This is to certify that the

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STUDIES ON INDUCED RESISTANCE IN CUCUMBER

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Sara Moray Rutter

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STUDIES ON INDUCED RESISTANCE IN CUCUMBER

By

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ABSTRACT

STUDIES ON INDUCED RESISTANCE IN CUCUMBER

By

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Heat shock (50°C, 45 seconds) treatment of scab susceptible, etiolated cucumber seedlings developed increased soluble peroxidase activity and enhanced activities of three anodic peroxidase isozymes by 14 hours after heat shock. This correlated with a previously determined time course of resistance to Cladosporium cucumerinum. Resistance was only partially effective when another heat shock was given to seedlings to suppress active metabolism prior to an inoculation of C. cucumerinum. Actinomycin D and cycloheximide treatments resulted in increased peroxidase activity and in the activity increase of the anodic peroxidase isozymes. Twelve hours between the initial exposure to cycloheximide and sampling was required for the activity increase of the anodic isozymes. Cycloheximide induced resistance in etiolated seedlings to C. cucumerinum but not systemic resistance in greenhouse grown cucumber plants to Colletotrichum lagenarium. Results of deuterium oxide labeling isopycnic equilibrium centrifugation experiments suggested that peroxidase was synthesized de novo after heat shock.

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GENERAL INTRODUCTION

Stermer and Hammerschmidt (8) reported that heat shocking of etiolated cucumber seedlings (50°C for 40 seconds) induced resistance to Cladosporium cucumerinum, the cause of cucumber scab, when the seedlings were inoculated at least three hours after the heat shock. Associated with the induction of disease resistance was an increase in total soluble peroxidase activity and an enhancement of anodic cell wall associated peroxidase isozymes by 24 hours after the heat shock. Induced systemic resistance in greenhouse grown cucumber plants has also been associated with the enhancement of total soluble peroxidase activity and of the anodic cell wall associated peroxidase isozymes (2, 7). Stermer found (9) that lignification did not play a significant role in heat shock induced resistance in etiolated cucumber seedlings, whereas enhanced lignification beginning at the time of a pathogen challenge (2, 3) was reported of greenhouse grown cucumber plants. Stermer also found (9, 10) that hydroxyproline, a moiety of the cell wall protein extensin, increased in cell walls of heat shocked seedlings over a period of three days after heat shock. The increase was enhanced and extended over time when a challenge inoculation of C. cucumerinum was made 24 hours after heat shock (9, 10).

Heat shock has been used in several studies in plant pathology to induce a susceptible state in a host plant to a pathogen to which the host is normally resistant (4, 5). In Section I of this thesis the use

of heat shock as an inducer of resistance and as an inducer of susceptibility was experimented with to determine 1) if active metabolism was required for disease resistance to be expressed after resistance was induced by heat shock, and 2) what similarities could be detected between resistance induced by heat shock and disease resistance induced by pathogens.

Heat shock results in a reduction of protein synthesis (6). In Section II, two other metabolic inhibitors, cycloheximide and actinomycin D, were given to etiolated cucumber seedlings in an attempt to suppress the increase in peroxidase activity after heat shock. I found that both of these inhibitors enhanced peroxidase activity. Further experiments were performed to determine if a chemical metabolic inhibitor would induce resistance to C. cucumerinum in etiolated cucumber seedlings as heat shock does.

Filner and Varner (1) first reported the use of density labeling techniques in a higher plant system to determine if de novo synthesis of alpha-amylase occurred in the barley-aleurone layer system. In Section III a density labeling experiment using deuterium oxide as the heavy label was performed on etiolated cucumber seedlings in an effort to determine if the increase in peroxidase activity in heat shocked seedlings was due to de novo synthesis of the enzyme. Newly synthesized enzyme will have a greater buoyant density when deuterium rather than hydrogen atoms are bonded to carbons than enzyme synthesized with hydrogen bonded to the carbons.

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SECTION I

HEAT SHOCK AND INDUCED RESISTANCE IN ETIOLATED CUCUMBER SEEDLINGS

INTRODUCTION

Heat shock has often been used as a tool to induce a susceptible interaction between a plant host and an incompatible pathogen. Heath (7) reported that a heat shock given just prior to inoculation reduced the nonhost resistance to rust pathogens in four plant species. The compatible interaction included in the study was not affected by heat shock. Heath hypothesized that an active metabolism was required for nonhost resistance but was not necessary for the compatible reaction. Hazen and Bushnell (6) reported that the hypersensitive response in barley to powdery mildew was completely suppressed by a 55°C, 45-second heat shock given just before the challenge inoculation. Stermer and Hammerschmidt (12) found that heat shocking a cucumber seedling of a cultivar normally resistant to Cladosporium cucumerinum then challenging immediately following the shock, allowed the fungus to ramify through the tissue in a manner similar to that found in a susceptible cultivar. Heat shocked cucumber seedlings were also susceptible to infection by Helminthosporium carbonum (a pathogen of corn but not of cucumber) when inoculation took place immediately after heat shock.

Yarwood et al (16) found that heat shocking corn seedlings in a 50°C waterbath for 20 seconds caused an increase in the production of anthocyanin in the seedlings and induced resistance to rust infection when inoculation occurred two days after the heat shock. Stermer and Hammerschmidt (11, 13) reported that dipping the hypocotyls of five-day-old etiolated cucumber seedlings in a 50°C water bath for 40 to 50 seconds induced resistance against C. cucumerinum within 15 to 21 hours after heat shock. Heat shock induced resistance in cucumber was correlated to enhanced soluble peroxidase activity (11, 13) and also

with increased levels of the cell wall hydroxyproline rich glycoprotein, extensin (13, 14). Systemically induced resistance and genetic resistance to C. cucumerinum had previously been correlated with enhanced peroxidase activity (4) and extensin (5), respectively. Cell wall preparations of heat shocked cucumber seedlings were found to be more resistant to enzymatic degradation by culture filtrates of C. cucumerinum than cell walls of nonshocked seedlings (14). Cell walls from heat shocked seedlings were not, however, protected from enzyme degradation by a general cell wall degrading enzyme preparation (Macerozyme) (14).

In experiments designed to test the nature of induced resistance in reed canarygrass, Vance and Sherwood (15) found that leaf discs inoculated with Botrytis cinerea to induce resistance against Helminthosporium avenae were still resistant when floated on a solution of cycloheximide and challenged with H. avenae. Without the resistance inducing inoculation, the leaf discs remained susceptible to H. avenae when floated on cycloheximide. The resistance inducing inoculation had caused the formation of lignified papilla and the enhanced activity of three enzymes involved in the formation of lignin; phenylalanine ammonia lyase, tyrosine ammonia lyase, and peroxidase. The cycloheximide treatment did not reduce the activities of the enzymes once they were induced.

A group of fast moving anodic cell wall associated peroxidase isozymes are correlated with systemically induced resistance in greenhouse grown cucumber (4). The same isozymes, judging by electrophoretic mobility, are found in heat shocked seedlings 24 hours after heat shock (11). The onset of resistance in heat shocked seedlings occurs between 15 and 21 hours after heat shock (11). To

determine if the onset of resistance could be correlated with the appearance of these isozymes in heat shocked hypocotyls, experiments were carried out to determine the time of appearance of the isozymes after a resistance inducing heat shock.

Since heat shock induced resistance is correlated with changes in the cell walls (14), possibly by making the cell walls less penetrable by fungi, an experiment was designed using heat shock to determine how much of the resistance to C. cucumerinum in heat shocked seedlings was a result of passive barriers to infection induced by heat shock and how much of the resistance required active defense mechanisms. Using heat shock to create a susceptible condition (6, 7) would preclude the possibility of an effect of the chemical on the growth of the pathogen used in the challenge inoculation.

Finally, in systemically induced resistance by Colletotrichum lagenarium in cucumber, a second "booster" inoculation following the first inducing inoculation causes an increase in soluble peroxidase activity and an increase in resistance over plants inoculated only one time (4). An experiment was done to see if heat shock induced resistance could be enhanced by a second heat shock.

MATERIALS AND METHODS

Plant Material

Cucumber seeds of a scab susceptible cultivar (Cucumis sativus L. cv. Marketer) were sown on moistened germination paper and grown in the dark for five days at 20 to 21°C.

Pathogen Material

Cladosporium cucumerinum Ell. and Arth. was cultured in Petri plates on potato dextrose agar at 20°C in the dark. Conidial suspensions were made from 7- to 10-day-old cultures by washing the cultures with distilled water while rubbing with a bent glass rod, followed by filtering the suspension through two layers of cheesecloth. Spore concentration was determined with a haemocytometer.

Inoculation of Plant

Conidial suspensions were adjusted by dilution to a concentration of 1×10^6 spores ml^{-1}. The suspensions were sprayed over the seedlings to runoff. Seedlings were kept between two rolled layers of moistened germination paper (3, 11) in the dark at 20 to 21°C.

Disease Assessment

The dark red discolorations caused by the formation of cladochrome when C. cucumerinum infects etiolated cucumber seedlings (9) were used to assay the extent of disease. The percent of the surface area of the apical 2 cm of the hypocotyl covered with discolorations or macerated was estimated and graded according to the following scale:
0 to 10% = 0, >10 to 30% = 1, >30 to 60% = 2, >60 to 100% = 3 (3, 11) (Figure 1). Disease assessment was made five days after the challenge inoculation. Each experiment was repeated four times.

Crude Peroxidase Enzyme Preparation

The apical 2 cm of the hypocotyl without the cotyledons was taken from each seedling and frozen at -20°C until assayed for peroxidase. Ten frozen sections were homogenized in a glass homogenizer with 1 ml of ice cold 0.01 M sodium phosphate buffer pH 6.0 with 0.5 M sucrose, then

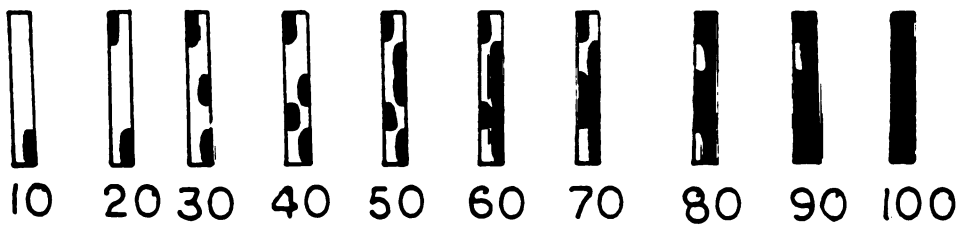

Figure 1. Disease assessment guide. Diagrams represent the apical 2 cm of the hypocotyl of scab susceptible cucumber seedlings inoculated with Cladosporium cucumerinum. Sections were scored according to percentage of surface area diseased: 0 to 10% = 0, >10 to 30% = 1, >30 to 60% = 2, >60% = 3.

centrifuged 10,000 x g for 20 minutes in 10°C. The supernatant was used for peroxidase activity assays, protein estimation, and discontinuous gel electrophoresis.

Heat Shock of Seedlings

Five-day-old cucumber seedlings were heat shocked by dipping the entire hypocotyl of each seedling in a 50°C waterbath for 45 seconds (11).

Soluble Peroxidase Activity Assay

Total soluble peroxidase activity was assayed using guaiacol as the hydrogen donor. The reaction mixture consisted of 100 μl of diluted crude enzyme extract, 1 ml of 0.28% w/v guaiacol in 0.1 M sodium phosphate buffer pH 6.0 with 0.3% hydrogen peroxide (10). The enzyme solution was diluted to give a change of absorbance of 0.1 to 0.2 absorbance units per minute at 470 nm. Activity was expressed as the change in absorbance at 470 nm $minute^{-1} \cdot mg^{-1}$ protein. Protein was estimated by the method of Bradford (1), using reagent prepared by BioRad.

Discontinuous Polyacrylamide Gel Electrophoresis

Discontinuous anodic polyacrylamide gel electrophoresis (PAGE) was performed using 7.5% polyacrylamide (pH 9.1) in 1.5 mm thick resolving gels with a 3.89% polyacrylamide (pH 6.7) stacking gel (8). Gels were run at 16 mamp constant current. Equivalent amounts of protein were loaded onto the gels for each treatment. Peroxidase isozymes were detected by soaking the gels in 200 ml of 0.05 M sodium acetate buffer pH 5.0 and staining with 11 ml of 0.36% 3-amino-9-ethyl carbozyl in N'N'dimethylformamide, with two to three drops of 30% hydrogen peroxide dropped from a Pasteur pipet (2). When bands appeared, the reaction was

stopped and the gel was fixed by transfer of the gel to a solution of methanol:water:acetic acid (50:40:10 v/v). The fixing solution was replaced by a 10% v/v solution of glycerol in distilled water prior to drying the gel.

Treatments

For time course studies on the increase in activity of the anodic peroxidase isozymes seedlings were heat shocked at time 0 and then kept in the dark in a beaker with distilled water around the roots. Starting at twelve hours after the heat shock, seedlings were sampled at two hour intervals up to 18 hours. The tissue was stored at -20°C until assayed.

In the double heat shock experiments, seedlings were heat shocked on day 1, then again on day 2, then either challenged with C. cucumerinum immediately after the second heat shock or challenged on day 3. Controls were seedlings not heat shocked, heat shocked on day 1 only or heat shocked on day 2 only.

RESULTS

Time Course

Gel electrophoretic separation of the anodic peroxidase isozymes showed an increase in activity of the fast moving anodic isozymes between 14 and 18 hours after heat shock. Activity was detected on the gel by 14 hours after heat shock, and continued to increase over the next 4 hours (Figure 2). Seedlings not heat shocked did not have detectable activity of these isozymes on the gel at either time sampled (0 and 18 hours).

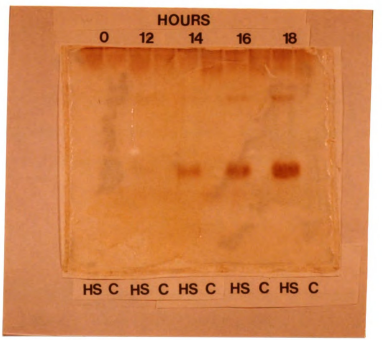

Figure 2. Time course of the enhancement of activity of the fastest moving anodic peroxidase isozymes after heat shock. Samples of heat shocked (50°C, 45 seconds) and nonshocked etiolated cucumber seedlings were prepared and electrophoresced in adjacent lanes. Equivalent amounts of protein (75 μg) of each treatment sample were loaded onto a 7.5% polyacrylamide (pH 9.1) 1.5 mm thick gel. Peroxidase isozymes were stained with 3-amino-9-ethyl carbozyl.

Heat Shock and Disease Resistance

Seedlings heat shocked on day 1 to induce resistance then heat shocked on day 2 to attempt to induce susceptibility prior to a challenge inoculation of C. cucumerinum did have some disease resistance when compared to seedlings with no heat shock induced resistance (Table 1). Total soluble peroxidase activity and the activities of the fastest moving anodic peroxidase isozymes were comparable between the two treatments with disease resistance inducing heat shocks on day 1, as were the activities of the two treatments with no resistance inducing heat shocks.

Seedlings heat shocked on two consecutive days, then sampled on day 3, did not exhibit more disease resistance or peroxidase activity than those seedlings heat shocked one time (Table 2).

DISCUSSION

Heat shock induced resistance appeared to be partly composed of changes in the plant tissue which did not require active metabolism once the changes had developed. One of these changes was the increase in activity of the cell wall associated peroxidase isozymes. The seedlings with heat shock induced resistance which were heat shocked again prior to challenge showed a resistance which was intermediate between seedlings with no induced resistance and those with induced resistance. The results presented here suggest that there is an active as well as a passive element in heat shock induced resistance in cucumber to respond to a fungal challenge. These results with cucumber differ from those of Vance and Sherwood's (15) with reed canarygrass where a metabolic

TABLE 1

Resistance to Cladosporium cucumerinum and peroxidase activity of cucumber seedlings heat shocked to induce resistance and heat shocked to induce susceptibility.

TREATMENT		DISEASE RATING[1]	RELATIVE PEROXIDASE ACTIVITY[2]
Day 1	Day 2		
HEAT SHOCK	NO HEAT SHOCK	2.10 ± .32 a	281 ± 35
HEAT SHOCK	HEAT SHOCK	2.46 ± .18 ab	294 ± 23
NO HEAT SHOCK	HEAT SHOCK	2.91 ± .07 b	129 ± 9
NO HEAT SHOCK	NO HEAT SHOCK	2.91 ± .07 b	100

[1]Disease ratings were made five days after challenge inoculation of C. cucumerinum (1×10^6 spores ml^{-1}) on day 2. Disease ratings were made as described in Figure 1. Treatments with a letter in common are not significantly different P = .99, l.s.d. = .78. All values are means and standard deviations of the mean of four replicates.

[2]Samples for peroxidase activity assays were taken after the treatment of day 2. Peroxidase activity is expressed as a percentage of the activity of the nonshocked control. Activity was measured as the change in absorbance at 470 nm $minute^{-1}$ mg^{-1} protein. All values are means and standard deviations of the mean of four replicates.

TABLE 2

Resistance to Cladosporium cucumerinum and peroxidase activity of cucumber seedlings heat shocked to induce resistance on two consecutive days.

TREATMENT		DISEASE RATING[1]	RELATIVE PEROXIDASE ACTIVITY[2]
Day 1	Day 2		
HEAT SHOCK	NO HEAT SHOCK	2.27 ± .20 a	607 ± 93
HEAT SHOCK	HEAT SHOCK	2.52 ± .14 ab	506 ± 60
NO HEAT SHOCK	HEAT SHOCK	2.05 ± .13 a	300 ± 54
NO HEAT SHOCK	NO HEAT SHOCK	2.93 ± .05 b	100

[1]Disease ratings were made five days after challenge inoculation of C. cucumerinum (1×10^6 spores ml^{-1}) on day 3 as described in Figure 1. Treatments with a letter in common are not significantly different P = .99, l.s.d. = .557. All values are means and standard deviations of the mean of four replicates.

[2]Samples for peroxidase activity assays were taken on day 3. Peroxidase activity is expressed as a percentage of the activity of the nonshocked control as described in Table 1. All values are means and standard deviations of the mean of four replicates.

inhibitor did not affect resistance once it was induced. The cucumber seedlings with no heat shock induced resistance which were heat shocked just before the challenge inoculation had the same amount of disease as nonshocked seedlings. These results are similar to Heath's (7) in that the susceptible interaction was unaffected by heat shock. Stermer found (13, 14) that lignification did not appear to be a factor contributing to heat shock induced resistance. He suggested that the crosslinking of extensin molecules in the cell wall by the increased amount of active peroxidase could account for the resistance. This may account for the resistance which withstood the second heat shock, the passive element of the induced resistance. What the active element (or elements) may be is unknown at the present time.

Over the 12- to 18-hour period after heat shock, total soluble peroxidase activity is not significantly higher in heat shocked seedlings than in nonshocked seedlings (11). However, the separation of the anodic isozymes by electrophoresis in this work showed a striking increase in activity of these isozymes during this period. The increase in activity correlates with the time of the onset of resistance estimated by Stermer and Hammerschmidt (11) and thus strengthens the correlation between induced resistance and the peroxidase isozymes.

The observation that the peroxidase isozymes and resistance were not enhanced by a second heat shock demonstrates a difference between heat shock induced resistance and induced resistance by a microorganism. In the latter form of induced resistance, a second inoculation of the pathogen was found to cause an additional increase in peroxidase activity and resistance (4).

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SECTION II

METABOLIC INHIBITORS AND HEAT SHOCK INDUCED RESISTANCE IN ETIOLATED CUCUMBER SEEDLINGS

INTRODUCTION

The metabolic inhibitors cycloheximide and actinomycin D have been used in plant physiological studies to help determine if processes such as increased enzyme activity (3, 18) or morphological changes (10, 29) are due to de novo protein synthesis. Cycloheximide inhibits protein synthesis in yeast cultures by interfering with the formation of the peptide chain on the 60 s ribosomal subunit (24). Actinomycin D binds to the minor groove of deoxyribonucleic acid thus interfering with transcription (22). Cycloheximide and actinomycin D have been used also in plant pathological studies to create susceptible interactions between plant hosts and pathogens (10, 11, 28). Heath (10) used cycloheximide and actinomycin D to suppress the nonhost resistance response between several plant species and incompatible rust fungi. Vance and Sherwood (28) used cycloheximide to suppress resistance in reed canarygrass to several incompatible pathogens.

In preliminary experiments conducted on etiolated cucumber seedlings (Cucumis sativus), in an attempt to block the enhancement of the activities of the fastest moving anodic peroxidase isozymes which occur after heat shock (27), I found that cycloheximide caused a similar increase in the activities of these isozymes. In the following work, experiments were performed to determine the effects on peroxidase activity of different concentrations of cycloheximide and actinomycin D on heat shocked and nonshocked seedlings, and to determine if cycloheximide could induce resistance in etiolated seedlings to Cladosporium cucumerinum, or in greenhouse plants to Colletotrichum lagenarium.

MATERIALS AND METHODS

Plant Material

For experiments using etiolated cucumber seedlings, seeds of Cucumis sativus L. cv. Marketer were sown on moist germination paper and kept in the dark at 20 to 21°C for five days. On the fifth day, the seedlings were used in experiments.

For one experiment, seeds of C. sativus cv. Marketer were sown in four-inch pots in Baccto Grower's Mix in a greenhouse and grown for 21 days.

Pathogen Material

Cultures of Colletotrichum lagenarium (Pass.) Ell. and Halst. race 1 and Cladosporium cucumerinum Ell. and Arth. were grown on potato dextrose agar in Petri plates in the dark at 20°C for 7 to 14 days. Conidial suspensions of both pathogens were made by flooding a culture with distilled water and gently rubbing the culture with a bent glass rod. The suspension was filtered through two layers of cheesecloth and the concentration of spores estimated using a haemocytometer.

Inoculation of Etiolated Seedlings

A conidial suspension of C. cucumerinum (1×10^6 spores ml^{-1}) was sprayed over the seedlings until runoff. Seedlings were placed between two moist layers of rolled germination paper (8, 27).

Inoculation of Greenhouse Plants

To induce resistance in the greenhouse plants, a conidial suspension of C. lagenarium (1×10^5 spores ml^{-1}) was infiltrated 10 times into the abaxial side of the first true leaf of 10 plants using a 3 ml syringe with the needle removed. Solutions of cycloheximide were infiltrated into the first true leaves of 10 plants each in the same

manner. Distilled water was used as a control. To challenge the green plants, one week after the inducing treatment a conidial suspension of C. lagenarium (5×10^4 spores ml^{-1}) was dropped 10 times onto the adaxial surface of the second leaf. Plastic bags were put over each plant for one day and the plants were kept out of direct sunlight. After one day the bags were opened and after another day the plants were returned to direct light out of the plastic bags. This experiment was replicated four times.

Heat Shock of Seedlings

Seedlings were dipped in a 50°C waterbath so that the entire seedling except the roots was submerged in the waterbath for 45 seconds.

Cycloheximide and Actinomycin D Treatments of Seedlings

Seedlings were dipped in solutions of cycloheximide (Sigma) or actinomycin D (Sigma) in concentrations of 0, 1, 10 or 100 μM dissolved in distilled water. The seedlings were then placed in glass 50 ml Erlenmeyer flasks or glass 100 ml beakers. The dipping solution was poured around the roots of the seedlings to provide moisture to the roots. The seedlings were kept in the dark for the duration of the experiment.

Disease Assessment

Disease on etiolated seedlings from C. cucumerinum was assessed on the fourth and fifth day after challenge. Disease was assessed as described in Section I, Figure 1. Disease on greenhouse grown plants from C. lagenarium was assessed by counting necrotic lesions. Maximum disease was scored at 10, no necrotic lesions present as 0.

Peroxidase Enzyme Extraction

The apical two centimeters of the hypocotyl of each etiolated seedling was taken for soluble peroxidase activity assays. Ten

seedlings were sampled for each treatment. The sections were stored at -20°C until processed. In greenhouse grown plants, one centimeter diameter discs were taken from the second leaf of each plant. Ten discs were sampled for each treatment, one disc from each plant. The hypocotyl sections and leaf discs were homogenized using a modified drill in ice cold 0.01 M sodium phosphate buffer pH 6.0 with 0.5 M sucrose (1 ml per 10 sections or discs). The samples were centrifuged in a microfuge at 13, 600 x g for five minutes. The supernatant was used as the crude enzyme preparation. Protein was estimated by the method of Bradford (4) in the actinomycin D and cycloheximide dose response on etiolated seedlings experiments. In all of the other experiments, protein was estimated by the method of Lowry et al (16).

Soluble Peroxidase Activity Assay

Total soluble peroxidase activity was assayed as described in Section I of this thesis.

Separation of Anodic Peroxidase Isozymes

Discontinuous polyacrylamide gel electrophoresis (PAGE) was performed as described in Section I of this thesis. Peroxidase isozymes were detected by staining with 3-amino-9-ethyl carbozyl as described in Section I of this thesis.

SDS PAGE

Protein denaturing gels were composed as in activity gel electrophoresis using a 7.5% or 10% polyacrylamide for the running gel with the addition of 0.1% sodium dodecyl sulfate (SDS) Samples were mixed with an equal volume of treatment buffer of 0.01 M sodium phosphate pH 6.0, 0.5% SDS, 2% v/v glycerol, 1% v/v 2-mercaptoethanol, and boiled for 90 seconds. The upper and lower buffers also contained 0.1% SDS (15). Low molecular weight markers (BioRad) were loaded onto

one lane to find the region where the peroxidase isozymes would be expected to migrate. These isozymes have been estimated to be 20,000 to 30,000 daltons in molecular weight (26). The gel was fixed with methanol, water, and acetic acid (50:40:10 v/v) overnight, then soaked in two changes of 2.5 mg of dithiothreitol in 500 ml of distilled water for one hour. The gel was then soaked in 500 ml of a 0.1% silver nitrate solution for another hour, rinsed rapidly with distilled water, followed by 300 ml of 3% $NaCO_3$. The gel was then soaked in 500 ml of 3% $NACO_3$ containing 100 μl of 37% formaldehyde. When the protein bands were sufficiently developed, the reaction was stopped by adding 12 g of granular citric acid. After 10 minutes, the gel was repeatedly rinsed in distilled water (20).

RESULTS

Cycloheximide Dose Response in Etiolated Seedlings

Etiolated seedlings treated with cycloheximide were stunted in size and appeared similar to heat shocked seedlings. Seedlings heat shocked and treated with cycloheximide were more stunted than heat shocked seedlings not treated with cycloheximide and nonshocked seedlings treated with cycloheximide.

Polyacrylamide gel electrophoretic separation of the fastest moving anodic peroxidase isozymes showed an increase in activity of these isozymes in nonshocked seedlings in response to increasing concentrations of cycloheximide. However, heat shocked seedlings treated with increasing concentrations of cycloheximide showed an inhibition of activity in these isozymes (Figure 1). Neither 7.5% or

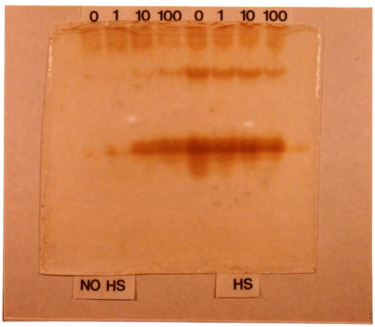

Figure 1. Activity gel of cycloheximide dose response. Polyacrylamide gel electrophoresis of the anodic peroxidase isozymes extracted from samples of seedlings not heat shocked (the four lanes on the left half of the gel) or heat shocked (the four lanes on the right half of the gel) and treated with 0, 1, 10, or 100 μM cycloheximide for 24 hours. Samples were taken 24 hours after the beginning of the experiment. Equivalent amounts of protein (75 μg) of each treatment sample were loaded in each lane of a 7.5% polyacrylamide 1.5 mm thick gel. Peroxidase isozymes were stained for activity with 3-amino-9-ethyl carbozyl.

10% polyacrylamide denaturing gels stained with silver nitrate revealed a disappearance or appearance of any major protein bands after 24 hours of cycloheximide treatment (Figure 2).

Actinomycin D Dose Response in Etiolated Seedlings

PAGE showed that actinomycin D had an effect similar to that of cycloheximide. An increasing concentration of actinomycin D resulted in an increase in the activity of the fastest moving anodic peroxidase isozymes in nonshocked seedlings but resulted in an inhibition of activity of the isozymes in heat shocked seedlings (Figure 3).

Length of Time Required in Cycloheximide for Enhanced Activity of the Peroxidase Isozymes

Polyacrylamide gel electrophoresis of samples from seedlings treated with 10 μM cycloheximide showed enhanced activity of the fastest moving anodic isozymes when the seedlings were exposed to cycloheximide for 2 hours and sampled 24 hours after the beginning of the experiment (Figure 4). The activity of the isozymes increased as the length of time in 10 μM cycloheximide increased to eight hours. The eight-hour treatment had a similar enhancement of the isozymes as that found in a 24-hour treatment (data not shown). Activity of the isozymes was greater in those seedlings exposed to 100 μM of cycloheximide than in those exposed to 10 μM of cycloheximide.

Time Course of Anodic Peroxidase Isozyme Activity Enhancement

Enhancement of the activities of the fastest moving anodic peroxidase isozymes was detected in seedlings treated with 100 μM of cycloheximide and sampled 16 hours from the start of the treatment (Figure 5). The greatest activity enhancement was seen in those seedlings kept in cycloheximide for 24 hours and sampled at that time.

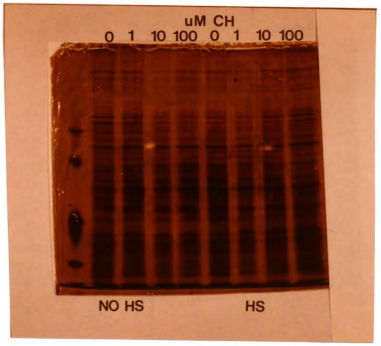

Figure 2. SDS PAGE of cycloheximide dose response. Polyacrylamide gel electrophoresis of denatured extracts of samples identical to those in Figure 1. The first lane on the left was loaded with 12 μg of protein of a mixture of low molecular weight markers. Equivalent amounts of protein (20 μg) of each treatment sample were loaded on to a 7.5% polyacrylamide 1.5 mm thick gel. Marker proteins are: phosphorylase B, 92.5 kD; bovine serum albumin, 66.2 kD; ovalbumin, 45.0 kD; and carbonic anhydrase 31.0, kD. A 10% polyacrylamide gel gave a similar result.

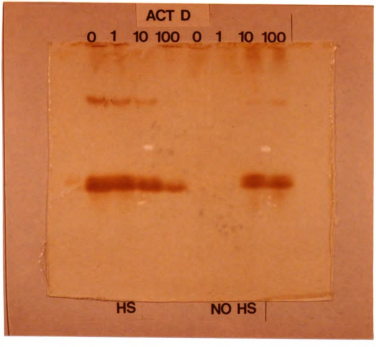

Figure 3. Actinomycin D dose response. Polyacrylamide gel electrophoresis of the anodic peroxidase isozymes extracted from samples of seedlings heat shocked (the four lanes on the left half of the gel) or not heat shocked (the four lanes on the right half of the gel) and treated with 0, 1, 10 or 100 μM actinomycin D for 24 hours. Samples were taken 24 hours after the start of the experiment. Equivalent amounts of protein (50 μg) were loaded onto a 0.75 mm thick 7.5% polyacrylamide gel. Peroxidase isozymes were stained for activity with 3-amino-9-ethyl carbozyl.

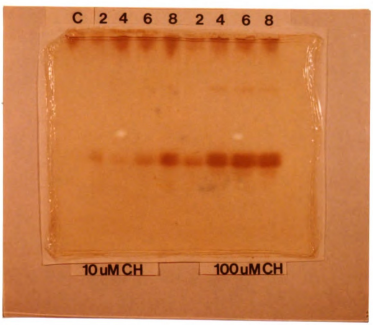

Figure 4. Length of time required in cycloheximide for enhancement of the activities of the anodic peroxidase isozymes. Seedlings were treated with 10 μM or 100 μM cycloheximide for 0, 2, 4, 6 or 8 hours. Seedlings were removed from cycloheximide at these times. Samples were taken 24 hours after the beginning of the experiment. Polyacrylamide gel electrophoresis was carried out by loading 75 μg of protein of each treatment sample onto a 7.5% polyacrylamide 1.5 mm thick gel. The peroxidase isozymes were stained for activity with 3-amino-9-ethyl carbozyl.

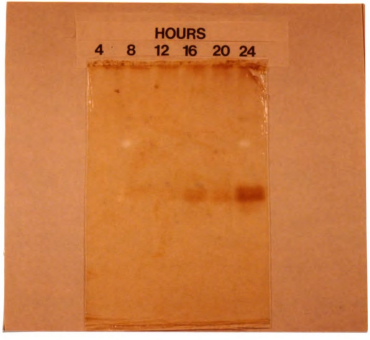

Figure 5. Time course of the enhancement of the activities of the anodic peroxidase isozymes. Seedlings were treated with 100 μM of cycloheximide for 4, 8, 12, 16, 20, or 24 hours. Equivalent amounts of protein (75 μg) of each treatment sample were loaded onto a 7.5% polyacrylamide 1.5 mm thick gel. The peroxidase isozymes were stained for activity with 3-amino-9-ethyl carbozyl.

Cycloheximide Induced Resistance to C. Cucumerinum in Etiolated Seedlings

The degree of disease in the susceptible cotyledons appeared to be the same in heat shocked seedlings as in cycloheximide treated seedlings. This indicated that cycloheximide that might have still been in the seedlings was not affecting the fungus to an observable degree. Total soluble peroxidase activity was enhanced, when compared to the water control, in the heat shocked seedlings and in the seedlings treated with cycloheximide. Seedlings treated with 100 μM cycloheximide for 24 hours showed resistance to C. cucumerinum (Table 1).

Effects of Cycloheximide Infiltrated into the First Leaf of Three-Week-Old Greenhouse Grown Plants

Cycloheximide (10 μM and 100 μM) caused an increase in soluble peroxidase activity that was greater than that found in the water control plants but less than that found in plants inoculated with C. lagenarium (Table 2). No enhanced disease resistance was detected in plants treated with cycloheximide six days after the challenge inoculation. The first leaves treated with cycloheximide showed necrotic lesions where the infiltrations were made. These lesions developed over a few days. The lesions from 100 μM cycloheximide were more severe than those from 10 μM cycloheximide (Figure 6).

DISCUSSION

There are several reports on the effects of cycloheximide and actinomycin D on the activity of enzymes. Birecka and Miller (2) found that actinomycin D (10 $\mu g\ ml^{-1}$) stimulated the activity of soluble peroxidase isozymes in sweet potato root discs due to injury and ethylene treatment. Cycloheximide (5 $\mu g\ ml^{-1}$) inhibited this enhancement,

TABLE 1

Cycloheximide induced resistance in etiolated cucumber seedlings to Cladosporium cucumerinum.

TREATMENT	RELATIVE DISEASE[1] Day 4	RELATIVE DISEASE[1] Day 5	PEROXIDASE ACTIVITY[2]
100 μM CYCLOHEXIMIDE	44 ± 18 a	69 ± 9 x	29 ± 2
HEAT SHOCK	50 ± 19 a	82 ± 6 x	45 ± 1
DISTILLED WATER	100 b	100 y	11 ± 1

[1]Disease ratings were made four and five days after challenge inoculation of C. cucumerinum (1 x 10^6 spores ml^{-1}). Disease was rated as described in Figure 1, Section I of this thesis. Data are expressed as percents of the water treatment. Student's t test was performed on each pair of treatments. Treatments with a letter in common are not significantly different at P = .95. All values are the means and standard deviations of the mean of four replicates.

[2]Samples for peroxidase activity assays were taken at the time of the challenge inoculation. Peroxidase activity is expressed as the change in absorbance at 470 nm $minute^{-1}$ mg^{-1} protein. All values are the means and standard deviations of the mean of four replicates.

TABLE 2

Effects of cycloheximide, infiltrated in the first true leaf of three-week-old cucumber plants, on the second leaf.

TREATMENT	RELATIVE PEROXIDASE ACTIVITY[1]	RELATIVE DISEASE[2]
10 μM CYCLOHEXIMIDE	140 ± 18 ab	109 ± 4 x
100 μM CYCLOHEXIMIDE	180 ± 40 ab	102 ± 3 x
C. LAGENARIUM	212 ± 45 b	80 ± 4 y
DISTILLED WATER	100 a	100 x

[1]Peroxidase activity was assayed seven days after the first inoculation. Peroxidase activity is expressed as a percentage of the water treatment (change of absorbance at 470 nm minute^{-1} mg^{-1} protein). Student's t test was performed on each pair of treatments. Numbers followed by the same letter are not significantly different at P = .95. All values are the means and standard deviations of the mean of four replicates.

[2]Disease was assayed seven days after the challenge inoculation of C. lagenarium (5 x 10^4 spores ml^{-1}). Data are expressed as percentages of the water treatment with standard deviations of the mean. Student's t test was performed on each pair of treatments. Numbers followed by the same letter are not significantly different at P = .95. The experiment was replicated four times.

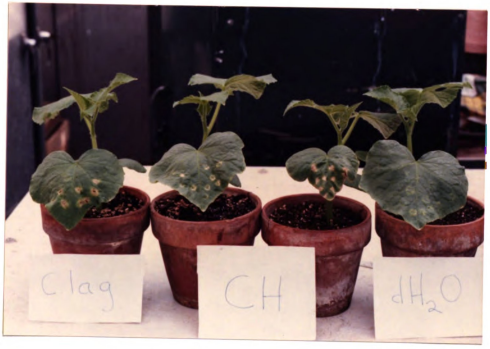

Figure 6. Greenhouse grown cucumber plants treated with Colletotrichum lagenarium, cycloheximide, or water. The first leaf of three-week-old plants was infiltrated with spores of C. lagenarium (1×10^5 spores ml^{-1}), 10 μM or 100 μM cycloheximide, or distilled water (pictured from left to right). Plants are pictured two weeks after treatment.

suggesting that the increase in peroxidase activity required de novo synthesis of the enzyme. Actinomycin D and cycloheximide did not affect the injury response enhancement of peroxidase activity in carrot roots. Birecka and Miller tentatively concluded that the increase in peroxidase activity in carrot root sections was due to the activation of previously synthesized protein. Attridge and Smith (1) found that cycloheximide in increasing concentrations (0 to 500 $\mu g\ ml^{-1}$) resulted in an increase in phenylalanine ammonia lyase (PAL) activity in etiolated three- to five-day-old cucumber seedlings within three hours of exposure. Because cycloheximide inhibited the enhanced PAL activity due to exposure to blue light but did not inhibit the increase in activity due to an environmental temperature shift (25°C to 4°C), the authors speculated that an inactive pool of PAL was the source of the enhanced enzymatic activity due to the temperature shift. Jones and Northcote (12), investigating PAL activity induction in bean cell suspension cultures as a result of an increase in the cytokinin to auxin ratio, found an expected decline in PAL activity, after the induction, was suppressed by actinomycin D. The authors hypothesized that PAL enzyme degradation was inhibited by the exposure to actinomycin D so that the increased activity of PAL was thought not to be due to de novo synthesis. Novacky and Wheeler (22) found that oat leaves exposed to actinomycin D (1 to 25 $\mu g\ ml^{-1}$) for 24 hours showed an enhancement of peroxidase isozymes identical to that found in wounded leaves and those treated with Helminthosporium victoriae toxin. Higher concentrations of actinomycin D inhibited the enhancement of activity of the isozymes due to the toxin. The authors suggested that the enhancement of activity due to exposure to actinomycin D might be due to the presence of stable messenger ribonucleic acids in sufficient concentration to maintain

protein synthesis in the presence of the inhibitor. Ridge and Osborne (24) found that actinomycin D (10 $\mu g\ ml^{-1}$) applied to pea seedlings 24 hours after the start of exposure to ethylene increased the ethylene enhancement of soluble peroxidase activity. In this work, evidence was presented of a diffusable inhibitor of peroxidase activity which was hypothesized to be sensitive to actinomycin D, thus resulting in an increase in peroxidase activity.

In the work presented here, actinomycin D and cycloheximide caused an enhancement of the activities of the fastest moving anodic peroxidase isozymes in etiolated cucumber seedlings. These isozymes are identical to those which are enhanced in cucumber in response to pathogen attacks, wounding, and senescence (30). The activity enhancement from heat shock, however, was inhibited by increasing concentrations of actinomycin D and cycloheximide. It is interesting that cycloheximide, an inhibitor of translation, and actinomycin D, an inhibitor of transcription, had such similar effects on the activity of these peroxidase isozymes.

Difficulties arise in interpreting results of experiments which involve exposing plants to chemicals when the effective site of action is not known. Cycloheximide has been reported to have varying effects on different species of higher plants (17). The results of the five papers described and of the work presented here show different effects of cycloheximide and actinomycin D on enzyme activity in different systems.

The enhancement of the anodic peroxidase isozymes due to 100 μM of cycloheximide followed a similar time course to that of the heat shock enhancement. There seems to be a requirement of a lag period for the

enzyme activity increase to occur. This suggests that a similar process leading to increased peroxidase activity is occurring in both treatments (Figure 4, Figure 5, and Figure 2 of Section I of this thesis).

Cycloheximide (100 μM) induced resistance to C. cucumerinum in etiolated cucumber seedlings in a manner appearing to be similar to that found in heat shocked seedlings. There have been reports on cycloheximide induced resistance in tomato plants and in wheat (6). In these earlier reports, antifungal activity was attributed to active derivatives of cycloheximide believed to be accumulated in the leaves of these plants. Given the evidence of enhanced peroxidase activity in the cucumber seedlings, a more likely source of resistance would seem to be physiological changes in the host.

Localized application of cycloheximide (10 or 100 μM) to the first true leaves of three-week-old plants resulted in a systemic increase in peroxidase activity without a concurrent increase in resistance to C. lagenarium. The increase in peroxidase activity in the cycloheximide treated plants, while not significantly different from that of the pathogen inoculated plants, was also not significantly different from the water treated controls because of the relatively large variation within the treatments. A possibility which must be considered is that a greater increase in peroxidase activity might result in some resistance to C. lagenarium being developed. Aging in leaves is also associated with an increase in activity of cell wall associated peroxidase isozymes (the fastest moving anodic isozymes in cucumber) (9) but senescence does not give resistance to C. lagenarium (14). Associated with an increase in peroxidase activity must be an increase in substrates such as hydrogen peroxide and molecules such as monohydroxyphenols to act as electron donor (7, 9, 14). These substrates were perhaps not produced

in sufficient concentrations in the second leaf of the cycloheximide treated plants.

Métraux and Boller (18) found that several salt solutions applied to the first leaves of three-week-old cucumber plants induced systemic resistance to C. lagenarium. The salt solutions caused necrotized areas to be produced as did the cycloheximide solutions used in the experiments presented here. However, the cycloheximide treatments did not induce systemic resistance. Nadolny and Sequeira (21) showed that an increase in peroxidase activity in tobacco plants did not necessarily lead to induced systemic resistance. When saprophytic or heat killed bacteria were injected into tobacco leaves, an increase in peroxidase activity was observed without a concurrent development of resistance. The correlation of induced resistance and enhanced peroxidase activity in cucumber plants demands more testing.

Cycloheximide is a phytotoxic compound which is probably affecting several systems in the host (e.g. protein synthesis, ion exchange, membrane integrity) (17) during the duration of the experiments. The interaction of a fungus and host is dynamic and depends on the environmental conditions that affect the growth of the pathogen and the physiological state of the host.

The work presented here raises questions concerning the increase in the activities of the anodic peroxidase isozymes. Are the isozymes newly synthesized, held in an inactive complex, held in an incompleted form, or degraded at a reduced rate after the stimulus? These questions must be answered to gain more understanding of the control mechanisms of induced resistance in cucumber.

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SECTION III

DEUTERIUM OXIDE LABELING AND ISOPYCNIC EQUILIBRIUM CENTRIFUGATION OF HEAT SHOCKED ETIOLATED CUCUMBER SEEDLINGS

INTRODUCTION

When etiolated cucumber seedlings are heat shocked at 50°C for 45 seconds, peroxidase enzyme activity increases three to four fold within 24 hours after heat shock (Section I and Section II of this thesis). The increase in peroxidase activity correlates with the development of disease resistance to Cladosporium cucumerinum (10, 11). Peroxidase activity was also found to increase in response to treatments with 1 to 100 μM of cycloheximide (Section II of this thesis). In order to investigate whether the increase in peroxidase activity after heat shock was due to de novo enzyme synthesis, a density labeling experiment was performed using deuterium oxide as the heavy isotope. Deuterium oxide introduced into a plant at the time of the inducing stimulus, in this experiment a heat shock, becomes a part of the amino acid pool by permanently exchanging with hydrogen atoms bonded to carbon atoms (6). Density labeling has an advantage over immunological and radiolabeling techniques in that enzymes do not need to be purified to determine if new protein is being synthesized as a result of a particular stimulus (6). Experiments using deuterium oxide as a label are designed so that samples from tissue fed the deuterium label and given an inducing stimulus are compared to samples from tissue fed the label but not given any inducing stimulus. The period of labeling should be shorter than the life of the enzyme being investigated. The crude enzyme extracts are loaded onto a solution capable of forming a stable gradient during centrifugation and of attaining high densities with low viscosity (e.g. cesium chloride, rubidium chloride, or potassium bromide) (5, 6). The gradients are fractionated after the proteins have reached their buoyant

density equilibrium. The parameters of interest in interpreting the results of comparative density labeling experiments are: 1) The bandwidth at 50% of the maximum activity of the curve of enzyme activity plotted against fraction number or buoyant density. This is a measurement of the amount of labeling of the enzyme which has occurred. A maximum bandwidth is obtained when 50% of the enzyme is labeled with the deuterium. The bandwidth also depends on the lifetime of the enzyme relative to the length of the labeling period. 2) The shift of the enzyme activity peak of induced labeled samples from the activity peak of induced unlabeled samples is compared to the shift of the activity peak of noninduced labeled samples from the activity peak of noninduced unlabeled samples (Figure 1), (1, 6, 7). To ascertain that the amount of labeling of the amino acid pool is unaffected by the given inducing stimulus, the activity of a second enzyme which is not affected by the stimulus is assayed (1, 6, 7). Another control which is included is an external marker added to each gradient so that treatments in separate gradients may be compared (1, 6, 7). Several authors have cautioned against inferring the mechanism of enzyme control from density labeling experiments (1, 6, 7). Because only an active enzyme can be detected, an increase in activity of unlabeled enzyme can be a result of an enzyme released from an inactivator complex or a reduction in the rate of enzyme degradation (C in Figure 1). A shift in the enzyme activity peak, on the other hand, can be a result of <u>de novo</u> synthesis of the enzyme or the activation of an inactive incomplete precursor (A in Figure 1) (7).

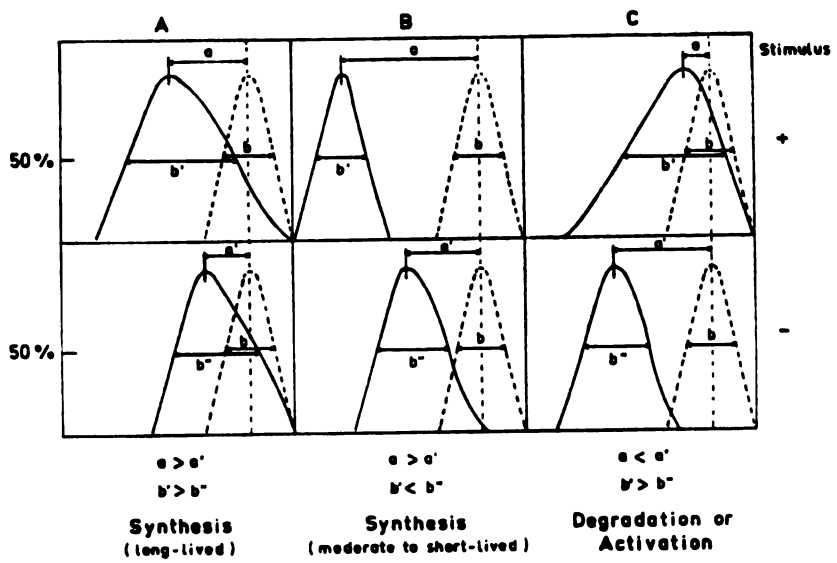

Figure 1. Examples of enzyme activity curves after deuterium oxide labeling, isopycnic equilibrium centrifugation, and fractionation. Activity curves of unlabeled samples (---) and labeled samples (——) are shown in the presence (+) or absence (–) of the activity inducing stimulus. Bandwidths b′ and b" at 50% of maximum activity depend on the amount of labeling which has occurred and the lifetime of the enzyme being labeled. Activity peak shifts a and a′ depend on the presence of de novo synthesis of the enzyme. Figure is taken from Acton et al (1).

MATERIALS AND METHODS

Plant Material

Seeds of Cucumis sativus L. cv. Marketer were sown on moist germination paper and kept in the dark at 20 to 21°C for five days.

Heat Shock of Seedlings and Labeling with Deuterium Oxide

The hypocotyl and cotyledons of five-day-old seedlings were dipped in a 50°C water bath for 45 seconds. Ten to 20 heat shocked seedlings were dipped into a solution of 60% v/v deuterium oxide (D_2O) (99.8% purity, Aldrich Chemical Company) then placed in a glass beaker, the roots moistened by the D_2O solution. Another set of heat shocked seedlings were dipped in distilled water. Nonshocked seedlings were dipped in distilled water or in the D_2O solution as controls. Seedlings were kept in the dark for 46 hours then sampled by taking the apical 2 cm of the hypocotyls. Samples were stored in -20°C until processed.

Extraction of Crude Peroxidase Enzyme Extract

The hypocotyl sections were homogenized in cold 0.01 m l sodium phosphate buffer pH 6.0 using a modified drill. The samples were centrifuged in a microfuge at 13,600 x g for five minutes. The Lowry assay (8) was performed on the clear supernatant to estimate protein content. The crude enzyme extract was stored in -20°C.

Isopycnic Equilibrium Centrifugation

Equal amounts of protein (100 μg) from each treatment sample were loaded into 6 ml polyallomer tubes (Sorvall Instruments) filled with 32% (w/w) cesium chloride dissolved in 0.1 M sodium phosphate buffer pH 6.0. The external marker enzyme, B-galactosidase (Sigma) was added to each tube in aliquots containing 9.18 units. Ultracentrifugation was carried out in a TV865 Sorvall Instruments vertical rotor at 15°C at 50,000 rpm for

30 hours. Two drop fractions (approximately 90 μl) were collected from the top of each tube by pumping a saturated CsCl solution colored with bromphenol blue into the bottom of each tube using a density gradient fractionator (Isco). Fifty microliters of each fraction were taken to assay for peroxidase activity. The remaining solution in all of the odd numbered fractions was used to assay for B-galactosidase activity, and in the even numbered fractions for acid phosphatase activity.

Peroxidase Enzyme Microassay

Fifty microliters of each fraction were pipetted into a microtiter plate. One hundred microliters of substrate reagent consisting of 2, 2-Azino-di(3 ethylbenzthiazoline sulfonic acid) (ABTS) dissolved in 50 mM sodium citrate buffer pH 4.0 (4.4 mg ABTS in 20 ml buffer) and 7 μl of 30% hydrogen peroxide, were pipetted into the microtiter plate. The plate was allowed to incubate at room temperature for 15 to 30 minutes. The reaction was stopped by the addition of 100 μl of a solution consisting of 70 μl 48% hydrogen fluoride, 120 μl 1 M sodium hydroxide and 20 μl 40% disodium ethylene diaminetetraacetic acid (EDTA) in a total volume of 20.01 ml (personal communication by Brian Terhune and D. T. A. Lamport). The microtiter plates were read in a Minireader II (Dynatech Laboratories, Inc.) at an absorbance of 630 nm.

B-Galactosidase Activity Assay

To every odd numbered fraction 400 μl of a reagent consisting of 0.1 M sodium phosphate buffer pH 7.2 with 10 mM 2-mercaptoethanol and 2 mM o-nitrophenyl B-D-galactopyranoside (Sigma) were added (4). The reaction was carried out in a 37°C waterbath for 30 to 60 minutes. The reaction was stopped by adding 800 μl or 1 N sodium hydroxide. Absorbance was read at 410 nm.

Acid Phosphatase Activity Assay

To every even numbered fraction 400 µl of a reagent consisting on 0.051% p-nitrophenyl phosphate (Sigma) dissolved in 0.02 M sodium acetate buffer pH 5.0 were added (2). The reaction was allowed to incubate at room temperature for 24 hours. The reaction was stopped by adding 800 µl of 1 N sodium hydroxide. Absorbance was read at 410 nm.

Graphical Representation of Results

The activity curves of peroxidase activity were made by transforming the minor peaks into the percent of the highest peak (9). The activity curves of acid phosphatase were made in the same manner.

RESULTS

Cucumber seedlings treated with 60% v/v D_2O for 46 hours were stunted compared to seedlings grown in water. Peroxidase activity in these seedlings was also elevated compared to unlabeled seedlings. Seedlings heat shocked and treated with D_2O also had increased levels of peroxidase activity when compared to heat shocked seedlings kept in water.

A 30-hour, 50,000 rpm ultracentrifugation was sufficient for the enzymes to reach their buoyant density equilibrium point. A centrifugation of 48 hours at 50,000 rpm did not change the position of the activity peak or the bandwidth at 50% maximum activity of the external marker enzyme B-galactosidase as measured in fraction number. Band widening of the peroxidase activity peak was detected in the D_2O labeled samples at 50% of the activity peak. Figure 2 shows results of a single representative run. Figure 2A represents samples from labeled and unlabeled heat shocked seedlings. Figure 2B represents samples from

labeled and unlabeled nonshocked seedlings. A slight shift in the peroxidase activity peak of samples of labeled seedlings from the activity peak of nonlabeled seedlings can be seen in both graphs. In order to have results which reflected only the effect of the heat shock stress without the stress of the D_2O labeling, the absorbance datum for each fraction of nonshocked labeled seedling samples was subtracted from the datum for each fraction of heat shocked labeled seedlings. The data were transformed to percent of the maximum difference. Data from unlabeled samples were treated in the same manner (Figure 3). A shift in activity peaks between the unlabeled and labeled samples can be seen. The results indicate that either new peroxidase was synthesized or that an incompletely synthesized enzyme was completed as a result of heat shock. Figure 4 shows the activity profiles of acid phosphatase in samples from heat shocked and nonshocked labeled seedlings. The results suggest that heat shock did not cause an increase in the labeling of the amino acid pool. In preliminary experiments, heat shock was found to have no effect on total acid phosphatase activity when measured 24 hours after heat shock.

DISCUSSION

The results of the density labeling experiments suggest that the increase in peroxidase activity due to heat shock is at least partially a result of newly synthesized enzyme. Because the peroxidase enzyme exists in heat shocked seedlings as four anodically migrating isozymes and, in addition, possibly three cathodically migrating isozymes (3) the increased activity is probably a combination of increased synthesis of particular isozymes while other isozymes may be activated from

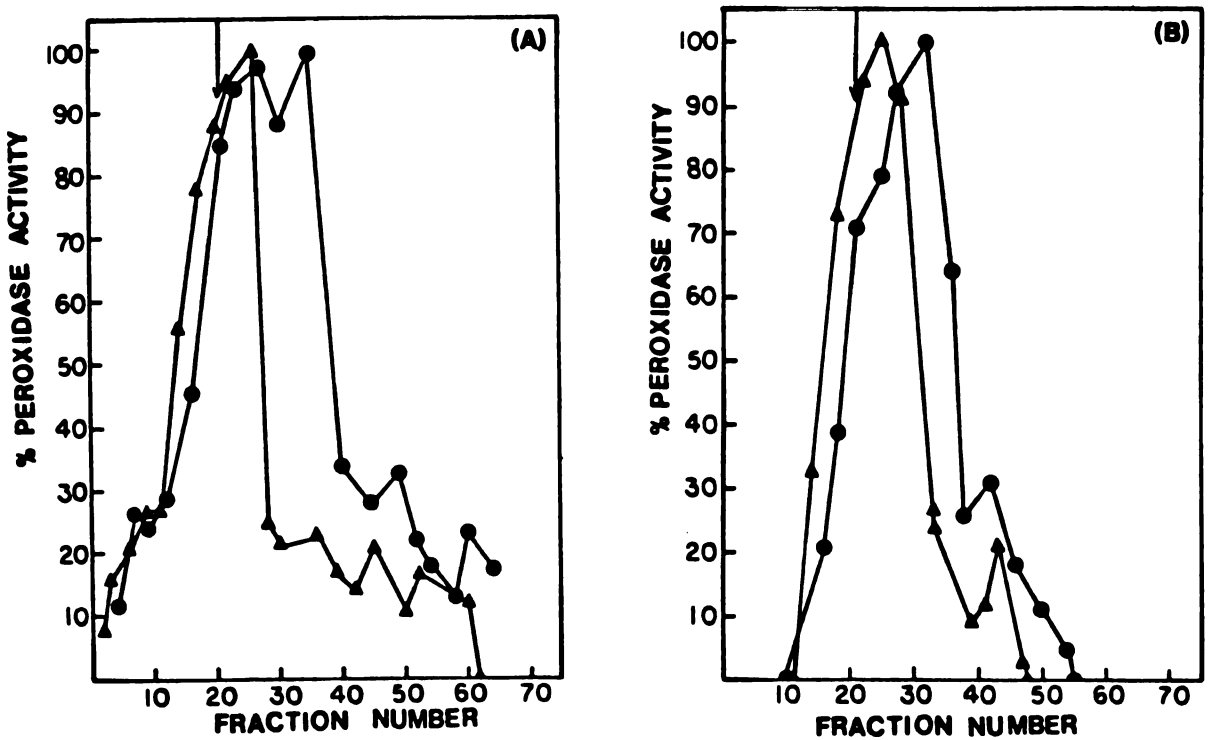

Figure 2. Relative peroxidase activity of heat shocked and nonshocked seedlings after density labeling and isopycnic equilibrium centrifugation. The activity peak of B-galactosidase, the external marker enzyme, is represented by the arrow. A) Relative peroxidase activity of heat shocked seedlings labeled with D_2O (●) and heatshocked seedlings unlabeled (▲). B) Relative peroxidase activity of nonshocked seedlings labeled with D_2O (●) and of unlabeled (▲) seedlings. Gradients were formed with those of Figure 2A.

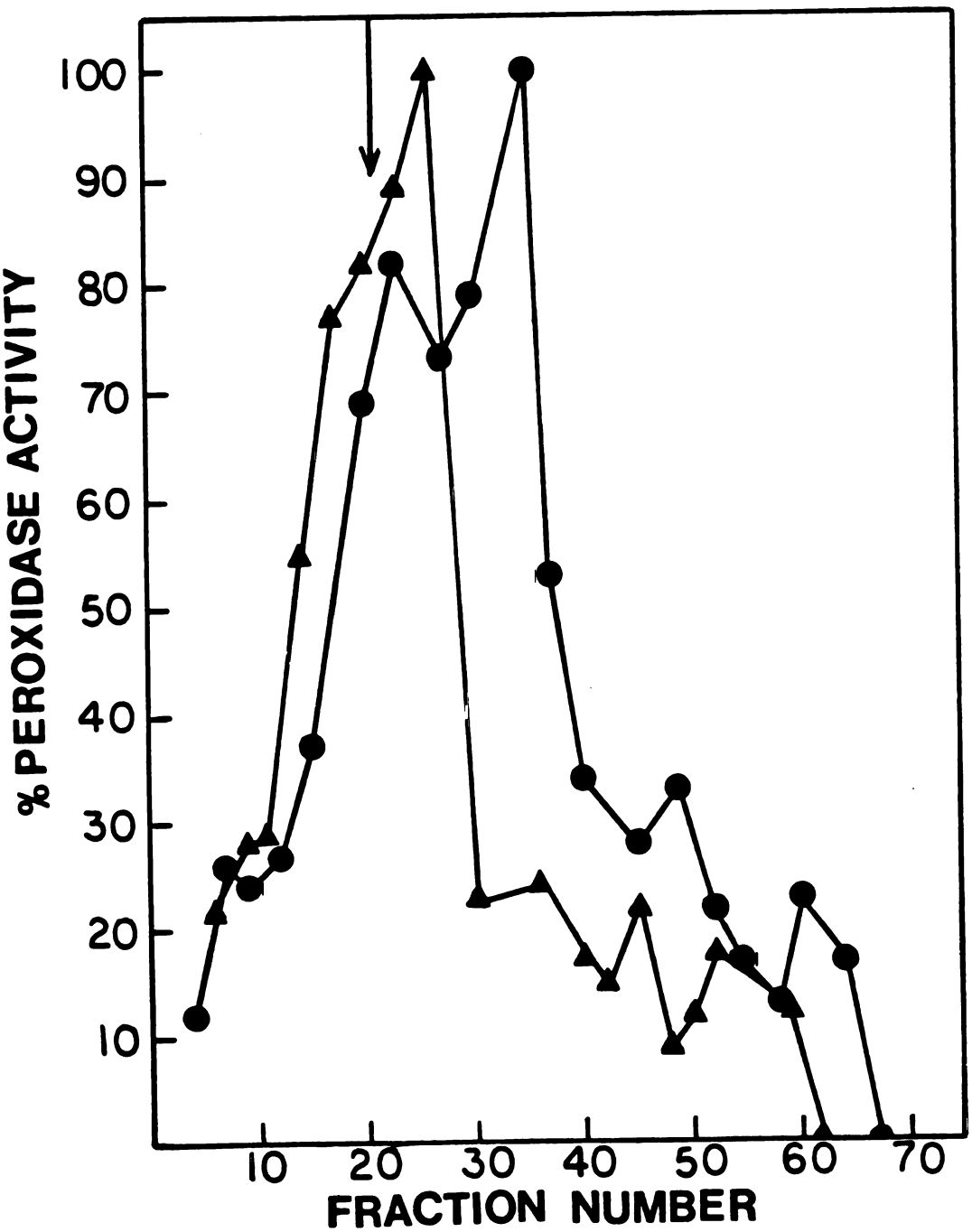

Figure 3. Relative peroxidase activity as a result of heat shock. Differences between the labeled (●) and unlabeled (▲) peroxidase activity curves (Figures 2A and 2B) were calculated and transformed to percent of the maximum differences in the labeled and unlabeled fractions.

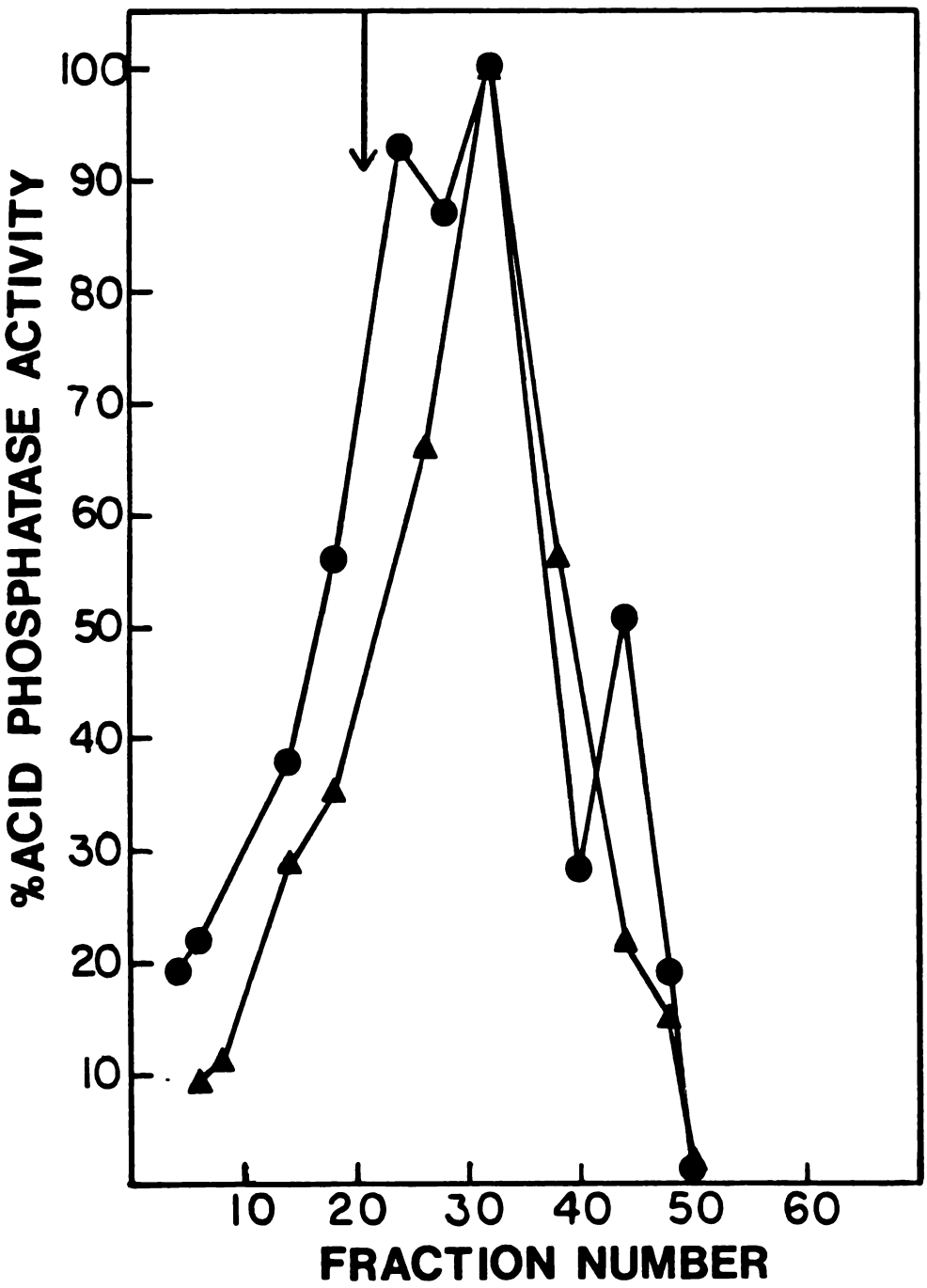

Figure 4. Relative acid phosphatase activity of heat shocked and nonshocked seedlings labeled with deuterium oxide. Labeled heat shocked samples (●) and labeled nonshocked samples (▲). The activity peak of B-galactosidase, the marker enzyme, is represented by the arrow. The enzyme assays were made of fractions taken from the gradients represented in Figure 2.

preexisting protein as a result of heat shock. This could give a smaller peak shift in the D_2O labeled samples. A difficulty in working with peroxidase enzyme activity is that it is highly variable depending to a large extent on the ontogenetic age of the plant. In the experiments presented here, the variables of growing conditions and age of the seedlings in days were kept constant. However, the process of labeling the seedlings increased peroxidase enzyme activity.

When separating enzymes on salt gradients, the shallower a gradient is the greater the separation is of the protein being investigated (5, 6). Johnson (6) and Hu et al (5) reported that many other salts such as rubidium chloride, potassium bromide, lithium bromide, or potassium acetate were more suitable for protein separation work because of the shallow gradients they formed compared to the cesium chloride gradient. Shallower gradients give better resolution and shorten the time needed in centrifugation to reach the equilibrium point (6). In preliminary experiments, I used potassium acetate gradients but found that the alkalinity of the salt interfered with the peroxidase enzyme assay. In future labeling experiments, selecting a salt such as potassium bromide or lithium bromide may give more easily interpreted results. In solutions of approximate density of 1.3 kg l^{-1}, potassium bromide forms a gradient 40% shallower while lithium bromide forms a gradient 12% shallower than the gradient formed by cesium chloride (6).

Quail and Varner (9) examined peroxidase isozyme development in germinating barley seeds. Separating crude enzyme extracts centrifuged on cesium chloride gradients into fractions and loading the fractions onto starch gels, they were able to determine that certain isozymes were

synthesized during germination and that others were present in the dry seed. This procedure may be one that could give interesting results in the heat shocked cucumber seedling system.

Since peroxidase activity is easily stimulated by a variety of stresses, a more profitable approach to resolving the question of de novo synthesis may be to look at the messenger ribonucleic acid population (mRNA) which codes for peroxidase. Presumably, an increase in mRNA for peroxidase would correspond to an increase in enzyme activity after the inducing stimulus was given.

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RECOMMENDATIONS

1. Investigate the role of ethylene in heat shock induced resistance using the ethylene synthesis inhibitor aminoethoxy-vinylglycine (AVG) and ethylene action inhibitors such as silver thiosulfate.
2. Investigate the role of 1-aminocyclopropane-1-carboxylic acid (ACC) in the development of induced resistance in cucumber by applying to greenhouse grown plants and etiolated seedlings. Use AVG as an inhibitor of the formation of ACC then add back ACC in the presence of silver thiosulfate.
3. Investigate induced resistance in cultivated tomato using heat shock and microorganisms.
4. Use polyclonal antibodies to the three anodic peroxidase isozymes in cucumber to isolate mRNAs which code for the isozymes. Establish a time course of accumulation of mRNAs after heat shock.
5. Determine the activity changes of enzymes, other than peroxidase, after heat shock in cucumber seedlings e.g. chitinase, phenylalanine ammonia lyase, B 1,3-glucanase.