THE CHEMICAL NATURE OF THE MELANINS FROM *Coprinus* spp.

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ABSTRACT

Among the Basidiomycetes the species of the genus *Coprinus* have black spores and hymenia that, at maturity, are transformed through autodigestion into a black or inky fluid that drops from the pileus and blackens the soil beneath the fruiting structures. The melanins from three different species of *Coprinus* have been characterized by spectroscopic techniques, including cross-polarization, magic-angle spinning carbon-13 nuclear magnetic resonance, and chemical and thermal degradations. The aliphatic moiety of these melanins is made up of fatty acids, polysaccharides, and proteins, and the aromatic part includes alkylbenzenes, alkylphenols, hydroxytoluenes, and more complex polycyclic aromatic hydrocarbons. It is very likely that the melanins of the Basidiomycetes, as represented by *Coprinus* species, contribute to soil humus formation.

INTRODUCTION

Many organisms, including fungi, actinomycetes, and a few bacteria, synthesize dark polymers. They excrete these into the growth media or incorporate them into cells or spores. The polymers resemble soil humic acids in many chemical characteristics, such as elemental composition, resistance to decomposition in soil, amino acids released upon hydrolysis, phenols recovered by degradative methods and low boiling points produced by pyrolysis (Martin et al. 1972; Saiz-Jimenez et al. 1979).

In recent years, the contribution of microscopic fungi to soil humus formation has been extensively investigated (Martin et al. 1972; Martin et al. 1974; Haider et al. 1975; Saiz-Jimenez et al. 1975). However, the possible importance of Basidiomycetes in this process has been neglected.

Among the Basidiomycetes, the species of the genus *Coprinus* have black spores and hymenia that through autodigestion, at maturity, are transformed into a black or inky fluid that drops from the pileus and blackens the soil beneath the fruiting structures; it may in this manner be incorporated into the soil humus.

The purpose of this paper is to characterize the melanins of different *Coprinus* species and discuss their possible contribution to soil humus formation.

MATERIALS AND METHODS

Coprinus atramentarius basidiocarps were collected at the University Park of Madrid, Spain, where the fungus was growing on *Populus pyramidalis* roots. *Coprinus comatus* basidiocarps were collected near Salamanca, Spain, along the border of a road. *Coprinus micaceus* fruiting structures were collected at Braunschweig, Germany, from a forest turf. The melanins were extracted from the hymenia by grinding with alumina 305 (Sigma) and extraction with 0.5 *N* NaOH solution. The mixture was filtered, and the extracts were centrifuged at 10 000 *g*. The supernatant was acidified to pH 1.5 with 1 *N* HCl, and the melanin precipitates were recovered by centrifugation at 5000 *g*. The melanins were redissolved in 0.1 *N* NaOH and centrifuged at 10 000 *g* to eliminate cellular debris. The supernatant was again acidified with HCl, and the precipitate was washed several times with 0.1 *N* HCl until the washings were colorless, dialyzed against distilled water until free of Cl^-, and dried at 60°C.

The elemental analyses were performed by the Organic Chemistry Institute of Barcelona, Spain. All the determinations were expressed on a dry, ash-free basis. Infrared spectra were recorded on a Perkin-Elmer 377 instrument, using the KBr technique. Electron spin resonance (ESR) measurements were made by a Varian E-

3 spectrometer as reported by Riffaldi and Schnitzer (1972), but employing a nominal operating frequency of 9.39 GHz.

A ^{13}C nuclear magnetic resonance (NMR) spectrum for the melanin was obtained by the cross-polarization and magic-angle spinning (CPMAS) technique, which has been described elsewhere (Miknis et al. 1979; Maciel et al. 1979; Hatcher et al. 1981 *a,b*). The spectrum was obtained on a Nicolet NT 150 at a spectrometer frequency of 37.74 MHz using 1 ms cross-polarization time and 1 s cycle time per scan. Approximately 50 000 scans were obtained. Aromaticity of the melanin was calculated by integrating peak areas assigned to aromatic carbons (110 to 160 ppm) and normalizing to total area.

The procedure for 6 *N* HCl hydrolysis has been described (Saiz-Jimenez et al. 1975). Alkali fusion was carried out by the method of Piatelli et al. (1965). The ether extracts were chromatographed on TLC as described by Saiz-Jimenez et al. (1975). The compounds were identified by cochromatography with known compounds and by color reactions with diazotized sulfanilic acid, fast blue B salt, and Erhlich reagents. The pyrolysis-mass spectrometry procedures have been described in previous papers (Haider et al. 1977; Saiz-Jimenez et al. 1979). The permanganate oxidation procedure was reported by Martin and Saiz-Jimenez (1978). The reaction products were extracted with methyl ethyl ketone, remethylated and purified by alumina column using toluene-ethyl acetate 1:1 as eluent. The recovered extract was evaporated to dryness, redissolved in methanol, and analyzed by direct injection into a gas chromatograph-mass spectrometer computer system (Hewlett Packard model 5992 B), using an SP-2250 column programmed isothermically at 150°C for 5 min and then from 150 to 270°C at a rate of 4°C/min. The identity of the compounds was confirmed by running the mass spectrum and by computer-searching a file of known spectra. No quantitative data are reported because accuracy cannot be ensured with the method employed (Martin et al. 1981).

RESULTS

The *Coprinus* melanins have properties similar to other fungal melanins. They are insoluble in water, dilute acids, and such organic solvents as acetone, chloroform, and ethanol. They are partially soluble in cold NaOH and soluble in hot NaOH solutions.

Elemental analyses

The C contents of fungal melanins have been reported to vary from 49.1 to 62.9% (Schnitzer et al. 1973; Saiz-Jimenez 1975). The C contents of untreated *Coprinus* melanins were within this range and varied from 54.7 to 59.2% (Table 1). After 6 *N* HCl hydrolysis, the C contents of the *Coprinus* melanins increased and were higher than most values reported for other melanins (60.7 to 65.1%). The N contents of fungal melanins range from about 1 to 11% and depend upon the species, the N source, and the quantity of available N in the growth medium. The *Coprinus* melanins contained 7 to 8.2% N. Losses of N upon acid hydrolysis ranged from 47 to 74% and weight losses from 61 to 67%. N contents of the hydrolyzable fractions were calculated according to Ishiwatari (1977) and varied from 9 to 17%, which is similar to that of proteins (14 to 19%). The hydrolysis of nonproteinaceous substances, such as polysaccharides, would account for the slightly lower N values. The atomic H/C ratios of untreated and hydrolyzed *Coprinus* melanins ranged from 1.4 to 1.7, which, according to van Krevelen (1950), indicates that from 55 to 75% of the C atoms are in aliphatic form.

Infrared spectra

The infrared spectra of *Coprinus* melanins show a broad band at 3390 cm^{-1} (H-bonded OH), well-defined groups of strong bands at 2950, 2920, and 2850 cm^{-1} (aliphatic CH), a shoulder at 1710 cm^{-1} (C=O of COOH), broad bands at 1650 cm^{-1} (aromatic C=C and/or amide I) and 1520 cm^{-1} (amide II), a medium band at 1450 cm^{-1} and a shoulder at 1390 cm^{-1} ($C\text{-}CH_3$), shoulders at 1225, 1140, and 1100 cm^{-1} (CO and OH of alcohols), a broad band centered at 1030 cm^{-1} (CO of polysaccharides), and shoulders at 910 cm^{-1} (aromatic CH and/or aliphatic CH) and 720 cm^{-1} (acyclic CH_2). The 6 *N* HCl hydrolysis caused some drastic changes in the spectra, mainly a strong decrease of the bands at 3390 and 1650 cm^{-1}, a decrease of the band at 1030 cm^{-1}, and the disappearance of the band at 1520 cm^{-1}. Bands at 2950, 2920, and 2850 cm^{-1} were intensified. A very important feature of the infrared spectra of the *Coprinus* melanins was

TABLE 1

Elemental analyses of untreated and 6 N HCl hydrolyzed melanins, H/C atomic ratios, and weight losses[a]

Samples	C		H		N		O		H/C		% Weight loss on hydrolysis	% Weight loss of nitrogen
	a	b	a	b	a	b	a	b	a	b		
Coprinus atramentarius	59.2	69.4	8.4	8.8	7.6	2.0	24.8	19.8	1.7	1.5	67	74
Coprinus comatus	54.7	66.5	7.5	7.8	8.2	3.5	29.6	22.2	1.6	1.4	65	57
Coprinus micaceus	55.3	66.8	7.2	7.5	7.0	3.7	30.5	22.0	1.6	1.4	61	47

[a] a, before acid hydrolysis; b, after acid hydrolysis.

the indication of high amounts of aliphatic structures.

ESR spectra

The spectra of the *Coprinus* melanins consist of single, symmetrical lines devoid of hyperfine splitting, which indicate that the free radicals are extremely complex in structure (Riffaldi and Schnitzer 1972). The spin concentrations of the untreated *Coprinus* melanins varied from 0.7 to 1.6×10^{17} spins/g (Table 2) and are of the same order of magnitude as those obtained for other fungal melanins. However, the free radical contents of the hydrolyzed melanins were from 6 to 31 times greater than those of the untreated melanins and higher than those fungal melanins, soil fulvic acids, and most of the humic acids reported by Riffaldi and Schnitzer (1972).

The g values of soil fulvic and humic acids and fungal melanins have been reported to vary from 2.0028 to 2.0040 (Riffaldi and Schnitzer 1972; Senesi and Schnitzer 1978). The g values of *Coprinus* melanins were within this range and varied from 2.0035 to 2.0040. However, the line widths of these melanins ranged from 4.21 to 6.30 and were higher than most values obtained for humic substances.

Alkali fusion

Alkali fusion of the *Coprinus* melanins yielded from 11 to 13 compounds, of which 8 were common to all three samples. None of the compounds gave coral pink to magenta color with Erhlich's reagent, indicating the absence of indoles and pyrroles among the degradation products. Five phenols, catechol, and salycilic, *p*-hydroxybenzoic, protocatechuic, and 3,5-dihydroxybenzoic acids were identified. All these have been obtained upon alkali fusion of other fungal melanins (Piatelli et al. 1965; Nicolaus 1968).

Pyrolysis-mass spectra

Figure 1a shows the pyrolysis-mass spectrum of the *Coprinus atramentarius* melanin, as representative for this group of melanins. The most prominent peaks were related to nitrogen-containing compounds, commonly observed during pyrolysis of proteins (Meuzelaar et al. 1974). They were a series of pyrroles (m/z 67, 81, 95), pyridines (m/z 79, 93, 107), and indoles (m/z 117, 131, 145), as well as an ammonia peak at

TABLE 2

ESR data for Coprinus *melanins*[a]

Samples	Spins/g, $\times 10^{-17}$		g Values		Line width, gauss	
	a	b	a	b	a	b
Coprinus atramentarius	0.7	7.8	2.0040	2.0035	5.47	5.04
Coprinus comatus	1.4	8.1	2.0038	2.0035	5.45	4.21
Coprinus micaceus	1.6	49.0	2.0040	2.0036	5.88	6.30

[a] a, before acid hydrolysis; b, after acid hydrolysis.

m/z 17. A further prominent series of homologous ions at m/z 68 (furan), 82 (methylfuran), 96 (furfural or dimethylfuran), 98 (furfuryl alcohol), and their partly alkylated derivatives at m/z 112 and 126, as well as peaks at m/z 114 and 128 (related to pentose and deoxyhexose, respectively), are evidence of complex polysaccharides in the molecule. Fragments from amino sugars appeared to be relatively important, as indicated by the series of peaks at m/z 32, 43, 59, 73, 85, 97, 109, 125, 137, and 151, which were the most prominent peaks in the spectrum from pure chitin (Meuzelaar et al. 1974). Peaks at m/z 34 (SH_2), 48 (CH_3SH), 62 (C_2H_5SH), and 64 (SO_2) showed the existence of sulfur-containing compounds. Alkenes were indicated by prominent peaks at m/z 28, 42, 56, 70, and 84 (ethene, propene, butene, pentene, hexene). They were probably derived from aliphatic hydrocarbons, as evidenced by the fragment series at m/z 43, 57, 71, and 85. Aromatic fragments were noted from peaks at m/z 78 (benzene), 92 (toluene), 104 (styrene), 106 (C_2-alkylbenzene), 120 (C_3-alkylbenzene), and 134 (C_4-alkylbenzene). Also phenols were present with peaks at m/z 94 (phenol), 108 (cresol), 122 (C_2-alkylphenol), and 136 (C_3-alkylphenol). Weak signals at m/z 124 (dihydroxytoluene) and 138 (trihydroxytoluene) were noted.

Figure 1b shows the spectrum from the 6 *N*

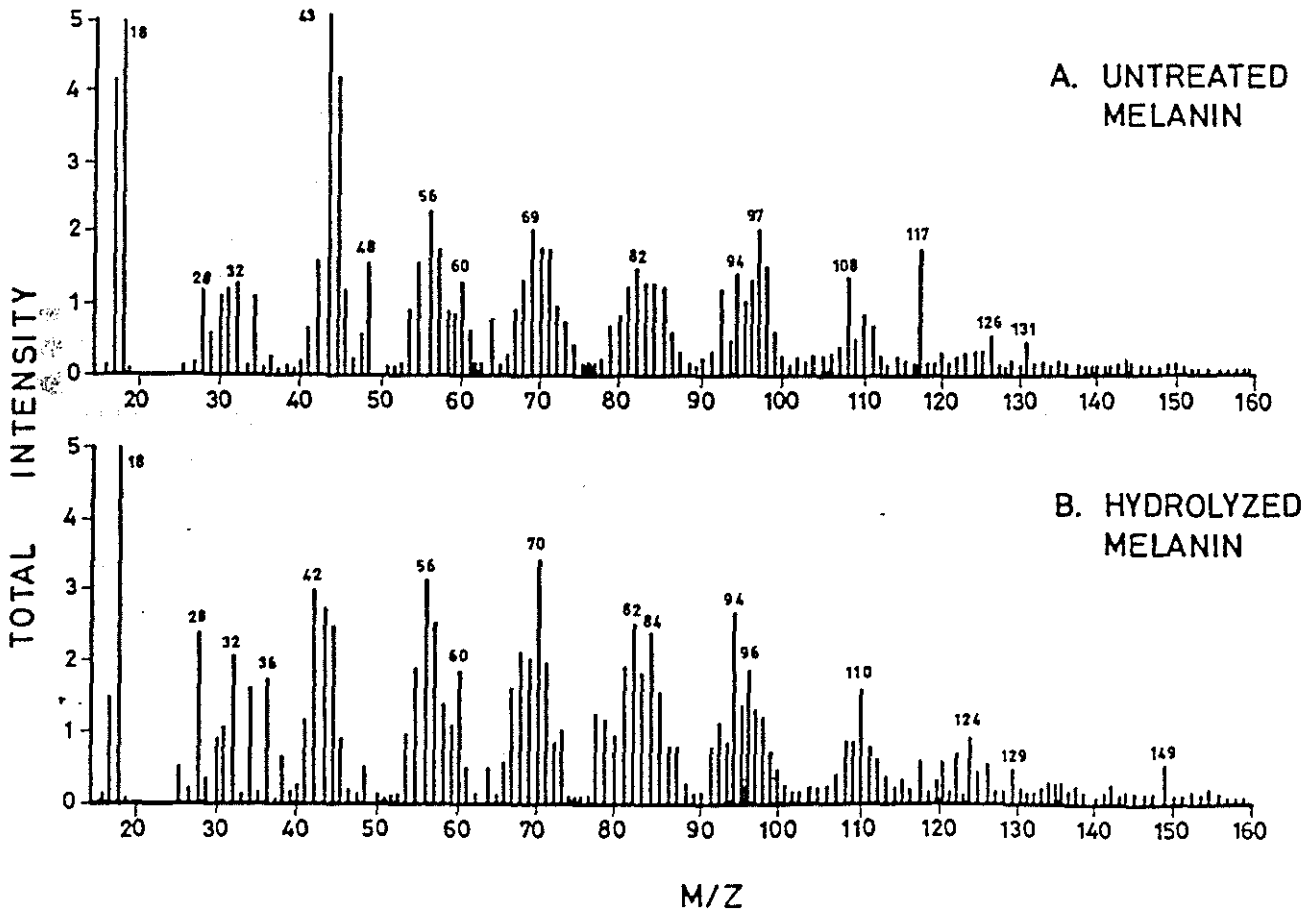

FIG. 1. a. Pyrolysis-mass spectrum of untreated *Coprinus atramentarius* melanin. b. Pyrolysis-mass spectrum of 6 *N* HCl-hydrolyzed *Coprinus atramentarius* melanin.

HCl-hydrolyzed *Coprinus atramentarius* melanin. Compared with the untreated sample, the acid-hydrolyzed residue gave strong peaks for pyrroles, pyridines, furans, alkenes, benzenoid, and phenolic compounds. Conversely, the intensity of the indole and complex polysaccharide peaks decreased.

Permanganate oxidation

The oxidation products obtained from the hydrolyzed and methylated melanin from *Coprinus micaceus* are listed in Table 3. Because they were methylated prior to the gas chromatographic separations, it is likely that most of the COOH and OH groups occurred in the initial material as carboxyl and phenolic hydroxyls, rather than as esters and ethers. The most abundant compounds isolated from the melanins were a homologous series of straight chain fatty acids ranging from C_{12} to C_{24}, with dominance of the n-C_{16}, n-C_{18}, and n-C_{20} fatty acids, as well as a series of saturated branched (*iso* and *anteiso*) fatty acids ranging from C_{14} to C_{22}. Several components of the series of monounsaturated fatty acids, probably in the same range, were identified as minor compounds. They were poorly separated from their saturated counterparts on the column used, as also reported by Boon et al. (1975). In addition, aliphatic dicarboxylic acids were found, the most abundant being adipic acid. Benzenecarboxylic acids, dialkylphthalates, and methylpyrazolcarboxylic acids were present in small quantities.

TABLE 3

Identified compounds produced by the oxidation of hydrolyzed and methylated Coprinus micaceus *melanin*

n-C_{12} to n-C_{24} fatty acid methyl esters
n-C_{12} to n-C_{24} unsaturated fatty acid methyl esters
Branched (*iso* and *anteiso*) C_{14} to C_{22} fatty acid methyl esters
Dimethyl adipate
Dimethyl pimelate
Dimethyl undecane 1, 11 dioate
Dimethyl tridecane 1, 13 dioate
Dimethyl tetradecane 1, 14 dioate
Dibutyl phthalate
Di-(2-ethylhexyl) phthalate
Benzenedicarboxylic acid dimethyl ester
Benzenetricarboxylic acid trimethyl ester
Dimethoxybenzenetricarboxylic acid trimethyl ester
Benzenetetracarboxylic acid tetramethyl ester
Dimethoxybenzenetetracarboxylic acid tetramethyl ester
Methoxybenzenepentacarboxylic acid pentamethyl ester
Benzenehexacarboxylic acid hexamethyl ester
2-Methyl pyrazol (3)-carboxylic acid methyl ester
2-Methyl pyrazol (3,4)-dicarboxylic acid dimethyl ester

CPMAS ^{13}C NMR spectra

The CPMAS ^{13}C spectra of humic substances have peaks in the aliphatic (0 to 50 ppm) and aromatic (110 to 160 ppm) regions (Hatcher et al. 1981*a*,*b*) similar to the CPMAS spectrum of the *Coprinus micaceus* melanin (Fig. 2), whose prominent signals can be assigned to specific carbons. Thus, the sharp peak at 30 ppm is characteristic of methylene carbons in long-chain hydrocarbons (Lindeman and Adams 1971), and the shoulder at 15 ppm indicates the presence of methyl carbons. Model compounds like fatty acids (Johnson and Jankowski 1972) show peaks of methyl carbons at 14 ppm, peaks of methylene carbons between 23 and 34 ppm, and a peak of carboxyl carbons at 180 ppm. The agreement between carbon signals in fatty acids and dicarboxylic aliphatic acids constituted the bulk of the permanganate oxidation products offer further support for assigning the signals between 0 to 50 ppm and about 178 ppm (COOH) in the melanin spectrum to fatty acid carbons.

Oxygen- and nitrogen-substituted aliphatic carbons resonate between 50 and 110 ppm. Because the nitrogen content of the melanin is low (7%) compared with the oxygen content (30.5%), contributions from N-substituted aliphatic carbons are expected to be small. Thus, the discrete peak at 56 ppm is probably that of methoxyl carbons. Polysaccharides (e.g., cellulose) show an intense peak at about 75 ppm that is associated with resonances of the nos. 2, 3, and 5 carbons. Further peaks at about 66, 86 to 90, and 106 ppm are assigned to the nos. 6, 4, and 1 carbons, respectively (Atalla et al. 1980; Bartuska et al. 1980). The peaks at about 61, 74, 85, and 103 ppm in the melanin spectrum agree well with those of cellulose. Because the complex polysaccharide pattern has been observed in the pyrolysis-mass spectra, and because evidence of

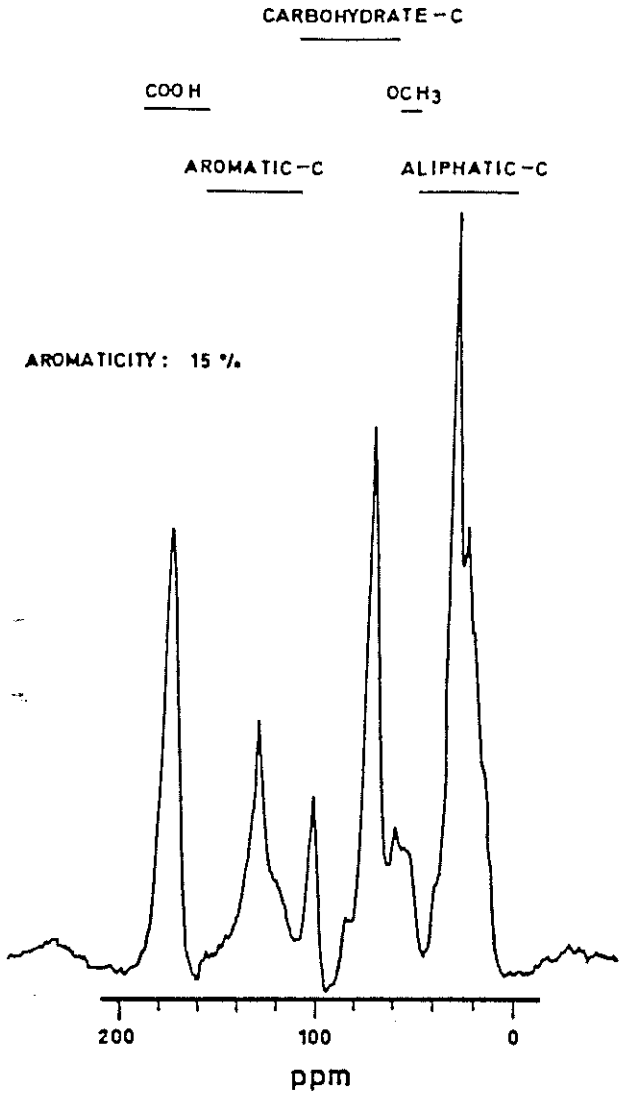

FIG. 2. CPMAS ^{13}C NMR spectrum of *Coprinus micaceus* melanin.

ppm could be assigned to polysaccharide carbons. Further, the ^{13}C NMR spectrum of cellulose (Miknis et al. 1979) is the same as that obtained for the *Coprinus* melanin in that region.

Hatcher et al. (1980, 1981*a*,*b*) reported that unsubstituted and alkyl-substituted aromatic carbons resonate between 120 and 130 ppm in humic substances, and that a peak at about 150 ppm is related to oxygen-substituted aromatic carbons of phenols. Alkylbenzenes are typical components that would yield resonances at 130 ppm. Alkylbenzenes and alkylphenols have also been identified as pyrolysis products, as have benzenecarboxylic and phenolic acids as permanganate oxidation products from the *Coprinus* melanins.

The *Coprinus micaceus* melanin NMR spectrum shows 18% of carboxyl carbons, 15% of aromatic carbons, 27% of polysaccharide carbons, 3% of methoxyl carbons, and 38% of aliphatic (fatty acids) carbons.

DISCUSSION

The *Coprinus* melanins appear to be complex polymers in which the aliphatic structures predominate over the aromatic ones, as shown by the H/C atomic ratios, weight losses upon 6 *N* HCl hydrolysis, products released upon permanganate oxidation, and infrared, pyrolysis-mass, and CPMAS ^{13}C NMR spectra. Important components of the molecule are polysaccharide (including chitin) and fatty acid materials. These substances may be chemically bound to the aromatic portion or they may have been extracted with the melanins from the cell walls by NaOH solutions. Many of the fungal melanins studied by Meuzelaar et al. (1974, 1977) and Saiz-Jimenez et al. (1979) also contained considerable amounts of proteins, polysaccharides, and aliphatic materials. Hydrolysis (6 *N* HCl) reduced the N content of the *Coprinus* melanins and also the polysaccharide components. However, the pyrolysis-mass spectrum of the residue shows that nitrogeneous compounds are still present. Because fungal melanins contain N that is not hydrolyzed to amino acid, it has been postulated that some amino groups of proteins are covalently bonded to the benzenoid nucleus to give linkages that resist acid hydrolysis (Haider et al. 1975). Therefore, it seems possible that the N-containing fragments in the pyrogram of the hydrolyzed melanin arose from free amino groups in dibasic amino acids or terminal amino acids of the protein chain bonded to the phenolic rings or both (Saiz-Jimenez et al. 1979). Furthermore, it has been noted that acid hydrolysis of chitin yields melanoidins, a black polymer formed by condensation of amino sugars, which might be responsible for the presence of some of the nitrogeneous fragments in the pyrogram of the acid-hydrolyzed melanin.

As early as 1954, Commoner et al. observed that melanins contained stable free radicals. The origin and role of the unpaired electrons in melanins seem to be related to some biological properties, e.g., radiation protection (Swartz 1972). In the *Coprinus* melanins an increase in free radicals has been found upon acid hydrolysis, which suggests that the treatment is able to produce some new free radicals, either by the release of hydrolyzable materials or by the formation of secondary free radicals brought about

by browning reactions. In fact, it has been proved that in strong acid conditions, melanoidins can be formed. Chitin subjected to 6 *N* HCl hydrolysis produces a melanoidin with a *g* value of 2.0034, a free radical concentration of 6.9×10^{17} spins/*g*, and a line width of 4.63. Due to the high polysaccharide (including chitin) and protein content of these melanins, it is highly probably that some of the additional free radicals measured in the hydrolyzed melanins could arise from the new browning reaction structures.

On the basis of alkali fusion products, Nicolaus (1978) proposed a chemical distinction between eumelanins (animal melanins) of the indole type, arising from the enzymic oxidation of tyrosine, and allomelanins (plant, fungi, and bacteria melanins) formed by the enzymic oxidation of nitrogen-free precursors, such as phenols. Some of the aromatic portions of *Coprinus* melanins consist of alkylbenzenes, alkylphenols, and hydroxytoluenes, as demonstrated by pyrolysis-mass spectrometry. This is further corroborated by the fragments released by permanganate oxidation. The presence of indoles and pyrroles as structural units could not be discerned by alkali fusion degradation. However, the existence of pyrroles, pyridines, and indoles as thermal degradation fragments is not in disagreement with the alkali fusion results, because these heterocyclic nitrogen compounds are common reaction products, originating during the heating of proteins and amino acids (Martin et al. 1977) and consequently cannot be definitely regarded as structural units of the *Coprinus* melanins.

The presence of different benzenecarboxylic and phenolic acids among the oxidation products of the *Coprinus* melanin suggests that the polymer has a complex aromatic moiety. This is demonstrated by the presence of benzenetetra-, penta-, and hexacarboxylic acids, which may arise either from dimethylnaphthalene, naphthacene, or pyrene building blocks (Hayes and Swift 1978) and even more condensed aromatic structures or alkyl-substituted benzene rings. The existence of polycyclic aromatic hydrocarbons in fungal melanins had already been pointed out by Nicolaus (1968). Further, the methoxy-substituted benzenecarboxylic acids indicate the presence of phenolic hydroxyls in the aromatic rings. Also, substituted pyrazoles were identified as degradation products. These compounds, as reported by Spiteller (1981), arise from a 1,3 dipolar addition of diazomethane to unsaturated compounds.

Hayatsu et al. (1981) have reported that buffer-controlled permanganate oxidation of coals yields mainly aliphatic dicarboxylic acids ranging from C_4 to C_{18}. From nonbuffered oxidations, although short-chain dicarboxylic acids (C_2 to C_8) were found in significant amounts, long-chain dicarboxylic acids were considerably depleted, because they are further oxidized to more stable short-chain dicarboxylic acids and CO_2. After 1 h of nonbuffered alkaline permanganate oxidation of nonane-1,9-dicarboxylic acid, only 14% of the starting acid was recovered, whereas for hexadecane-1,16-dicarboxylic acid, the remaining products amounted to only 4%, mainly consisting of C_8 to C_{13} aliphatic dicarboxylic acids with a small amount of the original compound.

The oxidation procedure employed in this work is close to the buffer-controlled oxidation of Hayatsu et al. (1981) and has been shown previously (Martin and Saiz-Jimenez 1978) to produce a great amount of short- and long-chain aliphatic compounds (alkanes, fatty acids, methyl ethyl ketones, and aliphatic dicarboxylic acids). The differences between both methods are in the buffer employed and the oxidation time, which obviously must be higher for coals. From the results, it can be assumed that in the present oxidative conditions there is no extensive degradation of the aliphatic dicarboxylic acids derived from the *Coprinus* melanin.

It is well known that fungal structures contain neutral and polar complex lipids (straight-chain saturated and unsaturated fatty acids, tryglicerides, sterols, natural methyl esters of long-chain fatty acids, etc.), as reported by Laseter et al. (1968), Weete et al. (1970), Mumma et al. (1971), Fisher et al. (1972), and Gunasekaran et al. (1972). Furthermore, basidiomycete mycelia, fruiting bodies, and certain pigmented surfaces are rich in lipid components, among which oleic and linoleic acids are the most abundant (Shaw 1967; Holtz and Schisler 1971). The presence of adipic acid as one of the most prominent compounds among the oxidation products is in agreement with the results of these investigators, because permanganate oxidation of oleic and linoleic acids will give rise to this aliphatic dicarboxylic acid.

Permanganate oxidation of soil humic acids yields fatty acids as major products with the n-

C_{16} and n-C_{18} chain lengths dominant (Schnitzer and Skinner 1974; Griffith and Schnitzer 1976, 1977; Martin et al. 1981). The distribution of the fatty acids and the fact that n-C_{16} and n-C_{18} compounds constitute a very large percentage of the total indicate a microbial origin for the bulk of the fatty acids isolated from soil humic acids. Furthermore, the lipid fraction of composted municipal refuse, as well as the humic fraction, have a similar composition. Dead and living microorganisms may constitute 25% of the weight of these composts (Gonzalez-Vila et al. 1982).

Humic substances and fungal melanins are extremely complex organic materials, and a wide variety of different types of aliphatic carbons (straight-chain, branched, cyclic, etc.) and aromatic carbons (unsubstituted, substituted, condensed, etc.) are expected to contribute to the NMR spectra. For this reason, spectra often are devoid of much fine structure. Recently, CPMAS ^{13}C NMR techniques have been applied to solid samples of humic fractions (Hatcher et al. 1980, 1981*a,b*). They demonstrated the usefulness of the cross-polarization technique for the quantitative structural analyses of humic and fulvic acids by showing that aromatic carbon signals can be distinguished clearly from those carboxyl, ether, methoxyl, and aliphatic carbons and that carbon aromaticity can be measured quantitatively. Aromaticity of the *Coprinus* melanin agrees well with the percentage of carbon atoms in aromatic form deduced from the atomic H/C ratio. Further, the ^{13}C NMR data support the conclusions that have already been drawn from other degradative methods. However, as reported by Miknis et al. (1979), the importance is that the NMR measurements are made directly and nondestructively on unaltered samples and provide valuable information on the carbon distribution between aromatic and nonaromatic structures in the sample.

Basidiomycetes comprise the second largest class of fungi, with some 13 000 species, and to it belong most of the large and conspicuous species found in fields and woods. Basidiomycetes that occur in grasslands, forest litter, and soils may contribute to soil humus formation. The following groups may be defined

Ectomycorrhizal fungi, which live in close association with higher plants. Tan et al. (1978) reported the formation of black polymers (humic acidlike compounds) in the culture medium of *Pisolithus tinctorius*, a fungus living symbiotically with pine trees in a soil environment rich in humus.

Wood rotting fungi, which decompose cellulose and lignin. The lignins undergo drastic changes, in that most of the larger lignin fragments continue to decompose over time and the smaller units become stabilized in the soil to a greater degree by linkage into microbial melanins or complex humic acid molecules (Martin and Haider 1980). Further, certain wood-destroying basidiomycetes produce humic acid-type substances in the fruiting structures. The attack on lignin gives rise to either aromatic precursors, which are converted into specific metabolic products (e.g., hispidin), or lignin breakdown compounds. In both cases, their polymerization by phenolases produces dark polymers confined to the sporophore. This process has been observed in *Phellinus igniarius* (Kirk et al. 1975), *Inonotus hispidus*, and *Poria obliqua* (Bu'Lock 1967).

Agaricales, in which the fruiting body is an ephemeral structure usually lasting only a few days, whereas the mycelium, living on organic matter in the soil, may last for years. The lysed fruiting bodies constitute a pool of organic materials (e.g., polysaccharides, proteins, lipids) that stimulate the soil microflora activity and the building of microbial humus. Furthermore, fairy rings, common in woodlands, grasslands, and lawns, are the result of the activity of soil-inhabiting fungi, mainly Agaricales. These fungi contribute to soil organic matter accumulation because the older mycelia die as the colony grows outward, and the soil benefits from the lysis of microbial tissues.

Among the Basidiomycetes (Agaricales), the genera *Coprinus*, *Psatyrella*, and *Paneolus* are cosmopolitan. Many species inhabit the drug of herbivorous and dead plant remains, including dead wood. They appear to be secondary invaders in the process of rotting wood, and their fungal cell components, including melanins, contribute to soil organic matter. In fact, it has been shown that resistant components of decomposed plant materials under attack and newly synthesized microbial tissues both enrich the organic matter of the soil and tend to be distributed in all three humic fractions, humic and fulvic acids and humin (Wagner 1968).

Although the *Coprinus* melanins are higher in fatty acids, polysaccharides, and proteins than some fungal melanins and soil humic acids, their general chemical properties and the fragments or components released by oxidative and pyrolytic degradation procedures are similar, and it would be difficult to differentiate them from soil humic polymers by these procedures. It is very likely that the dark pigments or melanins of the Basidiomycetes, as represented by *Coprinus* species contribute to soil humus formation.

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REFERENCES

Atalla, R. H., J. C. Gast, D. W. Sindorf, V. J. Bartuska, and G. E. Maciel. 1980. ^{13}C NMR spectra of cellulose polymorphs. J. Am. Chem. Soc. 102:3249–3251.

Bartuska, V. J., G. E. Maciel, H. I. Bolker, and B. I. Fleming. 1980. Structural studies of lignin isolation procedures by ^{13}C NMR. Holzforschung 34:214–217.

Boon, J. J., J. W. de Leeuw, and P. A. Schenk. 1975. Organic geochemistry of Walvis Bay diatomaceous ooze: 1. Occurrence and significance of the fatty acids. Geochim. Cosmochim. Acta 39:1559–1565.

Bu'Lock, J. D. 1967. Essays in biosynthesis and microbial development. Wiley, New York.

Commoner, B., J. Townsend, and G. E. Pake. 1954. Free radicals in biological materials. Nature 174:689–691.

Fisher, D. J., P. J. Holloway, and D. V. Richmont. 1972. Fatty acid and hydrocarbon constituents of the surface and wall lipids of some fungal spores. J. Gen Microbiol. 72:71–78.

Gonzalez-Vila, F. J., C. Saiz-Jimenez, and F. Martin. 1982. Identification of free organic chemicals found in composted municipal refuse. J. Environ. Qual. 11:251–254.

Griffith, S. M., and M. Schnitzer. 1976. The alkaline cupric oxide oxidation of humic and fulvic acids extracted from tropical volcanic soils. Soil Sci. 122:191–201.

Griffith, S. M., and M. Schnitzer. 1977. Organic compounds formed by the hydrogen peroxide oxidation of soils. Can. J. Soil Sci. 57:223–231.

Gunasekaran, M., W. M. Hess, and D. J. Weber. 1972. The fatty acid composition of conidia of *Aspergillus niger* V. Thiegh. Can. J. Microbiol. 18:1575–1576.

Haider, K. J., P. Martin, and Z. Filip. 1975. Humus biochemistry. *In* Soil biochemistry, vol. 4. E. A. Paul and A. D. McLaren (eds.). Dekker, New York, pp. 195–244.

Haider, K., B. R. Nagar, C. Saiz-Jimenez, H. L. C. Meuzelaar, and J. P. Martin. 1977. Studies on soil humic compounds, fungal melanins and model polymers by pyrolysis-mass spectrometry. *In* Soil organic matter studies, vol. 2. IAEA, Vienna, pp. 213–220.

Hatcher, P. G., D. L. VanderHart, and W. L. Earl. 1980. Use of solid-state ^{13}C NMR in structural studies of humic acids and humin from Holocene sediments. Org. Geochem. 2:87–94.

Hatcher, P. G., G. E. Maciel, and L. W. Dennis. 1981*a*. Aliphatic structures of humic acids: A clue to their origin. Org. Geochem. 3:43–48.

Hatcher, P. G., M. Schnitzer, L. W. Dennis, and G. E. Maciel. 1981*b*. Aromaticity of humic substances in soils. Soil Sci. Soc. Am. J. 45:1089–1094.

Hayatsu, R., R. E. Winans, R. G. Scott, and R. L. McBeth. 1981. Is kerogen-like material present in coal? 1. Buffer-controlled permanganate oxidation of coal. Fuel 60:158–161.

Hayes, M. H. B., and R. S. Swift. 1978. The chemistry of soil organic colloids. *In* The chemistry of soil components. D. J. Greenland and M. H. B. Hayes (eds.). Wiley, Chichester, pp. 179–230.

Holtz, R. B., and L. C. Schisler. 1971. Lipid metabolism of *Agaricus bisporus* (Lange) Sing: 1. Analysis of sporophore and mycelial lipids. Lipids 6:176–180.

Ishiwatari, R. 1971. Organic polymers in recent sediments. Dr. Sci. thesis. Tokyo Metropolitan University.

Johnson, L. F., and W. C. Jankowski. 1972. Carbon-13 NMR spectra. Wiley-Interscience, New York.

Kirk, T. K., L. F. Lorenz, and M. J. Larsen. 1975. Partial characterization of a phenolic pigment from sporocarps of *Phellinus igniarius*. Phytochemistry 14:281–284.

Krevelen, D. W., van. 1950. Graphical statistical

method for the study of structure and reaction processes in coal. Fuel 29:269–284.

Laseter, J. L., J. Weete, and D. J. Weber. 1968. Alkanes, fatty acid methyl esters and free fatty acids in surface wax of *Ustilago maydis*. Phytochemistry 7:1177–1181.

Lindeman, L. P., and J. Q. Adams. 1971. Carbon-13 nuclear magnetic resonance spectrometry: Chemical shifts for the paraffins through C_9. Anal. Chem. 43:1245–1252.

Maciel, G. E., V. J. Bartuska, and F. P. Miknis. 1979. Characterization of the organic material in coal by proton decoupled ^{13}C nuclear magnetic resonance with magic-angle spinning. Fuel 58:391–394.

Martin, F., and C. Saiz-Jimenez. 1978. Humic acids from lignite: 2. Alkaline permanganate oxidation. Fuel 57:353–356.

Martin, F., C. Saiz-Jimenez, and A. Cert. 1977. Pyrolysis-gas chromatography-mass spectrometry of soil humic fractions: 1. The low boiling point compounds. Soil Sci. Soc. Am. J. 41:1114–1118.

Martin, F., C. Saiz-Jimenez, and F. J. Gonzalez-Vila. 1981. The persulfate oxidation of a soil humic acid. Soil Sci. 132:200–203.

Martin, J. P., and K. Haider. 1980. Microbial degradation and stabilization of ^{14}C-labeled lignins, phenols, and phenolic polymers in relation to soil humus formation. *In* Lignin biodegradation: Microbiology, chemistry, and potential applications, vol. 1. T. K. Kirk, T. Higuchi, and H.-m. Chang (eds.). CRC Press, Boca Raton, Florida, pp. 77–100.

Martin, J. P., K. Haider, and E. Bondietti. 1972. Properties of model humic acids synthesized by phenol-oxidase and autooxidation of phenols and other compounds formed by soil fungi. Proc. Int. Meet. Humic Substances, Nieuwerluis. PUDOC, Wageningen, pp. 171–186.

Martin, J. P., K. Haider, and C. Saiz-Jimenez. 1974. Sodium amalgam reductive degradation of fungal and model phenolic polymers, soil humic acids, and simple phenolic compounds. Soil Sci. Soc. Am. Proc. 38:760–765.

Meuzelaar, H. L. C., P. G. Kistemaker, and M. A. Posthumus. 1974. Recent advances in pyrolysis-mass spectrometry of complex biological materials. Biomed. Mass Spectrom. 1:312–319.

Meuzelaar, H. L. C., K. Haider, B. R. Nagar, and J. P. Martin. 1977. Comparative studies of pyrolysis-mass spectra of melanins, model phenolic polymers, and humic acids. Geoderma 17:239–252.

Miknis, F. P., G. E. Maciel, and V. J. Bartuska. 1979. Cross polarization magic-angle spinning ^{13}C NMR spectra of oil shales. Org. Geochem. 1:169–176.

Mumma, R. O., R. D. Sekura, and C. L. Fergus. 1971. Thermophilic fungi: 2. Fatty acid composition of polar and neutral lipids of thermophilic and mesophilic fungi. Lipids 6:584–588.

Nicolaus, R. A. 1968. Melanins. Hermann, Paris.

Piatelli, M., E. Fattorusso, R. A. Nicolaus, and S. Magno. 1965. The structure of melanins and melanogenesis: 5. Ustilagomelanin. Tetrahedron 21:3229–3236.

Riffaldi, R., and M. Schnitzer. 1972. Electron spin resonance spectrometry of humic substances. Soil Sci. Soc. Am. Proc. 36:301–305.

Saiz-Jimenez, C. 1975. Caracterizacion del pigmento de *Eurotium echinulatum* Delacr.: 1. Datos analiticos. An. Edaf. Agrobiol. 34:829–839.

Saiz-Jimenez, C., K. Haider, and J. P. Martin. 1975. Anthraquinones and phenols as intermediates in the formation of dark colored, humic acid-like pigments by *Eurotium echinulatum*. Soil Sci. Soc. Am. Proc. 39:649–653.

Saiz-Jimenez, C., K. Haider, and H. L. C. Meuzelaar. 1979. Comparison of soil organic matter and its fractions by pyrolysis-mass spectrometry. Geoderma 22:25–37.

Saiz-Jimenez, C., F. Martin, and A. Cert. 1979. Low boiling point compounds produced by pyrolysis of fungal melanins and model phenolic polymers. Soil Biol. Biochem. 11:305–309.

Schnitzer, M., and S. I. M. Skinner. 1974. The low temperature oxidation of humic substances. Can. J. Chem. 52:1052–1080.

Schnitzer, M., M. I. Ortiz de Serra, and K. Ivarson. 1973. The chemistry of fungal humic acid-like polymers and of soil humic acids. Soil Sci. Soc. Am. Proc. 37:229–236.

Senesi, M., and M. Schnitzer. 1978. Free radicals in humic substances. *In* Environmental biogeochemistry and geomicrobiology, vol. 2. W. E. Krumbein (ed.). Ann Arbor Science, Ann Arbor, pp. 467–481.

Shaw, R. 1967. Fatty acids of fruiting bodies of basidiomycetes. Nature 213:86–87.

Spiteller, M. 1981. Substituierte Pyrazole als Bestandteile von Huminsäure-abbauprodukten. Z. Pflanzenernaehr. Bodenkd. 144:500–504.

Swartz, H. M. 1972. Cells and tissues. *In* Biological applications of electron spin resonance. H. M. Swartz, J. R. Bolton, and D. C. Borg (eds.). Wiley-Interscience, New York, pp. 155–195.

Tan, K. H., P. Sihanonth, and R. L. Todd. 1978. Formation of humic acid-like compounds by the ectomycorrhizal fungus, *Pisolithus tinctorius*. Soil Sci. Soc. Am. J. 42:906–908.

Wagner, G. H. 1968. Significance of microbial tissues to soil organic matter. *In* Isotopes and radiation in soil organic matter studies. IAEA, Vienna, pp. 197–205.

Weete, J. D., D. J. Weber, and J. L. Laseter. 1970. Lipids of *Rhizopus arrhizus* Fischer. J. Bacteriol. 103:536–540.