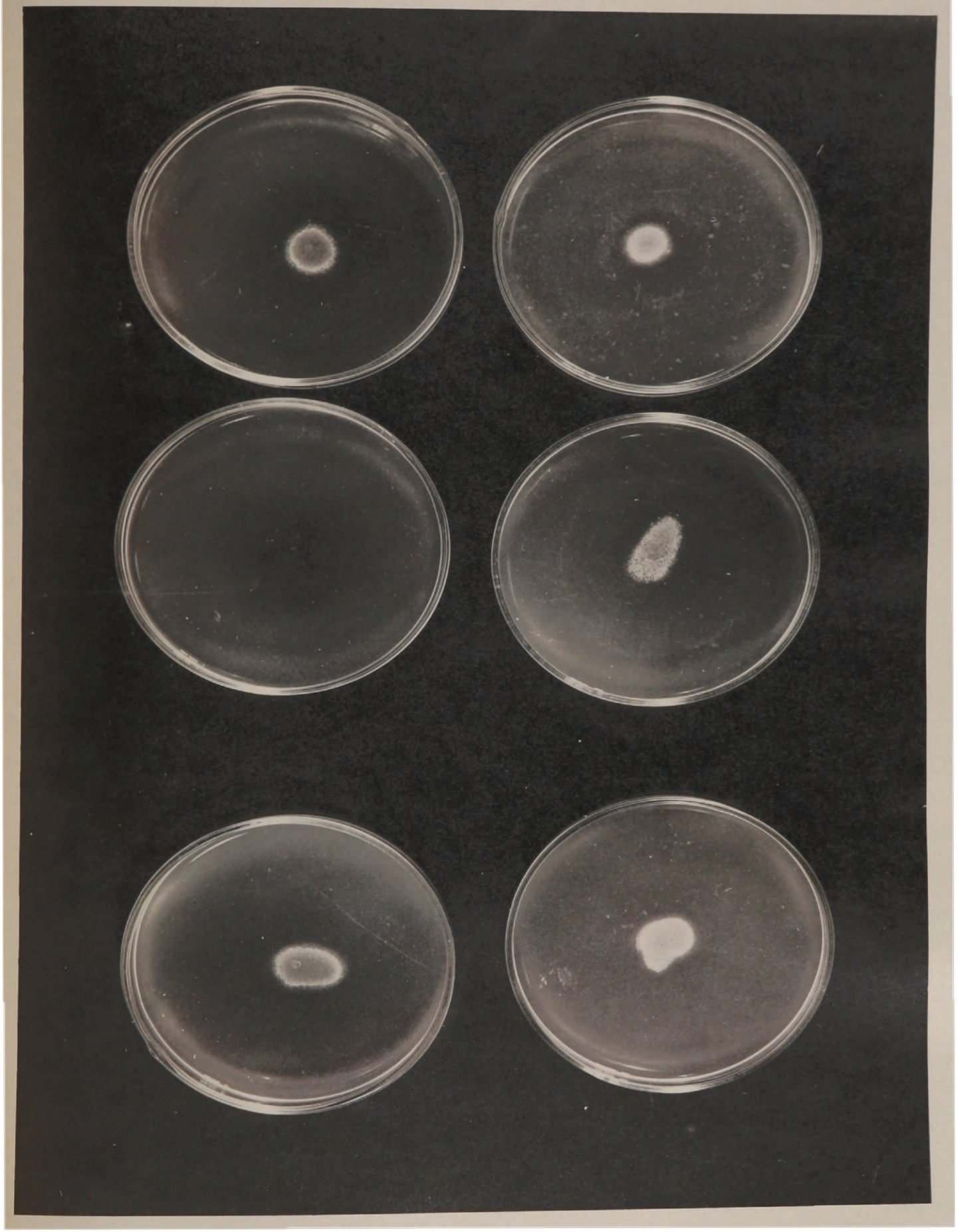
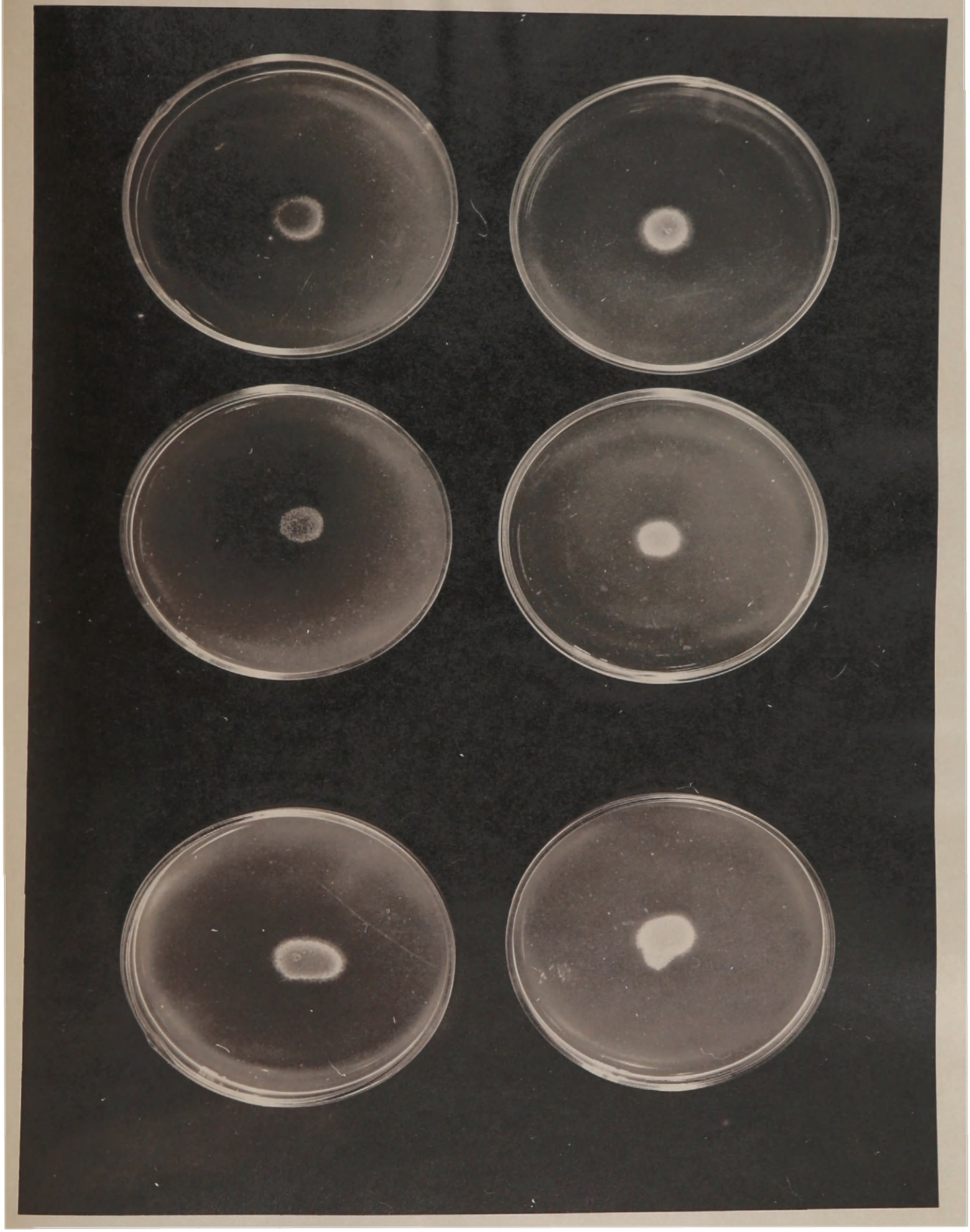


PLATE XVI.

Growth of spores of Glomerella cingulata which had been washed, brought in contact with various compounds for 24 hours, rewashed, and a drop of spore suspension from each treatment placed on potato dextrose agar plates for four days. Each compound in the treatments was present at 1 ppm.

Upper row left to right: distilled water control, sodium Omadine and ferric chloride, sodium Omadine and manganous sulfate.

Lower row left to right: sodium Omadine, ferric chloride, manganous sulfate.



CHAPTER IV

DISCUSSION AND CONCLUSIONS

In Vivo Screening of Antifungal Compounds

The Omadine salts, Panogen 15, hexachlorobenzene and Acti-dione exhibited the greatest activities of the antibiotic and synthetic organic materials screened against several organisms causing foliar diseases.

The higher temperatures encountered in later screening runs favored infection of *C. lindemuthianum* on bean plants, as is demonstrated in experiments 7 and 11 (Table III). The amount of infection was greater in all treatments except Panogen 15 which reduced the disease incidence to a trace at these higher temperatures. Increased control by Panogen 15 may be caused by its volatility and eradicant properties.

The treatment means of the Omadines and hexachlorobenzene at 200 ppm and Panogen 15 at 4.4 ppm showed highly significant control in all trials against *C. lindemuthianum* on beans, *P. lingam* on Chinese cabbage and *P. sorghi* on corn. Acti-dione at 3 ppm produced highly significant control in eight of the nine screening experiments in which it was employed. When the Omadines and hexachlorobenzene were diluted to 100 ppm and Panogen 15 to 2.2 ppm, an increase in the amount of infection was noted. However, all of the compounds tested except manganese Omadine still gave highly significant control. Sodium and copper Omadine and Panogen 15 gave the best over-all controls. Since copper

and mercury ions are toxic to many fungi, some increased activity is expected in their combination with organic compounds. The excellent control obtained with sodium Omadine may be partially explained on the basis of its water solubility for such a fungicide can be sprayed on foliage with uniform coverage since the particle size is molecular.

All of the antifungal materials tested allowed some infection by each pathogen. Heavy spore loads and incomplete coverage of inoculated hosts with fungicides accounted for some of the infections found in all treatments. A low pressure nozzle spray gun was employed, which did not create enough turbulence to produce complete foliar coverage, especially on the underside of some of the leaves.

A water-insoluble fungicide must be applied in the form of a suspension, in which case, the particle size contains a large number of molecules and the space between the particles on the foliage is greater than that of water-soluble fungicides. The coverage of a water-insoluble fungicide is therefore, not uniform which lessens the possibility of contact between the germinating spore and the compound and increases the percentage of infection. The water solubility of sodium Omadine therefore, probably contributed to its higher performance. Copper, manganese and zinc Omadine and hexachlorobenzene on the other hand were prepared as suspensions, which as explained did not provide as uniform a protective coverage.

Phytotoxicity

Diaphine HCl produced chlorosis and necrosis on all dicotyledoneous plants against which it was tested (55). The amount of injury was proportional to the concentrations of the material in the spray. At concentrations below 200 ppm the injury was limited to immature leaves. However, the leaves which developed after this initial injury were nearly free of injury. These observations seem to indicate that the action of this antibiotic was against some part of the maturation process since the meristematic areas were generally susceptable and the mature cells were much more resistant to this type of injury. This idea is further developed when it was learned that bean plants which were at the primary leaf stage had to be sprayed with an application of diaphine HCl at 200 ppm to cause necrosis of these mature leaves. However, the injury to older leaves differed from the injury to young tissues. Necrotic spots developed on sprayed mature leaves but young leaves became chlorotic after applications of diaphine HCl at 200 ppm and necrotic when higher concentrations were used. The chlorotic rings produced on cabbage and tobacco were similar to some virus symptoms.

Water soluble copper salts are known to be phytotoxic to higher plants (24,38). Increased phytotoxicity of copper Omadine over the sodium, manganese and zinc Omadines could therefore, be predicted. These studies have shown this to be the case, for the copper Omadine produced appreciable stunting on Chinese cabbage, but sodium, manganese and zinc produced only slight stunting. The non phytotoxic action of

Panogen 15 may have resulted from the relatively low concentration tested. Under conditions requiring single or widely spaced foliage applications of zinc, copper, manganese or sodium Omadine at the concentrations tested, phytotoxicity would probably be of no consequence. However, frequent applications or applications of higher concentrations would result in stunting and chlorosis.

Kenaga and Kiesling (29) reported that repeated application of sodium salt as foliar sprays and root drenches caused chlorosis and stunting on several economic plants. The roots of cucumber seedlings grown in sand treated with sodium Omadine were stunted and discolored. Roots did not regenerate their growing tips if severely damaged, but rather new roots were formed near or above the soil line. This substantiates Norman's claim (44) that root elongation and dry matter increase in cucumber and barley were inhibited by low concentrations of sodium Omadine.

Systemic Activity

Translocation of a substance within a plant may occur upward through the xylem and/or downward through the phloem. The translocation of a foreign compound such as the antifungal Omadine derivatives, presupposes the absorption of those compounds either by the root system or by the leaves. However, in the detached leaf culture trials with bean anthracnose, absorption of the compounds into the plant system by the roots was by-passed, since the zylem elements are in direct contact with antifungal compounds present in the liquid media. The compounds could

then be translocated throughout the xylem system of the leaf. In the detached leaf culture trials, the check solution containing eosine showed that the dye had moved into veinlets of the leaf within two hours after being placed in the solution. A similar movement of the antifungal materials Panogen 15, hexachlorobenzene and the four Omadine salts into leaf veinlets may therefore be assumed if the fungicide particle size is small enough to permit translocation. Since hexachlorobenzene and zinc, manganese and copper Omadine possessed only a very limited water solubility (roughly a few ppm), the translocation of these fungicides were also limited. In the case of Panogen 15 however, fumigation activity has been demonstrated (38). This partially explains the lower infection in the Panogen 15 treatment. Systemic activity for these same compounds was not demonstrated in the hydroponic experiments using bean plants inoculated with C. lindemuthianum in which the compounds had to be first absorbed by the roots and translocated to the infection site. Since no appreciable amount of control was obtained, the compounds were not absorbed through the root system, absorbed in quantities too small for protection against the causal organism, or the compounds were absorbed and deactivated by the plants.

Bioassay of sap from the freshly cut stems of cucumber seedlings grown in sand treated with sodium Omadine failed to demonstrate any biological activity. This test indicates that sodium Omadine was either not absorbed, or if absorption and translocation had occurred, deactivated by substances present in the roots and stems of cucumber seedlings. Bioassays of stem sections of tomato seedlings and of expressed sap

from cucumber, tomato and bean seedlings grown in treated sand also failed to demonstrate antifungal activity. These trials clearly demonstrate that the compound was not present in the test plants in an active state. The active compound was found in sand moisture at half the concentration which was applied 16 days previously. Therefore, ample amounts of the compound were available to the plants.

Antifungal activity was detected via bioassay in leaves and stems, but not in the roots of tomato seedlings whose foliage was sprayed with 100 and 200 ppm sodium Omadine. In similar experiments involving radish seedlings in which the foliage was sprayed with a concentration of 200 ppm sodium Omadine activity was found in the roots and hypocotyls, but not in the leaves. The activity recovered in all such experiments was never above 2 ppm. In some cases, such as the activity recovered from radish roots, the activity was below 1 ppm. Also, parts of untreated plants often exhibited antifungal activity equivalent to 1 ppm sodium Omadine, when bioassayed. Therefore, it was possible that sodium Omadine was not translocated in foliar treated plants and that the activity recovered via bioassay from extracts of treated plants was within the limits of experimental error.

When cross-sections of roots and stems of bean plants grown in hydroponic solutions containing 5 and 25 ppm sodium Omadine were bioassayed, biological activity was detected two inches from the root tips of plants in the 25 ppm treatments. Since the roots extended one and one-half inches into the solution, translocation or adsorption had occurred. It was possible that the compound was adsorbed to the roots,

and not removed by washing. However, biological activity was found only one-half inch from the root tips of plants grown in 5 ppm of the compound. If the activity was due to adsorption, it is probable that activity would have been recovered one and one-half or two inches from the root tips of these plants also. Since this was not the case, the compound was probably within the tissues of the roots. However, severe root injury was in evidence, at both levels of concentration of sodium Omadine. It is postulated therefore, that the root cells were killed and the inhibitor advanced into dead tissue, rather than being translocated in the active state through living tissues.

It was noted that sap exuded from the freshly cut stem-ends of cucumber seedlings grown in sodium Omadine treated sand when bioassayed, resulted in little or no bacterial contaminants appearing around the discs. This phenomenon was observed even though no antifungal activity was demonstrated in the sap. Considerable contamination was observed around the bioassay discs from untreated plants. Similar phenomenon were often observed when bioassays were run on the expressed juice from plants treated with both root and foliage applications. The expressed juice was bioassayed for antifungal and not antibacterial activity.

Stability of Sodium Omadine on Host Plants

The inhibition zones on bioassay plates obtained from leaf punches of bean leaves sprayed with 200 ppm sodium Omadine showed that activity is lost upon standing on the plant even when wash-off does not occur. Very little antifungal activity remained four days after application.

The factors causing this dissipation of activity are not fully understood; however, it is probable that adsorption and trans-cuticular absorption accounted for considerable loss of activity. If the compound was absorbed, it was partially deactivated which accounts for part of the reduction in activity. Decomposition of the compound by itself would not account for the dissipation (62).

Physiological Studies on the Influence of Sodium Omadine on Host Plants

The ultraviolet absorption curves for guttation water collected from cucumber seedlings grown at temperatures of 68° to 70° F in sodium Omadine treated sand differed from those grown in non-treated sand. The difference was found to be mainly one of concentration. Substances in the guttation water from plants grown in treated sand were lower in concentration than the substances in guttation water from control plants. However, after 60 to 84 hours from the treatment the absorption spectra curves approached those from the control plants, indicating recovery from the effects of the compound. This could be accounted for by root recovery and new growth and reduced concentration of the compound since the pot saucers were watered twice during this period.

The quantity of guttation water from treated plants was inversely proportional to the dosage of the drench treatment. Root injury was also increased with increased dosage of the compound. Therefore, the recorded changes in the quantity and ultraviolet absorption spectra characteristics of the guttation water from treated plants seems to be the result of root injury.

A temperature phenomenon was observed when the cucumber seedlings were grown at temperatures of 76° to 90° F. At these higher temperatures absorption spectra curves for guttation water from treated and untreated control plants were similar in all respects. Also, the characteristics of the absorption spectra curves for guttation water from plants grown at these higher temperatures as compared to those from plants grown at lower temperatures were entirely different as to slope and characteristic peaks. The reasons for this temperature phenomenon were not discovered. Attempts to characterize guttation water collected at both high and low temperatures as to amino acids and reducing sugars proved negative, i.e., no amino acids or reducing sugars were detected.

Inactivation of Sodium Omadine

Leaves of plants treated with foliar sprays of sodium Omadine were found to contain less reducing sugars than untreated controls, while plants treated with root drenches of the compound had higher concentrations of the reducing sugars than the controls. The decrease in reducing sugars in treated foliage was probably due to some change in ability of the treated leaves to produce a normal amount of simple sugars. The higher concentration of reducing sugars present in the foliage of plants treated with root-drench applications of sodium Omadine may be explained on the basis of root damage by the compound, disrupting downward translocation of food materials and perhaps altering the processes by which soluble sugars are converted into starch.

Reducing sugars as well as free amino acids present in juice expressed from untreated tomato, radish and cucumber plants were not quantitatively altered after contact with sodium Omadine up to 100 ppm. Sodium Omadine at concentrations up to 50 ppm was deactivated immediately upon contact with expressed juice from radish and tomato plant. Juice expressed from green portions of the plants produced the greatest deactivation. Substances other than reducing sugars and free amino acids present in the expressed juice were responsible for the deactivation of the inhibitor, since no change in the quantity of either was detected.

Ringel (53) found that D-glucose as well as other reducing sugars reduced the inhibitory properties of sodium Omadine in vitro. Employing the same ratio of D-glucose to sodium Omadine as Ringel, but at a much higher concentration and for a longer period of time, no reduction of inhibition was detected by bioassay against G. cingulata. Therefore, if a chemical reaction has taken place between sodium Omadine and D-glucose, it is not a strong chemical union. These results indicate that reducing sugars present within a plant do not play a roll in inactivation of the compound.

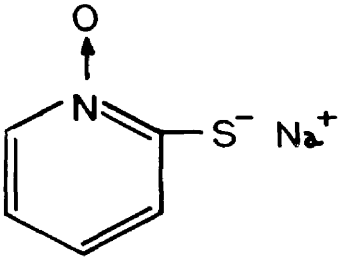
Chemical Reactions of the Omadine Derivatives

The prototype of the Omadine compounds exists in two tautomeric forms (45,62) and can therefore be named chemically as either 1-hydroxy-2(1H) pyridinethione or 2-pyridinethiol, 1-oxide (Figure III, B). The structural formula of sodium Omadine (Figure III, A) does not contain an -SH group, since the sodium has replaced the hydrogen of the

FIGURE III

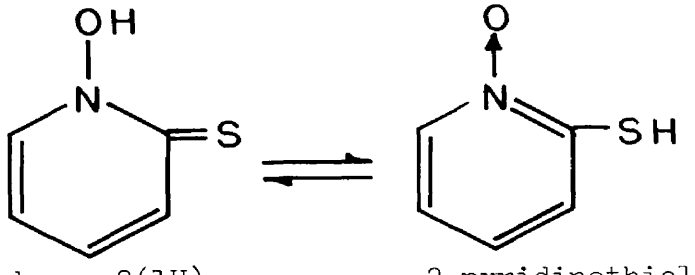
Structural formulas of sodium Omadine and the parent Omadine forms and two possible reactions of sodium Omadine.

A



Sodium salt of 2-pyridine-thiol, 1-oxide (sodium Omadine)

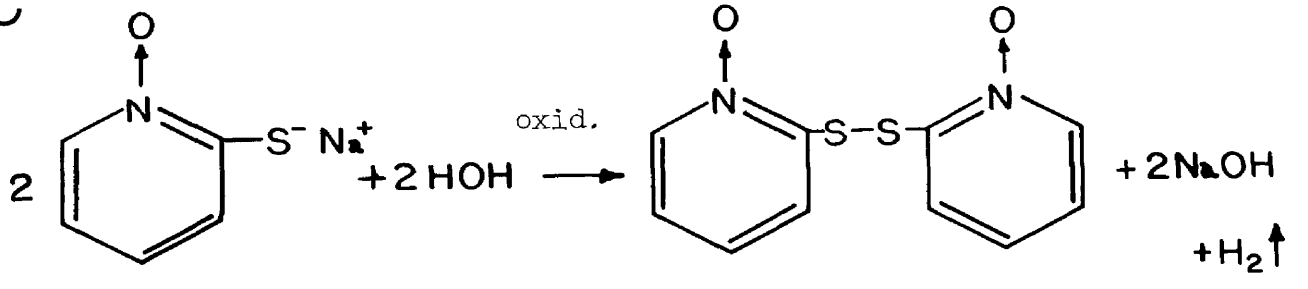
B



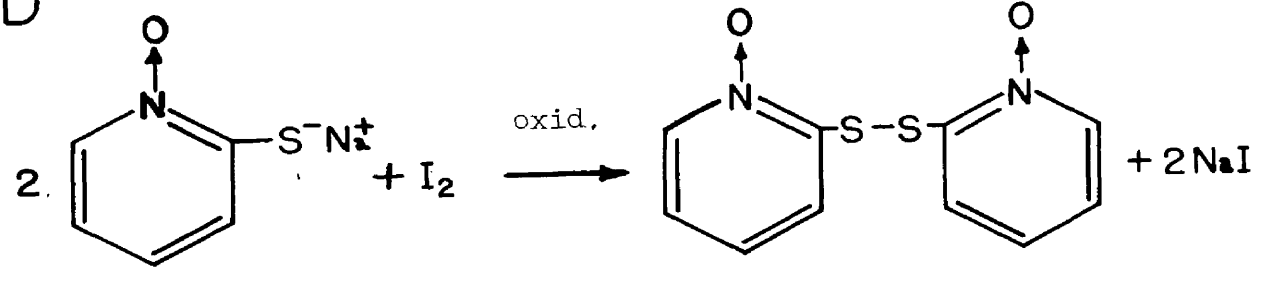
1-hydroxy-2(1H) pyridinethione (keto form)

2-pyridinethiol, 1-oxide (enol form)

C



D



hydrosulfide group of 2-pyridinethiol, 1-oxide. Chemical tests failed to indicate the presence of -SH groups on chromatograms of sodium Omadine. These tests were reliable for sulfur groups present in certain amino acids, but there was no way of determining if they would react similarly with compounds such as the sodium and disulfide Omadine derivatives. Sulfur groups are known to be present if the tests react in a positive manner, but if they do not react in a positive manner, the sulfur groups may be present nevertheless, as was the case with Omadine disulfide. It may also be that -SH groups were present in amounts too small for detection by these tests. Therefore, these tests did not substantiate the presence or absence of -SH or -SS groups in chromatograms of sodium Omadine.

The Squibb research group stated that the disulfide derivative was formed by treatment of the enol tautomeric form (2-pyridinethiol, 1-oxide), with mild oxidizing agents or merely upon standing in a solvent in which it was soluble (62). Therefore, the hypothesis was made that limited amounts of the -SH groups were present in aqueous solutions of sodium Omadine. This was further substantiated by chromatograms of sodium Omadine which yielded three active components, one of which was the sodium salt, and another the disulfide derivative. When the area or spot on the chromatogram containing the sodium salt was eleuted and re-chromatogramed, it yielded the same three active components as were obtained in the first chromatogram, proving that more disulfide was formed. It may be possible that sodium Omadine in aqueous solutions could be oxidized directly into the disulfide form without the addition

of an oxidizing agent. However, if this were the case, the sodium ion would have to be joined to -OH groups of water and this would result in liberation of free hydrogen (Figure III, C). The writer has found no evidence in the literature which would substantiate or negate the possibility of this reaction.

With the hypothesis that limited amounts of -SH groups were present in aqueous solutions of sodium Omadine, several chemical reactions could therefore be possible. Ringel (53) has presented a proposed scheme for the inactivation of "pyridinethiol" by a reaction with glucose in the formation of a thio hemi-acetal, also on the hypothesis that limited amounts of -SH groups were present in aqueous solutions of sodium Omadine. Although the writer was unable to substantiate the inactivation of sodium Omadine by D-glucose at a higher concentration, there was no reason to assume that such a reaction was not chemically feasible. However, other reactions would be just as chemically feasible.

The general type formula for the mercaptans is R·SH (12). The enol tautomeric form 2-pyridinethiol, 1-oxide, which would probably be the form of the existing groups bearing the -SH groups in solutions of the sodium salt, could very well fit this general formula type, and undergo some or all of the reactions which characterize the mercaptans. For instance, the hydrogen of the -SH groups of mercaptans may be replaced by sodium, forming a mercaptide, of which the following reaction is characteristic: $2\ R\cdot SNa + I_2 \longrightarrow R\cdot S\cdot S\cdot R + 2\ NaI$ (12). The general type formula of R·SNa could represent the sodium salt of 2-pyridinethiol, 1-oxide (sodium Omadine), with R representing the pyridine-1-oxide.

By the reaction formula above, the prediction could be made that the disulfide derivative would be formed if iodine were added to an aqueous solution of the sodium salt (Figure III, D). The disulfide derivative was obtained by this method, and was proven by means of paper chromatography. Furthermore, the Squibb research group (62) stated that vigorous oxidation of 2-pyridinethiol, 1-oxide would yield the sulfonic acid, one of the characteristic reactions of mercaptans (12). These reactions are presented as proof that the enol tautomeric form, 2-pyridinethiol, 1-oxide, could undergo some or all of the reactions characteristic of the mercaptans. Such reactions of the mercaptans include replacement of the hydrogen atom of the -SH group by reactions with organic acids, aldehydes and ketones, and replacement of the -SH group by reducing agents (12).

Mode of Action of the Omadine Derivatives

The first information concerning the mode of action of the Omadine derivatives in these studies came from the spore germination trials. The linearity of the DR curves indicated that there was only one mode of action for the sodium derivative, and very low dosages were required to produce complete inhibition. The swelling or vesicle formation at the tip of the germ tube of H. carbonum while under the influence of sub-lethal dosages of sodium Omadine is of considerable importance, for the action of the inhibitor must have taken place within the spore, or germ tube as the case may be, and not on the outside, since cellular growth was controlled within the cell. Of 41 compounds tabulated by Horsfall

(24) which cause deformities of germ tubes, only seven contained nitrogen groups, and none appeared similar to the structure of sodium Omadine. However, Horsfall (24) stated that the types of compounds which caused such deformities also cause mitotic aberrations. He concluded that the direct cause of such compounds was to produce nuclear monstrosities, and that cellular expansion which follows nuclear division in point of time caused resulting deformities. Since nuclear division does not take place, cellular division does not.

To fully comprehend the influence of sodium Omadine on the growth of L. minor, it should be compared to the effect produced by other compounds. The lethal threshold for growth of L. minor under the influence of Acti-dione, an antibiotic known for its phytotoxicity (18) was above 20 ppm (43), while the lethal threshold of sodium Omadine was between 0.5 and 0.1 ppm. The lethal threshold for growth of L. minor under the influence of Griseofulvin was between 10 and 20 ppm (43). Rimocidin at 20 ppm produced a 50 percent reduction of growth from control, while at this concentration thiolutin produced a 90 percent reduction (43). The growth response of L. minor to sodium Omadine substantiates the information from the spore germination tests as to the mode of action of this compound. It was reasoned that such minute amounts of the inhibitor produced such growth retardation that the compound must act within the cells of the plant, and not externally. These studies indicate that there is also only one mode of action of this compound on vascular plants. Only retardation of growth was observed down to as low as 0.005 ppm, which produced a 75 percent reduction of growth from control.

It was also reasoned that since minute amounts of the inhibitor produced such striking effects, the compound must disrupt one or more metabolic processes within the cells of the plant, or disrupt mitosis, making normal cellular division difficult, as was indicated in the spore germination studies.

The sodium salt is insoluble in most organic solvents, and very water soluble, existing in solution in the ionic state (45,62). It is doubtful that such a compound would be permeable to cell membranes, for in order to permeate a cell a compound must possess a balance of fat and water solubility, and an ionic compound does not possess fat solubility (6,69). Also, the polar groups in the semipermeable membranes possess electric charges, which would cause charged ions to permeate poorly as they would be adsorbed enroute. Yet, evidence has been furnished which indicates that sodium Omadine has permeated the cellular membranes. It was conceivable therefore, that the molecular structure of sodium Omadine was altered in some way so as to change the fat-water solubility balance allowing it to be permeable to the cell.

The Squibb research group stated (62) that water-insoluble Omadine salts such as the copper or the zinc salt were prepared by the treatment of an aqueous solution of sodium Omadine with an aqueous solution of a water-soluble metal salt. Confirmation of this was demonstrated in the laboratory. Therefore, when sodium Omadine was added to a solution containing a sufficient quantity of a soluble copper or ferric salt, the toxic compound was then copper or ferric Omadine as the case may be. In the replacement of sodium with one of the latter metals there has

been an alteration of the molecular structure. The copper, zinc, manganese and ferric Omadine derivatives all possess a better fat-water solubility balance than the sodium salt, and they act in the molecular state (45,47,48,49,62). The hypothesis was therefore made that the Omadine salts act as chelators, in which the preformed metal chelates possessing a fat-water solubility balance permeate the cellular membranes, and preform a toxic reaction within the cell.

To test this hypothesis, spores of G. cingulata which had been washed, were brought in contact with solutions of several Omadine derivatives and combinations of sodium Omadine plus water-soluble metal salts and appropriate controls, rewashed and tested for viability on culture plates. In these experiments, the spores of G. cingulata were washed with glass-distilled water several times, freeing them of as much exterior metal as possible (39). If little or no exterior metals were present in the surrounding medium and on the surfaces of the spores, then sodium Omadine should cause little change in spore viability from the control since replacement by other metals could not occur and the sodium salt was not permeable to cell membranes. When the experiment was run, the culture plates and the spore germination trials of the control and sodium Omadine treatments showed that the sodium Omadine treatment did not materially alter the spore germination, when compared to the control.

To further test the hypothesis, if washed spores of G. cingulata were brought in contact with water-soluble metal salts in solution with sodium Omadine, the sodium ion would be replaced by the metal in solution

and permeation of spores by the newly formed Omadine derivative should take place. The cellular permeation should cause a reduction in germination below that caused by both the sodium Omadine and metal salt controls. The zinc chloride:sodium Omadine treatment did not give a reduction of viability below the additive effects of the two compounds alone. However, the culture plates and spore germination trials show that a reduction in spore viability was produced for sodium Omadine solutions in combination with cupric chloride, ferric chloride and manganous sulfate which were below the additive effects of the compounds alone. The cupric and ferric salts in combination with sodium Omadine produced the greatest reduction in spore viability.

These results demonstrate that action of the copper, ferric and manganese Omadine derivatives took place within the spores, and that the ionized sodium Omadine did not permeate the spores and therefore was not toxic. Copper and ferric Omadine were also used in these experiments at concentrations of 1 ppm. However, an accurate dilution of these two metal salts is almost impossible, since the salts are near their limit of solubility (45,47,62). Therefore, the results shown in Plates XIII and XIV for the commercially prepared salts were at concentrations lower than 1 ppm.

Sodium Omadine reduced spore germination slightly. However, this is expected, since it is highly improbably that all excess metals were removed from the exterior of the spores. These metal ions replaced the sodium and permeation could occur. Also, it is possible that sodium Omadine molecules adhered to the surface of the spores, due to the

attraction from electric charges in the polar groups in the semipermeable membrane (6,69). If the spores come in contact with free metals, replacement of the sodium ion takes place, allowing permeation.

When sodium Omadine was used in these studies, it was employed in tests with other water-soluble salts present, and substitution of metals undoubtedly occurred. When distilled water was employed, as differentiated from glass-distilled water, sufficient metals were also present to allow substitution on the Omadine molecule.

Horsfall (24) stated that most nitrogen compounds are inherently toxic, but that many do not display activity as fungicides unless they contain a "shaped charge" to make them permeable. He further stated (24) that pyridine could be made into a fungicide by the addition of a styryl group in the number two position on the pyridine ring, or by a carboxy ester in the number three position. A fungicidal pyridine compound closely related to the Omadines is 3-pyridine thiol, the metal salts of which possess considerable fungitoxicity (61).

This study has shown that trace amounts of the Omadine derivatives produced remarkable growth retardation of L. minor and inhibition of fungal spores. The inhibitory reaction produced in all cases far exceeded the inhibitory reaction of the water-soluble metal salts. Therefore, the hypothesis was made that once the Omadine molecule enters the cell, it undergoes reduction, leaving the pyridine ring, which is the toxic property.

The zinc chloride:sodium Omadine treatment as previously discussed in the culture plate and spore germination tests, did not give a

reduction of viability below the additive effects of the two compounds alone. The fact that the zinc chloride caused some reduction in spore viability is evidence that zinc alone is toxic to these spores. It may be that spores of this fungus do not possess the ability to reduce the zinc chelate as quickly as other metal chelates, and therefore the toxic moiety of the compound is not released in sufficient quantities to cause the inhibition produced by other metal chelates.

CHAPTER V

SUMMARY

1. Acti-dione, Panogen 15, hexachlorobenzene and the Omadine salts of copper, zinc, manganese and sodium when applied as foliar sprays controlled P. lingam on cabbage, C. lindemuthianum on bean and P. sorghi on corn. The best overall control was obtained with sodium Omadine, copper Omadine and Panogen 15.

2. Diaphine HCl at a wide range of concentrations produced a marked chlorosis which on cabbage was similar in appearance to certain virus symptoms. Copper Omadine produced noticeable stunting on Chinese cabbage, while zinc, manganese and sodium Omadine produced only a slight stunting. Plants recovered quickly from any phytotoxic reactions produced by foliar applications of the Omadine compounds. The roots of cucumber seedlings grown in sand treated with sodium Omadine were stunted and discolored. They did not regenerate from their growing tips if injured severely, but new roots were formed near or above the soil line.

3. Concentrations of sodium Omadine necessary to produce total inhibition of the spores of C. lagenarium, C. phomoides and H. carbonum in distilled water were 0.05, 0.05 and 0.10 ppm respectively. Vesicles or swellings were occasionally produced at the tips of the germ tubes of H. carbonum at concentrations of 0.05 ppm.

4. The lethal threshold for growth of L. minor under the influence of sodium Omadine was between 0.5 and 0.1 ppm. Concentrations as low as 0.005 ppm produced a marked decrease in growth.

5. Sodium Omadine does not act as a systemic fungicide, and up to 50 ppm of the compound is inactivated immediately upon contact with extracts from radish and tomato.

6. Leaves of plants treated with foliar sprays of sodium Omadine were found to contain less reducing sugars than untreated controls, while plants treated with root drenches of the compound had higher concentrations of reducing sugars than the controls. It was thought that the lower amount of reducing sugars in treated leaves was due to some change in ability of the treated leaves to produce a normal amount of sugars. The greater amount of reducing sugars in plants treated with root drench applications was explained on the basis of root damage, disruption of the translocation, and perhaps an altering of the processes by which soluble sugars are converted into starch.

7. The ultraviolet absorption spectra of guttation water of cucumber seedlings grown at temperatures of 68° to 70° F in sand treated with sodium Omadine indicated a lower amount of inorganic substances were probably present than from control plants. At higher temperatures these differences were not present, and the absorption spectra curves for control plants from both high and low ranges of temperatures were entirely different.

8. The hypothesis was made that limited amounts of -SH groups were present in aqueous solutions of sodium Omadine, and that therefore

several chemical reactions characteristic of the mercaptans were possible. Circumstantial evidence was presented as the basis for the hypothesis, and evidence is presented to show that some of the reactions characteristic of mercaptans do take place.

9. Evidence was presented to the effect that in a solution containing water-soluble metal salts there is a replacement of the sodium ion attached to the Omadine molecule by the metal element in solution.

10. A second hypothesis was made that the Omadine salts act as chelators in which the preformed metal chelates, possessing a fat-water solubility balance, permeate the cellular membranes and preform a toxic reaction within the cell. Evidence is given in support of this hypothesis.

11. A third hypothesis was made that once the Omadine molecule enters the cell it undergoes reduction, leaving the pyridine ring which is the toxic property. Circumstantial evidence is presented in support of this hypothesis.

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A P P E N D I X

Multiple Spotting Apparatus for Paper Chromatography[1]

Considerable variability in paper chromatography may result from improper technique in application of samples to the paper. This variability is often multiplied several-fold when sample volumes of 1/10 ml or greater must be applied, for it necessitates repeated application of the sample in small and equal amounts to a particular point on the paper to build up the required volume. When multiple spotting must be done, in addition to being inaccurate, it becomes a time-consuming, and tedious chore.

Sample applicator-apparatus known to the author, appear very satisfactory for application of small volumes, but leave something to be desired when large volumes must be employed. Therefore, to circumvent this tedious task of multiple spotting of large volumes, a multiple spotting apparatus for paper chromatography was constructed (Plate A) which allows the simultaneous application of ten spots to various arrangements of filter papers held vertically on a separate adjustable drying rack. Up to 1 ml. of material may be applied per spot from matched tuberculin syringes fitted with No. 26 needles filed flush with the shank. The syringes are filled with the sample, and clamped in a parallel series one and one-half inches apart across a narrow bar, which is fastened at right angles to a lower framework. An electric motor geared down to a few rpm and mounted within the framework rotates a long threaded driving screw, the forward end of which is mounted in

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[1]The text of a paper presented at the forty-eight annual meeting of the American Phytopathological Society in conjunction with the Potato Association of America at Cincinnati, Ohio, in December, 1956.

ball-bearings below the cross-piece holding the syringes. A slide, mounted on aluminum right-angle pieces rides snuggly in a channel of similar right-angle pieces fastened to the framework on either side of the driving screw. The slide rides above the driving screw and moves down the channel by means of a "split-nut" held against the driving screw by a coil spring attached to the slide. At the forward end of the slide a metal crosspiece is mounted which contacts the syringe plungers. The motor turns the driving screw causing the split-nut-mounted slide to move forward, the slide cross-piece presses the syringe plungers resulting in the substance within each syringe to be exuded. The syringes are matched as to volume per given barrel length and therefore equal volumes are exuded from each syringe.

The electricity supplied to the motor is first connected to a 60-second-cycle percentage timer, which may be set to regulate the current supplied to the motor in various intervals of a minute, or in continuous operation.

A heat gun is mounted on the basal piece of the adjustable spotting rack (Plate B). A copper manifold attached to the drier runs just beneath and behind the paper or papers to be spotted. The filter paper sheets are fastened vertically at their upper and lower edges to adjustable glass-strips. For two-dimensional chromatograms, each filter-paper sheet is clipped vertically along one edge of the rack, folded back and fastened at its upper-corner to a heavy wire support which is attached to the verticle posts of the rack. The multiple-spotter is moved up to the drying rack to which the papers have been attached (Plate C).

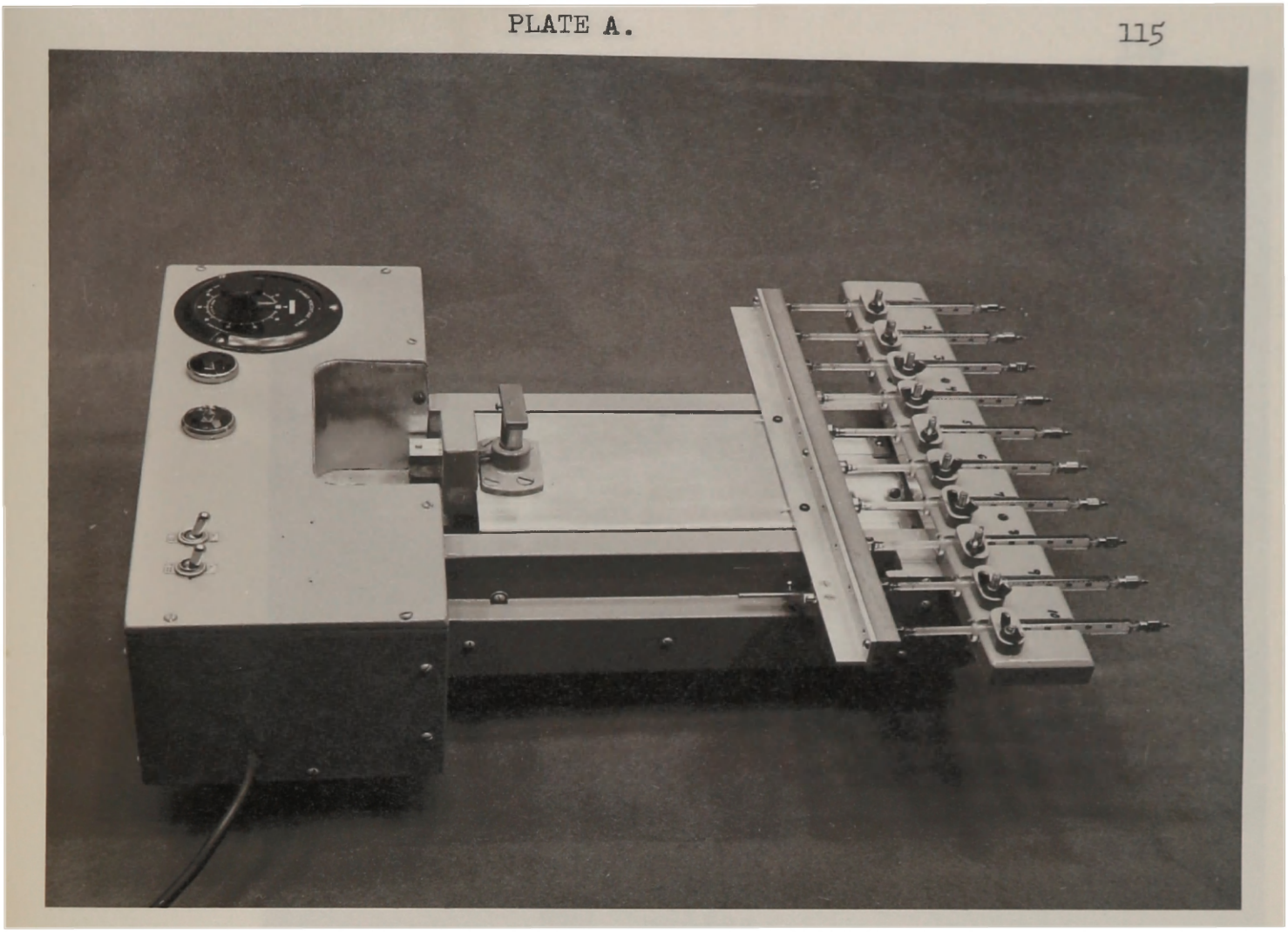
The blunt noses of the syringe needles are placed against the papers 1/2 inch above the lower glass strip. Drying of the samples is facilitated by hot or cold air passing from the manifold and directed on the spots. With the heating element of the heat gun turned on, the temperature of the air stream at the point of sample application is between 36-38° C over the entire length of the manifold.

Both the heat gun and the multiple-spotter are connected to an electric interval timer clock, which shuts off the apparatus after a predetermined length of time. By adjusting the percentage timer, the area of the applied spots may be accurately regulated (Plate D). A dilute solution of ink when applied to Whatman filter paper number 1 at a percentage timer setting of 5 percent produced sample spots of less than one-quarter inch in diameter. The sample spot diameters ranged from one-quarter inch up to three-quarters of an inch when a percentage timer setting of 100 percent (or continuous operation) was employed. Percentage timer settings of 40 percent through 100 percent resulted in sample spots which were slightly elongated due to the force of the heated air from the manifold. The size of the sample spots remained consistently even at all percentage timer settings.

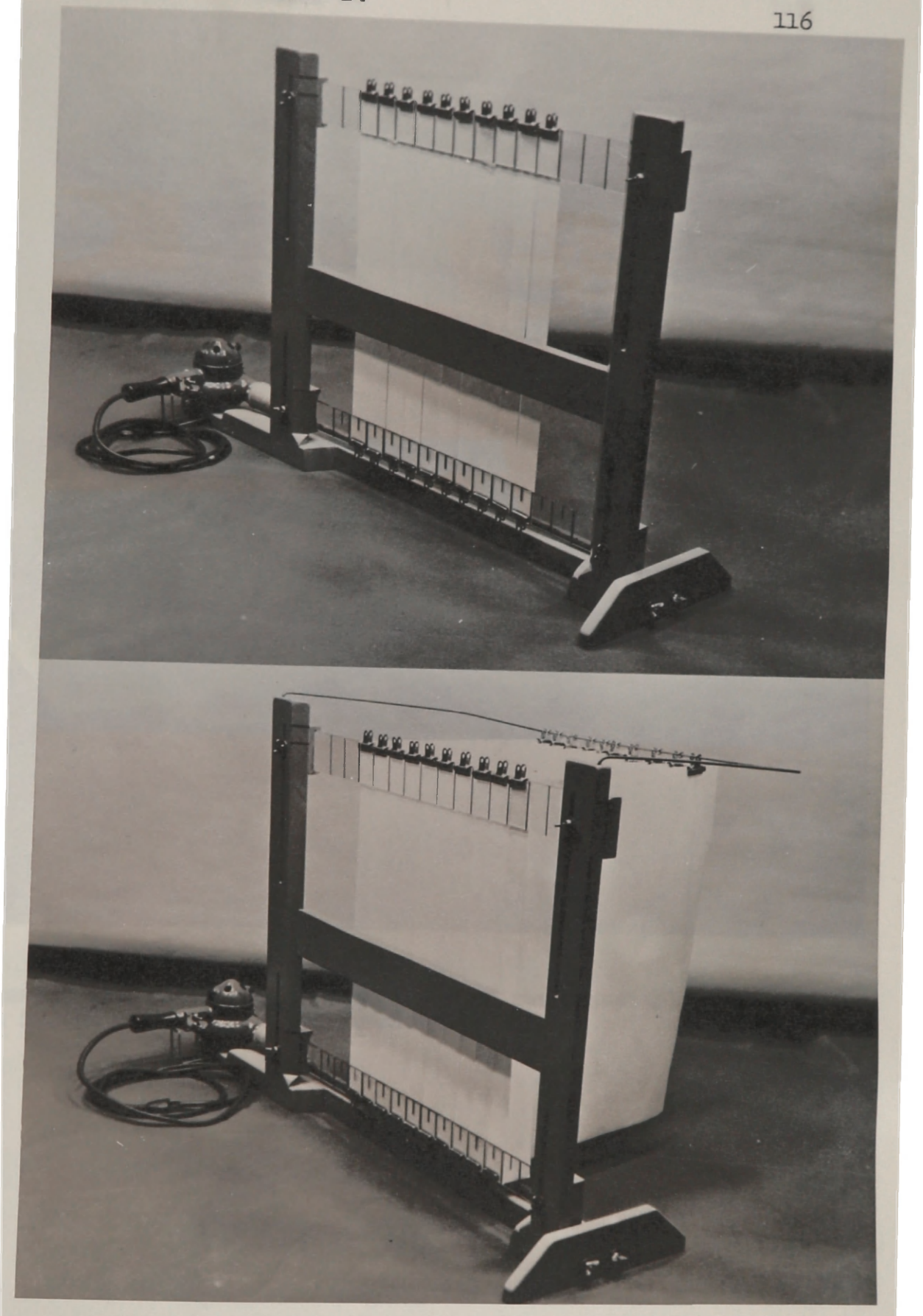
Calculations as to unit-time per unit volume for any percentage timer setting is easily computed.

By means of a photoelectric densitometer, one-dimensional chromatograms of amino acid mixtures applied in equal volumes were read as to maximum density for each ninhydrin reacting spot. On 36 replications the densitometer showed little variation among spots of the same amino acids.

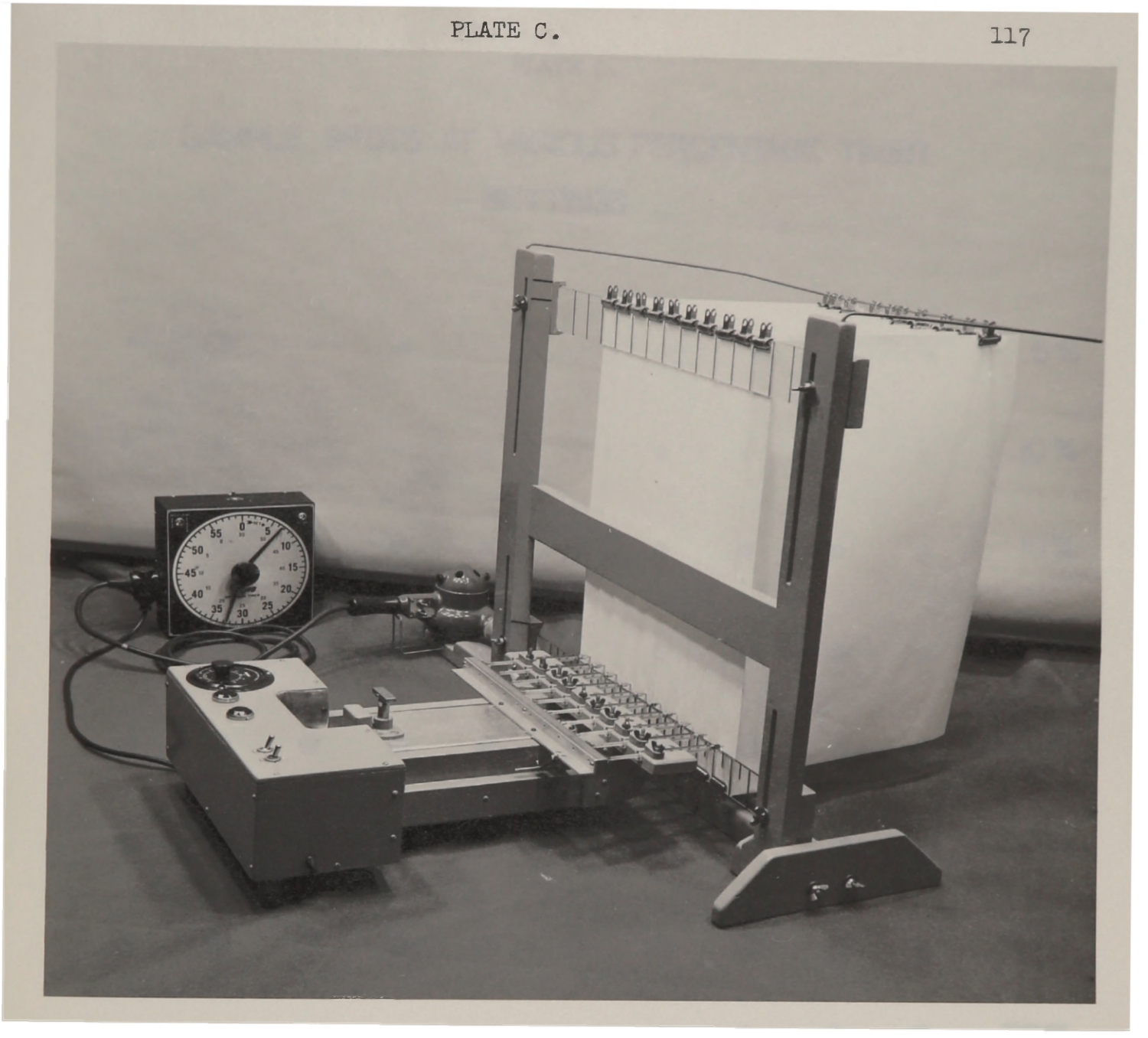
With this apparatus large sample volumes may be employed; as many as ten sample spots may be applied to any size filter paper or papers, including one spot per sheet for each of 10 sheets when two-dimensional chromatograms are to be used; the spots are uniform within each run and among replications; the area of the spot is easily controlled; and the sample may be dried in either hot or cold air streams. Therefore, when larger volumes are to be employed for paper chromatography, replications may be made with greater ease, accuracy, and at a considerable saving of time.



Multiple sample spotter for paper chromatography



Adjustable spotting rack (upper) set up for one-dimensional chromatograms; (lower) set up for two-dimensional chromatograms.



Multiple sample spotter in position in front of the adjustable spotting rack which is set up for two-dimensional chromatograms.

SAMPLE SPOTS AT VARIOUS PERCENTAGE TIMER SETTINGS

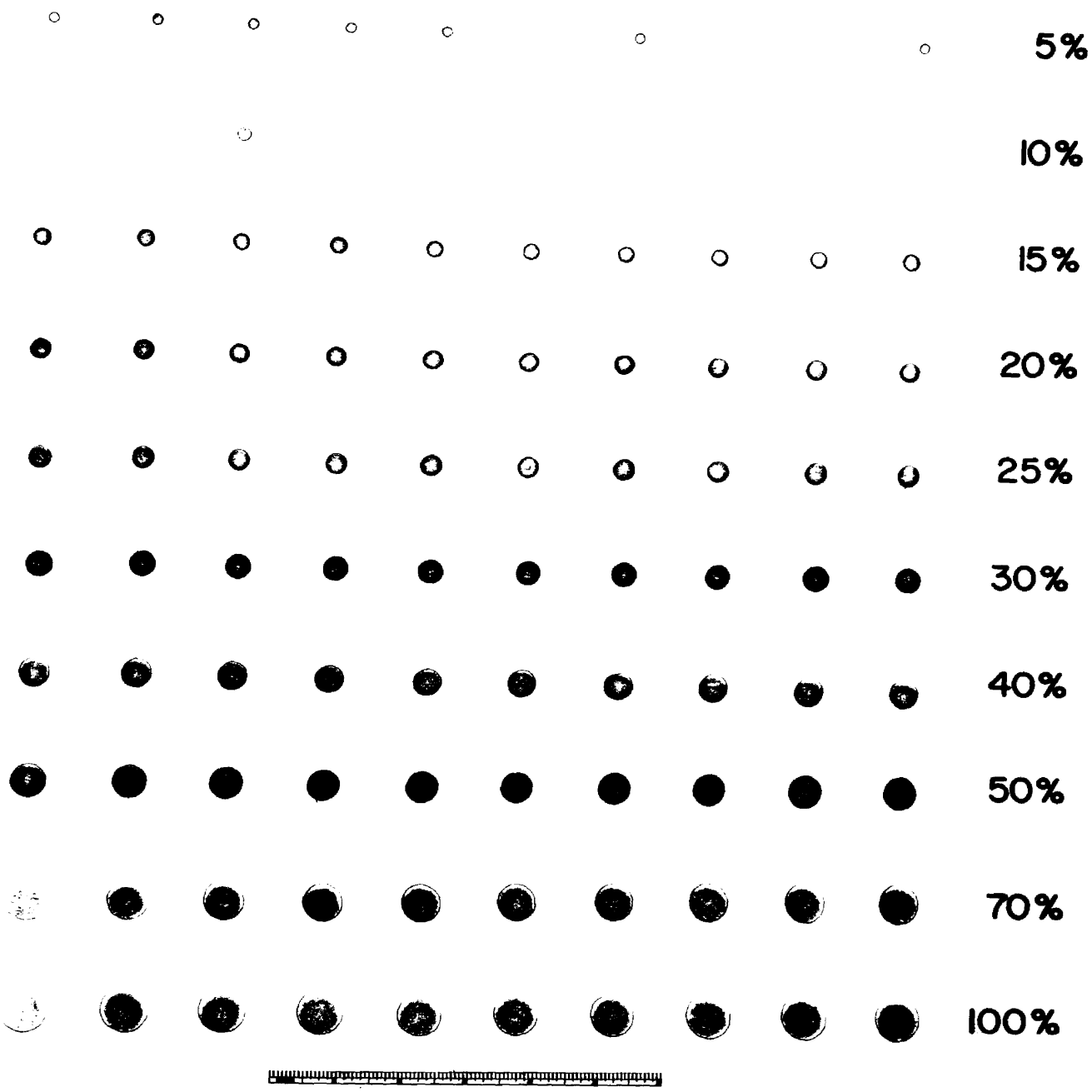


TABLE A. Composition of basal medium used in culture of Lemna minor.

KNO_3	0.002M	Thiamine	100 µg/l
$Ca(NO_3)_2$	0.003M	Pyridoxin	800 µg/l
KH_2PO_4	0.001M	Nicotinamide	800 µg/l
$MgSO_4$	0.001M	B	0.1 ppm
$CaCl_2$	0.003M	Mn	0.1 ppm
KCl	0.002M	Zn	0.3 ppm
$MgCl_2$	0.001M	Cu	0.1 ppm
Sucrose	2%	Mo	0.1 ppm
		Fe	0.5 ppm

TABLE B. Hoagland's complete nutrient solution.

Stock solutions:

No. 1	$Ca(NO_3)_2 \cdot H_2O$	227.7 gm/liter of solution
No. 2	KNO_3	109.70 gm
	KH_2PO_4	29.52 gm /liter of solution
	$MgSO_4 \cdot 7H_2O$	106.70 gm
	NaCl	7.05 gm
No. 3	H_3BO_3	0.282 gm
	$CuCl_2 \cdot H_2O$	0.004 gm
	$ZnCl_2$	0.003 gm /100 ml solution
	$MnCl_2 \cdot 4H_2O$	0.039 gm
	$FeCl_3 \cdot 6H_2O$	0.500 gm

Solution prepared: 40 ml of No. 1; 50 Ml of No. 2; 1 ml of No. 3 and brought up to 10 liters with distilled water.