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Abstract approved (Major professor)

This paper describes an attempt to ascertain the enzyme system responsible for melanin formation in a melanin-and-microsclerotia-forming strain of Verticillium albo-atrum Reinke and Berth. Previous work showed that near-UV radiation (3200-4000 Å) inhibits melanin synthesis and microsclerotia development in this strain and that catechol almost wholly reverses this inhibition. During the present study, additional chemicals were tested in living cultures. Only one of these, hydroquinone, almost consistently reversed the effects of near-UV, and it did so to a much lesser extent than did catechol. Chemicals which failed to induce the pigment formation include: aniline, ascorbic acid, chlorogenic acid, p-cresol, dopa, gallic acid, phenylalanine, p-phenylenediamine, pyrogallol, resorcinol, shikimic acid and tyrosine. Attempts to demonstrate

phenolase activity in extracts and whole cells revealed either weak activity or none. Manometric tests for phenolase demonstrated activity in potato and mushroom but little phenolase activity in Verticillium. A modified purpurogallin test indicated the presence of peroxidase activity in Verticillium extracts. With this information an assay for the catechol-oxidizing-system was developed. It involved mixing 1.0 ml of 0.1 M Tris-HCl buffer, pH 7.2, and 0.9 ml of distilled water with 0.4 ml of 1 M H_2O_2, 0.4 ml of 10^{-1} M catechol and 0.2 ml of 10^{-3} M Mn^{+2} in a test tube, bringing the reaction mixture to 30°C, then adding 0.1 ml of cell-free extract and incubating for ten minutes more. The O. D. of the dark red-brown pigment formed was measured at 490 mu. The system was saturated with each variable component to find the optimum concentration for the maximum reaction rate. Mg^{+2} and Ca^{+2} stimulated the reaction about one-half as much as Mn^{+2}. Other cations tested include: Co^{+2}, Cu^{+2}, Fe^{+2}, Ni^{+2}, and Zn^{+2}. Substrates oxidized by the cell-free system include: catechol, dopa, p-phenylenediamine and pyrogallol. It is concluded that the enzyme responsible for melanin formation in Verticillium is likely a peroxidase.

CHARACTERISTICS OF THE ENZYME SYSTEM RESPONSIBLE FOR MELANIN FORMATION IN VERTICILLIUM

by

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INTRODUCTION

Verticillium wilt causes over $3,000,000 in crop losses annually in Oregon. Farmers have difficulty controlling this soil-borne fungus pathogen because it produces microsclerotia, which resist chemicals and remain viable under conditions that are lethal to the hyphae and spores (37). The microsclerotia form a black pigment which is considered a type of melanin (3).

Since a strong correlation exists between melanin synthesis and microsclerotia development (3, 7), W. H. Brandt proposed a study of melanin synthesis as part of an investigation on microsclerotia formation. Melanin synthesis occurs widely among animals and plants, and enzymes of the phenolase group generally initiate melanin formation. Other enzyme systems that presumably could oxidize phenolic substrates to dark pigments are: cytochrome oxidase, laccase, and peroxidase.

Melanin synthesis as well as microsclerotia formation is inhibited by near-ultraviolet radiation (3200-4000 Å) (7). Catechol will reverse this inhibition (Brandt, unpub.). Manganese increases pigment formation in the dark but will not reverse the pigment inhibition by near-UV (6).

This is a report of research directed toward ascertaining some of the characteristics of the enzyme system responsible for melanin formation in Verticillium albo-atrum Reinke and Berth. The approach was twofold: to determine what substrates other than catechol can induce melanin synthesis in spite of near-UV radiation, and secondly, to determine some of the characteristics of the oxidative system in crude, cell-free extracts.

LITERATURE REVIEW

Relation of melanin synthesis to morphogenesis

Becker (3) and Brandt (7) have noted the high correlation of melanin synthesis and microsclerotia formation in Verticillium, and Isaac (16, p. 138) has alluded to the same correlation. Hirsch (12, 13, 14), Westergaard and Hirsch (44), and Barbsgaard and Wagner (2) have all documented the strong correlation between melanin synthesis and the formation of functional protoperithecia in Neurospora crassa. Environmental factors that affect one also directly affect the other. Horowitz, et al. (15), described tyrosinase induction in a tyrosinase-deficient, non-pigmented strain of N. crassa. This strain does not ordinarily form functional protoperithecia. Incorporation of tyrosinase inducers into the medium did not stimulate protoperithecia formation, but blackening of the mycelium (presumably by melanin) occurred.

Enzymes capable of initiating melanin synthesis

Cathechol stimulates the formation of a black pigment in dark-reared Verticillium cultures and induces pigment formation under near-ultraviolet radiation (Brandt, unpub.). Assuming that the oxidation of catechol to form the black pigment is an enzymatic reaction, this pigment, then, meets Mason's definition of melanin,

" . . . a pigment of high molecular weight formed by the enzymatic oxidation of phenols" (28, p. 277). Enzymes which might oxidize phenols, thus forming a black or brown pigment, include cytochrome oxidase, the phenolases, and peroxidase.

Cytochrome oxidase

The formation of melanin by cytochrome oxidase seems unlikely. Cytochrome oxidase activity is associated with mitochondria (33) and although mammalian melanin is formed in particles, the enzyme responsible is not cytochrome oxidase (38). Plant melanin synthesizing systems are reported to reside in soluble rather than particulate fractions of enzyme preparations (38). Oxidation of phenols in a cytochrome oxidase-cytochrome c system is by the coupled, non-enzymatic reduction of cytochrome c (5, p. 181; 4, p. 87). Melanin, to my knowledge, has not been formed by the cytochrome oxidase-cytochrome c system.

The phenolases

The group of enzymes that Mason calls phenolases includes catecholase, cresolase, dopa-oxidase, polyphenol-oxidase and tyrosinase (25, p. 106). In this thesis, laccase is included in this group. Each of these enzymes can oxidize phenols and the products could polymerize to form melanin.

Raper proposed a scheme of melanin synthesis from tyrosine in 1938 (34). He based his plan on the observations of Bourquelot and Bertrand, who worked with mushroom preparations in 1895, and on his own work with tyrosinase from mealworms (35). Since then there have been numerous investigations and many reviews (9, 10, 23, 25, 27, 28).

The naturally-occurring substrates for animal phenolases are dopa and tyrosine (10, 36). These two have been used or proposed as substrates for plant phenolases, as have many other phenolic compounds including catechol, ascorbic acid, protocatechuic acid, caffeic acid, p-cresol, chlorogenic acid, gallic acid, phenylalanine, hydroquinone and urushiol (1, 4, 5, 23, 28, 39, 46). The literature on melanin synthesis states and implies that enzymes of the phenolase group catalyze the initial oxidations which lead to the polymerized pigmented products.

Becker has demonstrated an aerobic oxidation of catechol by extracts from Verticillium (3). He called it phenolase activity, although he did not show data proving that oxygen was taken up.

Peroxidase

Mason (26), Nicholls (30) and Paul (32) have reviewed peroxidase systems.

James and Cragg (17), using barley extracts, found a

peroxidase that oxidized catechol and formed a brown pigment. Their reaction mixture contained barley extract, H_2O_2, and catechol in phosphate buffer at pH 6.4. They could not demonstrate a phenolase type of catechol oxidation.

Beevers (4, p. 78), Bonner (5, p. 179) and Hartree (11, p. 231) each indicated that peroxidase, in the presence of peroxide (H_2O_2), can oxidize a wide variety of monophenols, diphenols and aromatic amines, e. g., p-cresol, pyrogallol, hydroquinone, p-phenylenediamine, guaiacol and ascorbic acid.

Studies on the hydroxylation of aromatic substrates by peroxidase showed that Mn^{+2} stimulated the reaction (29). Some proposed intermediates in the peroxidation of aromatic donors can reportedly polymerize or form other complex compounds (45, p. 228).

Stutz (41) described an indole-3-acetic acid-oxidase which required a phenol for activity and which was stimulated by Mn^{+2}. It appeared to be a peroxidase linked to IAA oxidation through the peroxidation of a phenol.

Klapper and Hackett (22) stated that Mn^{+2} (at 1.3×10^{-3} M) was an absolute requirement for the peroxidase oxidation of hydroquinone. The authors stressed that Mn^{+2} stimulates the oxidative reaction with other hydroquinones and naphthohydroquinones, but is not required. They suggested that when oxidized quinone products accumulate, they inhibit further oxidation of hydroquinones and

naphthohydroquinones, and further suggested that Mn^{+2} acted by reversing this inhibition.

Kenten and Mann (20, 21) described peroxidase systems in which Mn^{+2} was oxidized to Mn^{+3} or Mn^{+4}. These workers demonstrated that this oxidation could be coupled to other enzyme systems which produce H_2O_2 (19).

Chance (8), proposed two mechanisms for peroxidase activity: first, an anaerobic peroxidation reaction; and secondly, an aerobic oxidation. The catalyst for the oxidase reaction appears to be a Mn^{+2}-activated H_2O_2-peroxidase complex.

Peroxidase in summary

The function of peroxidase, which no other enzyme system duplicates, is summarized by Nicholls (30, p. 301) as the peroxidation of oxidogenic donors (those which form radicals with oxidizing power after reacting with H_2O_2-peroxidase compounds) to form complex colored products.

MATERIALS AND METHODS

Organism

A melanin-and-microsclerotia-forming strain of Verticillium albo-atrum Reinke and Berth., isolated by J. D. Menzies from okra (Brandt's isolate H-13), was used in these experiments. Stock cultures from single, germinated spores grew in the dark for two weeks on 10 ml of sucrose-yeast extract agar in half-ounce perfume bottles, and were then refrigerated until used.

Media

Chemicals are reagent grade unless otherwise specified.

Sucrose-yeast extract agar

Sucrose	20.0 gm
Yeast extract (Difco)	3.0 gm
KH_2PO_4	2.5 gm
K_2HPO_4	2.5 gm
Agar (Bacto)	20.0 gm
Distilled Water	1000.0 ml

Sucrose-nitrate

(a)*	Sucrose	20.0 gm
	KNO_3	3.0 gm
	$MgSO_4 \cdot 7\ H_2O$	0.5 gm
	Microelement Solution (composition listed below)	2.0 ml
	Distilled Water	500.0 ml
(b)*	K_2HPO_4	1.0 gm
	Distilled Water	500.0 ml

*(a) and (b) are each autoclaved separately for 15 minutes at 15 pounds pressure and then mixed after cooling.

Microelement stock solution

$Fe(NO_3)_3 \cdot 9\ H_2O$	0.3617 gm
$ZnSO_4 \cdot 7\ H_2O$	0.2199 gm
$MnSO_4 \cdot H_2O$	0.1015 gm

Mix together in 300 ml distilled water, clear with not more than 2 ml of concentrated C. P. H_2SO_4 and make to 500 ml volume with distilled water.

Tests for ability to induce pigment formation under near-UV

For substrate tests on live cultures, spores were removed from the stock cultures by pipetting five ml of sterile distilled water into a stock culture bottle, gently shaking the bottle, then pouring the resulting spore suspension into 45 ml of sterile distilled water.

About one ml of this second spore suspension was aseptically pipetted onto four percent agar in petri dishes. These were kept in the dark at 24°C for 24 hours. Single germinated spores were removed under a binocular dissecting microscope by using the flattened tip of a dissecting needle, and were then transferred to petri dishes containing 30 ml of sucrose-yeast extract agar.

Cultures exposed to near-UV were placed about 70 cm below two 40-watt BLB near-UV fluorescent tubes (most radiation 3200-4000 Å, peak 3650 Å).

Substrates

Substrates were chosen on the basis of (1) their being proposed intermediates in melanin formation, (2) having been used by other researchers as substrates for phenolase enzymes, (3) being structurally similar to some of those in the above groups, or (4) being specific substrates for another type of oxidative system that might produce a black pigment. Catechol was considered the standard for comparison because of its known ability to induce pigment synthesis under near-UV. Other substrates used were aniline, ascorbic acid, chlorogenic acid, p-cresol, 3, 4-dihydroxyphenylalanine (dopa), gallic acid, hydroquinone, phenol, phenylalanine, p-phenylenediamine (for laccase), protocatechuic acid, pyrogallol (for peroxidase), resorcinol, shikimic acid, and tyrosine.

Substrate impregnated disks

One-half inch filter paper assay disks, Millipore filters and hypodermic needles were autoclaved separately in petri dishes, and the proper quantities of substrates were weighed into vials. Ten ml of distilled water added to the vials made 10^{-2} M solutions of the substrates. From these, 10^{-3} and 10^{-4} M solutions were prepared by dilution.

In a transfer box, substrates sterilized by Millipore filtration were added to the sterile disks. Using flamed forceps, saturated disks were added to the cultures. One disk of each concentration of substrate and one blank disk of sterile distilled water were added to each culture so that the sterile distilled water blank was over a wax pencil mark at 12 o'clock, the 10^{-2} M disk at 3 o'clock, the 10^{-3} M disk at 6 o'clock and the 10^{-4} M disk at 9 o'clock, when viewed from the top.

Then the cultures were returned to the light regime in which they had been growing.

Microscopic observations

For microscopic observations, small pieces of the colonies were removed and squashed in a drop of lacto-phenol (containing aniline blue) on glass slides.

Manometric and spectrophotometric analysis of crude enzyme preparations

Cultures

Liquid sucrose-nitrate medium was used to grow cells for the preparation of enzyme extracts. One ml of spore suspension, prepared as above, was added to approximately 100 ml of medium in 250 ml Delong culture flasks. These cultures were reared under near-UV (about 30 cm below two 20-watt BLB fluorescent tubes, most radiation 3200-4000 Å, peak 3650 Å) on a rotary shaker (about 280 oscillations per minute) for eight to ten days at 25°C.

Dark-reared cultures were grown in about 150 ml of medium in 500 ml Delong culture flasks. The inoculum was one ml of spore suspension. These cultures grew for four weeks at 24°C in a light-proof incubator without shaking.

Extracts

The cultures were harvested by centrifuging at 13, 500 x g. After decanting the medium, the cells were resuspended in cold distilled water and recentrifuged. The water was decanted, the cells removed, wrapped in aluminum foil and stored under refrigeration until used.

Ten gm of cells and ten gm of cold, powdered alumina were

mixed together in a precooled mortar in an ice bath. Five ml of buffer (0.1 M Tris-HCl, pH 7.2) were added and the mixture was ground for about ten minutes or until a homogeneous slurry resulted. Five ml more of buffer were added and the slurry ground again until uniform. This mixture was centrifuged and the supernatant was used as the enzyme extract.

Protein analysis

Protein was determined by the method of Lowry, et al. (24). Extracts contained between 1.33 and 1.55 mg of protein per ml.

Manometry

Studies of oxygen uptake were conducted in a Warburg respirometer. The water bath temperature was 25°C. Reaction mixtures contained: 1.0 ml of buffer, 0.5 or 1.0 ml of cells or extract, 0.5 ml of 10^{-2} M substrate in the side arm, enough distilled water to make 3 ml volume and 0.2 ml of 20 percent KOH in the center well with a filter-paper wick.

Purpurogallin test

The purpurogallin test of Sumner and Gjessing (42) was modified to provide a rapid qualitative test for peroxidase. The proportions of reactants were the same but the volumes were reduced by

one-half. The principle involved in this test was followed in developing an assay for the catechol-oxidizing-system. Hereafter COS will be used in place of catechol-oxidizing-system.

Assay for the Catechol-Oxidizing-System

Dr. H. J. Evans devised a test for the COS and, from this, the assay was developed. This assay consisted of adding 1.0 ml of buffer (0.1 M Tris-HCl, pH 7.2), 0.9 ml of distilled water, 0.4 ml of 1 M H_2O_2, 0.4 ml of 10^{-1} M catechol and 0.2 ml of 10^{-3} M Mn^{+2} to a small test tube. This mixture was incubated in a water bath at 30°C for five minutes, then 0.1 ml of extract was added with mixing. After ten more minutes' incubation a dark, red-brown color developed; this colored reaction mixture was transferred to a cuvette and the optical density (O. D.) read at 490 mu. A Beckman DB spectrophotometer was used for the readings. A blank of the reaction mixture without extract was incubated and read along with the experimental mixture. This value was subtracted to correct for autoxidation. A second blank of the complete reaction mixture plus extract, but without incubation, was subtracted to correct for turbidity due to the extract. Catechol and peroxide were prepared fresh for each day's use.

Standardization

Because the reaction mixture was saturated with the reactants, the problem arose of how to stop the reaction to be able to standardize it. Acid, thiourea and other reducing agents decolorized the pigment formed. Chilling would slow the reaction but would also fog the cuvette and interfere with the readings. It was decided to add the extract on a time schedule of one- or two-minute intervals, incubate for ten minutes, remove the reaction mixtures on the same time schedule and read immediately.

Absorption measurement

The wave length of 490 mu was chosen for the readings because it was the center of a broad peak of absorption as indicated by early experiments. Later the wave-length indicator on the spectrophotometer used for the routine analyses was found to be out of adjustment. After correction, 490 mu was on the edge of the peak. The readings were similar so the same point was used.

RESULTS

Macroscopic Observations

To ascertain what potential substrates stimulated pigment production, substrate saturated disks were added to the cultures when they were 12 days old. Observations of the cultures began three days after adding the substrate disks and continued for two weeks. Photographs were taken through the bottom of the cultures on the fifth day. Figures 1 through 21 are photographs of the results. Cultures with 10^{-2}, 10^{-3}, and 10^{-4} M catechol disks plus a distilled water disk and control cultures without any disks were included in each experiment. The tests were conducted both under near-UV and in darkness. In most cases the dark-reared test cultures did not differ significantly from the dark-reared control cultures. For this reason, and because the formation of pigment in the near-UV-reared cultures was a distinct and major reaction, only data on near-UV-reared cultures is presented in Table 1.

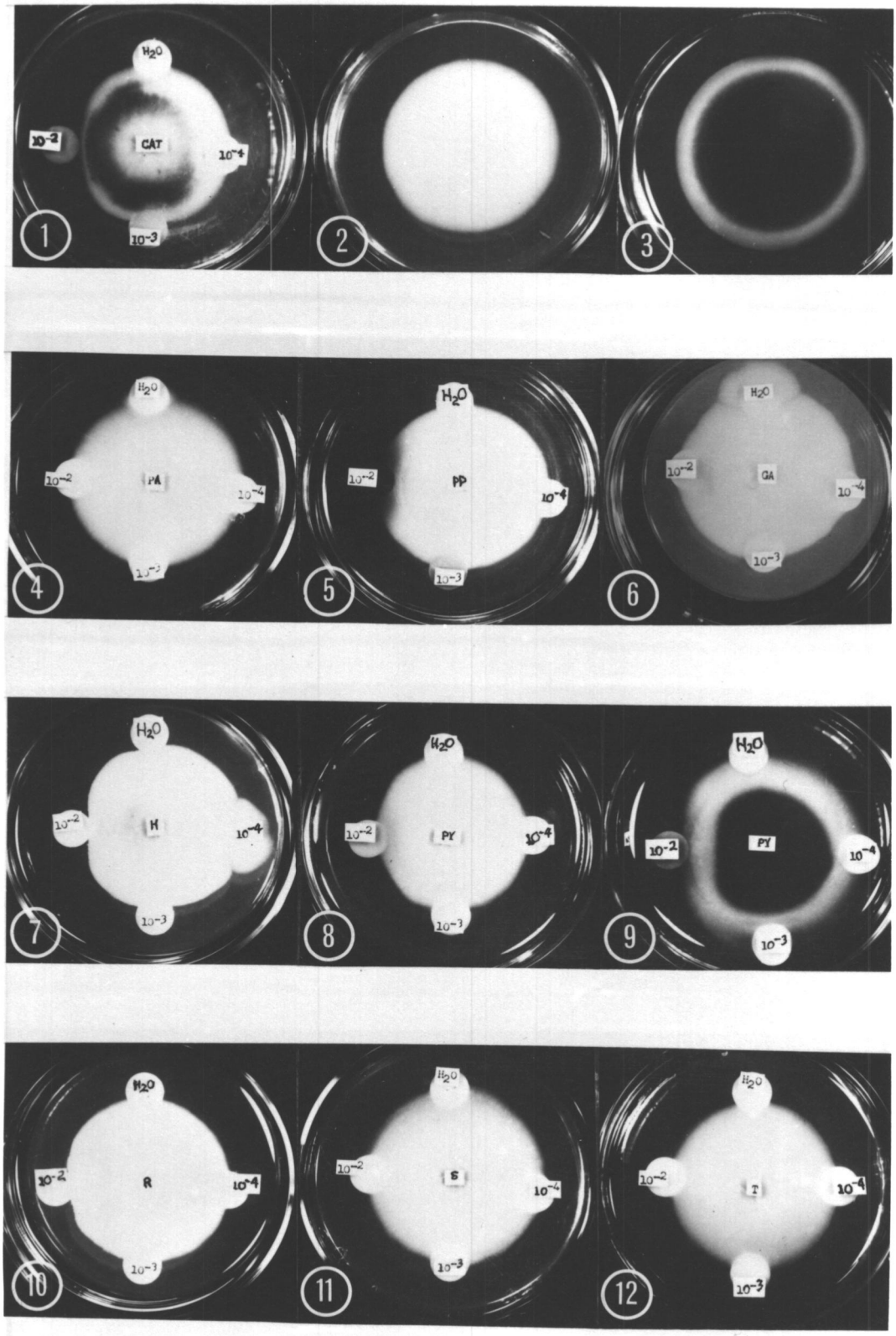
The macroscopic observations made after 13 days are presented in Table 1. All the catechol replications showed dark center pigmentation indicating pigment induction even under near-UV. Of the other substrates, p-cresol induced pigment formation in one out of eight cultures, phenylalanine in one out of seven, p-phenylenediamine in one out of nine and hydroquinone in eight out of ten. The

TABLE 1. Macroscopic observations of near-UV-reared cultures 13 days after adding substrate saturated disks.

Substrate	Number of Replications	Number showing Pigmentation
catechol	8	8
aniline	8	-
ascorbic acid	8	-
chlorogenic acid	3	-
p-cresol	8	1
dopa	3	-
gallic acid	7	1
hydroquinone	10	8
phenylalanine	7	1
p-phenylenediamine	9	1
pyrogallol	8	-
resorcinol	8	-
shikimic acid	3	-
tyrosine	4	-

Darkening was most pronounced near the 10^{-2} M substrate disk.

1. Near-UV-reared catechol standard.
2. Near-UV-reared control.
3. Dark-reared control.
4. Near-UV-reared culture with phenylalanine as the substrate.
5. Near-UV-reared culture with p-phenylenediamine as the substrate.
6. Near-UV-reared culture with gallic acid as the substrate.
7. Near-UV-reared culture with hydroquinone as the substrate.
8. Near-UV-reared culture with pyrogallol as the substrate.
9. Dark-reared culture with pyrogallol as the substrate.
10. Near-UV-reared culture with resorcinol as the substrate.
11. Near-UV-reared culture with shikimic acid as the substrate.
12. Near-UV-reared culture with tyrosine as the substrate.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CAT
10-4
10-3
1
2
3
H2O
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PA
10-4
10-3
4
H2O
10-2
PP
10-4
10-3
5
H2O
10-2
GA
10-4
10-3
6
H2O
10-2
H
10-4
10-3
7
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PY
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PY
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10-3
9
H2O
10-2
R
10-4
10-3
10
H2O
10-2
S
10-4
10-3
11
H2O
10-2
T
10-4
10-3
12

13. Dark-reared control culture.

14. Near-UV-reared control culture.

15. Near-UV-reared culture with catechol as the substrate.

16. Near-UV-reared culture with aniline as the substrate.

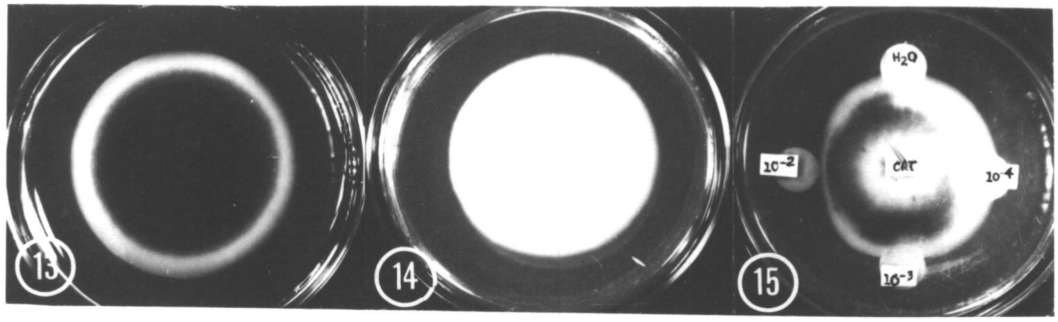
17. Near-UV-reared culture with ascorbic acid as the substrate.

18. Near-UV-reared culture with chlorogenic acid as the substrate.

19. Near-UV-reared culture with p-cresol as the substrate.

20. Dark-reared culture with p-cresol as the substrate.

21. Near-UV-reared culture with 3, 4-dihydroxyphenylalanine (dopa) as the substrate.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14
15
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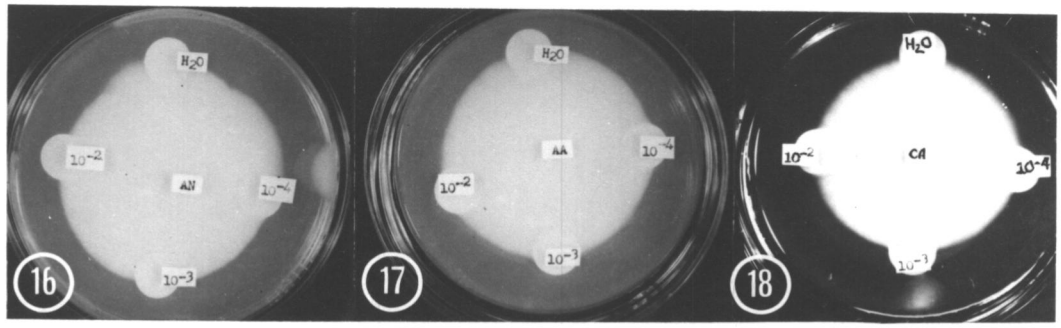
CRT
10^{-4}
10^{-3}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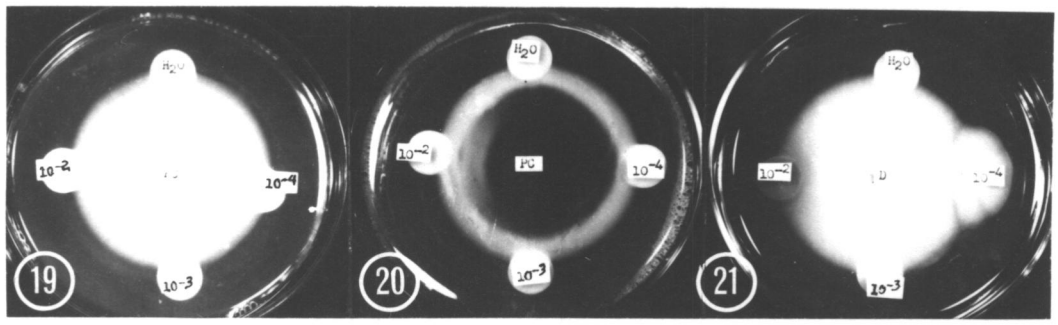
10^{-3}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10^{-4}
10^{-3}
20
H_2O
10^{-2}
PG
10^{-4}
10^{-3}
21
H_2O
10^{-2}
10^{-4}
10^{-3}

pigment induction by hydroquinone was not as rapid and not as complete as the induction by catechol. It occurred about 10-12 days after substrate was added and much less pigment developed.

Substrate reactions

Figure 1 illustrates the induction of pigment by catechol. A disk soaked in distilled water (H_2O) was included as a blank with each substrate test. Three concentrations of each potential substrate were used to provide a wide range of concentrations for the possible induction of pigment. The reaction with catechol was considered the standard. Catechol at 10^{-2} and 10^{-3} M effectively induced pigment formation. At 10^{-2} M, catechol also retarded growth. Catechol at 10^{-4} M and H_2O had little effect on pigment induction.

Figure 2 illustrates a near-UV-reared control culture with complete lack of melanin (black pigment) in the culture. Figure 3 shows a typical dark-reared control culture. The halo effect at the periphery of the colony is typical of this isolate. The edge of the melanized area is about three days older than the non-pigmented outer edge.

Autoxidation of the p-phenylenediamine can be seen around the 10^{-2} M disk in Figure 5 but no melanin was found. In cultures treated with hydroquinone (Figure 7), melanin formed much later than in those cultures which had catechol as the substrate and there

was less pigment. Autoxidation of the substrate, dopa, can be seen around the 10^{-2} M disk in Figure 21 but no melanin was found.

Thus, the results illustrated in the photographs and in Table 1 show that catechol alone consistently induced pigment formation in Verticillium cultures reared under near-UV. Hydroquinone caused pigment synthesis under near-UV most of the time but was much slower acting and induced much less pigment.

Microscopic Observations

Two weeks after adding the substrate disks, samples of the cultures were taken for microscopic observations. These were made on one or two representative cultures treated with each substrate to check whether or not microsclerotia or their forerunners were present.

Pigmented, dark-reared cultures were used as the standard for comparison. Typical structures included: colorless conidia, hyphae comprised of cells five to ten diameters long, microsclerotia and short segments that appeared to be forerunners of microsclerotia. Microsclerotia formation appeared to involve five steps: (1) the septation of the hyphae to form cells about one diameter in length; (2) the enlarging of these cells to about three times the diameter of mycelial cells; (3) the budding of these enlarged cells; (4) the formation of black pigment within these cells; and (5) the

thickening of the cell walls. The last two steps might proceed simultaneously; the result was a black cluster of enlarged cells.

In near-UV-reared control cultures, which produced no melanin, colorless conidia and hyphae were found. Hyphae, conidia, short-celled hyphae, enlarged cells, black pigment, cell-wall thickening and microsclerotia were chosen for comparing the effects of the various substrates on the cultures. The microscopic observations of near-UV-reared cultures are presented in Table 2.

When catechol was the substrate, all seven structures developed under near-UV. Near-UV-reared cultures treated with the other substrates typically contained only conidia and hyphae. One culture with p-cresol as the substrate contained all seven structures. At the time these observations were recorded, many of the cultures treated with hydroquinone showed all seven structures. Melanin, when present, was in microsclerotia. Tyrosine, phenylalanine and p-phenylenediamine induced a few forerunner structures. The dark-reared test cultures contained all of the structures.

These observations are consistent with those recorded in the photographs and Table 1. Thus, the results of these in vivo tests are uniform in showing that the effect of pigment induction under near-UV is quite specific for catechol.

TABLE 2. Microscopic observations of near-UV-reared cultures 14 days after addition of substrate disks.

substrate	conidia	hyphae	short-celled hyphae	enlarged cells	black pigment	cell-wall thickening	microsclerotia
catechol	+	+	+	+	+	+	+
aniline	+	+					
ascorbic acid	+	+					
chlorogenic acid	+	+					
p-cresol	+	+	+	+	+	+	+*
dopa	+	+					
gallic acid	+	+					
hydroquinone	+	+	+	+	+	+	+
phenylalanine	+	+	+				
p-phenylenediamine	+	+	+				
pyrogallol	+	+					
resorcinol	+	+					
shikimic acid	+	+					
tyrosine	+	+	+	+			
control	+	+					

*This response was in only one culture. The rest had only conidia and hyphae.

Manometry

Attempts to demonstrate oxygen uptake typical of a phenolase oxidation were not successful. Extracts prepared in phosphate buffer at pH 6. 0, 7. 0 and 7. 5 and in Tris-HCl at pH 7. 2 failed to consistently stimulate oxygen uptake upon the addition of catechol, p-cresol or chlorogenic acid. Separation of the extract into 18, 000 x g supernatant and pellet fractions gave no reproducible differences in either fraction upon incubation with catechol, hydroquinone, pyrogallol, chlorogenic acid or tyrosine. The rate of oxygen uptake was low and erratic and rarely exceeded 100 ul in 60 minutes.

Time of catechol-oxidizing-system production

A series of experiments to ascertain the time of production of the maximum catechol-oxidizing-system (COS) in both near-UV-reared and dark-reared cells was undertaken. Near-UV-reared cells were harvested when 6, 8, 10, 12 and 14 days old and tested within 24 hours. Dark-reared cells were harvested when 12, 15, 18, 21, 24 and 27 days old and tested similarly. These preparations were centrifuged at 3, 000 x g for five minutes. This low-speed centrifugation precipitated the alumina and large segments of hyphae, but the preparations were not cell free. Thus whole cells, as well as cell contents, could have catalyzed the oxidation of the substrates.

The results presented no consistent pattern and no singular time of maximum COS production. Due to endogenous respiration, the results were difficult to interpret. This endogenous rate was not reproducible and not appreciably affected by the presence of phenolase substrates. Boiled preparations and controls without preparation showed very low activity. Controls containing the preparation but without catechol showed nearly as much activity as the experimental reaction mixtures with catechol added. Figure 22, which shows oxygen uptake by a near-UV-reared cell preparation, is a typical example. Whole cells in the preparation may account for the high rate of endogenous respiration, but added catechol did not stimulate the rate of oxygen uptake to a degree approaching that of known phenolase sources (40). Thus, it appeared that either there was only a weak phenolase activity in these cells or that the method of assay was not suitable.

Comparison of phenolase from Verticillium, potato and mushroom

The irregular results prompted the use of known sources of phenolase, i. e., potato (31) and mushroom (40), to check the validity of the method used. Figure 23 shows a dark-reared Verticillium preparation compared to potato and mushroom preparations made in the same way and assayed at the same time. For the first 15 minutes (time of the first reading), the rate of oxygen uptake for potato

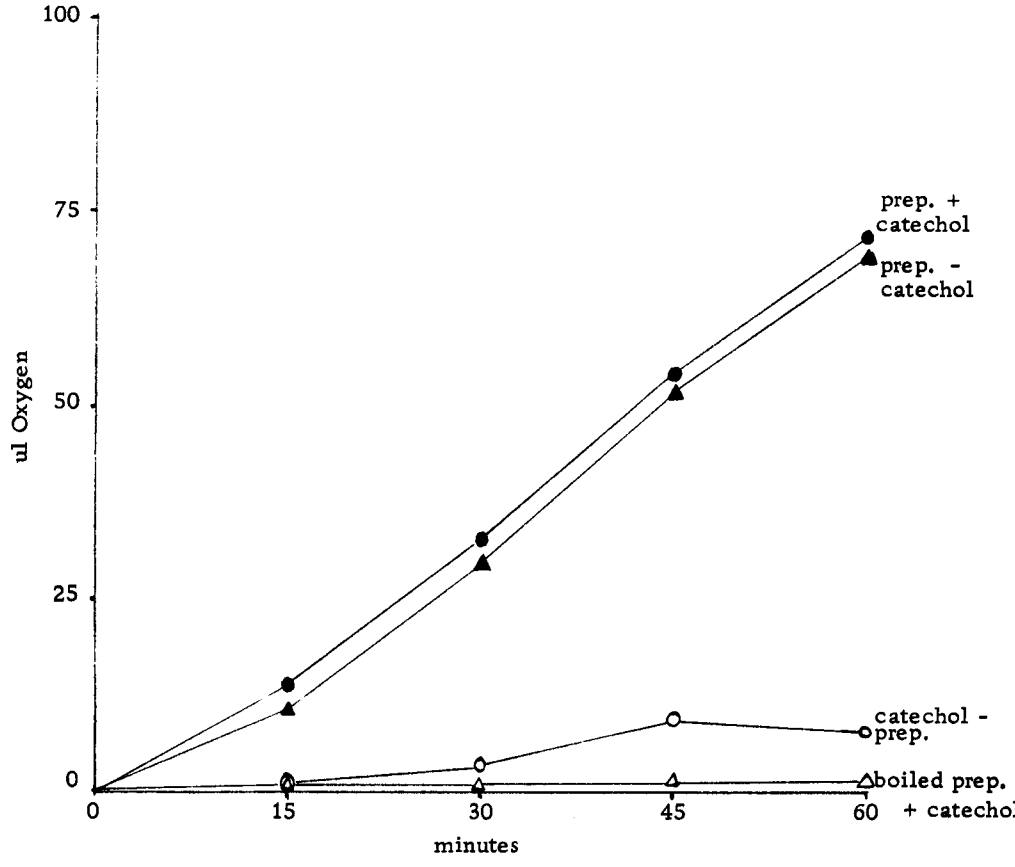

FIGURE 22. The effect of substrate on oxygen uptake by a crude preparation from near-UV-reared cells. (Complete reaction mixture is described in the methods section.)

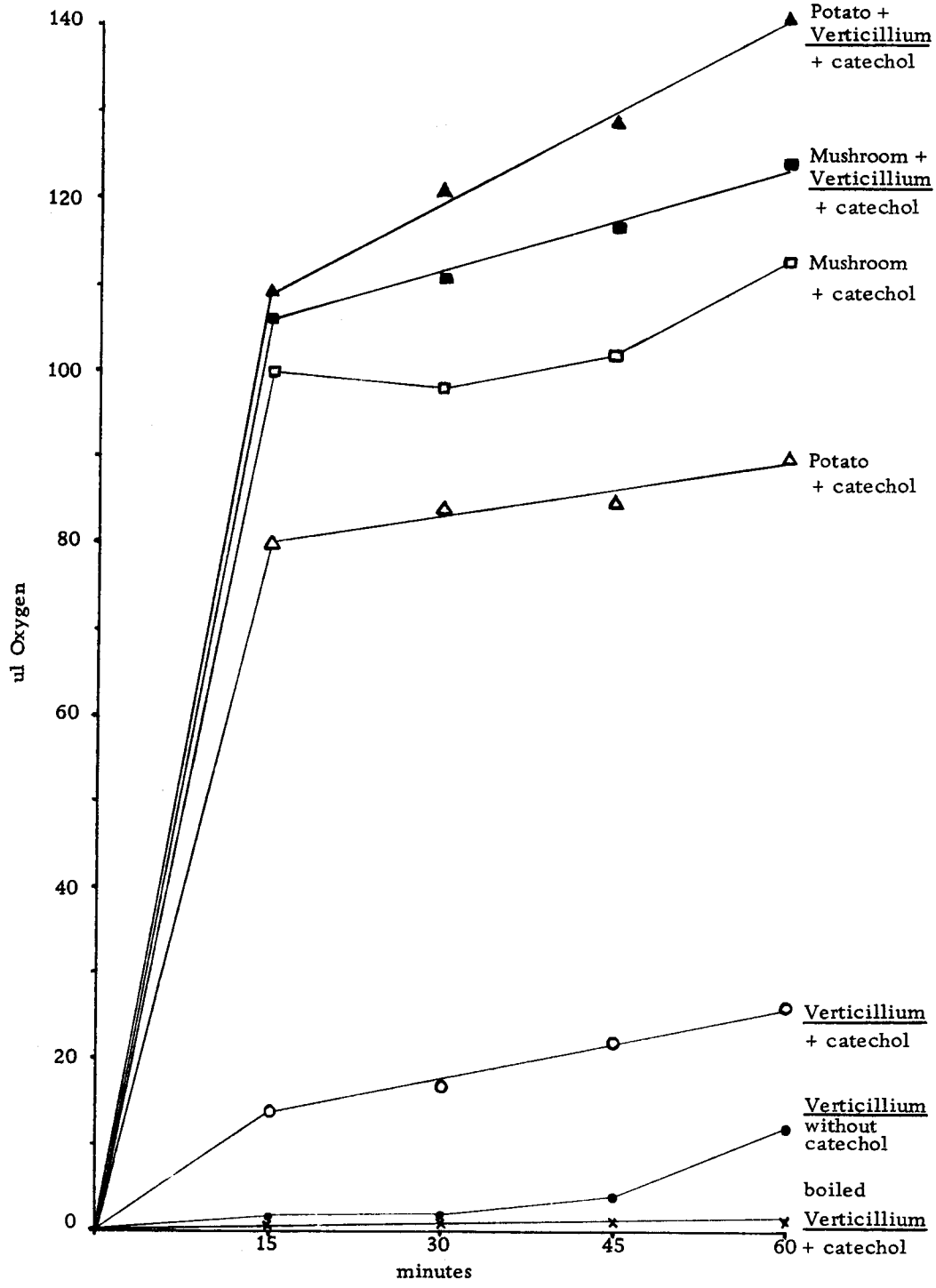

FIGURE 23. A comparison of oxygen uptake by extracts from Verticillium and known phenolase sources. (Prepared from 1 gm fr. wt. / 1 ml buffer).

was 5.3 ul/min. and for mushroom 6.6 ul/min. as compared to 0.93 ul/min. for the Verticillium preparation. After the first 15 minutes the rate diminished and the reduced rate was maintained for the rest of the test. Rapid oxygen uptake stopped after 15 minutes because the catechol was used up. To be completely oxidized, 5×10^{-6} moles of catechol require 3×10^{19} molecules (or 112 ul) of oxygen. As can be seen in Figure 23, 112 ul is close to the values actually recorded. Potato plus Verticillium preparations and mushroom plus Verticillium preparations were tested together to see if the Verticillium preparation contained an inhibitor to these known phenolases. There was no apparent inhibitor in the Verticillium preparation. These results indicated that the type of COS in Verticillium is not similar to the phenolase in either potato or mushroom.

Effects of iron added to the culture medium

Tests indicated the presence of peroxidase in the Verticillium extracts, thus, cells were grown in medium containing an additional 5×10^{-6} M chelated iron. The rate of oxygen uptake by crude preparations (3,000 x g for five minutes) of these cells was ascertained. The results presented in Figure 24 show that there was a fourfold increase in endogenous respiration rate (for the 60-minute test) of cells grown in medium with added iron as compared to cells grown in the standard culture medium (3.25 ul/min. to 0.83 ul/min.). The effect

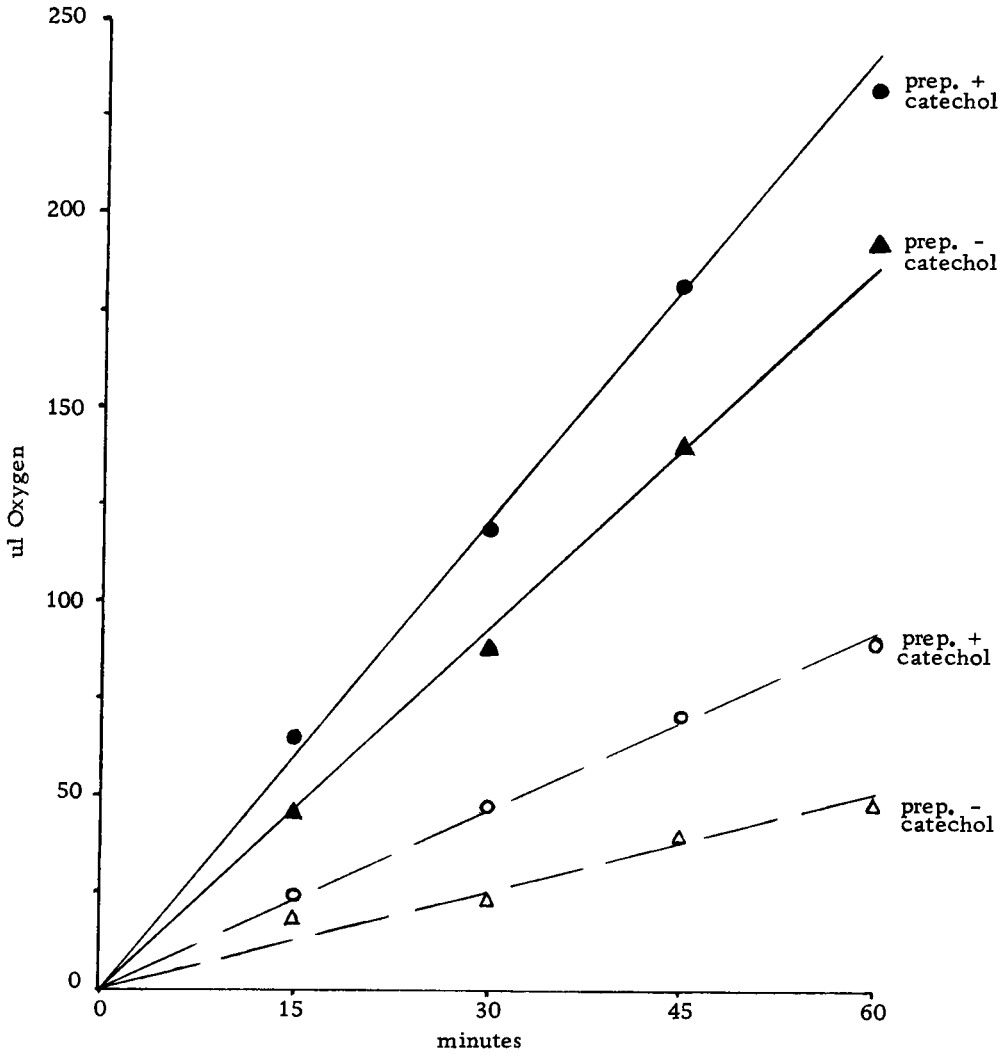

FIGURE 24. Effects of added iron in the culture medium on catechol oxidation by and endogenous respiration of crude Verticillium preparations from dark-reared cells. (Solid lines, from cells grown with 5×10^{-6} M added iron. Broken lines, from cells grown without added iron.)

on catechol oxidation was not as great. The rate of uptake (per 60 minutes) by preparations from cells grown with added iron was 3.86 ul/min. The rate by preparations from cells grown in the standard culture medium was 1.5 ul/min., which is little more than a twofold increase.

Endogenous respiration of near-UV-reared cell suspensions and the effect of added catechol are presented in Figure 25. Added catechol did not stimulate oxygen uptake. Neither did hydroquinone, tyrosine, dopa nor phenol. Cell-free extracts centrifuged at 27,000 x g for 20 minutes showed no activity toward catechol, hydroquinone, tyrosine, dopa or phenol and had no endogenous activity. This was further evidence that the COS in Verticillium was not a phenolase.

Colorimetry

The finding that the COS in Verticillium was probably not a phenolase prompted search for another enzyme system which might be the main initiator of melanin synthesis. Since manganese stimulated melanin synthesis (6), and peroxidase (which is activated by Mn^{+2}) could cause melanin synthesis, tests for peroxidase were undertaken.

The modified purpurogallin test showed the presence of peroxidase in both near-UV-reared and dark-reared Verticillium extracts. Since iron is required for peroxidase activity, cultures were

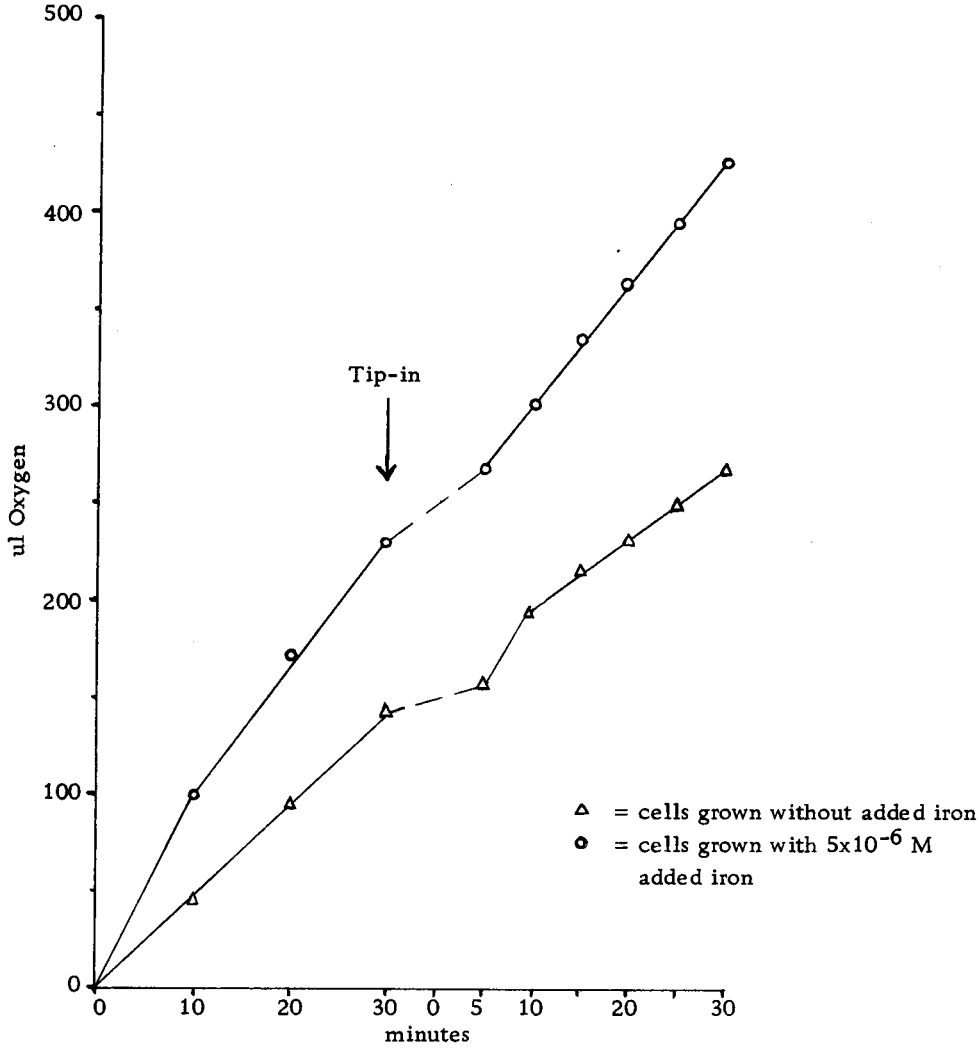

FIGURE 25. Oxygen uptake by suspensions of near-UV-reared Verticillium cells grown with and without iron added to culture medium.

grown with 5 x 10^{-6} M chelated iron added to the medium in order to determine whether this addition would increase the amount of peroxidase activity. Dark-reared cultures with iron added to the medium produced more melanin (by visual observation) and more peroxidase activity (as measured by this purpurogallin test) than similar cultures without iron added to the medium.

Catechol-oxidizing-system assay

The COS assay was developed using the purpurogallin test as a model. This assay depended upon cell-free extract, catechol, and hydrogen peroxide and was stimulated by the addition of Mn^{+2} (see Figure 26). To consistently produce cell-free extracts (as determined microscopically), fungus cells were ground with alumina and centrifuged at 3, 000 x g for five minutes to sediment the alumina and unbroken cells. The supernatant from this was centrifuged for 20 minutes at 27, 000 x g. No further fractionation was attempted. This could account for the low but definite amount of oxidation indicated by the control without H_2O_2. The O. D. indicated by the boiled control was due to turbidity. The control without catechol indicates the dependence of the reaction on a hydrogen donor such as catechol. The relatively high O. D. of the control without Mn^{+2} can be explained by the following: (1) the culture medium contained Mn^{+2}; (2) the alumina might contain Mn^{+2}; and (3) the extract was not dialyzed.

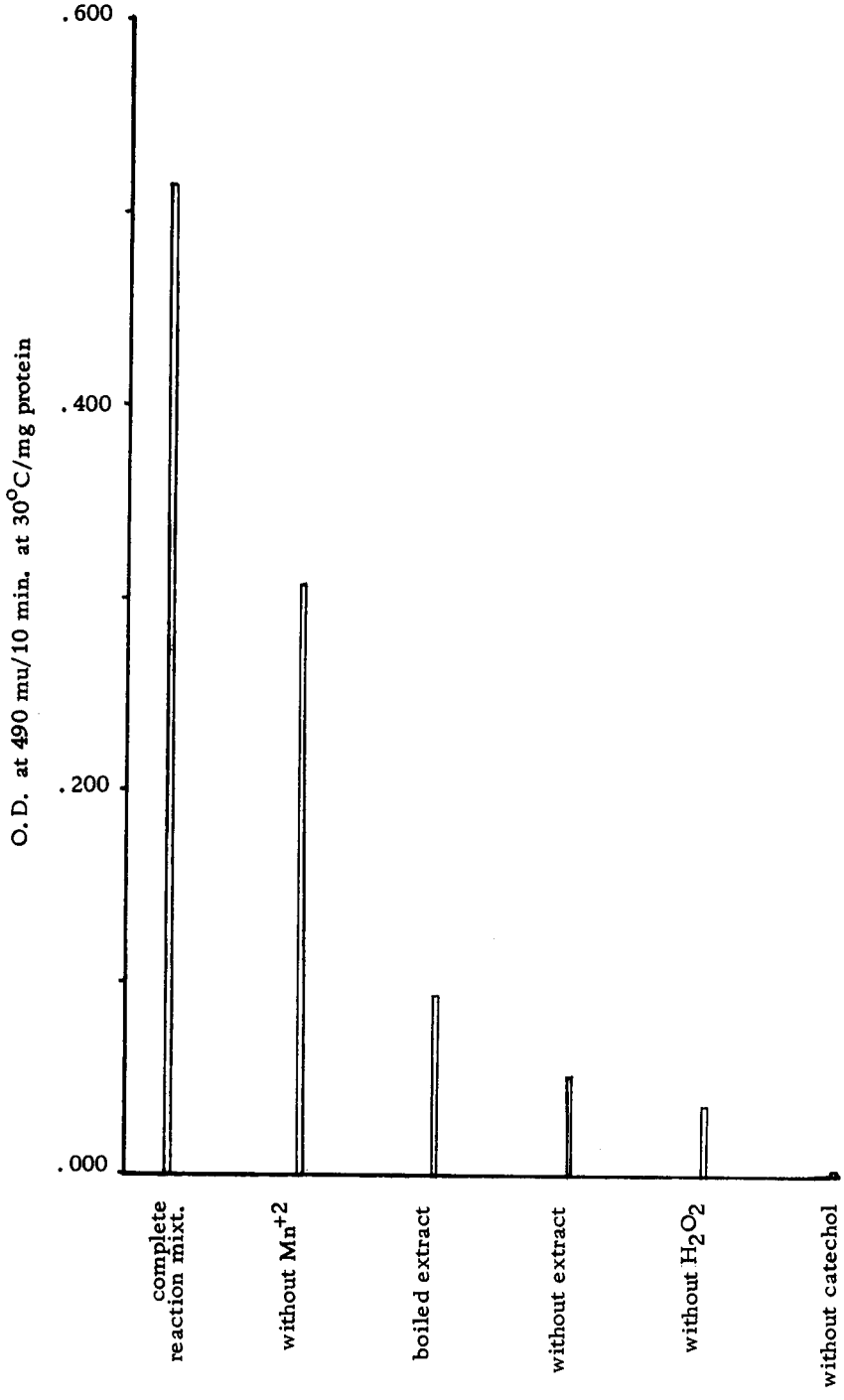

FIGURE 26. Essential components of the Catechol-Oxidizing-System in cell-free extracts from Verticillium cells. (For complete reaction mixture, see methods section.)

The absorption spectrum of the product has a broad peak between 460 and 490 mu (see Figure 27).

To determine the concentrations of the various reactants required for maximum reaction rates, all components in the reaction mixture, except the variable component in question, were maintained at high levels and the variable component was added in increasing amounts until a maximum reaction rate was obtained. These results are presented in Figures 28, 29, 30, and 31.

A concentration of H_2O_2 sufficient for complete saturation was not feasible because high concentrations of H_2O_2 caused autoxidation of substrate and could possibly denature the enzyme. For the reaction time used, 0. 133 M was sufficient. The system was saturated with catechol at 0. 0133 M. Without catechol the reaction was nil. Manganese stimulated the reaction but was not absolutely essential. This may be due in part to the presence of Mn^{+2} in the extract. The O. D. increased almost linearly with increasing amounts of extract. The volume of 0. 1 ml was chosen because it produced an O. D. of roughly . 500 in ten minutes at 30°C. The reaction curves of the near-UV- and dark-reared cell extracts were similar. The reactants for the catechol-oxidizing-system assay indicated that it actually was a catechol-peroxidizing system. Hereafter CPS will be used in place of catechol-peroxidizing system.

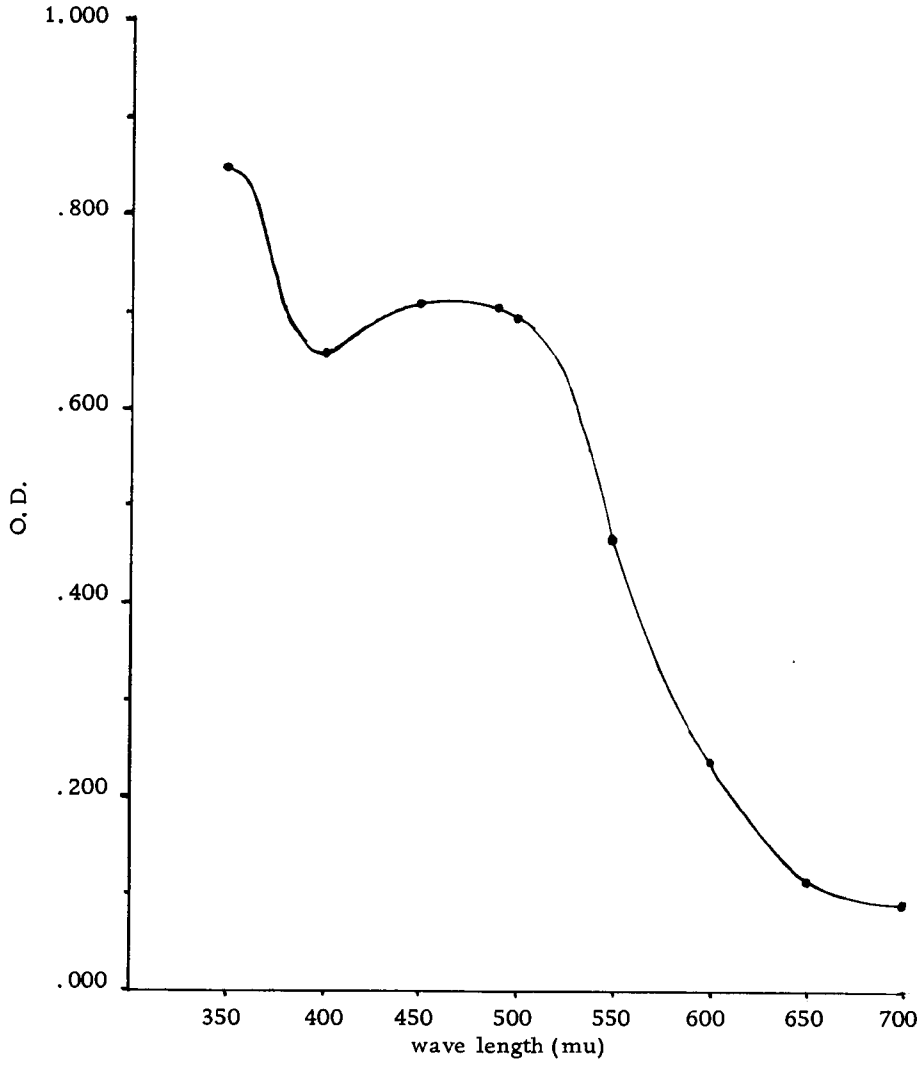

FIGURE 27. Absorption spectrum of oxidized catechol. (The reaction mixture contained: 0.4 ml of 1 M H_2O_2, 0.4 ml of 10^{-1} M catechol, 0.2 ml of 10^{-3} M Mn^{+2} in 0.9 ml of distilled water and 1.0 ml of 0.1 M Tris-HCl buffer pH 7.2, 0.1 ml of enzyme extract).

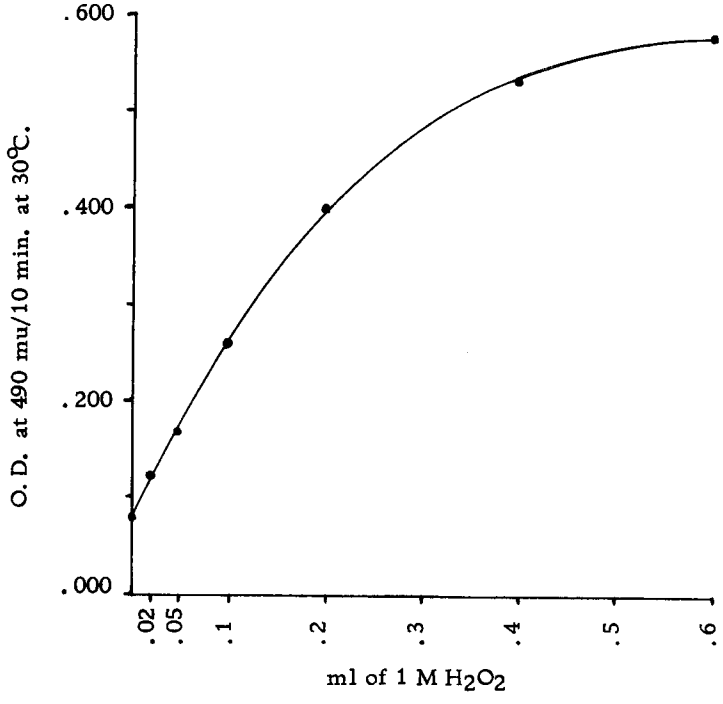

FIGURE 28. The effect of increasing concentrations of H_2O_2 on the catechol peroxidation reaction. (The standard reaction mixture was used with the volumes of H_2O_2 indicated.)

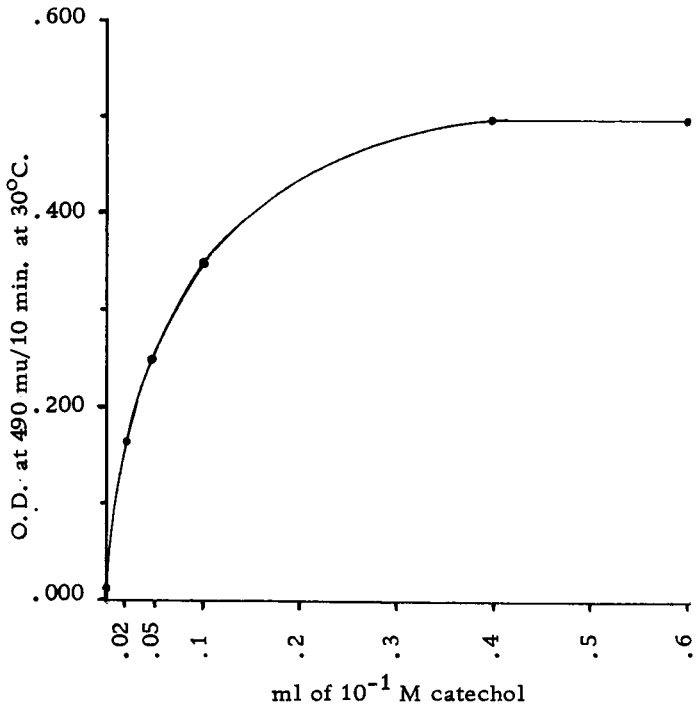

FIGURE 29. The effect of increasing concentrations of catechol on the catechol peroxidation reaction. (The standard reaction mixture was used with the volumes of catechol indicated.)

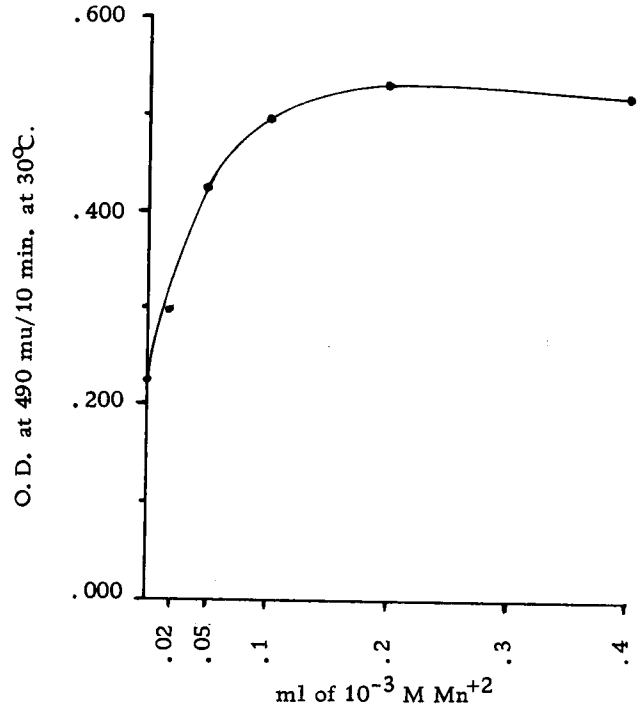

FIGURE 30. The effect of increasing concentrations of manganous sulfate on the catechol peroxidation reaction. (The standard reaction mixture was used with the volumes of Mn^{+2} indicated.)

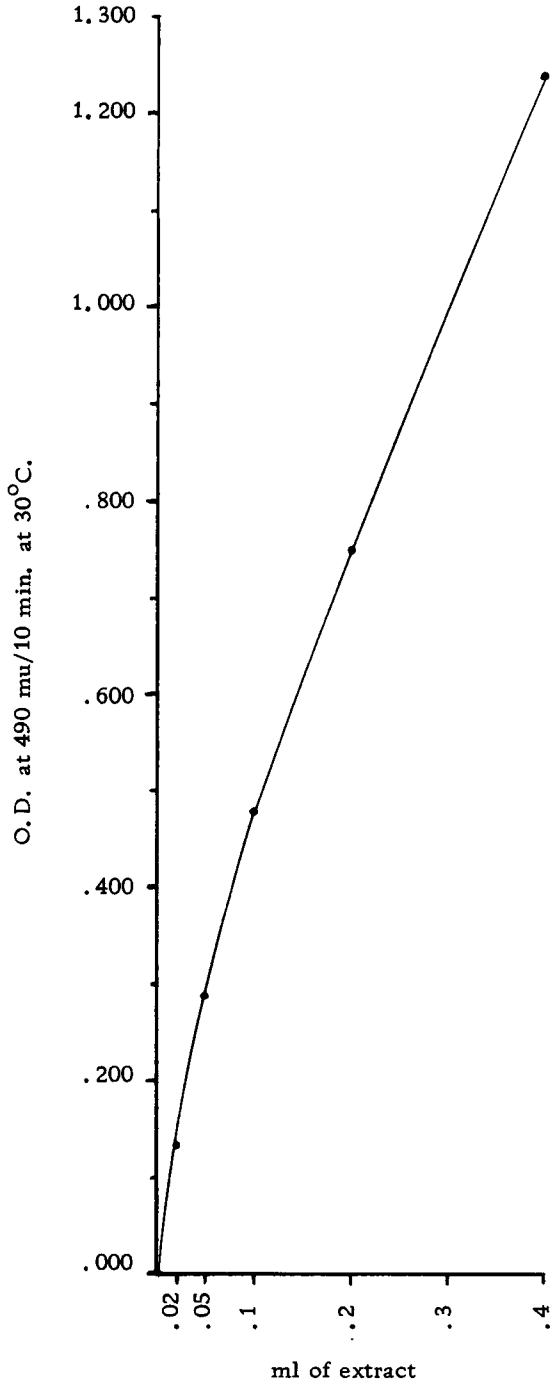

FIGURE 31. Relationship between the catechol peroxidase activity and the volume of extract in the reaction mixture. (The standard reaction mixture was used with increasing amounts of extract.)

Cation stimulation

Eight cations were tested to determine the types of metals that could stimulate the CPS and to what relative degree. Matthey's "Specpure" manganous sulfate was used in the standard reaction mixture. "Specpure" salts of zinc, copper, and iron were tested, as were "Baker analyzed" salts of cobalt, calcium and nickel and Mallinckrodt magnesium, all at 10^{-3} M concentration. The effects of these cations on oxidation of catechol by near-UV- and dark-reared cell extracts are illustrated in Figure 32. Cobalt caused considerable autoxidation of catechol during the five-minute equilibration before extract was added. When Cu^{+2} or Fe^{+2} was added to the reaction mixture there was rapid autoxidation. Although no specific cation stimulation of these crude extracts was observed, Mn^{+2} caused the most stimulation; Mg^{+2} and Ca^{+2} stimulated the CPS roughly one-half as much as Mn^{+2}.

Substrate tests

The same set of potential substrates as tested in vivo was tested in the cell-free CPS assay to ascertain which, if any, would be oxidized to form a dark pigment. Except for chlorogenic acid, the substrates were tested at 10^{-1} M concentrations with a catechol standard. Chlorogenic acid was tested at 10^{-2} M with a comparable

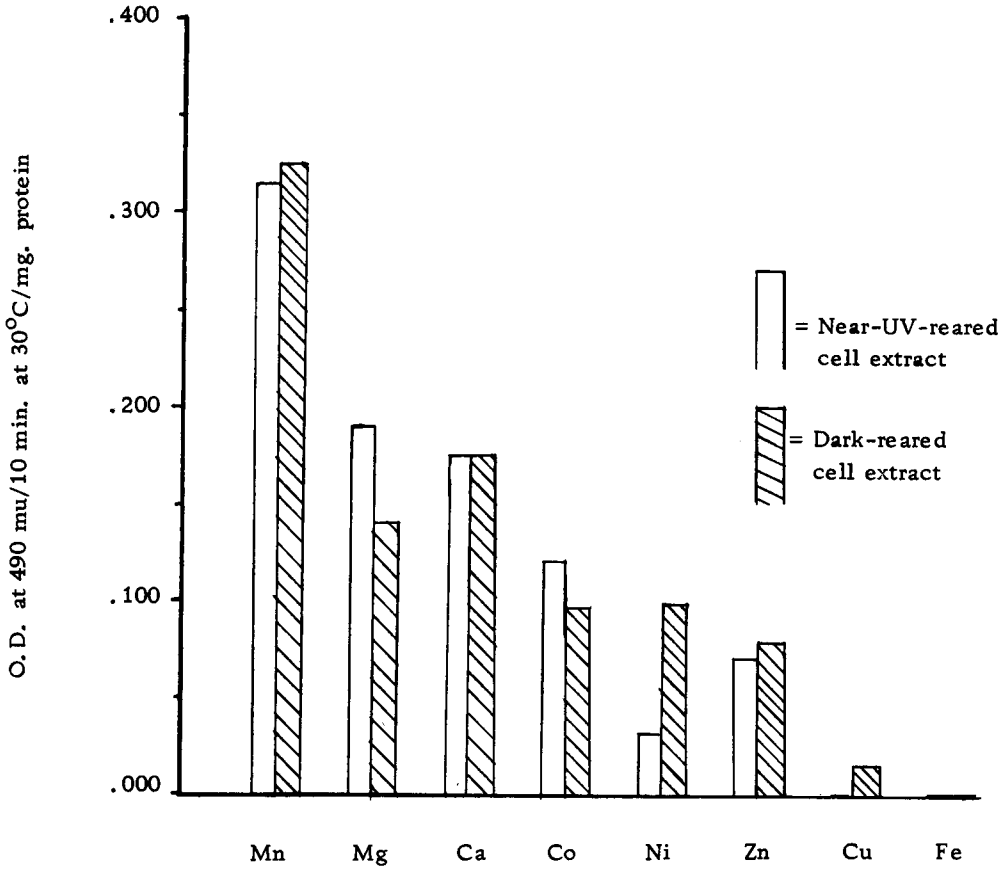

FIGURE 32. Cation stimulation of the Catechol-Peroxidizing-System from dark-reared and near-UV-reared Verticillium cell extracts. (When Co^{+2}, Cu^{+2} or Fe^{+2} was added to the reaction mixture, autoxidation of the catechol was rapid (without the extract). For the standard reaction mixture, see the methods section.)

catechol standard.

Other than catechol, only p-phenylenediamine and dopa were oxidized by this enzyme system to produce a pigment with absorbance at 490 mu (see Figure 33). The readings recorded for these two substrates were made on 1:3 dilutions of the reaction mixtures. The absorption spectra of oxidized p-phenylenediamine and dopa are presented in Figures 34 and 35.

Hydroquinone and pyrogallol also produced pigmented products in this reaction mixture. Oxidized hydroquinone showed the same absorption as catechol but in this case it was due to autoxidation. Oxidized pyrogallol had an altogether different absorption spectrum, characteristic of the yellow pigment (Figure 36). The purpurogallin test for peroxidase is based on this enzymatic oxidation of pyrogallol. The four compounds, p-phenylenediamine, dopa, hydroquinone and pyrogallol, are the only substrates, other than catechol, which form pigments in this reaction mixture.

The O. D. produced by the action of dark-reared cell extract on ascorbic acid, gallic acid and protocatechuic acid was not due to a pigment but rather to turbidity in the cuvette. Turbidity was a problem with the dark-reared cell extract because of a lipid component which clouded the walls of the cuvette. The O. D. recorded for shikimic acid was not due to a pigment nor turbidity. Thus, in this cell-free system, only p-phenylenediamine, dopa, and catechol were enzymatically oxidized to form a dark pigment.

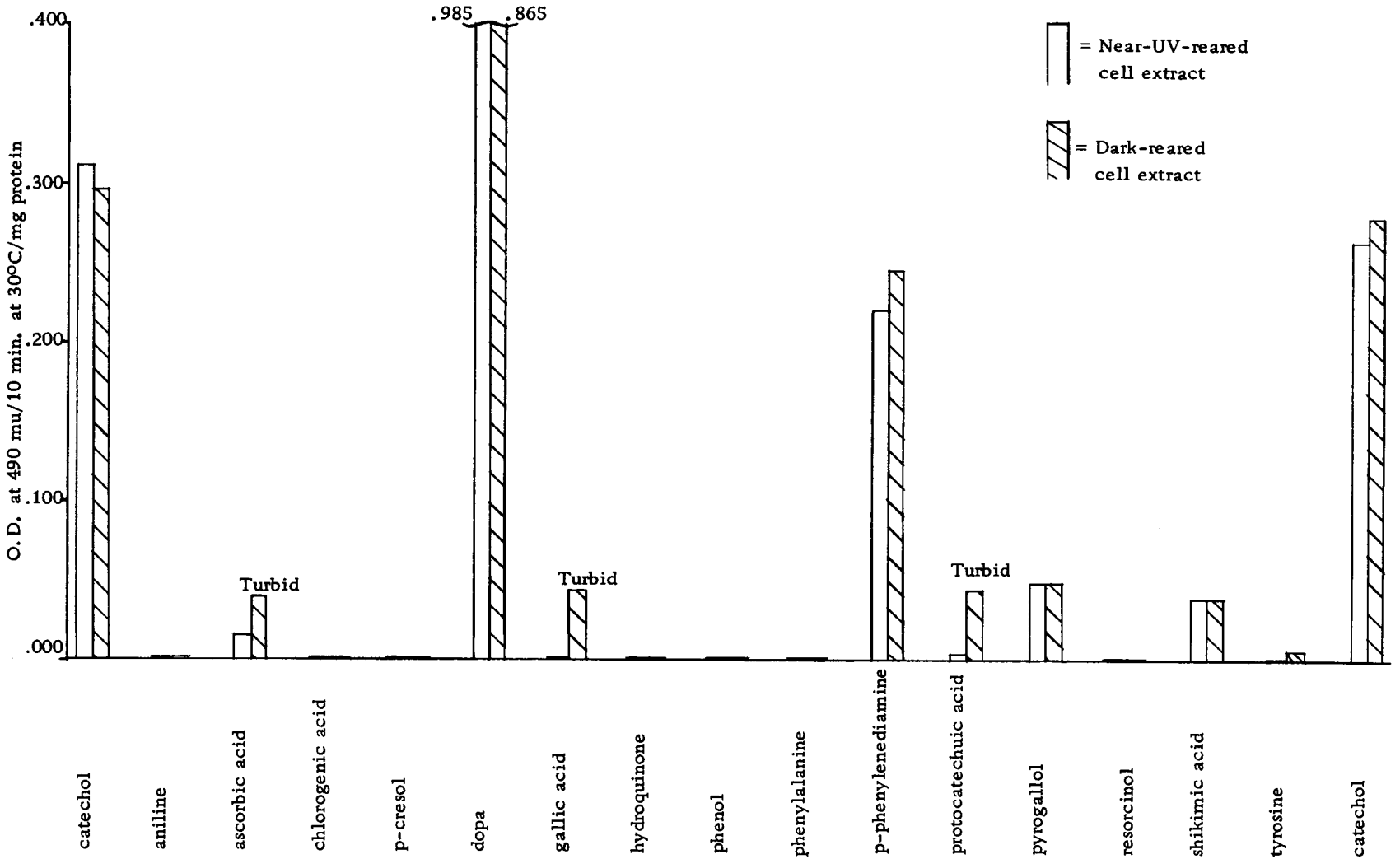

FIGURE 33. The effectiveness of various substrates in the Catechol-Peroxidizing-System. (The standard reaction mixture was used with the various substrates, except chlorogenic acid was tested at 10^{-2} M instead of 10^{-1} M.)

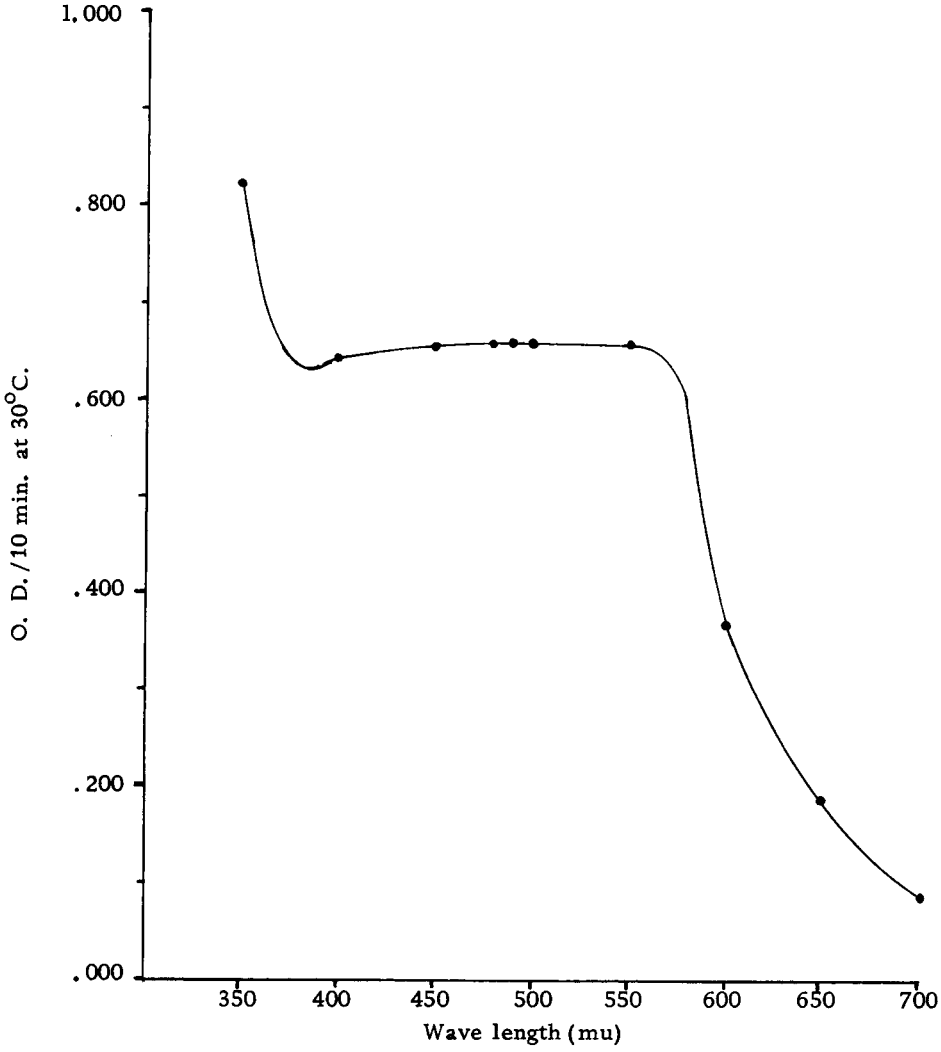

FIGURE 34. Absorption spectrum of oxidized p-phenylenediamine. (The standard reaction mixture was used except p-phenylenediamine was substituted for catechol.)

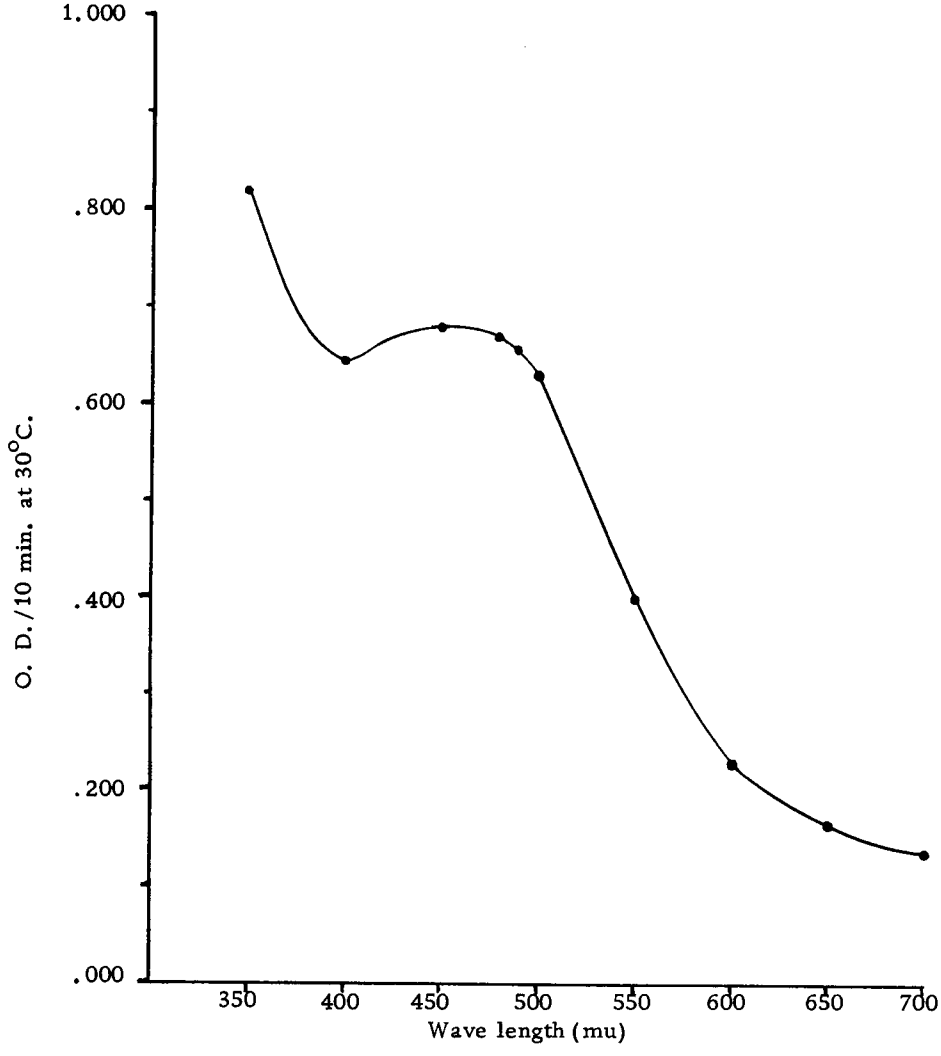

FIGURE 35. Absorption spectrum of oxidized 3, 4-dihydroxyphenylalanine (dopa). (The standard reaction mixture was used except that dopa was substituted for catechol.)

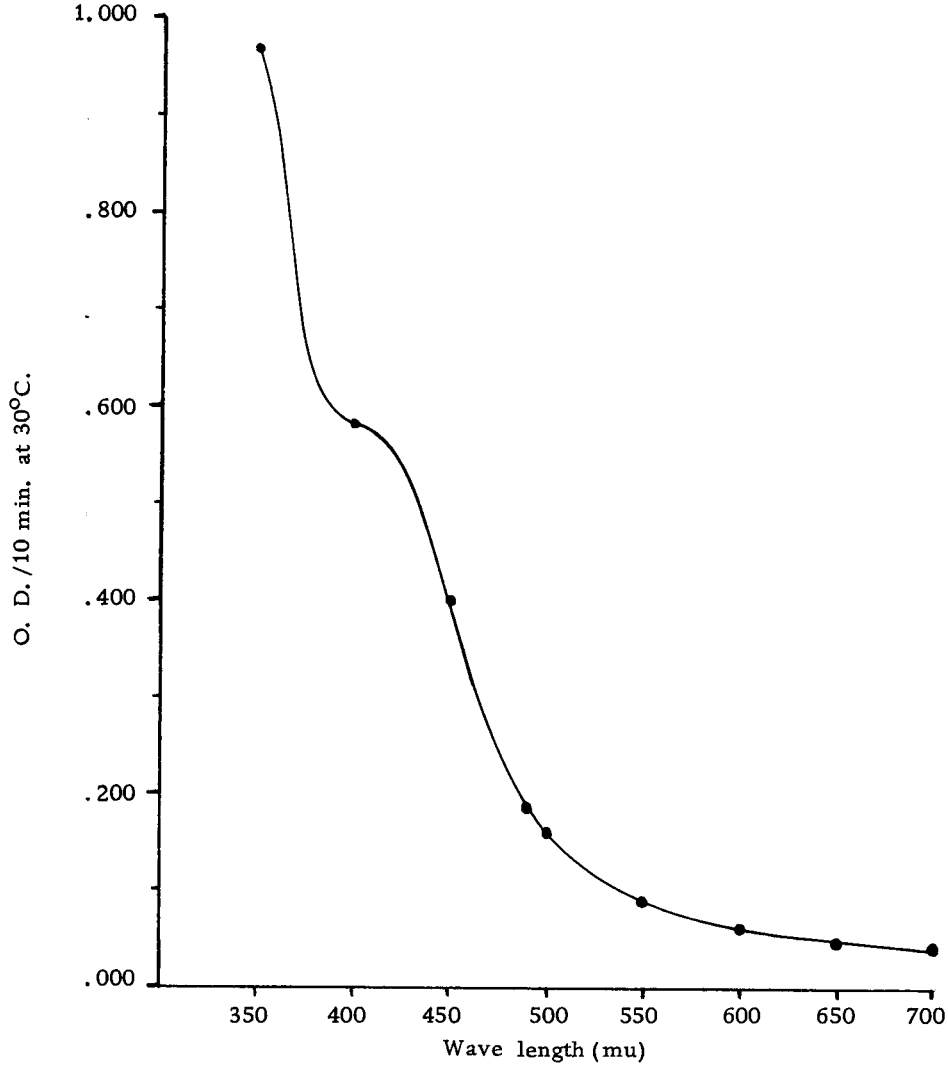

FIGURE 36. Absorption spectrum of oxidized pyrogallol. (The standard reaction mixture was used except that pyrogallol was substituted for catechol.)

DISCUSSION

In vivo substrate tests

Since catechol stimulates melanin formation in Verticillium and melanin results from the oxidation of phenols by phenolase, the presence of a phenolase was assumed to be responsible for melanin synthesis in Verticillium. Thus, a survey was conducted of other compounds which might serve as substrates for the induction of pigment formation under near-UV. Most of the usual phenolase substrates did not induce pigment formation; however, catechol and, to a lesser extent, hydroquinone did. This indicates a high enzyme specificity.

Efforts to demonstrate a phenolase

Attempts to demonstrate a typical phenolase using manometric techniques and the usual phenolase substrates, failed to consistently show stimulation of oxygen uptake. Varying the pH of the extracting buffer did not remedy this situation. Varying the time of harvest did not show a particular time of maximum phenolase activity. To test the method of extraction and assay, preparations from potato and mushroom were made and tested in the same way. The results (Figure 23) showed that, although the method was usable to assay a typical phenolase, it was not usable to assay for the COS in

Verticillium. Further, there was no apparent phenolase inhibitor in the Verticillium preparations. This evidence indicated that there was little or no phenolase activity in Verticillium.

However, in about one-half of the manometric tests, responses similar to those in Figure 24 occurred. These slight stimulations of oxygen uptake with added catechol may have indicated the presence of a weak phenolase in these crude preparations. However, results comparable to Figure 22 were just as common and indicated the absence of a phenolase. Further, catechol did not stimulate oxygen uptake when added to suspensions of Verticillium cells (Figure 25). Thus, I inferred that if a phenolase was present in Verticillium cells, it was weak and not like a typical phenolase. This inference agrees with the findings of Keith (18) and Patil (unpublished results), that there is no, or at best a weak, phenolase in Verticillium.

This inference appears at first to contradict Becker's conclusion that phenolase existed in Verticillium (3). But his assay was based on a colorimetric test changed drastically from that described by Sussman et al. (43). Becker used 1.0 ml of extract in 3 ml reaction mixtures and incubated them for one hour at 30°C before reading. Sussman et al., used 0.1 ml of extract in 3 ml reaction mixtures and read at ten-second intervals. Becker presented no oxygen uptake data; yet this is one of the most reliable and often-used techniques for phenolase assay, and it was the method used by

Sussman et al., to standardize their colorimetric test in the first place. The modifications Becker had to use in order to demonstrate an enzymatic catechol oxidation, indicate that the enzyme, if indeed it was a phenolase, was very weak.

Characteristics of the catechol-peroxidizing system

Because Mn^{+2} increased melanin formation in dark-reared Verticillium cultures (6) and Mn^{+2} often stimulates peroxidase (19)--which can oxidize phenols to form dark pigments -- peroxidase activity was investigated. The modified purpurogallin test showed the presence of peroxidase in both dark-reared and near-UV-reared cell extracts. This correlated well with the finding that Mn^{+2} stimulated melanin synthesis in Verticillium.

Since the usual phenolase assay, using oxygen uptake, proved futile for assay of the CPS, a different assay was developed. The required reactants for this assay proved to be: H_2O_2, catechol, and extract (see Figures 26, 28, 29 and 31). The extract activity was heat labile and manganese stimulated the reaction. These facts further suggested that the catechol-oxidizing-enzyme was peroxidase.

Additional tests showed that Mn^{+2} stimulated the reaction more than any of the other seven cations tested, i. e., Mg^{+2}, Ca^{+2}, Co^{+2}, Ni^{+2}, Cu^{+2}, Fe^{+2}, and Zn^{+2} (Figure 32). This is in agreement with Brandt's (6) findings that Mn^{+2} alone stimulated melanin

synthesis in dark-reared Verticillium cultures.

The fourfold increase in endogenous respiration and the two-fold increase in catechol oxidation by suspensions of cells grown in culture medium containing added iron, are further evidence that there is a peroxidase system in Verticillium.

Catechol and, to a much lesser extent, hydroquinone were the only substrates oxidized by the CPS in vivo. Looking at the results of the substrate tests with cell-free extracts, immediate differences are seen. In addition to catechol, dopa and p-phenylenediamine were enzymatically oxidized to form pigments with absorption similar to that of catechol (Figures 27, 34 and 35). It should be noted that both dopa and p-phenylenediamine autoxidize readily in distilled water, and would oxidize more in the reaction mixture. Pyrogallol was also oxidized but its spectrum was quite different (Figure 36). Hydroquinone was not enzymatically oxidized, although the autoxidation product was similar to that of catechol. Thus, although this assay did not show the same specificity as the in vivo tests, the narrow range of substrates was still indicated.

Comparison of dark-reared and near-UV-reared cell extracts

Extracts from cells grown in darkness and under near-UV-radiation were similar in almost all ways. The required reactants for the CPS and the saturation curves of these reactants were the

same. There was general similarity in the degree of CPS stimulation by the various cations, and the limited specificity of substrates was common to both. The different reactions of the two extracts to Mg^{+2} and Ni^{+2} (Figure 32) were probably not significant in view of their similar reactions with other cations. Likewise, the differences in O. D. between dark-reared and near-UV-reared cell extracts incubated with ascorbic acid, gallic acid and protocatechuic acid are probably not significant because of the lack of reaction in vivo and the infrequency of the difference in vitro.

SUMMARY

Of 13 potential substrates other than catechol, only one, hydroquinone, induced melanin formation in Verticillium cultures under near-UV-radiation; this induction was not nearly as great as that by catechol.

The other 13 compounds tested were aniline, ascorbic acid, chlorogenic acid, p-cresol, dopa, gallic acid, hydroquinone, phenylalanine, p-phenylenediamine, pyrogallol, resorcinol, shikimic acid, and tyrosine.

The usual manometric techniques for phenolase using known phenolase substrates indicated that Verticillium albo-atrum, isolate H-13, contains either no phenolase or weak phenolase.

Comparison of Verticillium preparations with those from potato and mushroom indicated that the catechol-oxidizing-system in Verticillium is unlike that in these known phenolase sources.

A modified purpurogallin test showed the presence of a peroxidase in extracts from both dark-reared and near-UV-reared Verticillium cells.

Chelated iron (5×10^{-6} M) added to the culture medium increased endogenous respiration of cell suspensions and increased peroxidase activity in cell-free extracts.

Reaction mixtures containing cell-free extract, peroxide,

Mn^{+2} and catechol formed a red-brown pigment which darkened upon standing.

The extract activity was heat labile. The reaction depended upon extract, H_2O_2, and catechol. Manganese stimulated the reaction.

Mn^{+2} stimulated the oxidation of catechol more than Mg^{+2}, Ca^{+2}, Co^{+2}, Ni^{+2}, Zn^{+2}, Cu^{+2}, or Fe^{+2}. Mg^{+2} and Ca^{+2} stimulated the reaction about one-half as much as Mn^{+2} and the others caused less stimulation than these two.

Of 15 substrates other than catechol assayed with the cell-free extract, only dopa, p-phenylenediamine and pyrogallol were enzymatically oxidized by this reaction mixture. The substrates tested included the 13 listed above plus phenol and protocatechuic acid.

These results indicate that the enzyme responsible for melanin formation in Verticillium is a peroxidase.

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