ELECTRON SPIN RESONANCE SPECTROMETRY OF FUNGAL MELANINS

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We investigated electron spin resonance (ESR) parameters (free radical concentrations, *g* values, and linewidths) of a variety of fungal melanins. The influences of oxygen, culturing conditions, methylation, solvent extraction, acid hydrolysis, and persulfate oxidation on ESR parameters were also studied. Our major objective was to gain a better knowledge of the similarities of fungal melanins with soil humic acids. We suggest that these biopolymers are different from soil humic acids with regard to ESR parameters.

Soil humic materials are highly heterogeneous mixtures formed through numerous chemical and biological reactions. They contain lipid, polysaccharide, protein, and lignin components, which can be cleaved by relatively mild treatments. It appears that these classes of bioorganic matter, which are found in living systems and associated with the humic "core," may be relatively young and may originate from organic materials present in the environment as a result of biological actions.

In fact, microbial products are rich in lipids, proteins, and polysaccharides. Furthermore, microorganisms can degrade lignin. Phenols released from lignins or microbially synthesized partially degraded lignins, and other reactive compounds could undergo autooxidation or enzymatic polymerization to produce humiclike molecules. In addition, microorganisms, especially fungi, synthesize intra- and extracellular melanins, which are considered similar to soil humic acids. These polymers resemble humic acids in some characteristics, e.g., elementary composition, exchange capacity, resistance to decomposition, infrared spectra, phenols recovered after Na-amalgam reduction, and in the amino acids released upon hydrolysis with 6 *N* HCl (Martin et al. 1972*a*; Saiz-Jimenez et al. 1975). However, Saiz-Jimenez et al. (1979) believe that the similarities between pyrograms of the low boiling compounds of fungal melanins and soil humic acids cannot be regarded as conclusive evidence for similarities in the chemical structure of these materials. The low boiling compounds identified upon pyrolysis are derived from proteins, carbohydrates, and aliphatic structures associated with the polymers. These structures, when present in humic materials, may have a microbial origin and give only partial information about the humic components. Therefore, in the comparative studies of these materials, we must recognize the importance of the aromatic moiety.

Soil humic compounds contain high concentrations of free radicals of the semiquinone type, which are stable in air for many years and can survive the geochemical processes of humification and diagenesis. Under anaerobic environmental conditions microbial populations can utilize humic substances as electron pathways (Senesi and Schnitzer 1978). Recently, several papers have been published on the free radicals in humic and fulvic acids (Senesi and Schnitzer 1977; Senesi et al. 1977; Senesi and Lèvesque 1979), but only very few data are available on fungal melanins (Schnitzer and Skinner 1969; Riffaldi and Schnitzer 1972*a*). Therefore, it is interesting to study the free radical content in a variety of fungal melanins as a means to gain a better knowledge of their similarities with soil humic acids.

MATERIALS AND METHODS

The melanins synthesized by the fungi *Aspergillus niger*, *Aspergillus sydowi*, *Coprinus atramentarius*, *Coprinus comatus*, *Coprinus micaceus*, *Drechslera catenaria*, *Eurotium echinulatum*, *Hendersonula toruloidea*, and *Stachybotrys chartarum* were used. The melanins from *Coprinus* spp. were isolated from basidiocarps, and the remaining fungi were cultured on either glucose-asparagine or Czapek-Dox media. Chemical characteristics of the melanins are described elsewhere (Martin and Haider 1969;

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Haider and Martin 1970; Martin et al. 1972*b*; Saiz-Jimenez et al. 1975; Saiz-Jimenez et al. 1979; Saiz-Jimenez 1983).

Solvent extraction was accomplished in a Soxhlet apparatus for periods of 72 h, using hexane, benzene, ethyl acetate, and methanol. Methylation was achieved with diazomethane. The procedure for 2 *N* and 6 *N* HCl hydrolysis has been described (Saiz-Jimenez et al. 1975), as was that for persulfate oxidation (Martin et al. 1981).

Electron spin resonance (ESR) spectra were obtained using a Varian E-3 spectrometer at X-band frequencies, as reported by Riffaldi and Schnitzer (1972*a*). Data acquisition was carried out with a Tektronix 4051 graphic computer system and a Tektronix plotter/digitizer. The magnetic field at the sample was calibrated with diphenylpicrylhydrazil (DPPH). Spin concentrations were estimated by comparison with known concentrations of DPPH diluted with powdered KCl. Numbers of radicals were assumed to be proportional to signal height times signal widths squared. The differences in field between maxima in the derivative signals were taken as linewidth. Spectroscopic splitting factors (g values) were computed from values of the magnetic field (H) at which resonance occurred for the sample (H_1) and for a standard (H_2) of known g values (DPPH), using the relationship $H_2/H_1 = g_u/g_k$, where the subscripts u and k stand for unknown and known, respectively (Schnitzer and Ghosh 1982).

RESULTS AND DISCUSSION

ESR spectra of fungal melanins

A representative ESR spectrum of the melanins studied is shown in Fig. 1. This spectrum,

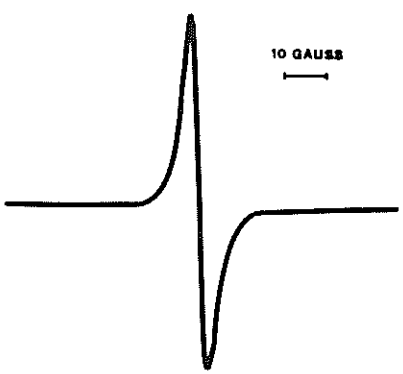

FIG. 1. ESR spectrum of *Eurotium echinulatum* melanin (glucose-asparagin medium).

TABLE 1

Range of ESR parameters for fungal melanins, model phenolic polymers, and soil humic fractions

	g Values	Linewidths, G	Spins, $g \times 10^{-17}$
Fungal melanins	2.0035–2.0041	2.9–5.9	0.7–19.0
Phenolic polymers	2.0036–2.0039	3.2–4.6	0.9–3.4
Soil humic acids	2.0034–2.0035	3.2–5.0	1.2–2.8
Soil fulvic acids	2.0034–2.0036	3.8–5.9	0.2–0.7

as shown for soil humic and fulvic acids (Riffaldi and Schnitzer 1972*a*), also consists of a single symmetrical line devoid of any hyperfine splitting, which indicates that the free radicals are extremely complex in structure.

ESR parameters in fungal melanins

The ESR spectral parameters of interest in fungal melanins are the g values, free radical concentration, linewidth, and lineshape (Table 1).

The g value is a useful parameter in terms of structural information, because a sufficient number of g values are catalogued in the literature so that some information on the radicals in fungal melanins can be obtained by comparison with the g values of compounds expected to be present. If the free radical electrons in fungal melanins are associated with a condensed-ring aromatic system containing only hydrogen and carbon, g values close to 2.0027 would be expected, because variations in g values for such radicals are quite small. The high g values found for the melanins suggest that some element other than carbon and hydrogen plays a role in the free radical species.

Elements present in melanins include carbon, hydrogen, oxygen, nitrogen, and sulfur. The g values in Table 1 correlate poorly with either the nitrogen ($g \sim 2.0031$) or sulfur ($g \sim 2.0080$) radicals, but well with the oxygen-containing free radicals. Methoxybenzene radicals are known to have g values around 2.0035 to 2.0040 and semiquinones from 2.0038 to 2.0047 (Blois et al. 1961), and the measured g values in fungal melanins all range between 2.0033 and 2.0041. Furthermore, several authors have reported that free radicals in soil humic and fulvic acids were most likely semiquinones (Steelink and Tollin 1967; Senesi and Schnitzer 1977).

It is obvious that phenolic polymers as fungal

melanins should contain a high amount of free radicals. The melanins studied have radical concentration similar to those of model phenolic polymers (with the exception of those from *Eurotium echinulatum* and *Drechslera catenaria*), and both groups, in many cases, have higher concentrations than soil humic substances (Table 1). Riffaldi and Schnitzer (1972*a*) stated that the free radical contents, *g* values, and linewidths of two melanins were of the same order of magnitude as those for fulvic acids. However, when working with a greater number of samples, as in this study, these similarities are not apparent, and fungal melanins were found to contain more free radicals than any of the humic fractions from Spanish soils or from Argentinian, Canadian, and Japanese soils (Riffaldi and Schnitzer (1972*a*).

According to Singer and Lewis (1978) three main factors contribute to the ESR linewidth of organic free radicals: hyperfine interaction with both aromatic protons and aliphatic substituents; electron delocalization, which is related to the degree of condensation and molecular size, and intermolecular exchange, which is dependent on free radical concentration.

Lineshapes are classified either as Lorentzian or Gaussian. They are distinguishable by the ratio of the half-width of the absorption curve to the peak-to-peak distance of the derivative curve. The Lorentzian shape has the property of approaching the baseline very slowly, whereas the Gaussian shape is narrower and drops off very quickly. The latter is common in biological systems and may result from a system in which each paramagnetic entity resonates at a slightly different magnetic field, most commonly because the apparent line is really an envelope of unresolved hyperfine components of narrower intrinsic linewidths, in contrast to the Lorentzian line shape that usually implies that all radicals are resonating at the same field (Bolton et al. 1972).

The shape of the ESR curves is Gaussian for most of the melanins, although *Aspergillus sydowi*, *Coprinus atramentarius*, and *Coprinus micaceus* show a line with good approximation to the Lorentzian type.

Effect of oxygen on ESR parameters

Gaseous oxygen has been found to reversibly alter the ESR properties of chars and coals. Probably the main effect of oxygen is to decrease the spin-lattice relaxation time of the spins (Singer, personal communication). Usually the original ESR properties can be restored by simply removing the oxygen (Singer 1963).

To understand the effects of oxygen (air) on the ESR parameters of fungal melanins, four different samples from *Aspergillus sydowi*, *Drechslera catenaria*, *Eurotium echinulatum*, and *Stachybotrys chartarum* were measured under air, after evacuation at 10^{-5} mm Hg for up 2 h and again after air exposure. In the three cases the free radical contents, *g* values, and linewidths remained unchanged, so all the subsequent samples were not evacuated prior to ESR measurements.

Influence of culturing conditions on melanins

The ESR parameters reported by Riffaldi and Schnitzer (1972*a*) for *Aspergillus niger* and *Stachybotrys chartarum* were different from those studied in this paper for the same fungi (see Table 4). To investigate the possible influence of laboratory methods (culture media, isolation, extraction, etc.) in the properties of melanins, subcultures of the same original strain of *Eurotium echinulatum* (Saiz-Jimenez 1976) were employed in two different laboratories for melanin synthesis. Differences in *g* values, linewidths, and free radical concentrations were evident in most of the cases, as well as differences in the melanins when incubated in distinct culture media. Furthermore, the melanins from *Hendersonula toruloidea* isolated from culture media and from cells were dissimilar (Table 2). Because ESR parameters are related to the chemical structure, the differences observed presumably indicate different structural compositions of the melanins, induced by the culture conditions and extraction procedures. The results may also point out a possible change in the organism that leads to physiological degeneration, because the physiological potentialities of any culture are always changing during periods of active growth. Thus, Foster (1949) stated that all investigations dealing with specific metabolic functions of a fungus sooner or later encounter physiological degeneration manifested by progressive loss of the function of interest. As a matter of fact, *Eurotium echinulatum* was cultured in 1973, at which time the fungus showed the ability to synthesize about 50 phenols and anthraquinones (Saiz-Jimenez and Haider 1975; Saiz-Jimenez et al. 1975). In a subsequent survey 2 years later, the fungus nearly lost the capacity to synthesize phenols, but retained the capacity to produce anthraquinones.

TABLE 2

Effect of laboratory procedures on the ESR parameters of fungal melanins

Fungi	*g* Values	Linewidths, *G*	Spins, $g \times 10^{-17}$
Eurotium echinulatum			
Glucose-asparagin medium			
Sevilla	2.0037	4.2	5.4
Riverside[a]	2.0036	4.2	10.0
Czapek-Dox medium			
Sevilla	2.0041	5.0	1.3
Riverside[a]	2.0035	4.2	5.4
Hendersonula toruloidea			
Glucose-asparagin medium	2.0037	3.8	4.7
Czapek-Dox medium	2.0037	2.9	3.7
Cells	2.0036	3.4	1.8

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TABLE 3

Effect of methylation and solvent extraction on ESR parameters of fungal melanins

Fungi	*g* Values	Linewidths, *G*	Spins, $g \times 10^{-17}$
Eurotium echinulatum	2.0037	4.2	5.4
Eurotium echinulatum methylated	2.0035	5.0	12.0
Eurotium echinulatum extracted[a]	2.0036	4.2	6.9
Hendersonula toruloidea	2.0037	3.8	4.7
Hendersonula toruloidea methylated	2.0036	3.4	4.8
Hendersonula toruloidea extracted[a]	2.0039	3.4	6.9
Drechslera catenaria	2.0039	3.8	19.0
Drechslera catenaria methylated	2.0035	3.4	19.0
Drechslera catenaria extracted[b]	2.0036	3.4	21.0
Aspergillus sydowi	2.0039	4.2	1.7
Aspergillus sydowi methylated	2.0038	6.3	3.6

[a] Hexane, benzene, ethyl acetate, and methanol extractions.

[b] Methanol extraction.

Methylation and solvent extraction of melanins

It has been reported that methylation of humic acids decreased *g* values and linewidths and increased concentration of free radicals (Riffaldi and Schnitzer 1972*a*). In fungal melanins, *g* values decreased and free radicals increased upon methylation. However, these samples showed a contradictory behavior with respect to linewidth, which increased significantly in *Eurotium echinulatum* and *Aspergillus sydowi* and decreased slightly in *Hendersonula toruloidea* and *Drechslera catenaria* (Table 3).

The above-mentioned authors do not give explanations for the increase in free radicals and believe that the decrease in linewidths may be the result of the greater mobility of unpaired electrons. We are unable at this time to offer explanations for the distinct behavior of the melanins. However, as the linewidths are constitutional parameters, an increase in linewidths might be related to the decrease of the atomic C/H ratio or the increase of aliphatic substituents or both.

Solvent extraction of melanins released mainly *n*-alkanes and *n*-fatty acids, as well as anthraquinones in the cases of *Eurotium echinulatum* and *Drechslera catenaria*. There are a wide variety of responses in the melanins to the solvent extraction, which indicate varying amounts of extractable materials and differences in chemical structures. Thus, while the

Eurotium echinulatum ESR parameters do not change, except for an increase in some free radical contents, there is a slight linewidth decrease in *Drechslera catenaria* and *Hendersonula toruloidea*. Also the *g* value increased in *Hendersonula toruloidea* and decreased in *Drechslera catenaira*.

Acid hydrolysis and mild oxidation of melanin

Hydrolysis with 6 *N* HCL removed amino acids, carbohydrates, metals, and other adsorbed compounds from humic acids and melanins (Riffaldi and Schnitzer 1973; Saiz-Jimenez 1975). Riffaldi and Schnitzer (1972*b*) evaluated the effects of acid hydrolysis on the ESR parameters of humic acids. Free radical content for samples that had been subjected to acid hydrolysis were in all cases higher than those for untreated samples. Linewidth tended to increase slightly after hydrolysis in three of the four samples, but *g* values decreased or did not change.

Table 4 shows the effects of acid hydrolysis on fungal melanins. These biopolymers had the same behavior as humic acids with respect to free radical content and *g* values. It is noteworthy that the spin content doubled or tripled in 2 *N* HCl hydrolysis, while 6 *N* HCl hydrolysis caused an increase of 6 to 31 times. The linewidths, in some cases, showed a slight increase (*Aspergillus niger*, *Coprinus micaceus*, *Eurotium echinulatum*-6 *N* HCl) and, in others, a slight decrease (*Coprinus comatus*, *Eurotium echinulatum*-2 *N* HCl, *Stachybotrys chartarum*).

According to Riffaldi and Schnitzer (1972*b*), the enhancement of the spins has two possible explanations: either the materials lost contain few free radicals or none at all, so that the residue becomes enriched (hypothesis valid only for the 2 *N* HCl hydrolysis, in this case) or the hydrolysis produces additional free radicals. As acid hydrolysis leads to increased condensation or polymerization, and also perhaps aromatization (see Saiz-Jimenez 1983), the molecular complexity increases and so does the free radical concentration. In this concentration, Singer and Lewis (1978) reported that free radical content in chars increases as the polymerization process proceeds further and the atomic C/H ratio increases. A similar increase in this ratio has been noted for fungal melanins upon acid hydrolysis (Saiz-Jimenez 1975, 1983).

Furthermore, the observed decrease in *g* values may be explained either by the loss of oxygen-containing structures in which the unpaired spins are associated with aromatic molecules (e.g., loosely held and adsorbed phenols and quinones) or because melanins become more "aromatic hydrocarbonlike" upon acid hydrolysis.

Senesi et al. (1977) reported that H_2O_2, Ag_2O, and $NaIO_4$ oxidations of fulvic acids showed no significant changes in *g* values, but a broadening of linewidths was observed. Also very strong decreases in free radical concentrations were noted. Persulfate oxidation has been described by Martin et al. (1981). This method seems to release loosely held and adsorbed materials in amounts of up to 40% of the total original

TABLE 4

Effect of acid hydrolysis and mild oxidation on ESR parameters of fungal melanins

Fungi	*g* Values	Linewidths, *G*	Spins, $g \times 10^{-17}$
Aspergillus niger	2.0036	3.4	0.7
Aspergillus niger 2 *N* HCl	2.0035	3.8	1.8
Stachybotrys chartarum	2.0040	4.7	1.5
Stachybotrys chartarum 2 *N* HCl	2.0038	4.6	4.3
Coprinus comatus	2.0038	5.5	1.4
Coprinus comatus 6 *N* HCl	2.0035	4.2	8.1
Coprinus micaceus	2.0040	5.9	1.6
Coprinus micaceus 6 *N* HCl	2.0036	6.3	49.0
Eurotium echinulatum	2.0037	4.2	5.4
Eurotium echinulatum 2 *N* HCl	2.0035	3.8	10.0
Eurotium echinulatum 6 *N* HCl	2.0034	4.6	35.0
Eurotium echinulatum oxidized[a]	2.0035	3.8	22.0
Drechslera catenaria	2.0039	3.8	19.0
Drechslera catenaria oxidized[a]	2.0036	3.4	11.0

[a] After methylation.

weight, which indicates that it is milder than other oxidations previously reported (Schnitzer and Khan 1972).

Table 4 also shows the ESR parameters of the melanins before and after persulfate oxidation. A decrease was observed in the *g* values, linewidths, and free radicals in the two samples, except for the free radical content of *Eurotium echinulatum*, in which the spins were enhanced four times. These results can be understood if we keep in mind that this oxidation releases part of the aliphatic material, as reported by Martin et al. (1981) (decreasing the linewidth), and possibly an important portion of the aromatic moiety in the case of *Drechslera catenaria* (decreasing the *g* value and spins, corresponding to the loss of quinones). *Eurotium echinulatum* seems to release only a few oxygenated aromatic components (very slight decrease in *g* value and increase of spins, due to enrichment of free radicals in the residue).

Chemical structure of melanins

The biosynthesis of phenols by fungi is based either on acetyl-CoA and malonyl-CoA condensation reactions—acetate pathway—or from nonaromatic metabolites—shikimic acid pathway (Turner 1971). Through these pathways orsellinic, cresorsellinic, methylsalycilic, and *p*-hydroxycinnamic acids and acetylphloroglucinol are synthesized and transformed to numerous other phenolic compounds by decarboxylation, hydroxylation, alkylation, and oxidation of methyl groups. The resulting phenols are oxidized either by enzymes or autooxidation, and the oxidative coupling of phenols and quinones results in the formation of a phenolic polymer or melanin, which exhibits an electron spin resonance due to the presence of phenoxyl and semiquinone radicals. During the polymerization process other materials, such as alkanes, fatty acids, polysaccharides, and proteins, present in the culture media or in the cells are incorporated into the developing polymer.

There are no definitive explanations for the ESR phenomena observed in complex organic materials. A sensible way to approach such materials is to compare their spectra with those of pure model compounds or model polymers that might be relevant. In the laboratory, model phenolic polymers can be synthesized either by autooxidation or enzymatic oxidation (Martin et al. 1972*a*). The properties of these polymers, with or without proteins, are very similar to fungal melanins, including the ESR parameters.

CONCLUSIONS

From the data presented in this paper, it appears that fungal melanins are different from soil humic acids with regard to the free radical content, which is higher than phenolic polymers. As far as is known, the soil humic acids cannot be considered true phenolic polymers, although they contain these compounds as part of their building blocks, because other widely diverse structures, such as polycyclic aromatic hydrocarbons and benzenepolycarboxylic acids, are also present in the molecule. The resulting humic acid is produced by a long process of humification that takes many years. For instance, Scharpenseel (1971) reported radiocarbon dating of soil humic fractions ranging up to 6000 years old. Thus, free radicals in soil humic substances reflect the constitutional changes that occur during the humification process. Conversely, fungal melanins are considered to constitute young or new humus, in which functional groups may be relatively free of metal ions or interactions with other organic compounds (Zunino and Martin 1977*a*). When incorporated into soils, the melanins and microbial polymers may play important roles through polymerization-depolymerization reactions with humic substances (Suflita et al. 1891); through reactions with inorganic (Zunino and Martin 1977*b*) or organic compounds, such as pesticides and toxic pollutants (Fruh et al. 1977); and through the physiological effects that these substances are known to exert (Mato 1976). However, apparently it takes long processes before fungal melanins are converted to soil humic acids.

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