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# Analysis of the Structure of Synthetic and Natural Melanins by Solid-Phase NMR†

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ABSTRACT: The structures of one synthetic and two natural melanins are examined by solid-state NMR using cross polarization, magic angle sample spinning, and high-power proton decoupling. The structural features of synthetic dopa melanin are compared to those of melanin from malignant melanoma cells grown in culture and sepia melanin from squid ink. Natural abundance $^{13}C$ and $^{15}N$ spectra show resonances consistent with known pyrrolic and indolic structures within the heterogeneous biopolymer; $^{13}C$ spectra indicate the presence of aliphatic residues in all three materials. These solid-phase experiments illustrate the promise of solid-phase NMR for elucidating structural information from insoluble biomaterials.

Melanins are a class of pigments widespread in the animal and plant kingdoms (Nicolaus, 1968). A subcategory known as eumelanins are unique solid biopolymers derived from tyrosine and characterized by their insolubility, intense dark color, resistance to hydrolysis, lack of molecular regularity, and paramagnetism. After years of study, no generally accepted molecular structures have been defined for these materials. The single periodic structural feature observed in melanins is a 0.34-nm spacing believed to correspond to the adventitious parallel stacking of aromatic units in randomly oriented local domains (Nicolaus, 1968; Blois, 1978; Swan, 1974).

As materials, melanins have been described as both insulators and semiconductors (Swan, 1974; Pullman & Pullman, 1961), ion-exchange resins (Lindquist, 1986), redox polymers (Froncisz et al., 1980; Sarna et al., 1980) and free-electron scavengers (Packer et al., 1981). Their biological functions range from an obvious role as pigmentation agents in skin, hair, and feathers to protective agents against light-induced damage (an empirically evident action that still needs further mechanistic clarification) (Pathak et al., 1976), as possible participants in one- and two-electron reduction systems (Felix et

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al., 1978), and as sound-absorbing substances in the inner ear (Kaplan & Holasek, 1983). The reasons for the presence of the biopolymer in other tissues (the substantia nigra of the human brain for example) and its unusual involvement in certain disorders (Addison's disease, schizophrenia, and Parkinsonism) still defy explanation (Swan, 1974; Strzelecka, 1982). The paucity of structural knowledge about melanins inhibits the elucidation of their functions.

Because of insolubility and opacity, melanins resist structural analysis by the conventional techniques of UV–visible and infrared spectroscopy. X-ray diffraction powder patterns indicate the presence of randomly localized structural units (Blois, 1971; Thathachari & Blois, 1969). The free-radical content of melanins has been explored extensively by EPR[1] (electron paramagnetic resonance) spectroscopy (Felix et al., 1978; Angiolillo et al., 1985; Mason et al., 1960; Sealey et al., 1982a,b), and X-ray photoelectron spectroscopy (XPS) can define the chemical natures of nitrogen and sulfur atoms (Williams-Smith et al., 1976), but neither can yield structural details. High-resolution NMR in the liquid phase is precluded by the insolubility of eumelanins, although spectra of the alkali-soluble fungal melanins in 0.1 N NaOD have been reported (Ludemann et al., 1982).

Most structural information has been inferred from analyses of soluble fragments resulting from heating and oxidative degradation of the polymer. Additional insight has been obtained from synthetic studies using isotopically enriched precursors, but results of these attempts have not always yielded unequivocal interpretations (Blois, 1978; Swan, 1974). Identified structural elements within melanin include substituted hydroxyindoles, indolequinones, and pyrroles, free carboxylic acid groups, phenolic hydroxyls, and carbon-based free radicals. Still at issue is the presence of uncyclized aliphatic chains, originating from the monomer precursors: quantitative experiments using isotopic labels indicate the absence of uncyclized chains (Hearing et al., 1980); degradative analyses (Nicolaus et al., 1964) and other labeling studies in which retention of isotopes in specific structural loci was evaluated (Swan, 1963) support the presence of uncyclized moieties.

Recently, the NMR techniques of cross polarization (Pines et al., 1973), magic angle sample spinning (Andrew et al., 1959; Lowe, 1959), and high-power proton decoupling (Mehring, 1983) have been applied to structural studies of natural solid materials like coals and soil fractions (Vassallo et al., 1987). Solid-state NMR has also been recently used to probe the molecular conformation and crystalline structure of a polymer of pustulan, a glucan of known primary structure (Stipanovic et al., 1985). This report describes the first application of solid-state NMR to the evaluation of melanins of natural and synthetic origin: synthetic dopa melanin, melanoma melanin produced by malignant melanoma cells in culture, and sepia melanin obtained from the ink sacs of the squid *Sepia officinalis*. The results of this study reconfirm the polyindolic nature of the eumelanins and verify the presence of uncyclized material in the biopolymer from all three sources.

## Materials and Methods

*Materials.* Acids, bases, and organic solvents used were reagent grade. L-Dopa was also reagent grade and purchased from Sigma, as was melanin from *S. officinalis*. Melanoma melanin was collected from Fortner malignant melanoma (MMI) cells grown in culture in a blend of three parts of RPMI 1640 and one part of L15 (Liebowitz's) supplement with 20% fetal calf serum; it was a gift from Dr. David V. Woo and Harry L. Walton of Hahnemann University, Philadelphia, PA.

*Preparation of Dopa Melanin.* Dopa melanin was prepared from L-dopa according to the method of Arnow (1938) with the slight alteration that, after precipitation and washing, the melanin was vacuum dried for 6 h at 60 °C.

*Treatment of Melanoma Melanin.* The spent culture medium from which Fortner MMI cells were harvested was treated with an equal volume of concentrated HCl and refluxed for 3–5 days. The solution was then centrifuged, the supernatant decanted from the precipitated melanin, and the solid washed five times with 0.005 N HCl, twice with acetone, and once with diethyl ether. The solid was dried under vacuum overnight at 60 °C. The recovery of dried melanin was typically 100 mg for every 800 mL of spent cell culture medium.

*NMR Data Acquisition.* Solid-state NMR spectra were obtained on a General Electric NMR Instruments GN-300 wide-bore spectrometer. A Doty Scientific 5-mm probe equipped with a high-speed spinning system was used, and all experiments employed cross polarization (Pines et al., 1973), magic angle sample spinning (Andrew et al., 1959; Lowe, 1959), high-power proton decoupling techniques (Mehring, 1983), and quadrature detection. Sample size was ~80 mg.

Typical experimental parameters included 2.0-ms contact time, 10-s recycle delay, and from 2000 to 5000 transients for $^{13}C$ and up to 15000 transients for $^{15}N$ signal-averaged spectra. The Doty Scientific spinning system allowed spinning speeds of 10.0 kHz for $^{13}C$ experiments and 5.0 kHz for the $^{15}N$ spectra. The only exception to these spinning speeds was the 4.8 kHz used for the solid-phase $^{13}C$ spectrum of the L-dopa monomer. The higher speeds for the polymers placed all rotational sidebands outside the region of overlap with any spectral features of interest. External TMS was the shift reference for $^{13}C$ and external $^{15}N$-labeled $NH_4Cl$ for $^{15}N$. The spectrometer observe frequencies were 75.46 and 30.41 MHz, respectively. Exponential line broadening of up to 200 Hz was applied to all spectra to improve signal-to-noise ratios. Chemical shifts reported here are accurate to ±1 ppm in the best melanin spectra; in some spectra, the resolution is considerably poorer due to the breadth of the resonances and the signal-to-noise limitations.

*EPR Data Acquisition.* EPR measurements were carried out at 9.4 GHz with an X-band homodyne detection EPR spectrometer. Magnetic field modulation amplitude was on the order of 1 G with microwave power of ~3 μW under nonsaturating conditions. Sample size was ~80 mg, and spectra were obtained in quartz tubes at room temperature.

Table I: Spectral Features of $^{13}C$ and $^{15}N$ CP/MASS Spectra of Melanin Samples

| | $^{13}C$ resonances | | | $^{15}N$ resonances | |
|---|---|---|---|---|---|
| | carbonyl | arom | aliph | pyr/ind | aliph |
| dopa melanin | 172 | 143<br>118 | 55<br>35 | 130–80 | 0 |
| *melanoma melanin* | 173 | 125 | 53<br>33 | 130–80 | |
| sepia melanin | 173 | 140–110 | 70–30 | 130–95 | |

## Results and Discussion

The assignments of spectral features for the solid-state $^{13}C$ and $^{15}N$ spectra of the three melanin samples are summarized

[1] Abbreviations: NMR, nuclear magnetic resonance; CP/MASS, cross polarization, magic angle sample spinning; EPR, electron paramagnetic resonance; TMS, tetramethylsilane; L-dopa, 3-(3,4-dihydroxyphenyl)-L-alanine.

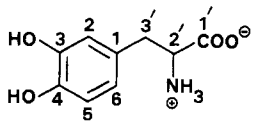


FIGURE 1: Structure of L-dopa, monomeric precursor of synthetic dopa melanin and of natural eumelanins; numbering refers to chemical shift assignments (see Figure 2).

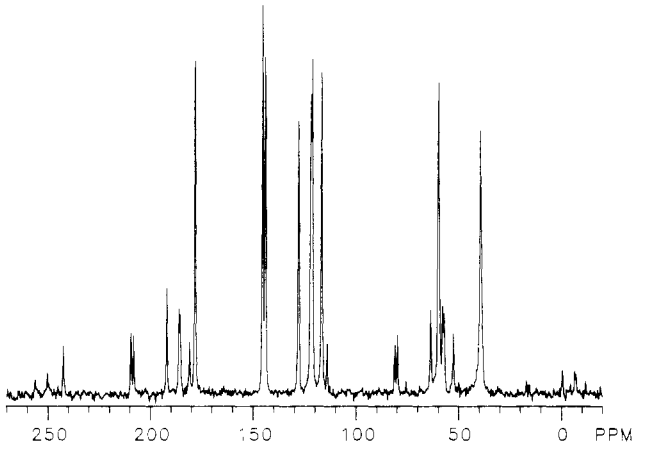


FIGURE 2: Solid-state $^{13}C$ spectrum of L-dopa, centerband assignments: C(1) 127, C(3) 144, C(4) 142, C(2), C(5), C(6) 121–116, C(1′) 177, C(2′) 59, C(3′) 38 ppm. The less intense resonances are rotational sidebands separated from the centerband resonances by multiples of the spinning speed (4.8 kHz); 64 transients were averaged and 10-Hz exponential line broadening was used in processing the spectrum.

in Table I. For the purpose of comparison, peak assignments were initially made for the solid-phase spectrum of L-dopa (Figure 1), the monomeric precursor of dopa melanin, by analogy to the spectrum of the same compound in the liquid phase (Lai et al., 1985). The less intense features in the L-dopa spectrum are rotational sidebands separated from the assigned centerbands by integral multiples of the spinning speed (Herzfeld & Berger, 1980).

The $^{13}C$ spectrum of synthetic dopa melanin (Figure 3a) shows five broad centerband resonances, melanoma melanin (Figure 3b) shows four broad resonances, and sepia melanin (Figure 3c) shows three. In all three samples, carbonyl peaks in the region associated with carboxylic acid resonances are readily apparent (ca. 173 ppm), as are peaks in and near the aromatic region consistent with aromatic carbons bearing oxygen (140–155 ppm) and indole and pyrrole structures (110–130 ppm). Resonances centered at 55 and 35 ppm result from uncyclized aliphatic chains in the polymer. It cannot be ruled out that these resonances may be due to unreacted monomer entrapped within the melanin matrix.

The spectra of dopa melanin and melanoma melanin are very similar, indicating that the same types of carbon resonances exist in both the synthetic and natural polymers. The sepia melanin shows a broad resonance containing many overlapping peaks in the region from 80 to 20 ppm; these features are set against a high background noise. In all three spectra, broad features may indicate heterogeneity in the polymer, a well-known aspect of melanin structure.

The $^{15}N$ spectra of all polymers (Figure 4a–c) show absorbances characteristic of indolic and pyrrolic nitrogens centered around 100 ppm (Levy & Lichter, 1979). The broad lines are further evidence for complex and variable cross-linking among the rings in the polymer network.

Spectral features arising from aliphatic material are also evident in the $^{15}N$ spectrum of synthetic melanin (Figure 4a). The resonance at 0 ppm may be assigned to the nitrogen of

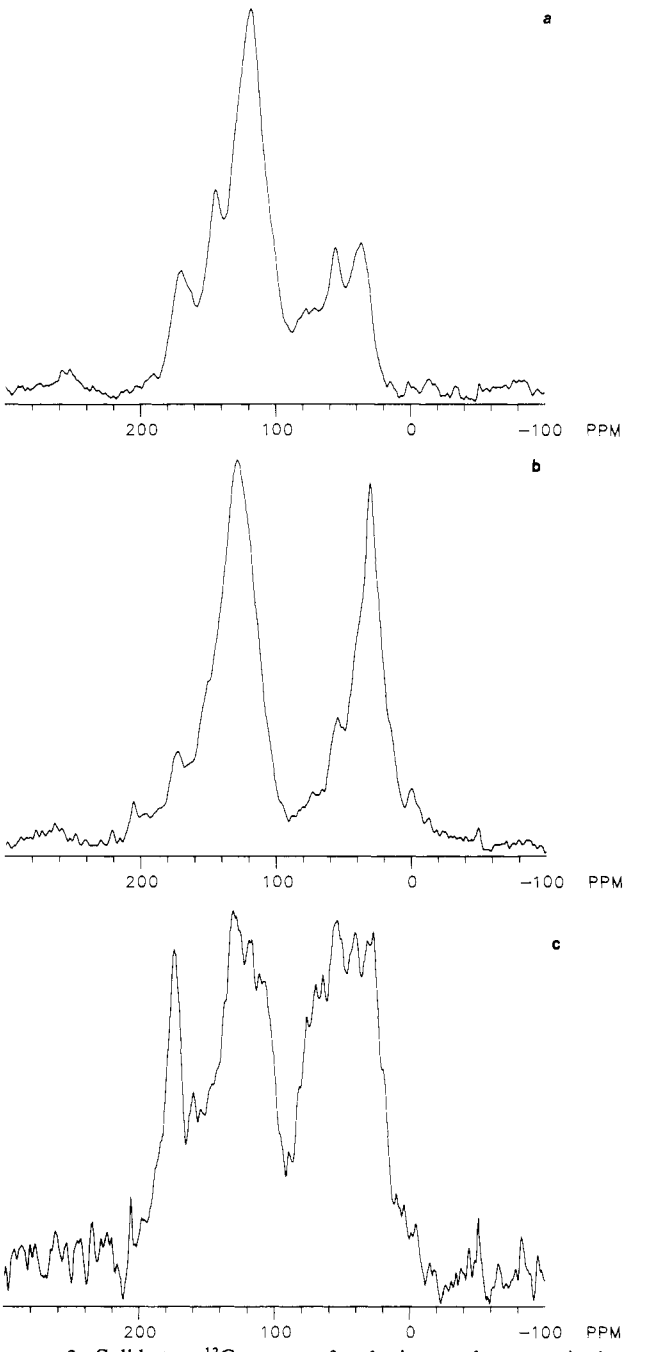


FIGURE 3: Solid-state $^{13}C$ spectra of melanin samples at a spinning speed of 10 kHz with 100-Hz line broadening, all showing carboxylic acid, aromatic, and aliphatic resonances: (a) synthetic dopa melanin, 4392 transients; (b) melanoma melanin, 1976 transients; (c) sepia melanin, 2784 transients. The poorer signal-to-noise ratio in spectrum c may be due to the higher free-radical content of sepia melanin noted in EPR studies.

a primary aliphatic amine. The corresponding spectra of melanoma and sepia melanin (Figure 4b,c) have no such obvious peaks in the aliphatic region, but the poorer signal-to-noise and the fact that cross polarization is not as efficient to nitrogen as it is to carbon will tend to obscure resonances.

EPR measurements of the three melanin samples showed a strong resonance absorption with a $g$ value of $\sim 2.004$ and a line width of $\sim 10$ G peak-to-peak in agreement with literature values (Mason et al., 1960). The spectrum from the sample of sepia melanin indicated a 2- to 3-fold higher free-radical content than that of the dopa melanin or the melanoma melanin. This factor, in addition to greater structural heterogeneity in the polymer, could explain the poorer signal-to-

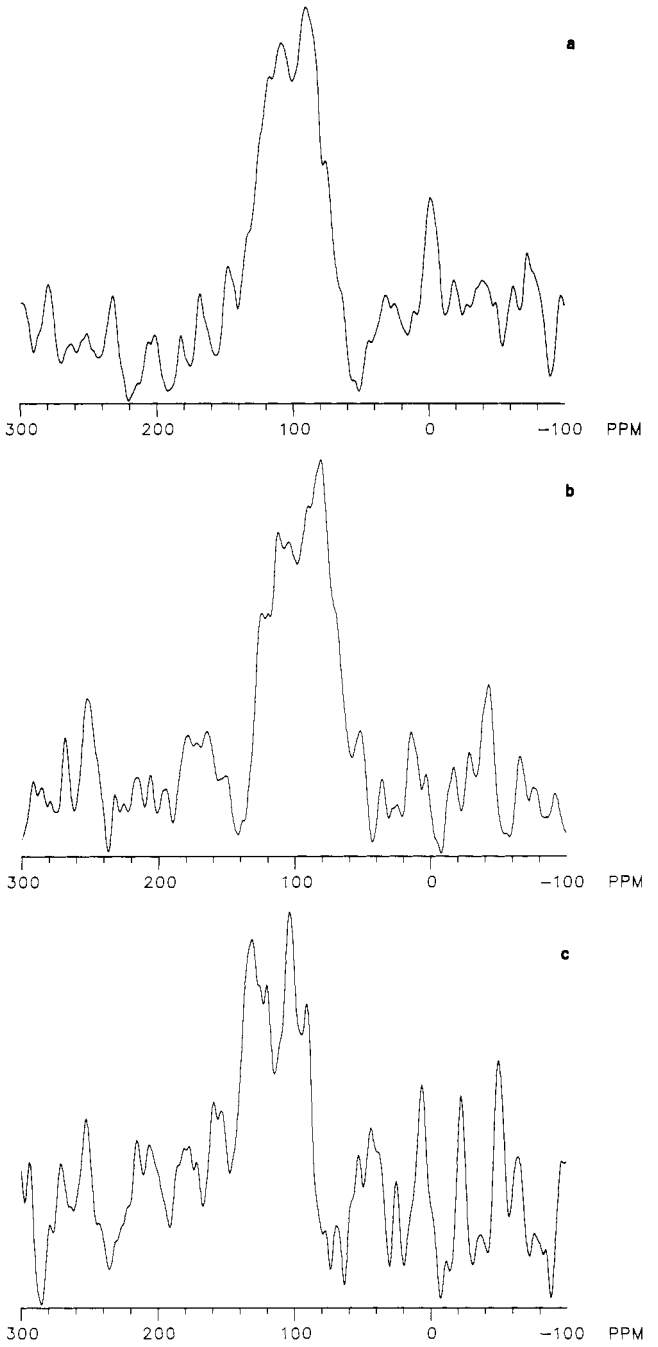


FIGURE 4: Solid-state $^{15}N$ spectra of melanin samples at a spinning speed of 5 kHz with 150-Hz line broadening, all showing resonances due to indolic and/or pyrrolic nitrogens. Only the synthetic melanin (a) shows evidence (peak at ca. 0 ppm) of an aliphatic primary amine. (a) synthetic dopa melanin, 7024 transients; (b) melanoma melanin, 2784 transients; (c) sepia melanin, 15 544 transients.

noise ratio in the NMR spectra of sepia melanin as a result of dipolar line broadening caused by the unpaired electrons.

The most important conclusions drawn from the solid-phase $^{13}C$ and $^{15}N$ spectra are that aliphatic carbons are present in all three types of melanin and that synthetic dopa melanin is similar in structure to natural melanin derived from malignant melanoma cells. This similarity has been previously claimed and used to advantage in studies of melanin binding since the seminal study of Potts on that topic (Potts, 1964), but these solid-state spectra provide the first direct confirmation of the structural likenesses. The sepia melanin has the same essential structural features as the other two, but its spectrum is more difficult to compare with confidence because of the poorer signal-to-noise ratio and the polymer's apparently greater heterogeneity in the region of resonances due to saturated carbons.

The $^{15}N$ spectrum of dopa melanin further confirms the presence of aliphatic material by showing the resonance due to a primary aliphatic amine. That this feature is absent from the other two polymers may stem from difficulties inherent in the observation of natural abundance $^{15}N$ and not from the samples themselves.

Finally, by use of the techniques of cross polarization, magic angle sample spinning, and high-power proton decoupling, solid-phase NMR of natural abundance $^{13}C$ and $^{15}N$ has proven to be a useful tool for the elucidation of structural features of the melanins that have previously been unobservable by any other spectroscopy. Although not as detailed as high-resolution spectra in the liquid phase, such solid-phase experiments show great promise for assisting in the clarification of structure of other insoluble solid biomaterials.

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# A Calorimetric Analysis of Human Plasma Fibronectin: Effects of Heparin Binding on Domain Structure†

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ABSTRACT: Fibronectin domain structure, as influenced by interaction with heparin, calcium, or chondroitin sulfate C, was analyzed by differential scanning calorimetry. A complex thermal denaturation transition was observed with a large sharp endotherm at 63 °C, a broad endotherm between 70 and 80 °C, and an exotherm at 80–90 °C. Analysis of the denaturation profiles revealed the existence of four thermal transitions, 59.1, 62.2, 67.3, and 74.3 °C, and an exotherm at 83.9 °C. The calorimetric enthalpies of the four endotherms are 1146 ± 259, 866 ± 175, 1010 ± 361, and 676 ± 200 kcal/mol, respectively. In all cases, the calorimetric to van't Hoff enthalpy ratio was greater than 1.0. Computer analysis of the primary structure of fibronectin revealed 29 ± 8% homology among the type I homology units and 28 ± 7% homology among type III homology units, suggesting that different structural domains could arise from the same homology type. This may explain why more thermal transitions are observed for fibronectin than there are homology types. Addition of heparin to fibronectin in varying molar ratios, i.e., 10:1 to 30:1, resulted in a larger calorimetric enthalpy for the first type of structural domain ($T_m$ = 59.1 °C) of fibronectin. At higher heparin to fibronectin ratios (40:1 or 75:1), the enthalpy of this domain decreased, while the others remained unchanged. In the presence of 5 mM calcium chloride, fibronectin thermal denaturation occurred at lower temperatures and was associated with precipitation of fibronectin. With both calcium chloride (5 mM) and heparin (20:1), a decrease in the total enthalpy of denaturation of fibronectin was observed, precipitation did not occur, and the $T_m$'s were comparable to fibronectin in the absence of $Ca^{2+}$. Chondroitin sulfate C resulted in a large endotherm at 61.2 °C and aggregation above 65 °C, which was unlike the response to heparin. Thus, fibronectin has multiple heparin binding sites which differ in their affinities for heparin and sensitivity to calcium. These calorimetric assessments of the structural domains of fibronectin may provide insight into the structure–function relationships of the molecule.

Fibronectin is a large, adhesive, glycoprotein which exists in both plasma and tissue (Mosher, 1984; Ruoslahti et al., 1981). The concentration of fibronectin in human plasma is between 300 and 400 μg/mL (Mosesson & Umfleet, 1970). Plasma fibronectin is an opsonic molecule that promotes phagocytosis of nonbacterial particulates (Rourke et al., 1984). Fibronectin is also a component of the extracellular matrix and involved in wound healing and fibroblast adhesion to substratum (Saba, 1982; Yamada, 1983). Cell adhesion, cell–cell interaction, and integrity of the vascular barrier (Cohler et al., 1987) are promoted by fibronectin in tissues (Klebe et al., 1977; Kleinman et al., 1981; Yamada, 1983; Yamada & Olden, 1978). After trauma, burn, or starvation, the plasma fibronectin levels decrease in association with depression of reticuloendothelial phagocytic function (Deno et al., 1984; Saba, 1982; Saba et al., 1984), implying an important function for fibronectin in host defense following injury. While differences in primary structure exist between plasma and tissue fibronectin, the two forms are structurally very

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