APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1980, p. 145-155
0099-2240/80/07-0145/11$02.00/0
Vol. 40, No. 1

Degradation of Melanin by *Aspergillus fumigatus*

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A strain of *Aspergillus fumigatus* from composted coffee and garden wastes utilized natural deproteinized insect, banana, hair, octopus, and synthetic tyrosine and dopa melanins as sole sources of carbon. With a sucrose supplement, degradation was essentially complete after 50 days in Czapek medium, pH 6.5 at 30°C. The catabolic rate differed for each substrate pigment, as did the molecular weight distribution of products accumulating in the medium. After incubation with L-[*U*-^{14}C]melanin, over 50% was recovered in a dark fungal pigment, the remainder appearing as cell protein, chitin, lipid, CO_2, and polar metabolites. When grown on melanin, the normally pale mycelia darkened with the production of a fungal allomelanin, with infrared spectrum and alkali fusion products differing from those of the substrate pigment. Isotope distribution in amino acids for *A. fumigatus* grown on labeled melanin supplemented with sucrose suggested separate pools for synthesis of cell proteins and melanoproteins. Deposition of allomelanin increased resistance of conidia, sterigma, and conidiophores to lytic carbohydrases as judged by scanning electron microscopy.

Natural melanins from plants, microorganisms, and animals consist of a polyaromatic backbone with proteins attached by covalent bonds (23, 24, 28). The complex is subject to slow degradation by soil flora, with carbon and nitrogen being released from the protein moiety at a rate slightly higher than from the benzenoid pigment component (12). Melanin of low protein content prepared by incubation of [*U*-^{14}C]tyrosine with polyphenol oxidase released only 9% of the isotope as labeled carbon dioxide in the course of 8 weeks of incubation with potting soil (2). Similarly, a natural melanin deproteinized by treatment of humic acid with 6 M HCl afforded only 2% of the residual pigment as carbon dioxide after 30 weeks of exposure to a sandy loam (17). Although a portion of the nonprotein component of melanoproteins is clearly resistant to degradation, the more labile aromatic components of fungal melanins and plant lignins contribute significantly to the formation of humic acids (7, 15, 16). Melanins from cultures of *Eurotium echinulatum* and *Stachyobotrys chartarum* release phenols and anthraquinones into the media which are subsequently recycled for synthesis of humic acid-like materials (14, 23). Together with wall material from heavily melanized fungi, these polymers constitute a significant portion of the stable components of soil humus (23). The origin of this fraction is not confined to lignified tissues and fungal debris, however. Melanins occur widely in both marine and terrestrial animals, with composition varying in the ratio of indolic and phenolic substituents (1, 21). The effect of this structural diversity on mineralization, catabolism, and the identity of the organisms contributing to the initial stages of polymer degradation has not been reported. This contribution addresses these issues by reporting the fate of deproteinized melanins of animal and synthetic origin after exposure to soil and to cultures of *Aspergillus fumigatus* isolated from these soils.

In addition to its role as a humin precursor, melanin influences the processing of soil organic matter by conferring resistance to enzymatic degradation of associated polysaccharides and proteins. Mixtures of β-(1,3)-glucanase and chitinase hydrolyzed the melanin-free hyaline hyphae of *A. phoenicia* but failed to act on melanized sclerotia (2). The resistance of *A. nidulans* to these carbohydrases was in proportion to the melanin/polysaccharide ratio, and addition of purified melanin to casein extended the time required for proteolysis by subtilisin (14). The organism described in the present study, *A. fumigatus*, develops a black color in the course of melanin degradation, providing the opportunity to compare susceptibility to lysis in colored and uncolored variants of the same strain. We examined mycelia by scanning electron microscopy after incubation with depolymerizing carbohydrases. Extensive damage to the uncolored population was revealed but litte change in the melanized samples was evident, confirming chemical studies with pigmented and unpigmented strains isolated from natural sources (2).

MATERIALS AND METHODS

Isolation of organisms. *A. fumigatus* NRRL 6463 was isolated from a compost heap in Watertown, Mass., consisting of garden wastes and a significant

amount of spent coffee grounds. Identity was verified by Margaret Bigelow, Department of Botany, University of Massachusetts/Amherst, and by the Taxonomy Group of the U.S. Department of Agriculture Fermentation Laboratory, Peoria, Ill. This strain was isolated in the course of a screening program in which soils and composts were added at a level of 2 g/150 ml to 0.8% nutrient broth fortified with 1.5% sucrose in 500-ml flasks. The flasks were incubated at 23°C for 48 h without agitation, after which inocula were transferred to Czapek broth supplemented with insoluble insect melanin, 5 mg/2.5 ml. The broth contained, per liter of distilled water: NH_4Cl, 3 g; K_2HPO_4, 1 g; $MgSO_4$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 0.01 g; and yeast extract, 1 g, pH 6.5. The cultures were incubated without agitation for 6 weeks at 23°C. Melanin utilization was monitored by dark coloration of the medium, which was initially straw yellow, and by disappearance of the insoluble melanin pellet. Samples yielding the highest rate of melanin utilization were streaked on Czapek agar, and individual colonies were again tested for melanin utilization by the qualitative method described above. The plating step was repeated with those cultures capable of melanin catabolism. Isolates from the second platings were free of contaminants as assessed by streaking on nutrient agar. In all tests for melanin utilization, parallel replicates, one with inoculum but prepared without melanin, and one with melanin but not inoculated, served as controls. Melanin remained insoluble in the former group, and pigmented cells failed to appear in the latter cultures.

Utilization and analysis of [*U*-^{14}C]melanin. Degradation was followed for 5 weeks in a 500-ml Gledhill shake flask containing 120 ml of Czapek medium fortified with 3% sucrose and 40 mg of melanin prepared from [*U*-^{14}C]tyrosine (3.6×10^7 cpm, total radioactivity). An uninoculated control failed to release CO_2 or show evidence of microbial growth during the sampling period. The design of the flask permitted free exchange of filtered air for the CO_2-free gases above the culture surface as well as sampling of both the liquid medium and CO_2 (9). Carbon dioxide was recovered at weekly intervals from the gas phase by withdrawal and replenishment of the Hyamine trapping solution (Packard Instrument Co., Inc.) suspended in a vial above the culture by a syringe equipped with a no. 13 needle, 15 cm long. Additional radioactive CO_2 was recovered by distillation from 20-ml samples withdrawn from the medium, and the values for dissolved CO_2 were added to those recovered from the Hyamine trap. Recovery of CO_2 was quantitative as determined with Na_2-$^{14}CO_3$. Total nonvolatile radioactive metabolites were assessed after evaporation. Nonvolatile metabolites in the liquid medium of low molecular weight were isolated by molecular sieving in polyacrylamide gels. Concentrated supernatants from the media clarified by sedimentation at 10,000 $\times$ *g* were taken up in 0.5 M ammonium acetate and chromatographed on Bio-Gel P-4 (2 by 30 cm) in the same buffer. Metabolites eluting between the void volume and the peak containing inorganic salts were pooled and lyophilized in preparation for chromatography on Bio-Gel P-2 (2 by 50 cm). The column was standardized with aromatic markers and peptides ranging from 80 to 13,000 daltons. Column development in 0.5 M ammonium acetate, pH 7.8, substantially eliminated sorption of benzenoid derivatives by the gel.

Distribution of radioactivity in mycelial components released by hydrolysis in 6 M HCl (0.01% phenol, wt/vol) under N_2 in a sealed tube at 100°C for 18 h was monitored by chromatography on a sulfonic acid resin according to a program designed for amino acid analysis (25). A stream splitter facilitated collection of amino acids for counting. Chitin was quantified as glucosamine. Materials not retained by the column at pH 2 were classed as acidic metabolites. Additional radioactivity eluted from the cation exchange after arginine in 0.2 M NaOH was considered to be associated with basic metabolites. Lipid was isolated in chloroform/methanol (2:1).

Determination of melanin. Each variable was assessed by including two controls with each treatment. The first tube consisted of the experimental medium (2.5 ml) plus 5 mg of insoluble pigment and the fungal inoculum; the second contained a similar amount of pigment but no inoculum; and the third was devoid of pigment but was inoculated. Experiments were replicated at least four times. Disappearance of substrate was assessed by spectrophotometric assay of insoluble melanin remaining at the bottom of the cultures at the end of the incubation period. Fungal pigment was quantified separately. After incubation, the cultures were diluted with 10 ml of 1% NaCl and mixed by mechanical blending. When centrifuged at 2,500 $\times$ *g*, all of the residual substrate melanin sedimented, whereas the finely dispersed mycelia were confined to the supernatant solution. The pelleted melanin was washed twice by sedimentation in water and 0.05 M acetic acid and solubilized for spectrophotometry by heating for 2 h to 100°C in 5 M NaOH containing 0.15 M sodium borohydride (5). Absorbance was measured at 310 nm, and values were compared with a standard pure melanin of the same derivation. Frequent checks of the spectrophotometric procedure by gravimetry or nitrogen determination showed concordance of values. Recovery of melanin from uninoculated controls was quantitative, and material responding to the spectrophotometric assay for melanin was absent from the culture when melanin was omitted.

A. fumigatus is normally silver-yellow and devoid of dark pigment unless grown on melanins or melanogens. When pigmented mycelia were present, interference with melanin determination was prevented by destruction of allomelanin with NaOH-borohydride. When recovery of the fungal pigment was desired, the saline supernatant with the blended cells was lyophilized and extracted first for 48 h in 15 ml of 2 M NaOH and then for 6 days in 15 ml of 6 M HCl (both treatments at 20°C). The insoluble material harvested by sedimentation at 1,000 $\times$ *g* was hydrolyzed for amino acid analysis at 110°C for 3 h in 6 M HCl under N_2 in a sealed tube. The dark residue was pelleted, washed, and dried for gravimetry, nitrogen determination, alkali fusion, and infrared and ultraviolet spectrophotometry.

Preparation of melanins. Pigments were prepared from puparia of the fleshfly *Sarcophaga bullata* (8), aged banana skin (21), black human hair (24), and

inksac of *Octopus bimaculoides* (22), or were synthesized from L-dihydroxyphenylalanine (L-dopa) with alkaline copper sulfate (5). Melanin labeled with ^{14}C was prepared from L-[*U*-^{14}C]tyrosine with tyrosinase purified from *Psalliotia arvense* (20, 26). The reaction medium contained: L-tyrosine, 0.5 μmol; L-[*U*-^{14}C]tyrosine, 85 μCi; and tyrosinase (28 U/mg), 10 mg in 10 ml of phosphate buffer, pH 6.8. After incubation for 90 h at 25°C, the insoluble pigment was sedimented, washed, and extracted twice with 20 ml of 2 M HCl at 80°C, again washed with water, and dried. Specific activity was 9×10^5 cpm/mg. L-[*U*-^{14}C]tyrosine (specific activity, 75 mCi/mmol) was purchased from New England Nuclear Corp., Boston, Mass. Natural and synthetic melanins were subjected to elemental analysis and examined in an infrared spectrophotometer as KBr disks. Excellent agreement with published values was observed (21).

Analytical methods. Bound phenolic and indolic substituents were identified by alkali fusion (11) followed by thin-layer chromatography on Whatman cellulose 300 CM sheets irrigated with *n*-butanol–acetic acid–water (60:15:25, vol/vol). Identities were verified by high-voltage electrophoresis in 0.2 M pyridine acetate, pH 4.5. Compounds were visualized by fluorescence quenching at 254 nm or by the Folin-Ciocalteu reagent for phenols. Phenols and indoles were revealed with $FeCl_3$ in ethyl alcohol and with diazotized sulfanilic acid (6). Amino acids were detected in a Beckman model 119C automatic analyzer (25). Radioactivity was measured in a Packard radiochromogram scanner and a Tri-Carb scintillation spectrometer with 10 ml of Aquasol (New England Nuclear Corp.); the counting efficiency was 64%. Samples were corrected for quenching by addition of an internal standard. Fractions from molecular sieves were scanned in the ultraviolet portion of the spectrum in a Cary recording split-beam spectrophotometer and in the infrared as KBr disks.

Enzymatic hydrolysis of fungal cell walls. Pale or darkly colored mycelia (100 mg, dry weight) were treated with 10 mg of a 1:1 mixture of commercial cellulase (Sigma Chemical Co., St. Louis, Mo.) and chitinase (Gallard-Schlesinger, Long Island, N.Y.) for 30 min at pH 6.8 at 25°C in 10 ml of 0.1 M ammonium acetate. Samples were prepared for scanning electron microscopy by coating with silver conducting paint. After addition of a drop of antistatic formulation and drying, the stubs were immersed in liquid nitrogen and examined in an American Metals Research instrument, model AMB 1000.

RESULTS

Isolation and growth characteristics of *A. fumigatus*. Soil microflora from garden, forest, and compost were first enriched on sucrose-nutrient broth and then examined for utilization of melanin in Czapek medium devoid of sucrose. Organisms from a dark compost fortified with coffee grounds consumed 10% of the insect melanin in 5 weeks, after which the rate increased until 60% was consumed over the next 3 weeks. Lesser rates were maintained by organisms from an acid loam rich in pine needles; 15% of the pigment was utilized in 8 weeks. Streak plating of the culture derived from coffee compost afforded a colony capable of relatively rapid degradation of melanin. This isolate was subsequently identified as *A. fumigatus*. Microflora of alternate soil samples were not investigated further, although significant melanolysis characterized several other specimens. A significant increase in the rate of melanin utilization by *A. fumigatus* was recorded when sucrose-nutrient broth was replaced with Czapek broth, which favors fungal development; over 80% of the substrate was rendered soluble in 6 weeks. Mycelial growth was generally restricted to the meniscus; the liquid broth remained transparent with gradual darkening. Rate and hue were characteristic for each type of melanin. Cultures devoid of melanin remained clear buff. Microscopic examination of the melanin particles at ×400 magnification revealed a pale thin coat of mycelia surrounding each granule of substrate. Optimum conditions for growth of *A. fumigatus* on insect melanin were assessed by culture in Czapek medium adjusted from pH 4.5 to 9.5. Cells at each pH increment were grown at 23, 30, and 37°C to establish the temperature preference (Fig. 1). Utilization was greatest at 30°C, pH 6.5; hence, these conditions were maintained in subsequent studies. Substitution of molar equivalents of ammonium nitrate for ammonium chloride, or asparagine for sucrose, did not enhance catabolism. Slow agitation with a glass bead improved pigment breakdown, although the amount of mixing was insufficient to displace the granules from the bottom of the tube or the fungal mat from the meniscus. Disappearance of the substrate was not hindered by accumulation of dark cleavage products in the medium; active melanolysis characterized the entire growth interval.

Consumption of different melanins by *A.*

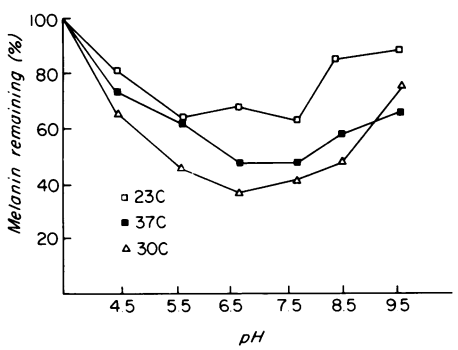

FIG. 1. *Growth of A. fumigatus on insect melanin in sucrose-Czapek broth at selected pH and temperatures. Time of incubation, 8 weeks.*

fumigatus. The ability of diverse melanins to sustain growth was ascertained with five pigments of widely different origin and composition. In agreement with published values for percentage of nitrogen, the range included: insect, 6.8%; hair, 4.0%; banana, 0.74%; octopus, 6.33%; and L-dopa (synthetic), 8.4%. Polymer individuality was also confirmed by ultraviolet and infrared spectra and by the products of alkali fusion as revealed by thin-layer chromatography. Purification included reflux in strong base or acid which effectively reduced amino acid titers to 10% or less as verified by amino acid analysis. All melanins examined were degraded by the fungus when presented on the basis of equal weights per incubate (5 mg/2.5 ml) (Fig. 2). With the exception of banana black, rate was uniform from weeks 5 to 8; synthetic (L-dopa) melanin sustained the most and insect pigment sustained the least catabolism. The relative position of the curves did not vary, although the rate of utilization for any one melanin fluctuated ±10% between experiments. It is evident, furthermore, that reduced breakdown was a consequence of omission of sucrose and that the decline was greater for L-dopa melanin than for blowfly pigment. The color of the liquid medium was not related directly to the solubilization rate; products of L-dopa melanin degradation imparted a red-beige coloration, whereas insect and banana afforded chocolate or black.

On incubation with melanin generated from L-[*U*-^{14}C]tyrosine with mushroom tyrosinase, substantial quantities were converted to $^{14}CO_2$, probably at the expense of soluble metabolites (Fig. 3). Whereas insoluble radioactive pigment was converted to soluble materials at a fairly constant rate for the entire 5-week interval, CO_2 production was enhanced fourfold from weeks 3 to 5. When the uninoculated control was examined for melanin at the end of the experiment, 95% of the starting particulate material was recovered, but the flask with the fungus contained only 8%. In both cases, specific radioactivity of purified substrate melanin recovered after incubation was unchanged from that of the starting material.

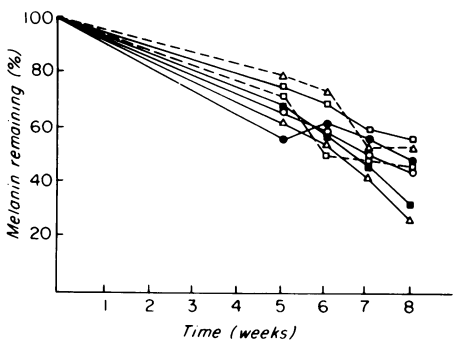

FIG. 2. *Degradation of various melanins by A. fumigatus. Symbols:* (——) *complete medium with sucrose added;* (- - -) *medium without sucrose;* (■) *insect;* (△) *dopa;* (□) *hair;* (●) *banana;* (○) *octopus.*

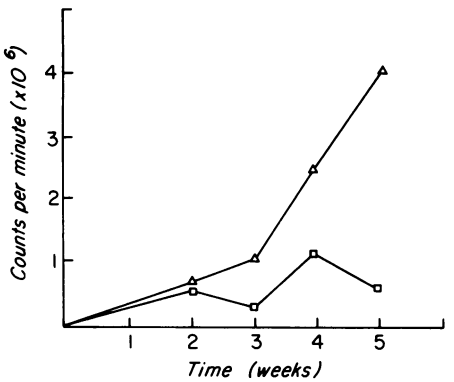

FIG. 3. *Utilization of [U-*^{14}*C]melanin (tyrosine) by A. fumigatus. Total counts initially, 3.6 × 10*^{7} *cpm. Symbols:* (△) $^{14}CO_2$; (□) *nonvolatile soluble metabolites.*

Chemical studies on products of melanin degradation. Radioactive melanin synthesized with mushroom tyrosinase was converted to CO_2 and soluble metabolites to the extent of 7 and 24%, respectively. Of the remaining label, 56% could be accounted for in the mycelial mat. Fractionation of the radioactive fungus (Fig. 4) revealed 55% of the radioactivity in the cells to be localized in the dark fungal pigment synthesized in the course of development. Fungal lipid retained 4% of the label, with insoluble chitin and soluble protein containing 5 and 6%, respectively. Compounds not retained by sulfonic acid cation-exchange columns and not reacting with ninhydrin, including neutral sugars, are reported as unidentified acidic components, and represented about 10% of the label recovered from the fungus. Strongly basic ninhydrin-reactive components were eluted with 0.2 M NaOH and comprised an additional 10% of the label.

Incorporation of isotope into fungal melanin and protein-derived amino acids is recorded in Table 1. The degree of incorporation into amino acids could be divided into two groups, those with specific activity less than 100 and those of 2,200 or greater. Specific activity of the deproteinized cellular pigment exceeded that of amino acids from protein by an order of magnitude or more. Although half the total radioactivity was recovered in the fungal pigment (Fig. 4), the bulk of the carbon in the depot derived from sucrose as revealed by a 10-fold diminution of

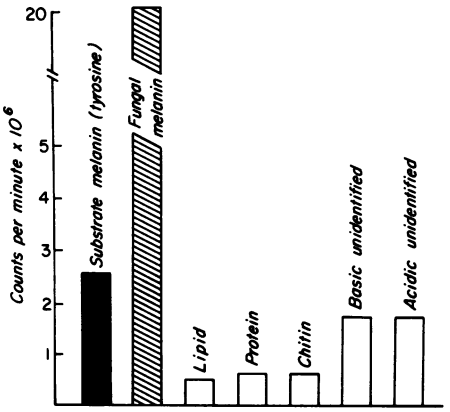

FIG. 4. *Distribution of radioactivity in A. fumigatus cultured for 5 weeks on enriched media with [U-^{14}C]melanin (tyrosine). For preparation of fractions, see text. Total radioactivity in original melanin, 3.6 × 10^7 cpm.*

TABLE 1. *Amino acid composition and specific activity of A. fumigatus allomelanin-protein complex grown on [U-^{14}C]melanin (tyrosine)*[a]

Constituent	Amt (mol/10^5 daltons)	Sp act (cpm/μmol)
Substrate melanin (tyrosine)		152,000[b]
A. fumigatus melanoprotein		
Aspartic acid	110	4,920
Threonine	62	4,200
Serine	68	4,050
Glutamic acid	110	60
Proline	34	9,520
Glycine	101	50
Alanine	90	42
Half-cystine	79	40
Valine	42	40
Methionine	63	5,710
Isoleucine	19	40
Leucine	20	30
Tyrosine	18	4,880
Phenylalanine	24	40
β-Alanine	27	3,660
Histidine	22	6,250
Lysine	68	2,210
Arginine	30	5,300
Glucosamine	9	2,700
Allomelanin pigment		14,000[b]

[a] Procedures for separating amino acids from pigment are described in the text.

[b] Based on assumed residue weight of 150 daltons.

specific activity. On the basis of low specific activity, less than 100 cpm/μmol, the contribution of sucrose to the carbon pool of the protein fraction exhibited noteworthy selectivity; glutamic acid, glycine, alanine, valine, leucines, cystine, and phenylalanine originated almost entirely from exogenous carbohydrate. On the other hand, aspartic acid, serine, threonine, proline, methionine, tyrosine, β-alanine, histidine, lysine, and arginine were partly derived from substrate melanin, since specific activity ranged from 2,210 to 9,520 cpm/μmol for these residues. Although tyrosine furnished carbon directly to substrate melanin via enzymatic synthesis, specific activity did not exceed that of other labeled residues in protein.

Approximately 80% of the radioactive melanin, 29 × 10^6 cpm, was recovered as cellular constituents (Fig. 4). An additional 10% was present in the medium in soluble form at the end of the incubation period. The average molecular weight of the latter fraction was assessed as 250 by chromatography on Bio-Gel P-2 (Fig. 5A). Thin-layer chromatography of peak I revealed two components of equal radioactivity at R_f 0.35 and 0.63; the former was fluorescent, and the latter responded to color tests indicative of indoles. For the component exhibiting R_f 0.35, λ_{max} was 261 nm and λ_{min} was 280 nm; for the component exhibiting R_f 0.63, λ_{max} was 280 nm

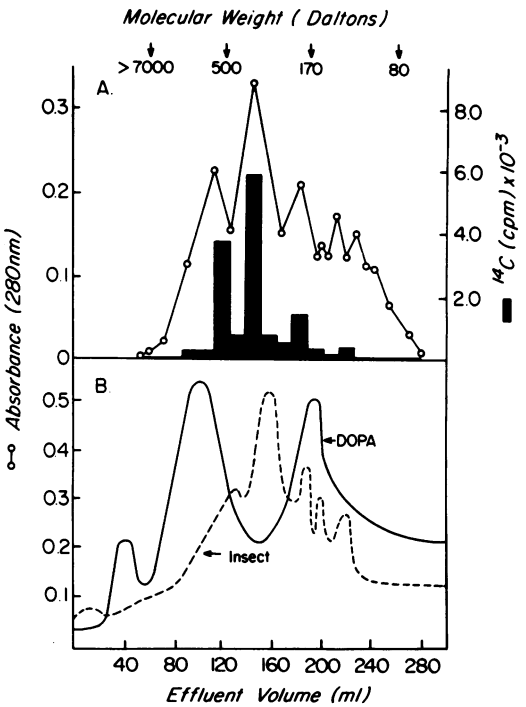

FIG. 5. *Chromatography on Bio-Gel P-2 (2 by 50 cm) of metabolites accumulating in media from cultures incubated on diverse melanins with 0.5 M ammonium acetate buffer. (A) L-[U-^{14}C]tyrosine melanin; (B) insect or L-dopa melanin.*

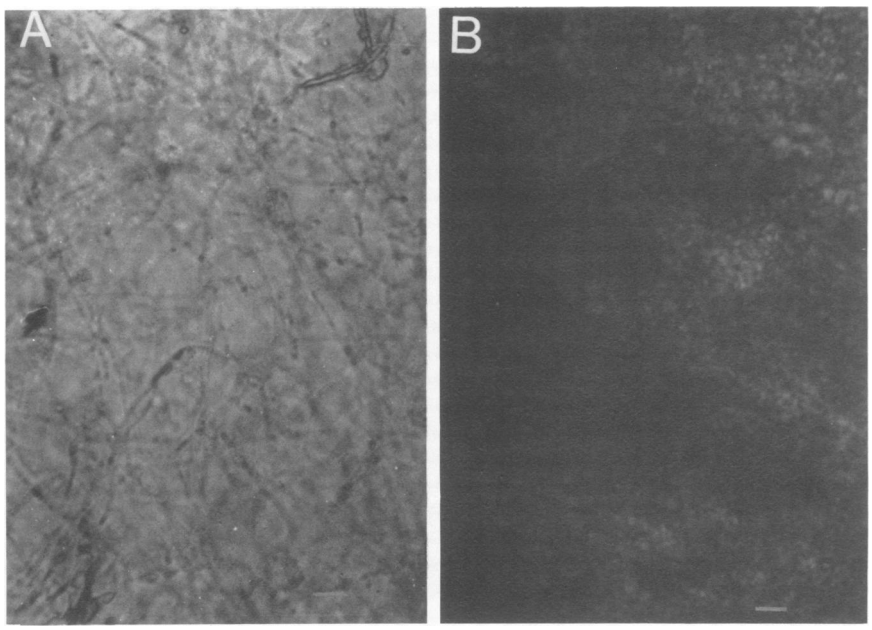

FIG. 6. *Mycelia grown (A) without and (B) with insect melanin (×400); bar represents 50 μm. Photographed at equal sample thickness and development and exposure times.*

and λ_{min} was 330 nm. Both substances reacted positively with reagents such as $FeCl_3$ and diazotized sulfanilic acid, indicating the presence of phenolic hydroxyl groups. Differences in fungal metabolites were revealed by chromatography of products accumulating in the medium after growth on melanins of separate origin (Fig. 5A). Insect melanin afforded two fractions on chromatography, one component issuing from the column at the void volume and a smaller entity of about 170 daltons. On the other hand, very little high-molecular-weight material characterized the products of L-dopa or tyrosine melanins, the elution patterns of which were similar and in keeping with the close chemical relation between the two precursors (Fig. 5B).

Properties of mycelial pigments from cultures grown on melanins. Light microscopy revealed that darkening of the fungal mat originated from deposition of pigment granules within the mycelial strands (Fig. 6). Transfer of spores from pigmented cultures reared on melanin to Czapek broth or slants prepared without melanin resulted in vigorous growth of pigment-free mycelia. The purified mold pigment differed from substrate in solubility in alkali and in a lighter brown color. The pigment formed by *A. fumigatus*, from [^{14}C]melanin (tyrosine), although highly radioactive, differed significantly in infrared spectral features from the starting material, especially at 3.5, 6.5 and 7.2 μm (Fig. 7). It is also evident from infrared scans that fungal pigment varied in composition with the source of precursor material. When grown on insect melanin, a deep inflection was evident at 6.6 μm that was absent from pigment derived from tyrosine melanin. Noteworthy differences were also recorded in the hydroxyl band at 3 μm. Thin-layer chromatography on cellulose sheets irrigated with *n*-butanol–acetic acid–water (60:15:25, vol/vol) revealed 5,6-dihydroxyindole (R_f 0.65) and 3,4-dihydroxybenzoic acid (R_f 0.81) as products of alkali fusion of both substrate melanins, but not of the corresponding fungal pigments.

Fungal susceptibility to enzymatic lysis. Scanning electron microscopy clearly showed that melanized mycelia were more resistant to 30-min digestion by β-(1,3)-glucanase and chitinase than cultures grown in the absence of melanin. There was extensive pitting of the conidiophore and loss of sculptured detail on sterigmata and conidia from pale specimens grown on melanin-free media (Fig. 8B). When enzyme treatment was prolonged to 90 min, many features of the darkened mycelia were still recognizable, whereas controls were reduced to thin amorphous nodules. Mycelia developing on tyrosine melanin and insect melanin could not be distinguished on the basis of stability to lytic carbohydrases in spite of differences in chemical structures.

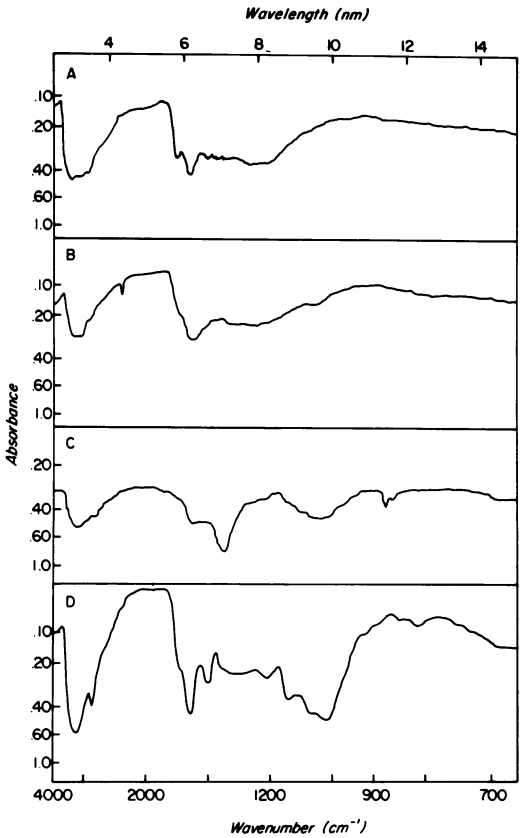

FIG. 7. *Infrared spectra of substrate and fungal melanins. (A) Substrate melanin (insect); (B) substrate melanin (tyrosine); (C) fungal allomelanin from insect melanin; (D) fungal allomelanin from tyrosine melanin.*

DISCUSSION

As pH 4.5 to 8.5 and 23 to 37°C, *A. fumigatus* readily degraded melanins of widely different origins and compositions over the course of 4 to 8 weeks on Czapek medium (Fig. 1 and 2). The culture from mixed coffee and garden compost was chosen for further study on the basis of significantly higher lytic activity compared with other sources; nevertheless, melanin degradation was measured in six different soil samples from the Boston area. The rate of substrate utilization was comparable with that of other arylated phenolic polymers joined by carbon-carbon bonds between adjacent rings, the resonance stabilization of the benzenoid substituents and free-radical longevity protecting not only cyclized loci but side chains as well (14, 24). For example, forest soils required 700 h for oxidation of 5% of the rings and side chains of hemlock lignin, approximately 1/10 the rate sustained by endogenous structural polysaccharides (4). High-molecular-weight tannins from pine bark retained more than half the polymer weight after 10 days of exposure to a strain of *Penicillium adametzi* capable of degrading the (+)-catechin precursor and procyanidin dimers and trimers essentially to completion in 50 h (10). Substantial levels of humic acids persisted in cultures of *Aspergillus*, *Arthrobacter*, *Pseudomonas*, and *Penicillium* after 7 weeks (19). In several instances, melanogenic phenols were released, suggesting a ready exchange of carbon between the two polyanions.

A. fumigatus developed optimally at 30°C, pH 6.5, when cultured in Czapek medium (Fig. 1). Visual inspection ascertained that fungal growth was essentially complete in 2 weeks, probably coinciding with exhaustion of sucrose. That melanin could be utilized as a carbon source was indicated by disappearance of 40 to 50% of the substrate after 8 weeks in medium with sucrose omitted (Fig. 2). In general, a heavier mycelial mat was observed with sucrose added, suggesting that the increased melanin catabolism in the presence of this auxiliary carbon source resulted from a greater number of active organisms rather than a higher titer of melanoclastic enzymes per cell. Although a lag period of 5 or 6 weeks usually preceded the interval with greatest utilization on minimal medium, a linear rate was representative of cultures offered sucrose initially.

Eumelanins show great diversity with respect to structure, molecular weight, and association with protein when in the native state (24). Pigments from hair, insect, octopus, banana, and the two synthetic polymers derived from tyrosine and L-dopa were consumed by the fungus after removal of 80 to 85% of the protein by prolonged extraction with concentrated HCl or NaOH. Growth, therefore, was at the expense of the benzenoid and indolic polymer constituents. The identity of the substrates was confirmed by examination in the infrared, by elemental analysis, and by alkali fusion where the differences were manifest, principally in the ratio of the two most stable cleavage products, 3,4-dihydroxbenzoic acid and 5,6-dihydroxyindole (10). Regardless of the origin of the polymer, *A. fumigatus* subsisted vigorously on the pigment, converting more than 80% to cellular components such as protein, polysaccharide, fungal pigment, and soluble by-products accumulating in the medium (Fig. 3 and 4). Whereas slow evolution of CO_2 from tyrosine melanin added to potting soil could be construed as decarboxylation of peripheral phenolic acids (2, 14), in the present study conversion of 90% of the polymer to CO_2, soluble metabolites, and other cellular constituents at-

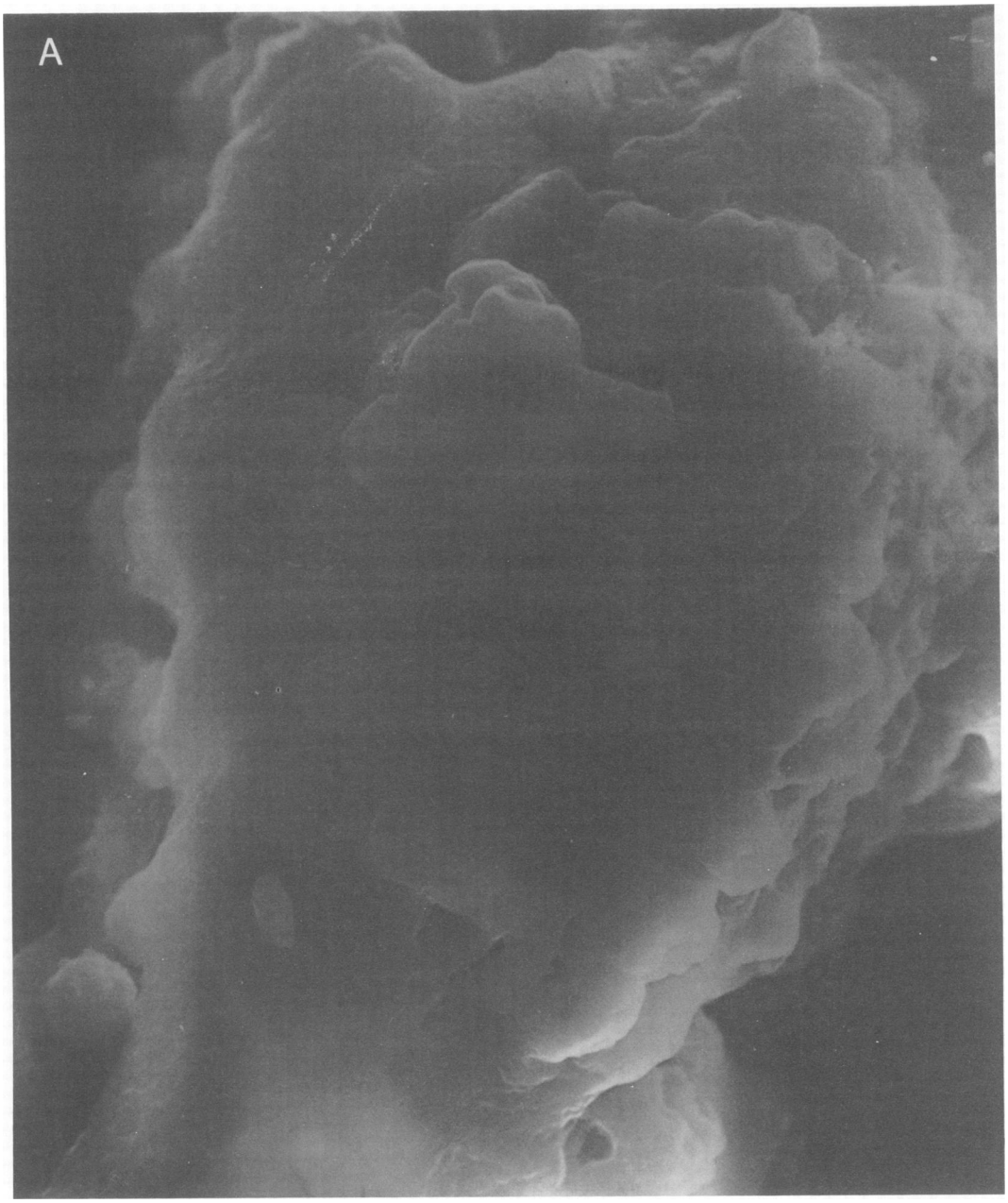

FIG. 8. *Effect of β-glucanase and chitinase on exterior fine structure of A. fumigatus with normal and melanized pigmentation. Scanning electron microscopy (×1,600). (A) Czapek-sucrose grown; (B) same medium plus insect melanin. For details, see text.*

tests to appreciable depolymerization and ring scission by *A. fumigatus*. Gel filtration established that the bulk of the radioactivity in the medium was in the molecular weight range of 150 to 250 (Fig. 5). Ultraviolet-absorbing material from the major fraction, peak I, comprised the bulk of the counts applied to the column and also responded to the $FeCl_3$ test for hydroxyindoles. The ultraviolet spectrum, infrared scan, and mobility on thin-layer chromatograms were similar to those of authentic hydroxyindoles, although definitive characterization was impossible because of instability of both fungal products and standards. Soluble metabolites could

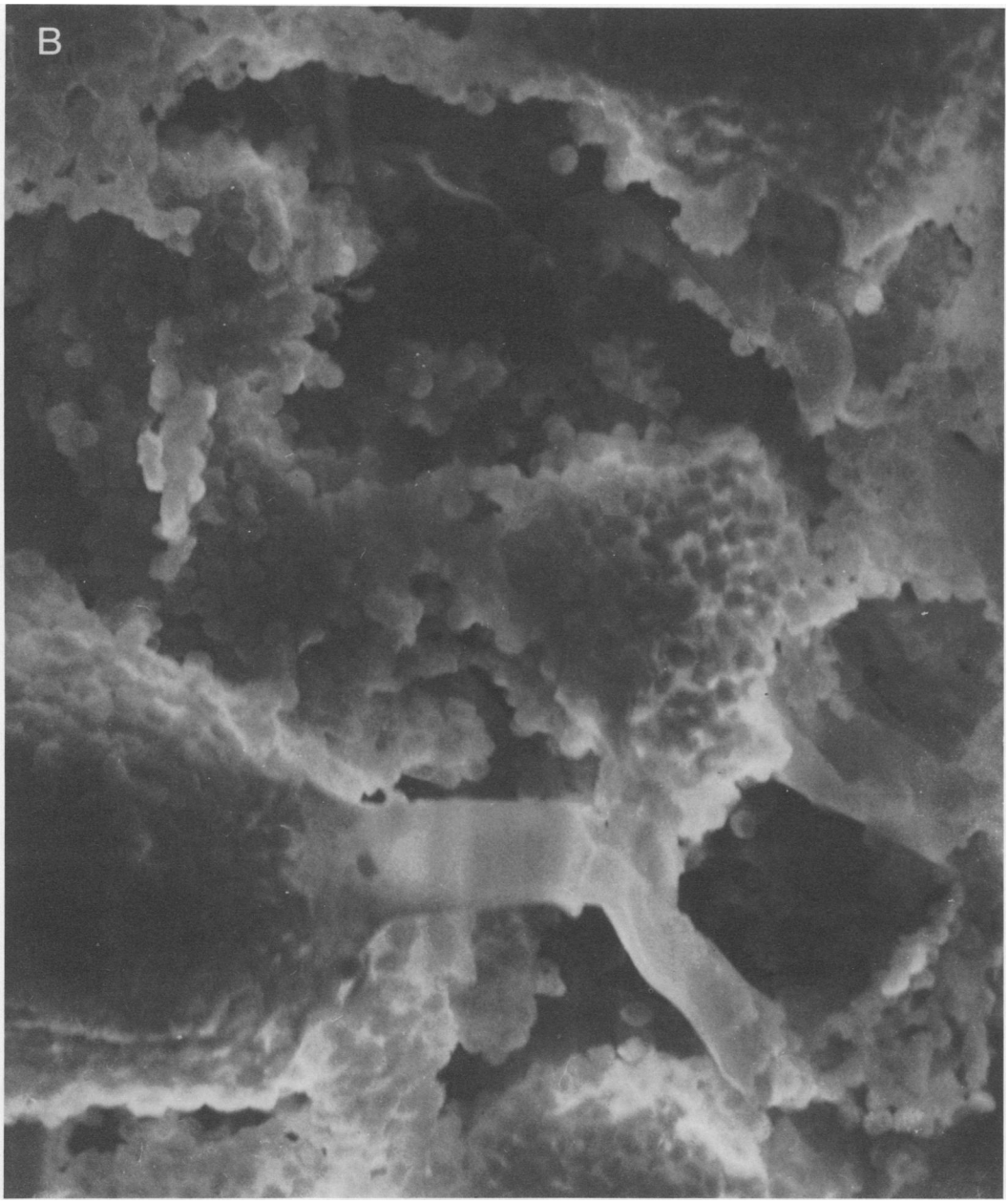

FIG. 8B.

originate directly from polymer scission or could be secondary products of fungal metabolism. With the exception of CO_2, the products accumulating in the medium probably did not represent initial catabolites, the majority of which may have been highly reactive and prone to spontaneous oxidation and rearrangement under the culture conditions.

Tyrosine melanin uniformly labeled with ^{14}C contributed materially to cell composition (Fig. 4). Protein plus chitin accounted for 11% of the radioactivity, lipid accounted for 4%, and unidentified basic and acidic components accounted for 10% each. The highly charged acidic and basic fractions included aromatics judged by absorption maxima between 250 and 280 nm, probably consisting of phenolic acids, aminophenols, and indoles. The mole ratios of the

amino acids in the fungal hydrolysate showed a preponderance of aspartic and glutamic acids and little histidine, in many respects resembling a similar fraction from *E. echinulatum* (23). After deproteinization, the bulk of the radioactivity (55%) was recovered as a dark fungal pigment, insoluble in HCl and resembling allomelanins from *A. niger* and pigmented *Verticillum* and *Penicillium* in hydrolysis products (27). Production of fungal pigment exceeded exogenous melanin sixfold, establishing that de novo synthesis accompanied melanolysis. The ready transfer of radioactivity from substrate to fungal pigment, therefore, was not the consequence of engulfment or occlusion, but did result from induction of an enzyme system in the unpigmented cells transforming the products of melanin catabolism to a new polymer. This system was moderate in specificity, producing dark fungal polymers with different infrared records depending on the melanin of origin (Fig. 7).

The pathway of melanin catabolism in the transfer of carbon to allomelanin and cell protein is obscure. In the presence of sucrose, significant randomization of label was observed in aspartic acid, serine, threonine, methionine, tyrosine, β-alanine, histidine, lysine, arginine, and glucosamine as judged by a diminution of specific activities compared with the substrate. The virtual absence of radioactivity in glutamic acid, glycine, alanine, and cystine indicates distinct compartments for enzyme systems responsible for synthesis of melanoproteins and cell proteins in the presence of exogenous carbohydrate. Such a distribution would obtain if both peptide and pigment moieties of allomelanin were synthesized solely from radioactive substrate melanin, amino acids associated directly with the fungal pigment consisting of those listed above as characterized by appreciable radioactivity. Synthesis of those residues in the cell protein, on the other hand, could derive principally from sucrose and ammonium salts, affording amino acids of low specific activity on hydrolysis.

A. niger, *A. nidulans*, *A. phoenicia*, and other dark species release lesser amounts of cell wall carbohydrate than do unpigmented strains after exposure to chitinase and glucanase (2, 3, 14). These observations have been extended to *A. fumigatus*, using microscopy as a criterion. Evidence of extensive modification of unpigmented mycelia can be deduced from Fig. 8; the damage included fusion of conidial protuberances, invagination of interconidial regions, and blistering of the sterigma and conidiophore. Digestion was rapid and essentially complete in 90 min. Inhibition of hydrolases by exogenous melanin has been ascribed to free radicals trapped by the pigment interacting with sulfhydryl or other sensitive groups on the enzymes (3, 14). Under natural conditions, rates of melanin degradation would be influenced not only by soil pH and the availability of nutrients, but also by the presence of other organisms utilizing intermediates released from the melanoprotein complex by *A. fumigatus*.

ACKNOWLEDGMENTS

We thank Margaret Bigelow, of the University of Massachusetts/Amherst, and the Taxonomy Group of the U.S. Department of Agriculture Fermentation Laboratory, Peoria, Ill., for taxonomic advice. Jerome Woodinsky, of Brandeis University, generously provided inksacs from *Octopus bimaculoides*. Invaluable assistance with isolation of melanin catabolites was provided by E. Zomer, P. Banda, and Keith Strout.

This investigation was supported in part by National Science Foundation grant PCM 75-22123.

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