Pergamon

0031-9422(94)00837-X

Phytochemistry, Vol. 39 , No. 1, pp. 49–55, 1995

0031-9422/95 $9.50 + 0.00

A COMPARISON OF NATIVE AND SYNTHETIC MUSHROOM MELANINS BY FOURIER-TRANSFORM INFRARED SPECTROSCOPY

JOHN A. PIERCE* and DORA M. RAST†

Institute of Plant Biology, University of Zürich, Zollikerstrasse 107, CH-8008 Zürich, Switzerland

(*Received* 16 *August* 1994)

Key Word Index—*Agaricus bisporus*; Agaricaceae; common mushroom; melanin; structure; synthesis; γ-glutaminyl-4-hydroxybenzene; Fourier-transform infrared spectroscopy; cross-links; phenolase.

Abstract—The diffuse reflectance Fourier-transform infrared spectra of the spore of the cultivated mushroom, *Agaricus bisporus*, of the melanin isolated therefrom and of various synthetic melanins related to the native material were recorded. These were prepared from γ-glutaminyl-4-hydroxybenzene (GHB; the presumed precursor of melanin in this fungus) as well as from simple phenol analogues of GHB, through auto- or enzyme-mediated oxidation and with, or without, potential non-phenolic co-reactants. The spectrum of the spore is dominated by distinct IR absorptions due to melanin, chitin and protein, while the isolated pigment is characterized mainly by the melanin absorption band at 1600 cm^{-1}, which can be attributed primarily to aromatic groups whose signal is enhanced by phenol functionalities and, to a lesser extent, by amido groups. In addition, pronounced alkyl and carbonyl absorption bands were observed, and there is evidence of extensive C–O and C–N bonding in the biopigment. The spectrum of the melanin made from GHB through enzyme catalysis (phenolase, EC 1.14.18.1/1.10.3.1) most closely matched that of the biopigment. Use of 4-aminocatechol (AC) instead of GHB afforded a product which was similar to the native melanin, whereas the pigment obtained by air oxidation of 4-aminophenol (AP) was largely dissimilar. Cross-linking of the reacting phenol with protein was apparent in the melanin formed enzymatically from AC, but no evidence for such reactivity was present in the AP-derived melanin. Preformed chitin did not become incorporated into either synthetic melanin. The fungus does not synthesize melanin from AP, nor directly from GHB, but from AC in a proteinaceous environment represented in part by the phenol oxidase itself.

INTRODUCTION

The melanin of the common mushroom *Agaricus bisporus* (Lange) Sing., a basidiomycete, appears to be distinct from that of ascomycetous fungi inasmuch as γ-glutaminyl-4-hydroxybenzene (GHB), and/or its deglutaminyl derivative, 4-aminophenol (AP), serves as the phenolic precursor educt [1–4]. The pigment is located in the wall of the spore [5], where it occurs in the form of electron-dense grains of restricted size range [6]. Since there are a variety of potential co-reactants present at the site of melanogenesis, including chitin, β-1,3-glucan and protein [5], the natural pigment material presumably contains covalently bound non-phenolic residues, even when present in an extensively solvent-purified form. Because of its complex heteropolymeric nature and extremely poor solubility, detailed structural features of a melanin cannot be successfully elucidated by straightforward, classical methods alone (see ref. [7]). The analysis of synthetic products related to the relevant native material represents another, complementary approach in tackling this problem and has been performed previously with tyrosine- and tryptophan-derived melanin [8–13]. Neither of these types of melanin occurs in fungi [3, 4]. The present study was carried out with the following aims: (i) to gain further insight into the identity of the phenolic melanogenic educt of *A. bisporus*, (ii) to provide additional evidence for the tent [14] that phenolase-catalysed synthesis of melanin results in covalent binding of the enzyme to the polymeric reaction product, and (iii) to analyse whether the 'melanin reactivity,' as described in ref. [15] for example, also involves other components of the wall. The choice of analytical method, DR FT-IR spectroscopy, was guided by its advantages over similar techniques; namely, detection of extremely low amounts of solid insoluble material, obviation of problems of sample preparation [16] and previous application to a variety of complex biomaterials such as coal [17, 18].

*Present address: Department of Environmental Health, School of Public Health, Loma Linda University, Loma Linda, CA 92350, U.S.A.

†Author to whom correspondence should be addressed.

RESULTS

The IR spectrum of the whole spore material compared to that of the purified melanin clearly demonstrates the

efficacy of the isolation procedure (Fig. 1), as shown by the disappearance of the intense features centred at 1060 cm^{-1} in the spectrum of the spore and in a diminution of the relative intensity of the bands between 1700 and 1500 cm^{-1}. In fact, one of the least intense significant features in the IR spectrum of the spore, at *ca* 1200 cm^{-1}, becomes one of the most prominent characteristics in the spectrum of the isolated native melanin. The spectrum of the purified melanin also displays a large relative absorbance in the 1500–1100 cm^{-1} region, attributable to substantial C–O and C–N functional groups. In addition, the presence of aromatic species with extensive phenolic substituents is probable, as are amino and amide groups, highly conjugated quinone species, and carboxylate functionalities, all of which would contribute both to the relatively intense 1600 cm^{-1} band and to additional absorption features in the fingerprint region (2000–600 cm^{-1}). The presence of aliphatic material is evident in both spectra by the set of bands centred at 2950 cm^{-1} (due to C–H stretching) amid the broad absorbance due to O–H and N–H bond stretching. Additionally, there are distinct absorption bands in the spectrum of the purified melanin near 1400 cm^{-1} that are also due to aliphatic groups.

The procedure proposed by Piatteli *et al.* [19] to isolate melanin from fungi involves treatment with 20% HCl for an extended period and leads to removal of non-polymerized material and degradation of most of the biopolymers, with the exception of the structures that possess a covalent acid-resistant linkage to the phenolic polymer. The possibility, therefore, exists that the final isolate could contain (some) residual protein as well as chitin (see Introduction). This was queried by analysing the IR spectra of phenolase and chitin from *A. bisporus* (Fig. 2). Both spectra possess strong absorbance bands

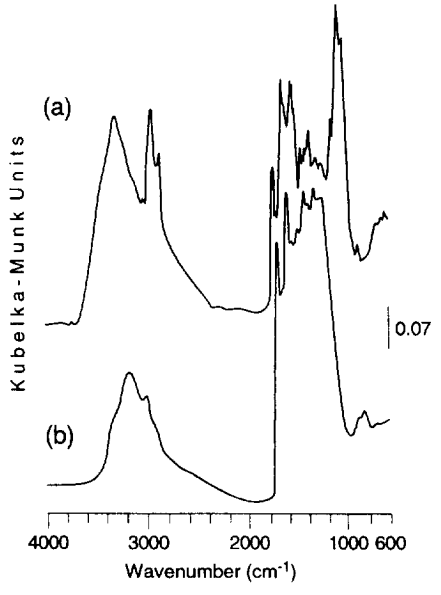

Fig. 1. DR FT-IR spectrum of (a) the *A. bisporus* spore, and (b) the isolated melanin therefrom.

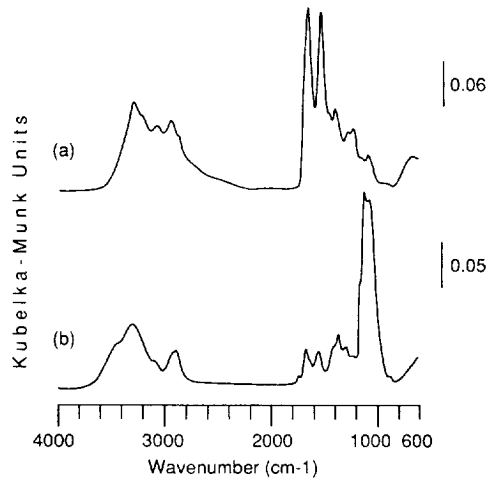

Fig. 2. DR FT-IR spectra of polymers present at the site of melanin deposition *in vivo* (the wall of the mushroom spore) and in the incubation mixture used to synthesize melanin *in vitro* (see also caption to Figs 4 and 5): (a) phenolase and (b) chitin, both from *A. bisporus*.

which can also be seen in the spectrum of the native melanin (Fig. 1b). This is particularly noticeable in the strong amide absorption bands of the enzyme protein at 1550 and 1650 cm^{-1}. Chitin, on the other hand, displays one intense feature centred at 1060 cm^{-1} due to stretching of the C–O bond in the *N*-acetylglucosamine residue, while the spectrum of the isolated material—in contrast with that of the spore (Fig. 1a), which shows absorption features due to chitin—displays only limited absorbance at this wavenumber. Thus, whereas the native melanin definitely contains protein, there is a negligible amount of residual chitin present. The C–H, N–H and O–H stretching frequencies from 3600 to 2400 cm^{-1} also show some, minor, similarities between the spectra of isolated melain and phenolase and chitin (compare Fig. 1b with Fig. 2), indicating the presence of aliphatic, amino, alcoholic, phenolic, and carboxylic acid groups.

The IR spectra of the three phenol compounds, namely AP, AC (4-aminocatechol) and GHB, used to prepare the synthetic melanins are given in Fig. 3. Besides the sharp absorption features in the 1700–1500 cm^{-1} part of the fingerprint region (note the large relative absorption caused by the amide bond in the spectrum of GHB), these spectra display characteristic amine absorption bands in the region 3300–2300 cm^{-1}. This is most apparent in the doublet centred at 3250 cm^{-1} in the spectrum of AP, indicating the primary amine: the corresponding doublet for AC is only partially observable, being obscured by strong background absorption of O–H bonds, while the single band at 3250 cm^{-1} in the spectrum of GHB denotes the presence of the secondary amine.

It is apparent from Fig. 4, with AP as the melanogenic phenol, that—regardless of whether the conditions for melanin synthesis are autoxidative or enzyme-catalytic or whether protein and chitin are present or whether there is a large molar excess of AP relative to BSA (bovine serum

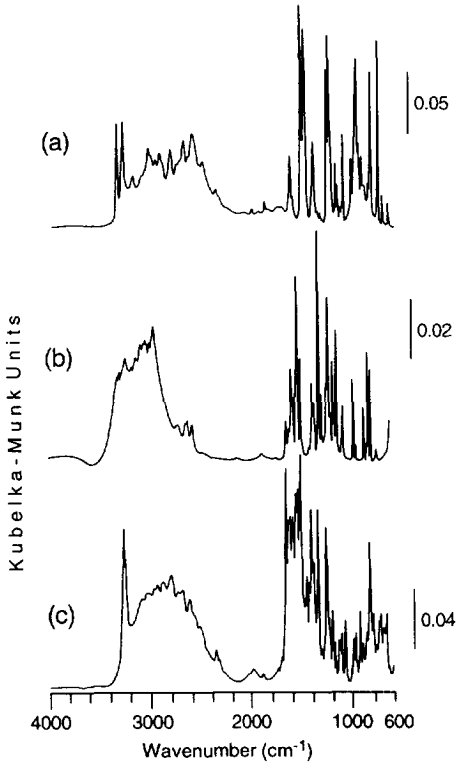

Fig. 3. DR FT-IR spectra of the phenolic educts used to prepare synthetic melanin (see also captions to Figs 4 and 5): (a) 4-aminophenol (AP), (b) 4-aminoacetechol (AC), and (c) γ-glutaminyl-4-hydroxybenzene (GHB).

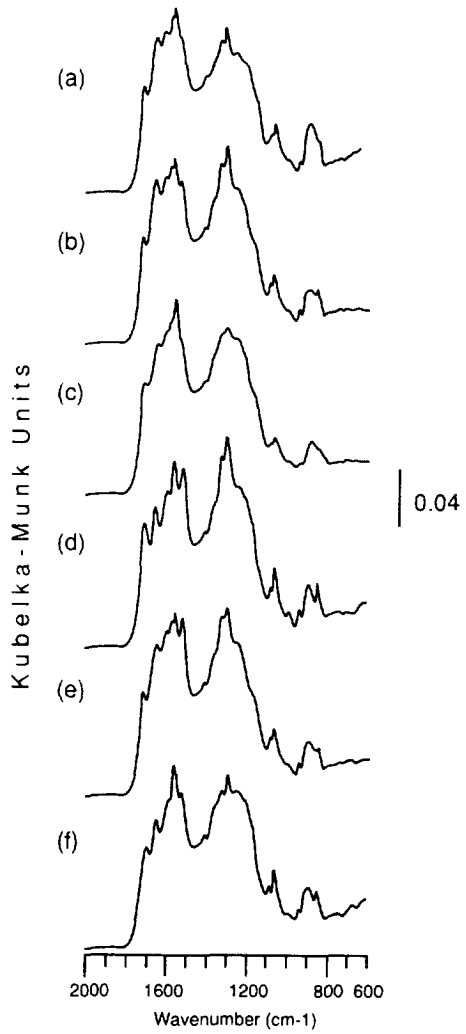

Fig. 4. DR FT-IR spectra of synthetic melanins prepared by using AP under various conditions: (a) autoxidation of AP (200 mg); (b) as (a) but in the presence of protein (BSA, 200 mg); (c) as (b) but in the presence of chitin (15 mg, fine suspension); (d) enzyme-catalysed oxidation (phenolase, 15 mg) of AP (200 mg); (e) as (d) but in the presence of protein (BSA, 200 mg); (f) as (e) but in the presence of chitin (15 mg, fine suspension).

albumin)—the synthetic melanins thus formed consistently result in the same chemical structure as judged by their DR FT-IR spectra. (Only the fingerprint region is shown, as the C–H, N–H and O–H stretching region does not afford any detailed information for purposes of comparison.)

The *ortho* hydroxylation of any phenol, particularly if non-enzymatic, is known to be a slow process [20], but once the catechol has been formed a variety of quinoid products are rapidly generated resulting in an augmented ability to randomly polymerize to a multitude of slightly different melanogenic species. The melanin prepared autoxidatively with AC does not, therefore, exhibit any distinct features in its DR FT-IR spectrum (Fig. 5a), but rather shows broad unresolved bands that culminate in a maximum absorption at approximately 1250 cm^{-1} (see Fig. 5a). Nevertheless, there is some resemblance to the spectrum of native melanin (Fig. 5e), with the exception of the intense band at 1600 cm^{-1}. When phenolase-catalysed melanin synthesis with AC (molar ratio 1300:1, phenol–protein, M_r = 110000 [21]) takes place in the presence of a metallothionein (molar ratio 110:1, AC–protein, M_r = 2000 [22]) the reaction intermediates generated from the diphenol could be surmised to become cross-linked with the metallothionein, as well as with the enzyme itself. This can be assessed by comparing the resultant spectrum (Fig. 5b) with that of chemosynthetic AC-derived melanin (Fig. 5a): an increase in the absorption in the 1600 cm^{-1} region (amide bonds) is apparent, indicating covalent linkage with the protein. The presence of finely dispersed chitin in the enzyme-catalysed melanization reaction mixture with AC produces, however, a melanin whose IR spectrum (Fig. 5c) is much more similar to that of the autoxidatively prepared AC-melanin (Fig. 5a), with the exception of a relative increase in the 1600 cm^{-1} region (due to enzyme protein incorporation). In particular, the large absorption at 1060 cm^{-1} present in the spectrum of the purified fungal chitin (Fig. 2b) is not seen. Finally, when GHB serves as the substrate for the phenolase, the product obtained resembles the native melanin (compare Fig. 5d with 5e).

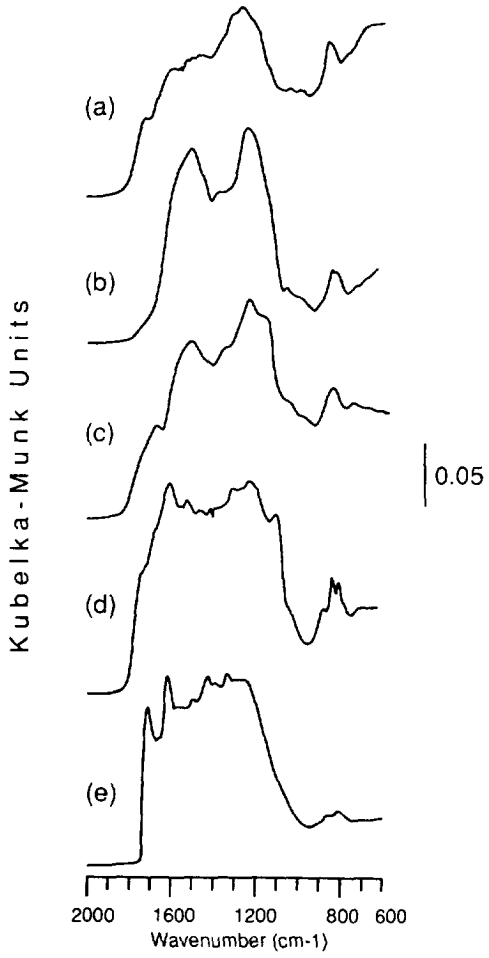

Fig. 5. DR FT-IR spectra of various synthetic melanins. Products prepared by: (a) autoxidation of AC (80 mg); (b) enzyme-catalysed oxidation (phenolase, 15 mg) using AC (200 mg) and metallothionein (10 mg); (c) as (b) using chitin (15 mg, fine suspension) instead of metallothionein; (d) enzyme-catalysed oxidation (phenolase, 15 mg) using GHB (300 mg); and (e) purified mushroom melanin.

However, the two spectra are still different in the 1700–1600 cm^{-1} region in that the strong carbonyl absorbance in the spectrum of the native melanin is absent in that of the synthetic GHB-melanin. Hence, *in vivo* additional material must become incorporated into the product *in statu nascendi*, to provide absorbance between 1700 and 1600 cm^{-1}. Conversely, the spectrum of native melanin does not display the distinct band seen at 1100 cm^{-1} in the spectrum of the melanin prepared enzymatically with GHB, a feature that is attributable to C–O or C–N bonds.

DISCUSSION

Fourier-transform infrared spectroscopy (see Introduction and Experimental) has, indeed, proved to be the method of choice in obtaining spectra of melanin (compare Figs 1, 4 and 5) that are well resolved, and thus amenable to detailed structural analysis. To appreciate this, these spectra should be compared with previous records obtained with dispersive infrared spectrometers operating in the transmission mode, whether relating to melanins of fungal [2, 23–25] or animal [8–10] origin, in which the deleterious effects of IR band saturation are seen.

Structure of the isolated native melanin considering the identity of the melanogenic phenols

One of the strongest absorption bands in the spectrum of the natural mushroom melanin is the feature at 1600 cm^{-1} (Fig. 1b). This can arise from a variety of possible functional groups (amine, carboxylate, conjugated quinone, or aryl enhanced by phenolic hydroxyls). Based on knowledge of the likely substrate (an amine-containing phenol, see Introduction), on IR spectroscopic features of melanins in general [2, 8–10, 23, 24] and on DR FT-IR studies of coals [26] (where carboxylate groups are thought to predominate), we conclude that the 1600 cm^{-1} band results mainly from an aromatic ring system with extensive phenolic groups, and to a lesser extent from amide groups (the N content of this pigment (8.8%) is relatively high [2]), rather than from carboxylate groups, as is the case in tyrosine-based melanin [27]. Furthermore, if the band at 1600 cm^{-1} were due to a carboxylate group there would normally be an almost equally intense band at 1400 cm^{-1}; this is not the case. It is clear that other reactions must occur during melanogenesis resulting in a multitude of substructures of the product which together afford its complexity, such as the significant absorbance at and below 1650 cm^{-1} (see above). This is undoubtedly due to the quinone intermediates and extensive conjugation generated in the course of phenolic radical polymerization. Still further bands can appear as a result of polymerization, attributable to a variety of ethers, with absorbance in the vicinity of 1200 cm^{-1}.

In contrast to the natural melanin, one of the least intense absorption bands in the spectrum of the potential melanogenic phenols (AP, AC or GHB) is this same feature at 1600 cm^{-1}. These monomers do possess distinctive absorption bands (Fig. 3) which would contribute to the IR absorption profile of the isolated native melanin (Fig. 1b) if polymerization did not occur, an important distinction. For example, the amide functional group in GHB imparts bands (Fig. 3c) that would account for the prominent absorption in the 1650–1500 cm^{-1} range and in the vicinity of 1250 cm^{-1} in the spectrum of the native melanin; although the presence of just an aryl-N linkage, as in AP and AC, as well as in GHB, would also suffice for the absorption at the lower wavenumber (Fig. 3a and b). In addition, the methylene groups in GHB would give rise to a band near 1400 cm^{-1}, as observed in the biomaterial. However, the spectrum of this native melanin is quite unlike that of any of the possible precursors, i.e. the

spectrum of any one of the monomers tested is insufficient to explain the absorption profile of the native melanin.

On the other hand, the information gained through the analysis of melanins prepared from AP, AC or GHB proved to be more revealing. Regardless of the experimental conditions for synthesis, namely autoxidation or enzyme catalysis and presence or absence of non-phenolic components in the medium, the set of AP-derived melanins afforded a group of very similar spectra (Fig. 4). There are only slight intensity and bandwidth fluctuations, which are probably due to minute particle size variations of the absorbing materials, which include the alkali halide support matrix, as well as the products themselves (compare with ref. [28]). As the bands observed are inherently narrow, even small changes in their width due to such differences in particle size would have a large effect on their intensity. The similarity of the spectra (Fig. 4a–f) suggests that homoreactivity of AP is more favourable than other possible reactions, such as to preclude any 'cross-reactivity' with non-phenolic components present in the reaction medium, not even with the phenolase, which, as a suicide enzyme [14], is normally entrapped into its melanin product and probably covalently linked to this. Oxidation of AP in aqueous solutions has been the subject of an earlier study [20], but the products identified therein (under the same experimental conditions used here) are eliminated by the action of HCl and subsequent water washings used in the work-up [19] of the melanin analysed. The structure of the resultant AP melanin suggests some type of repeating sub-unit rather than a random orientation of the AP molecules within the synthetic melanin matrix. This conclusion is based upon the appearance of distinct absorption features in the region from 1700 to 1500 cm^{-1} (compare Fig. 3a with Figs. 4a–f), which can be assigned to C=O, C=N, –NHCO–, aryl–NH–, C=C or aromatic groups. These absorption features thus discount any random orientation of the AP in the synthetic melanin. As such, a role for AP in the formation of melanin *in vivo* is doubtful. In contrast to that of AP, the polymerization of the corresponding diphenol (AC) apparently proceeds in a random, non-specific fashion (compare Fig. 4b–f with Fig. 5b–c). Even though the melanin made by autoxidation with AC (Fig. 5a) resembles the native melanin to a certain degree, the absorption in the 1600 cm^{-1} region is, however, still of relatively low magnitude when compared to that of the native product. This difference is possibly due to the absence of (i) protein (see below), (ii) significant amounts of phenol groups or (iii) the –NHCO– group evident in the spectrum of GHB (compare Fig. 3c) as well as in that of the GHB-derived melanin (cf. Fig. 5d). Indeed, the melanin prepared using GHB (Fig. 5d) most closely resembles the native pigment (Fig. 5e). The distinct band seen at 1100 cm^{-1} in Fig. 5d is of note as it only occurs in the spectrum of GHB-derived melanin and not in the spectra of the other synthetic melanins, or in the spectrum of the native melanin. Its origin must therefore be attributed to the glutamyl portion of GHB [since the spectrum of the autoxidation product of AC (Fig. 5a) does not exhibit this feature]. This represents evidence in favour of the view [3] that the glutamyl portion of GHB is detached immediately before melanization commences; the phenolic conjugate, therefore, serving only as the storage form of the much more reactive direct melanogenic precursor phenols AP and, thence, AC.

Structure of native mushroom melanin considering the presence of non-phenolic constituents

The IR spectra of proteins are dominated by absorbances due to the amide bonds, as the amino acid side groups contribute little to the overall spectrum (cf. ref. [29]). The main reason for using non-enzymatic proteins such as metallothionein in these experiments consisted in the enhanced ability of the side groups to react with a specific reagent, such as AC. This effect is evident when metallothionein is present during enzyme (phenolase)-catalysed oxidation with AC, as many new bands develop centred at 1050 cm^{-1} that are attributable to incorporation of protein (compare Fig. 5b with a). Furthermore, when using AC as the substrate for the phenolase, the enzyme itself is incorporated into the melanin, as slight differences at 1700 and 1050 cm^{-1} and a reduction in the relative intensity at 1500 cm^{-1} between this spectrum (Fig. 5b) and that of the material resulting from substituting chitin for metallothionein in the phenolase-catalysed oxidation attest (Fig. 5c). Although the additional presence of chitin in the latter experiment could explain the increase in absorption near 1050 cm^{-1} (Fig. 5c), the feature at 1700 cm^{-1} and the differences at 1500 cm^{-1} can only be due to incorporation of protein (phenolase) (compare Fig. 5c and a). Furthermore, the similarities between Fig. 5b and c and the differences from Fig. 2b show that preformed chitin does not become covalently bound to the phenolase-catalysed AC-melanin.

In the 850–800 cm^{-1} region in the spectrum of the native melanin (Figs 1 and 5e) there are two features at 840 and 800 cm^{-1}. That these bands could be due to the presence of a trisubstituted alkene cannot be ruled out, but amine groups in two different chemical environments could also explain this result. Interestingly, the higher wavenumber feature is only present in the melanin derived from AC and AC plus protein, whereas both of the absorption bands are evident in the AP and GHB melanin as well as in the native melanin (the lower wavenumber band being split in the GHB melanin). The possibility of the presence of amine groups in at least two chemical environments within the natural melanin is even more plausible when one considers the substrates involved (amine-containing phenol and protein) and the multitude of possible reactions which would occur during polymerization. In a recent study of an animal melanin [13], using ^{13}C solid-state NMR spectroscopy, it has been demonstrated that covalent linkages are formed in the pigment *in statu nascendi* between the pigment and protein, the resulting product thus being an insoluble complex heteropolymer. Using DR FT-IR spectroscopy, this has now also been shown to hold for a fungal melanin.

EXPERIMENTAL

Fungal materials. Spores of *Agaricus bisporus* were collected as described previously [3]. Melanin was isolated according to ref. [19]; for details, see ref. [2]. The purification of chitin from mycelium of the mushroom is described in ref. [30].

Preparation of melanins. To obtain chemosynthetic melanin, the following procedure was adopted [31]: dissolution of phenol in K-Pi buffer (50 mM, pH 9.0), aeration of incubation mixture at room temp. for 24 hr, acidification with 6 M HCl to pH 2.0, centrifugation of sample (20000 g, 20 min, room temp.), suspension of pellet in 20% HCl followed by refluxing for 120 hr, collection of insoluble material by centrifugation (as before), resuspension of melanin in H_2O and recentrifugation until supernatant was neutral. The air-dried product was placed under vacuum (10^{-4} Torr, 9 hr) and stored in a desiccator over P_4O_{10} until use. Enzyme-catalysed synthesis of melanin was performed as described earlier [2], followed by the same work-up as for chemosynthetic melanin.

Chemicals/biochemicals. GHB was synthesized and its purity checked according to ref. [32], the *p*-hydroxybenzyloxyaniline was of commercial origin. AC was prepared as described in ref. [33], except that filtration occurred under an atmosphere of argon. All other chemicals were from Fluka (Switzerland). Phenolase (tyrosinase; from *A. bisporus* fruit bodies) and metallothionein (from equine kidney; used as an analogue to the corresponding protein from *A. bisporus* [34]) were purchased from Sigma.

IR spectroscopy. The DR FT-IR spectra were obtained on a Digilab FTS-15 spectrometer fitted with a drop-in diffuse reflectance attachment and a deuterated triglycine sulfate room temp. detector. The spectra are displayed as in the absorption mode, but are expressed as Kubelka–Munk units [35]. Liq. N_2 boil-off was used to purge the instrument of water vapour. Powdered analytical grade KBr was used as reference, samples (500 mg) were mixed with KBr before measurement. One-thousand transients were co-added for the reference and five hundred for each sample, both measured at 4 cm^{-1} resolution.

Acknowledgements—Financial support by the Swiss National Science Foundation (grant No. 31-26961.89) is gratefully acknowledged. J.A.P. thanks Prof. W. von Philipsborn, University of Zürich, for permission to use the FT-IR spectrometer, and Dr Brian M. Pierce, Hughes Aircraft, for useful discussions. Part of this paper was edited while D.M.R. spent a sabbatical leave at Oxford University as a Visiting Scholar of Green College.

REFERENCES

1. Rast, D. M., Stüssi, H. and Zobrist, P. (1979) *Physiol. Plant.* **46**, 227.
2. Stüssi, H. and Rast, D. M. (1981) *Phytochemistry* **20**, 2347.
3. Rast, D. M., Stüssi, H., Hegnauer, H. and Nyhlén, L. H. (1981) in *The Fungal Spore: Morphogenetic Controls* (Turian, G. and Hohl, H. R., eds), p. 507. Academic Press, New York.
4. Wheeler, M. H. and Bell, A. A. (1988) in *Current Topics in Medical Mycology* (McGinnis, M. R., ed.), Vol. 2, p. 338. Springer-Verlag, New York.
5. Rast, D. M. and Hollenstein, G. (1977) *Can J. Bot.* **55**, 2251.
6. Hegnauer, H., Nyhlén, L. H. and Rast, D. M. (1985) *Exp. Mycol.* **9**, 221.
7. Prota, G. (1992) *Melanins and Melanogenesis.* Academic Press, San Diego.
8. Bridelli, M. G., Capelletti, R. and Crippa, P. R. (1980) *Physiol. Chem. Phys.* **12**, 233.
9. Bardani, L., Bridelli, M. G., Carbucicchio, M. and Crippa, P. R. (1982) *Biochem. Biophys. Acta* **716**, 8.
10. Bilinska, B., Stepien, K. and Wilczok, T. (1987) *Stud. Biophys.* **122**, 47.
11. Seraglia, R., Traldi, P., Elli, G., Bertazzo, A., Costa, C. and Allegri, G. (1993) *Biol. Mass Spec.* **22**, 687.
12. Hervé, M., Hirschinger, J., Granger, P., Gilard, P., Deflendre, A. and Goetz, N. (1994) *Biochem. Biophys. Acta* **1204**, 19.
13. Peter, M. G. and Förster, H. (1989) *Angew. Chem.* **101**, 753.
14. Escribano, J., Tudela, J., Garcia-Carmona, F. and Garcia-Canovas, F. (1989) *Biochem. J.* **262**, 597.
15. Menter, J. M., Townsel, M. E., Moore, C. L., Williamson, G. D., Soteres, B. J., Fisher, M. S. and Willis, I. (1990) *Pigm. Cell Res.* **3**, 90.
16. Griffiths, P. R. and Fuller, M. P. (1982) in *Advances in Infrared and Raman Spectroscopy* (Clark, R. J. H. and Hester, R. E., eds), Vol. 9, p. 153. Heyden, London.
17. Painter, P. C. and Coleman, M. M. (1979) *Fuel* **58**, 301.
18. Pierce, J. A. (1986) Ph.D. thesis. University of California, Riverside, CA.
19. Piattelli, M., Fattorusso, E., Nicolaus, R. A. and Magno, S. (1965) *Tetrahedron* **21**, 3229.
20. Brown, K. C. and Corbett, J. F. (1979) *J. Chem. Soc. Perkin Trans II* 308.
21. Robb, D. A. and Gutteridge, S. (1981) *Phytochemistry* **20**, 1481.
22. Kojima, Y., Berger, C., Vallee, B. L. and Kägi, J. H. R. (1976) *Proc. Natl Acad. Sci. U.S.A.* **73**, 3413.
23. Russell, J. D., Jones, D., Vaughan, D. and Fraser, A. R. (1980) *Geoderma* **24**, 207.
24. Russell, J. D., Vaughan, D., Jones, D. and Fraser, A. R. (1983) *Geoderma* **29**, 1.
25. Sinesi, N., Miano, T. M. and Martin, J. P. (1987) *Biol. Fertil. Soils*, **5**, 120.
26. Painter, P. C., Starsinic, M., Squires, E. and Davis, A. A. (1983) *Fuel* **62**, 742.
27. Swan, G. A. (1974) *Fortschr. Chem. Org. Naturst.* **31**, 521.
28. Fuller, M. P. and Griffiths, P. R. (1978) *Anal Chem.* **50**, 1906.
29. Wharton, C. W. (1985) in *Spectroscopy of Biological*

Molecules (Alix, A. J. P., Bernard, L., and Manfait, M., eds), p. 155. Wiley, New York.
30. Hänseler, E. (1983) Ph.D. thesis. University of Zürich, Switzerland.
31. Wheeler, M. H., Tolmshoff, W. J., Bell, A. A. and Mollenhauer, H. H. (1978) *Can. J. Microbiol.* **24**, 289.
32. Weaver, R. F., Rajagopalan, K. V., Handler, P., Rosenthal, D. and Jeffs, P. W. (1971) *J. Biol. Chem.* **246**, 2010.
33. Iorio, M. A. (1959) *Ann. Chim.* **49**, 370.
34. Münger, K. and Lerch, K. (1985) *Biochemistry* **24**, 6751.
35. Kubelka, P. and Munk, F. (1931) *Z. Tech. Phys.* **12**, 593.