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THE BIOSYNTHESIS OF MELANIN IN *ALTERNARIA*

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Key Word Index—*Alternaria*; fungi; acetate; biosynthesis; *p*-hydroxybenzoic acid; melanin; shikimic acid.

Abstract—A melanin which is insoluble in strong alkali has been isolated from *Alternaria* mycelium. Alkali fusion of the pigment produced *p*-hydroxybenzoate and a number of other unidentified phenols. Fungal growth, in light and dark, has been measured and related to melanin formation, phenolase activity and mycelial phenols. Both acetate and shikimate pathways may be involved in the biosynthesis of this melanin. Pigment, derived from [1-^{14}C]-acetate, on alkali fusion yielded only labelled 'neutral' phenols whereas pigment produced from [U-^{14}C]-phenylalanine gave mainly 'acidic' phenols, including *p*-[^{14}C]-hydroxybenzoate.

INTRODUCTION

Infection by fungi often causes a blackening of the wheat crop in the U.K. particularly when the period just prior to harvesting is wet [1]. The greyish flour derived from infected grain is difficult to bleach and the colour is carried over into products of the baking industry. Of the pigment-producing fungi which infect wheat, *Alternaria* is one of the most difficult to control. We have now examined the chemical nature and biosynthesis of the pigment produced by this organism.

RESULTS AND DISCUSSION

A pigment-producing strain of *Alternaria* isolated from infected wheat grain was grown in batch culture on a simple glucose–inorganic salts medium. Exhaustive extraction of the mycelium with organic solvents and acid yielded a black, amorphous solid (Found: C, 57.0; H, 4.3; O, 24.1; N, 2.6; ash 2%). The elemental analysis is similar to those quoted in the literature for melanins from other fungi [2] except that the nitrogen content is intermediate between the characteristic values given for animal [3] and plant [4] melanins.

The IR spectrum of the *Alternaria* pigment was also similar to those of melanins from various sources [4, 5] with the only band of real significance occurring at about 1600 cm^{-1} which is indicative of a conjugated quinonoid structure [5]. The *Alternaria* pigment behaved like most melanins in that it was insoluble in organic solvents. However, unlike other melanins, it was completely insoluble in strong aqueous alkali. Solution, with obvious degradation of the pigment, was only achieved with 12M HNO_3.

The *Alternaria* pigment was degraded, with difficulty, by alkali fusion and PC of the products revealed at least six compounds in very low yield which, from their colour reactions and UV spectra, appeared to be phenolic. Two of the compounds (F_1 and F_2) were immobile on paper electrophoretograms at pH 5.2 whereas F_3–F_6, inclusive, exhibited mobilities of between 0.7 and 1.0 relative to salicylic acid. This suggested that F_1 and F_2 were 'simple' phenols and the remaining four were phenolic acids. However, none of these compounds could be identified on paper chromatograms as the characteristic fusion products obtained from melanins (i.e. indole and pyrrolic acid derivatives from animal melanins or catechol derivatives and salicylic acid from plant melanins [2]). F_3 gave the same colour reactions and cochromatographed (4 solvents) and coelectrophoresed (3 buffer systems) with *p*-hydroxybenzoic acid. This is the first time that this compound has been reported as a degradation product from a melanin. *Alternaria* melanin appeared not to be degraded by oxidation using H_2O_2 or $KMnO_4$, which again demonstrates the exceptional stability of this pigment.

Studies on the biosynthesis of *Alternaria* melanin were initially hindered by the insolubility of the pigment in alkali which made the assay procedure difficult. A reliable method based on 'solution' of the pigment in 12M HNO_3, was eventually developed, however. Pigment and acid were allowed to stand for 5 hr with periodic shaking. The solution was then centrifuged and diluted with water and the colour measured at 450 nm. Under these conditions there was a linear relationship between absorbance and weight of melanin up to 40 mg.

Studies on melanin production by *Alternaria* in shake culture in the light at 27° showed that pigmentation approximately followed the growth rate up to 90 hr and then continued to increase after the glucose content of the medium had been depleted and growth had stopped. Melanin production was delayed by the absence of light, although growth was unaffected (Fig. 1) and with the initial pH of the medium above 7.0 or below 4.0 pigmentation was negligible although there was significant growth under these conditions (Fig. 2).

Phenolase, which is generally believed to be involved in the oxidation reactions leading to melanin formation, was assayed in the mycelium during the growth of *Alternaria* in shake culture. The total activity of the enzyme paralleled the fungal growth curve but the specific

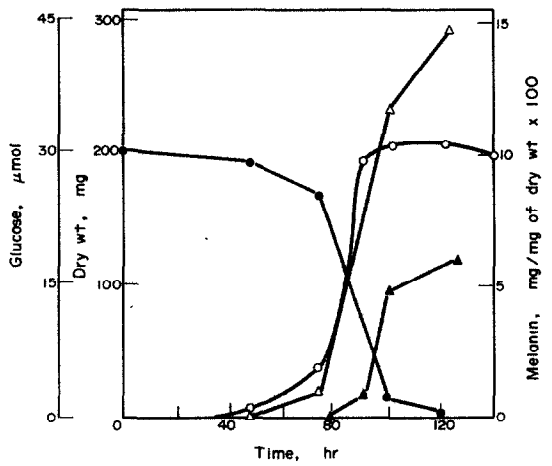

Fig. 1. Pigment production by *Alternaria* in light and dark. (○), growth curve and (●), glucose utilization, both in dark and light. (△), melanin production in the light; (▲), melanin production in the dark. For details see Experimental.

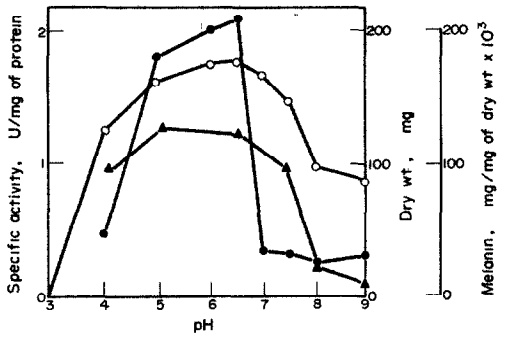

Fig. 2. The effect of pH on pigmentation of *Alternaria* mycelium. The pH of the medium prior to inoculation was adjusted with dilute NaOH or HCl. (○), Mycelial dry wt; (●), melanin formation; and (▲), phenolase activity; were measured after 4 days of growth; further details are given in the Experimental.

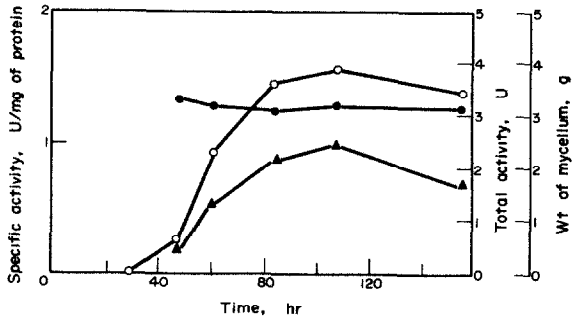

Fig. 3. Phenolase activity in *Alternaria* mycelium. Details are given in Experimental, (○) growth curves; (●), total phenolase activity; (▲), specific activity of phenolase.

activity in terms of mycelial protein, remained constant throughout the growth period (Fig. 3). When the fungus was grown in media with varying hydrogen ion concentrations (Fig. 2) the specific activity of mycelial phenolase was directly related to growth. Under acidic (initial pH 4.0) and neutral or alkaline conditions (initial pH 7–8) where growth of mycelia possessing phenolase with a relatively high specific activity did occur, there was negligible pigmentation. This suggests that at unfavourable hydrogen ion concentrations pigment formation may be inhibited by lack of the necessary phenolic substrates.

When an aqueous ethanolic extract of a 4-day mycelial growth (initial pH 6, in the light) was examined in a spectrophotometer, absorbance peaks at 270 nm and 310 nm, which are characteristic of phenolic derivatives, were observed. Paper chromatographic analysis of the extract showed that it contained several compounds which gave colour reactions with diazotized *p*-nitroaniline–NaOH reagent. Absorbance measurements, at 270 and 310 nm, with extracts from mycelia at various stages of growth showed that the total soluble phenol content rose to a maximum at 3 days (Fig. 4). This corresponds with the onset of melanogenesis (see Fig. 1). After this time the phenol content fell rapidly which could, in part, be due to the polymerization of the low molecular weight compounds to melanin.

An attempt was next made to compare the relative importance of the acetate and shikimic acid pathways in melanogenesis in *Alternaria*. Phenylalanine ammonia lyase activity has been detected in this fungus where increasing activity of the enzyme in the light parallels melanin formation [6]. The organism can also effect β-oxidation of cinnamic acid and its derivatives and hydroxylation of the resulting benzoic acids [7].

[^{14}C]-Melanins were isolated from *Alternaria* cultures in the presence of a number of labelled metabolites: the relative efficiencies of ^{14}C-incorporation into the pigment are shown in Table 1. The percentage incorporation was highest in the case of [1-^{14}C]-acetate and this value was not changed significantly when unlabelled phenylalanine

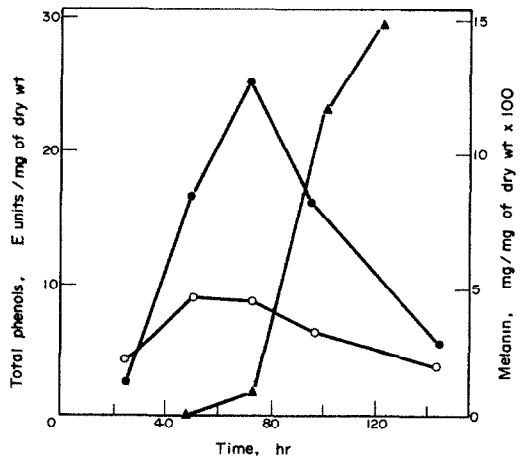

Fig. 4. The relationship between mycelial phenols and melanin formation in *Alternaria*. Absorbances at 270 nm, (○) and 310 nm, (●); Melanin formation (▲). For details, see Experimental

or cinnamic acid were added together with the labelled acetate to the media. Incorporation of label from acetate was however, largely blocked by benzoic acid and to a lesser extent by *p*-hydroxybenzoic acid. It is, therefore, clear that the acetate can serve as a precursor of melanin in *Alternaria*. Benzoic acid and *p*-hydroxybenzoic or structurally related compounds may be intermediates on the pathway from acetate to melanin although such compounds are normally derived from phenylalanine via cinnamic acid [8]. Dilution of ^{14}C-incorporation by benzoic and *p*-hydroxybenzoic acids is, perhaps, more likely to result from inhibition of enzymes involved in the pathway to the pigment.

Evidence for the participation of the shikimic acid pathway in *Alternaria* melanin synthesis was first obtained by feeding [U-^{14}C]-glucose alone and then comparing incorporation in the presence of unlabelled shikimic acid (Table 1). In the latter case, there was a marked dilution (82%) of the labelling of the pigment. Furthermore, the percentage incorporation from [U-^{14}C]-phenylalanine was higher than from [U-^{14}C]-glucose and a dilution of the radioactivity of the melanin occurred when unlabelled cinnamic acid, benzoic acid or *p*-hydroxybenzoic was added to the medium containing labelled phenylalanine. Protocatechuic acid, an ene-diol which is a degradation product from some melanins [2] and a phenolase substrate, produced a dilution significantly lower than the three other unlabelled compounds, which suggests that it is not a direct precursor of this pigment. The relatively low dilution of incorporation from [^{14}C]-phenylalanine which resulted from inclusion of unlabelled *p*-coumaric acid in the medium can perhaps be explained by the fact that *p*-coumarate is not a metabolite of *Alternaria* and that the direct formation of *p*-hydroxybenzoic acid by β-oxidation of this cinnamic acid derivative is blocked [cf 7]. It is unlikely that the labelled pigment produced in the presence of [^{14}C]-phenylalanine is derived from [^{14}C]-acetate, resulting from successive phenylalanine ammonia lyase and β-oxidation reactions, as unlabelled phenylalanine did not dilute incorporation from [^{14}C]-acetate.

Alkali fusion of the radioactive pigments produced in the presence of [1-^{14}C]-acetate and [U-^{14}C]-phenylalanine yielded six labelled phenols which were examined by paper electrophoresis. The melanin derived from [^{14}C]-acetate gave only labelled 'neutral' phenols (F_1 and F_2) whereas two radioactive acidic fractions, one of which was indistinguishable from *p*-hydroxybenzoic acid (F_3), were obtained from the fusion products of melanin formed in the presence of [^{14}C]-phenylalanine.

The tracer studies allow the conclusion that acetate and shikimate can both serve as precursors of *Alternaria* melanin although whether both mechanisms operate *in vivo* is not known. It is likely that part of the pathway from phenylalanine to melanin is a series of reactions essentially similar to those occurring in higher plants [8], i.e.

$$\text{phenylalanine} \xrightarrow{\text{PAL}} \text{cinnamate} \rightarrow \text{benzoate} \rightarrow p\text{-hydroxybenzoate}$$

p-Hydroxybenzoate could be further metabolized to an *o*-dihydroxyphenol before incorporation into the pigment but some *p*-hydroxybenzoate appears to be incorporated into the melanin intact, presumably by free radical reactions. These latter molecules may only occur as peripheral residues of the polymer and hence would readily be removed by alkali fusion. Phenylalanine ammonia lyase may well control the path of carbon into *Alternaria* melanin as the enzyme is subject to inhibition by cinnamic and benzoic acids [9].

EXPERIMENTAL

Materials. Radiochemicals were purchased from the Radiochemical Centre, Amersham, Bucks, U.K., and all other chemicals were from B.D.H. Ltd., Poole, Dorset, U.K.

Fungal cultures. The original culture of *Alternaria* sp., isolated from infected wheat, was supplied by RHM Research Ltd., High Wycombe, Bucks. Stock cultures were maintained on potato-dextrose agar slopes at 6–7°C. Fungal mycelium for all experiments was grown in shake culture at 27°. The medium contained (g): glucose, 5.0; KNO_3, 3.5; $K_2H_2PO_4$, 1.75; $MgSO_4$, $7H_2O$, 0.75; in H_2O to 1 l. (final pH 6). Dry wts of mycelium were obtained by filtering off the hyphae, washing with H_2O and drying at 70° to constant wt. Glucose in the medium was determined by using alkaline 3,5-dinitrosalicylic acid reagent [10].

Assay for melanin. 12 M HNO_3 (10 ml) was added to known wts of mycelium which were left at room temp. for 5 hr after mixing well. The solns were then centrifuged and 1 ml aliquots

Table 1. Incorporation of ^{14}C-labelled substrates into melanin

Labelled substrate	Unlabelled compound (5 μM)	% Incorporation of ^{14}C into melanin ± S.E.M.	% Dilution of incorporation
D-[U-^{14}C]-glucose	—	1.5 ± 0.10 (2)	—
	shikimic acid	0.27 ± 0.06 (2)	82
L-[U-^{14}C]-phenylalanine	—	2.2 ± 0.15 (2)	—
	cinnamic acid	0.4 ± 0.10 (2)	81.9
	p-coumaric acid	0.69 ± 0.10 (2)	68.6
	benzoic acid	0.36 ± 0.08 (2)	83.6
	p-hydroxybenzoic acid	0.34 ± 0.02 (2)	84.5
	protocatechuic acid	0.91 ± 0.13 (2)	58.7
Sodium [1-^{14}C]-acetate	—	3.5 ± 0.20 (2)	—
	phenylalanine	3.6 ± 0.20 (2)	—
	cinnamic acid	3.5 ± 0.15 (2)	—
	benzoic acid	1.2 ± 0.10 (2)	65.8
	p-hydroxybenzoic acid	2.8 ± 0.14 (2)	20.0

of the melanin extracts diluted to 20 ml with H_2O. The A_{450} of the resulting solutions were measured and the concentration of melanin (mg/mg mycelial dry wt) determined from a standard curve constructed for a purified sample (see below) of *Alternaria* melanin.

Pigment extractions. Alternaria mycelium was grown for 14 days at 27°, separated from the medium by filtration, washed thoroughly with H_2O and then macerated with H_2O to a uniform blend. The disintegrated tissue was filtered off, washed and air dried. Continuous Soxhlet extraction was then carried out with petrol (bp 60–80°, 12 hr) followed by EtOH (20 hr). The crude pigment was dried and suspended in 12 M HCl at room temp. for 4 days. The suspension was diluted with H_2O and centrifuged and the residue washed with M HCl (3 ×) and then resuspended in conc HCl for a further 4 days. The pigment, obtained by centrifugation, was washed with HCl, H_2O and EtOH and then, again, continuously extracted with EtOH and Me_2CO and finally refluxed with 5 M HCl for 5 days. The purified pigment was filtered, washed with acid, H_2O and EtOH and air dried (yield, *ca* 2% of the dry wt of mycelium). Elemental analysis of the pigment was carried out by the Alfred Bernhardt Mikroanalytisches laboratorium, W. Germany and IR spectra recorded using KBr discs.

Chromatography and electrophoresis. Descending PC was carried out on H_2O-washed Whatman No. 3 paper in the following solvents: *A*, EtOAc–HOAc–H_2O (9:2:2); *B*, *n*-BuOH–EtOH–H_2O (40:11:19); *C*, *n*-BuOH–HOAc–H_2O (6:1:2); *D*, EtOAc–HOAc–HCO_2H–H_2O (18:3:1:4). For ascending 2D PC on Whatman No. 1 paper solvent *E*, C_6H_6–HOAc–H_2O (6:7:3, upper phase) was used in the first direction and solvent *F*, HCO_2Na–HCO_2H–H_2O (10:1:200, w/v) in the second direction. TLC was carried out on cellulose plates in solvent *G*, *i*-PrOH–*n*-BuOH–*t*-BuOH–NH_4OH–H_2O (4:2:2:1:2). Phenols were detected on chromatograms with: UV light in both the presence and absence of NH_3 vapour; diazotized *p*-nitroaniline solution [11] and oversprayed with M NaOH soln, and $FeCl_3$/$K_3Fe(CN)_6$ reagent [12]. Electrophoresis was carried out on Whatman 3 mm paper at 33V/cm in the following buffers: 0.2 M NaOAc, pH 5.2; 0.2 M Na borate, pH 10; 0.2 M NaPi, pH 7.5; 0.008 M Na molybdate, pH 5.2.

Degradation of melanin. (a) *Alkali fusion*: This was achieved by the method of Hackman and Goldberg [13]. The products were resolved into 2 major fractions by preparative PC using solvent *A*; these were eluted from the paper with H_2O and concnd. The faster moving fraction was separated into 5 components (F_2–F_6) by preparative paper electrophoresis in NaOAc buffer (pH 5.2). These compounds were further purified by 2D PC using solvents *E* and *F*. The slower moving fraction (F_1) from the original preparative chromatogram was purified by PC using solvent *B*. The UV spectra of the purified compounds were recorded in ethanolic solution. (b) *Oxidation of melanin*: This was attempted with H_2O_2 [14] and with $KMnO_4$ [15]. In both instances the pigment was recovered apparently unchanged. In the latter case PC analysis revealed no low MW products.

Extraction and assay of phenolics from fungal mycelium. Shake cultures of *Alternaria* were set up as previously described and duplicate flasks taken 1, 2, 3, 4 and 5 days after inoculation and the contents filtered through sintered glass funnels. The mycelial samples were washed thoroughly with H_2O and dried and equal weights of the samples then ground up in 80% aq. EtOH using a cooled pestle and mortar. After filtration the EtOH extracts were individually concnd to dryness and the residues redissolved in EtOH (0.1 ml). PC examination of the phenols was achieved using solvents *A* and *B*. The EtOH extracts were also analysed for 'total phenols' (expressed as absorbance units/g dry wt of mycelium) by measuring absorbances at 270 and 310 nm.

Extraction and assay of phenolase. Me_2CO powders of mycelia were extracted with 0.05 M NaOAc buffer, pH 5.4, and centrifuged at 38 000 g for 1 hr. The resulting supernatant solns were dialysed for 4 hr against 2 changes of 0.01 M NaOAc buffer, pH 5.4. Phenolase activity was determined by use of a Clark O_2 electrode. Reaction mixtures contained 0.05 M NaOAc buffer, pH 5.4 (0.6 ml), 0.1 M catechol (0.1 ml) and enzyme (0.2 ml). The electrode was calibrated with air-saturated H_2O at 30° and the reaction was allowed to proceed at 30°. Protein concns were measured by the method of Lowry *et al.* [16] and phenolase activities expressed as μmol O_2 utilized min/mg protein.

Tracer experiments. Alternaria cultures were grown as described above for 85 hr and then presterilized (15 psi for 15 min) D-[U-^{14}C]-glucose, (3 mCi/mmol), L-[U-^{14}C]-phenylalanine (492 mCi/mmol) or Na [1-^{14}C]-acetate (57 mCi/mmol) (1 μCi of each) was aseptically added to appropriate flasks. In the dilution expts, unlabelled compounds (final conc 5 μM) were sterilized and administered together with the ^{14}C-tracers. All cultures were then allowed to grow for a further 72 hr. The radioactive melanin produced was extracted as previously described, carefully ground to a fine powder using an agate pestle and mortar and then suspended in EtOH. Different volumes of the suspension were spotted onto weighed Al planchets, the EtOH evaporated off with an IR lamp and the planchets then reweighed. Radioactivity was counted with a Tracer-lab Multimatic gas-flow counter. Over the range used there was a linear relationship between melanin weight and radioactivity.

Degradation of radioactive melanins. Labelled melanins were degraded by alkali fusion and the products separated by paper electrophoresis in 0.2 M NaOAc buffer, pH 5.2. The radioactivity was then located using a Tracer-lab 4π scanner and the appropriate paper strips cut from the electrophoretograms. The radioactivity on the strips was measured with a Beckman CPM 100 Scintillation Counter using 2,5-diphenyloxazole in toluene as scintillator.

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