Biosynthetic and Structural Studies on Pheomelanin. 2

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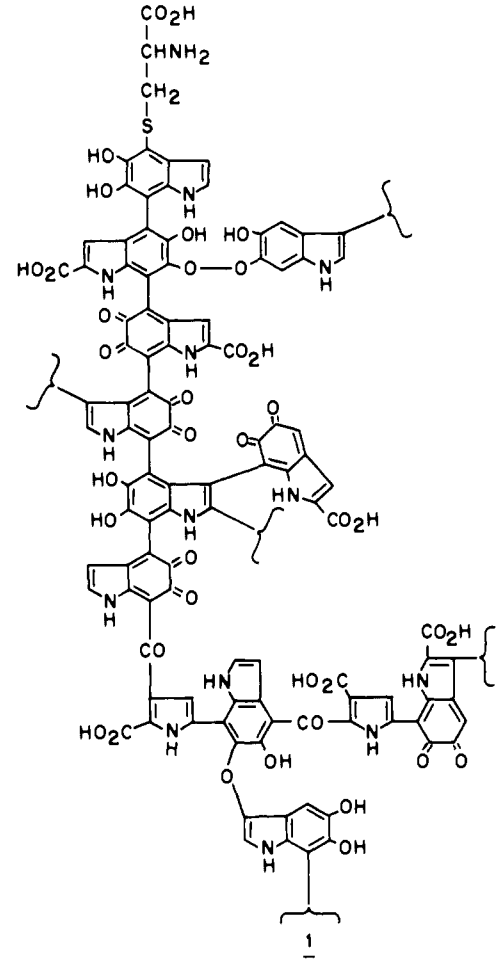
Abstract: Pheomelanin, the red-brown polymeric pigment present in the hair and skin of fair-skinned humans, is produced by the tyrosinase-mediated copolymerization of L-3,4-dihydroxyphenylalanine (L-dopa) and L-cysteine. Cysteinyldopas are the first isolatable intermediates along the biosynthetic pathway, and their individual polymerization by the action of tyrosinase produces model pheomelanin polymers well suited for structural studies. A reinvestigation of the structure of pheomelamin employing carbon-13, carbon-14, tritium, and sulfur-35 labeled cysteinyldopas is reported. Results indicate that (1) pheomelanins produced from individual cysteinyldopas are more homogenous than those from dopa and cysteine, (2) during the biosynthesis there is a significant loss of tritium from the benzylic carbon of the cysteinyldopas which, by carbon-13 NMR, corresponds to formation of an sp^2 carbon in the alanyl side chain, and (3) small amounts of degradation of both the alanyl and cysteinyl side chains occur during biosynthesis.

Melanins are one of the major biological polymers responsible for the diversity of coloration found in the animal kingdom. Interest in melanins stems from a combination of their widespread occurrence in biological systems (e.g., the skin, hair, choroid and iris of the eye, and inner ear of mammals, the substantia nigra of primates, bird feathers, insect cuticles, and the ink sack of cuttlefish) and, with the possible exceptions of photoprotection and camouflage, a lack of understanding of their biological function. In addition, transformation of the cell responsible for production of melanins in humans, the melanocyte, leads to a serious form of cancer, malignant melanoma.

Epidermal melanin pigments are believed to be the single most important factor in the protection of skin from damage caused by sunlight. There are two types of melanins responsible for the color of human skin: the black or brown eumelanins and the reddish-brown pheomelanins. Traditionally, these two melanins were considered to be chemically distinct, but more recent evidence has shown that the two melanins are biogenetically related and exist as intimate mixtures in epidermal pigmentation.[2] Photochemically the two pigments differ, and it has been proposed that rapid photodegradation of pheomelanin is responsible for the increased susceptibility of fair-skinned individuals to the deleterious effects of sunlight.[3]

Epidermal melanins consist of a polymeric chromophore covalently bound to a protein fraction. Neither the sequence nor nature of this attachment is known. With the exception of electron spin resonance spectroscopy,[4] spectroscopic analysis has heretofore revealed little structural information. Biosynthetic studies and harsh chemical degradative methods were used to determine that the eumelanin chromophore is an amorphous, irregular polymer composed of several major monomer units (structure **1**).[5] Less work has been done on the structure of the pheomelanin polymer; however, chemical degradative studies indicate that pheomelanin, which is produced by the tyrosinase-catalyzed copolymerization of L-3,4-dihydroxyphenylalanine (L-dopa) and L-cysteine, *may* be composed of 6,7,8,9-tetrahydro-4-hydroxythiazolo[4,5-*h*]isoquinoline-7-carboxylic acid (**2**) monomers.[6-8] However, the degradative methods used were sufficiently harsh that one must question whether the products isolated were artifacts and whether the structure deduced from such products accurately represents the intact polymer. We therefore decided to reinvestigated the structure of pheomelanin employing precursors labeled with both radioactive isotopes and ^{13}C, which was monitored by NMR spectroscopy.

In our initial paper on these investigations, we reported results on the studies of pheomelanin produced from L-dopa and L-cysteine,[9] using carbon-14 and tritium-labeled dopas and sulfur-35-labeled cysteine. We demonstrated that (1) pheomelanin is



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(1) NIH Research Career Development Awardee, 1982-1987.
(2) Rorseman, H.; Agrup, C.; Hansson, C.; Rossengren, A.-M.; Rosengren, E. In "Pigment Cell"; Klaus, S. N., Ed.; Karger: Basel, Switzerland, 1979; Vol. 4, pp 244-252.
(3) Agin, P. P.; Sayre, R. M.; Chedekel, M. R. *Photochem. Photobiol.* **1980**, *31*, 359-362.
(4) Sealy, R. C.; Felix, C. C.; Hyde, J. S.; Swartz, H. M. In "Free Radicals in Biology"; Pryor, W. A., Ed.; Academic Press: New York, 1980; Vol. IV, pp 209-259.
(5) Swan, G. A. *Fortschr. Chem. Org. Naturst.* **1974**, *31*, 522-582.
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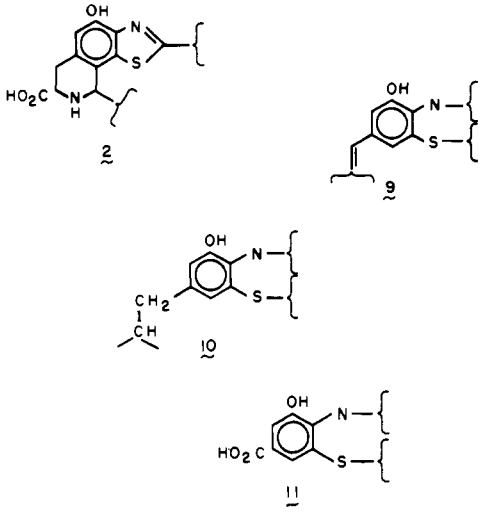
not a uniform polymer but rather a mixture of a number of polymers, (2) pheomelanin is not strictly a 1:1 copolymer of dopa and cysteine, and (3) during the course of the biosynthesis up to 50% of the dopa residues may suffer decarboxylation. In the current paper we have examined the preparation of pheomelanin from cysteinyldopas, the first isolable intermediates in its biosynthesis.

Materials and Methods

Electrophoresis grade acrylamide, ammonium persulfate, *N,N,N',N'*-tetramethylenediamine (TEMED), sodium dodecyl sulfate (NaDodSO$_4$), and electrofocusing gel were purchased from Bio-Rad Laboratories. Ampholytes were prepared synthetically as previously described.[10] Polyphenol oxidase (E.C. 1.14.18.1) was purchased from Sigma Chemical Co. and assayed by literature methods.[11] All other biochemicals were purchased from Sigma Chemical Co. Radioactive compounds were purchased from Amersham, New England Nuclear, or Research Products International. Organic chemicals were from Aldrich and scintillation cocktail was prepared in the laboratory.[12]

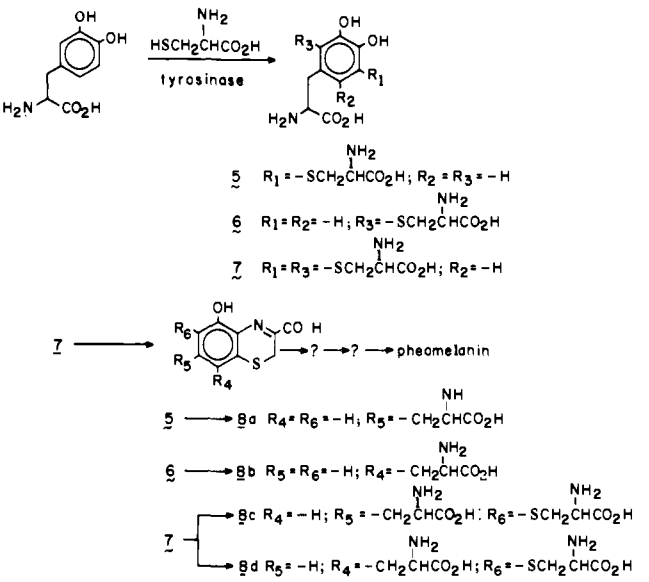
Proton and carbon NMR spectra were obtained on either a Perkin-Elmer R-600 or an IBM NR80 nuclear magnetic resonance spectrometer. Infrared spectra were recorded on a Perkin-Elmer R-298 infrared spectrometer, and absorbance spectra were obtained on a Varian Cary 219 spectrophotometer. Levels of radioactivity were determined with a Beckman LS5000 scintillation counter, using an internal standard and chanels-ratio method to correct for quenching. L-[3-^{3}H]dopa was synthesized by literature methods.[9]

Synthesis of D,L-[13-^{13}C]dopa. 4-Bromoveratrole (10.85 g, 50.0 mmol) was converted to the magnesium Grignard reagent. This solution was then attached to a sealed system, in which $^{13}CO_2$ had been generated by dropping concentrated H_2SO_4 on $Ba^{13}CO_3$ (10.0 g, 50.5 mmol, 99% enrichment), and the stirred solution was allowed to take up $^{13}CO_2$. After most of the $^{13}CO_2$ was consumed, the mixture was acidified, extracted with $CHCl_3$ (4 × 50 mL) and dried ($MgSO_4$). The solvent was removed in vacuo to yield [7-^{13}C]-3,4-dimethxybenzoic acid (**3**) (6.2 g, 68% yield).



In a three-neck flask equipped with condenser, argon inlet, and septum, 2.0 g of **3** (11.0 mmol) was dissolved in 75 mL of dried THF. To this solution, under argon, was added 11 mL of a 1 M BH_3·THF solution (11.0 mmol), and the reaction mixture was allowed to stir at room temperature for 7 h. The solvent was removed in vacuo, the resultant white residue was hydrolyzed by stirring in 150 mL of saturated $NaHCO_3$ for 15 min and extracted with $CHCl_3$ (4 × 75 mL), and the combined organic layers were dried over Na_2SO_4. The solvent was removed in vacuo to yield 1.9 g of crude product which, upon flash chromatography over silica gel (50 g; column,- 1.5 cm × 120 cm; eluant, hexane/EtOAc 50/50), yielded [7-^{13}C]-3,4-dimethoxybenzyl alcohol (**4**) (1.5 g, 81% yield).

Scheme I



The benzyl alcohol (**4**) (1.5 g, 8.9 mmol) was converted to D,L-[3'-^{13}C]dopa (0.6 g, 38%) as previously described.[9]

Preparation of Cysteinyldopas. The following radiolabeled cysteinyldopas were prepared according to literature methods:[13] (2) 3-[((*R*)-2-amino-2-carboxyethyl)thio]-5-hydroxy-(*S*)-tyrosine-β-^{13}C (**5a**); (ii) 2-[((*R*)-2-amino-2-carboxyethyl)thio]-3-hydroxy-(*S*)-tyrosine-β-*I* (**6a**) (S.A. 4.98 × 10^8 dpm/mmol), 3-[((*R*)-2-amino-2-carboxyethyl)thio]-5-hydroxy-(*S*)-tyrosine-β-*I* (**5b**) (S.A. 5.25 × 10^8 dpm/mmol); (iii) 2-[((*R*)-2-amino-2-carboxyethyl)thio]-3-hydroxy-(*S*)-tyrosine-β-^{14}*C* (**6b**) (S.A. 2.89 × 10^8 dpm/mmol), 3-[((*R*)-2-amino-2-carboxyethyl)thio]-5-hydroxy-(*S*)-tyrosine-β-^{14}*C* (**5c**) (S.A. 2.80 × 10^8 dpm/mmol); (iv) 2-[((*R*)-2-amino-2-carboxyethyl)thio]-3-hydroxy-(*S*)-tyrosine-α-^{14}*C* (**6c**) (S.A. 1.40 × 10^8 dpm/mmol), 3-[((*R*)-2-amino-2-carboxyethyl)thio]-5-hydroxy-(*S*)-tyrosine-α-^{14}*C* (**5d**) (S.A. 1.45 × 10^8 dpm/mmol); (v) 2-[((*R*)-2-amino-2-carboxyethyl)thio]-3-hydroxy-(*S*)-tyrosine-*carboxy*-^{14}*C* (**6d**) (S.A. 4.79 × 10^8 dpm/mmol), 3-[((*R*)-2-amino-2-carboxyethyl)thio]-5-hydroxy-(*S*)-tyrosine-*carboxy*-^{14}*C* (**5e**) (S.A. 4.98 × 10^8 dpm/mmol); (vi) 2-[((*R*)-2-amino-2-carboxyethyl)thio-^{35}*S*]-3-hydroxy-(*S*)-tyrosine (**6e**) (S.A.. 7.23 × 10^8 dpm/mmol), 3-[((*R*)-2-amino-2-carboxyethyl)thio-^{35}*S*]-5-hydroxy-(*S*)-tyrosine (**5f**) (7.29 × 10^7 dpm/mmol); (vii) 2-[((*R*)-2-amino-2-carboxyethyl-*1*-^{14}*C*)thio]-3-hydroxy-(*S*)-tyrosine (**6f**) (S.A. 8.50 × 10^7 dpm/mmol), 3-[((*R*)-2-amino-2-carboxyethyl-*1*-^{14}*C*)thio]-5-hydroxy-(*S*)-tyrosine (**5g**) (S.A. 7.93 × 10^7 dpm/mmol).

Preparation of Synthetic Pheomelanin. An adaptation of an earlier method was employed for preparation of pheomelanin.[14] This pheomelanin was fractionated into bands by preparative isoelectric focusing (IEF) on a granulated gel bed.[9] The deeply colored bands along with the focused ampholytes were eluted from the gel bed with 0.01 M Na_3PO_4, and when necessary, the ampholytes were removed by gel filtration over a short column of Bio-Gel P-2 (0.01M Na_3PO_4 eluant).

Preparation of Cysteinyldopa Pheomelanins (CP). Approximately 25 mg of one of the cysteinyldopas was added to a stirred 20-mL solution of 0.1 M phosphate buffer (pH 6.8) at 37 °C. To this solution was added 2.5 mg of polyphenol oxidase (dissolved in 3 mL of phosphate buffer), and the reaction was allowed to stir for 24 h at 37 °C. The reaction was stopped by adjusting the pH to 3 with concentrated HCl, and the resultant precipitate collected by centrifugation and washed several times with a pH 3 HCl solution. Typically, 20 mg of a brown solid was obtained. CP were fractionated into its major bands by preparative IEF on a granulated gel bed as previously described.[9]

Results and Discussion

Radiochemical Studies. A polymeric chromophore that has similar, if not identical, structural properties to the naturally occurring eu- and pheomelanin is produced by in vitro incubation of polyphenol oxidase with the appropriate precursors.[5,14] Fur-

(7) Fattorusso, E.; Minale, L.; DeStafano, S.; Cimino, G.; Nicolaus, R. A. *Gazz. Chim. Ital.* **1968**, *98*, 1443–1463.
(8) Minale, L.; Fattorusso, E.; Cimino, G.; DeStefano, S.; Nicolaus, R. A. *Gazz. Chim. Ital.* **1969**, *99*, 431–449.
(9) Deibel, R. M. B.; Chedekel, M. R. *J. Am. Chem. Soc.* **1982**, *104*, 7306–7309.
(10) Righetti, P. G.; Drysdale, J. W. In "Laboratory Techniques in Biochemistry and Molecular Biology, Isoelectric focussing"; Work, T. S., Ed.; North-Holland Publishing Co.: New York, 1976; pp 358–367.
(11) Bergmeyer, H. U. In "Methods of Enzymatic Analysis"; Bergmeyer, H. U., Ed.; Academic Press: New York, 1974; pp 674–682.
(12) Scintillation cocktail was prepared by mixing dioxane (946 mL), naphthalene (94.6 g), PPO (5.62 g) and POPOP (0.47 g).
(13) Ito, S.; Prota, G. *Experientia* **1977**, *33*, 1118–1119.
(14) Fattorusso, E.; Minale, L.; DeStefano, S.; Cimino, G.; Nicolaus, R. A. *Gazz. Chim. Ital.* **1969**, *99*, 969–992.

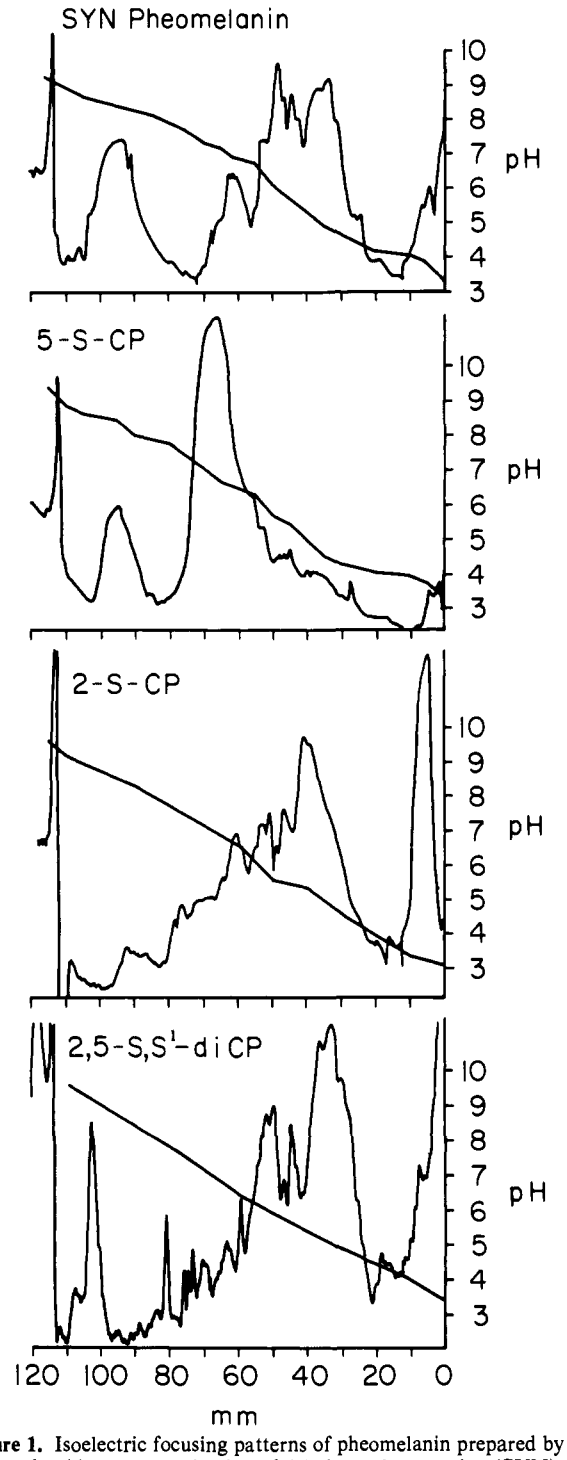


Figure 1. Isoelectric focusing patterns of pheomelanin prepared by polyphenol oxidase polymerization of (a) dopa plus cysteine (SYN), (b) 5-*S*-cysteinyldopa (5-*S*-CP), (c) 2-*S*-cysteinyldopa (2-*S*-CP), and (d) 2,5-*S*,*S*′-dicysteinyldopa (2,5-*S*,*S*′-diCP).

thermore, we have shown that pheomelanin produced in this manner can, after purification by literature methods, be further fractionated by isoelectric focusing (IEF). It has been demonstrated that cysteinyldopas **5–7**, the first isolable intermediates in the biosynthesis of pheomelanin, arise from the addition of cysteine to oxidized dopa.[13] The cysteinyldopas are then presumably converted to benzothiazines, **8**, which are subsequently polymerzied through a series of steps to ultimately afford pheomelanin (see Scheme I).[15]

In our initial paper we reported results on the biosynthesis of pheomelanin produced from dopa and cysteine (synthetic pheomelanin, SP). Knowing that the first step in this biosynthesis results in the production of 5-*S*-cysteinyldopa (5-*S*-CD) (**5**), 2-*S*-cysteinyldopa (2-*S*-CD), (**6**), and 2,5-*S*,*S*′-dicysteinyldopa (2,5-*S*,*S*′-CD) (**7**) in a ratio of 15:3:1,[13] we reasoned that by using each of these cysteinyldopas individually to produce the corresponding cysteinyldopa pheomelanins (CP) we would be able to prepare pheomelanins which would be more homogeneous, be easier to analyze, and serve as good model systems for further studies.

(15) Prota, G.; Thomson, R. H. *Endeavour* **1980**, *35*, 32–38.

Table I. Radiochemical Analysis of the Four Major Bands of 5-*S*-CP

band no.	% retention of tritium from **5b,c**	% retention of carbon from **5b,d**	% retention of dopa group from **5b,e**
1	62 ± 2	79	79 ± 2
2		83	
3		91	
4		88	

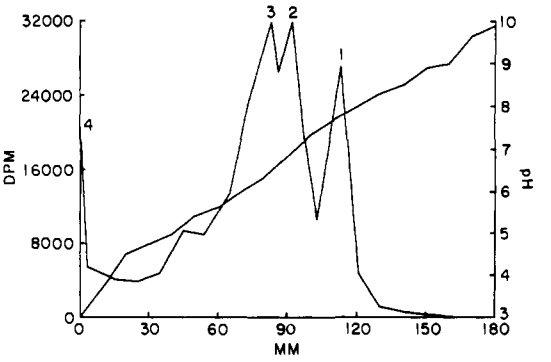


Figure 2. Typical isoelectric focusing pattern of pheomelanin prepared by the action of polyphenol oxidase on 5-*S*-CD.

By employing standard procedures, each of the CPs were prepared from the corresponding cysteinyldopa, subjected to IEF, and their IEF patterns were compared and contrasted with one another and with that of SP (see Figure 1). From the IEF patterns it is apparent that (1) none of these model systems is identical with SP and (2) 5-*S*-CP is the most homogeneous of the polymers containing one predominant band at an apparent pK_a of 6.6.

We first performed radiolabeled experiments involving the conversion of cysteinyldopas to CPs by using dual-labeled 5-*S*-CD as the precursor, the rationale being that 5-*S*-CP was the most homogeneous CP and therefore would provide a simpler system to study than 2-*S*-CP or 2,5-*S*,*S*′-CP. To do this, we have employed a combination of carbon-14- and tritium-labeled 5-*S*-CD as well as tritium- and sulfur-35-labeled 5-S-CD to probe the structure of 5-*S*-CP. In this system, we employed the same methodology that was employed in our initial studies,[9] namely that while degradation of the alanyl side chain may occur during pheomelanin biosynthesis, it was unlikely that the C-3 atom would be lost from the polymer (i.e., the benzylic carbon of cysteinyldopa). Incubations of 5-*S*-CD labeled with tritium and carbon-14 at the β-position of the alanyl side chain with mushrooms tyrosinase afforded, after IEF, four major pheomelanin bands, (Figure 2) each of which had lost 38 ± 2% of the tritium (see Table I). This is in contrast to earlier experiments where SP exhibited a loss of tritium in its various bands ranging from 13% to 53%. Therefore, with regard to tritium loss, 5-*S*-CP is much more uniform than SP, and tritium labeling at the β-position can serve as a reliable internal standard.

Incubations of 5-*S*-CD labeled with tritium at the β-position and carbon-14 at the α-position of the alanyl side chain with mushroom tyrosinase afforded pheomelanin bands that exhibited a small loss of the α-carbon relative to the β-carbon (see Table I). This is in contrast to no detectible loss in the case of SP. However, due to the greater homogeniety of the 5-*S*-CP polymer with regard to the tritium standard we were more reliably able

Table II. Radiochemical Analysis of the Four Major Bands of 5-*S*-CP

band no.	% retention of sulfur from **5b,f**	% retention of carbon from **5b,g**
1, 2, 3, 4	86 ± 2	81 ± 2

Table III. Radiochemical Analysis of the Six Major Bands of 2-*S*-CP

band no.	% retention of tritium from 6a,b	% retention of carboxy group from 6a,d
1	74 ± 2	79
2		80
3		82
4		88
5		86
6		87

to document small losses of carbon-14 in CP as compared to the more heterogeneous SP system. Therefore, using our present system, we have been able to demonstrate a small amount of degradation of the alanyl side chain. Similar experiments using 5-*S*-CD labeled with tritium at the β-position and carbon-14 on the carboxy group of the alanyl side chain, demonstrated that relative to the β-carbon there is a 21% loss of the carboxy group (see Table I) during biosynthesis. Once again this loss is uniform from band to band and supports the use of cysteinyldopa pheomelanin polymers as homogeneous model pheomelanins.

We next addressed the fate of the cysteinyl side chain during the polymerization process. Incubation of either 5-*S*-CD labeled with tritium at the β-position of the alanyl side chain and sulfur-35 in the cysteinyl side chain or 5-*S*-CD labeled with tritium at the β-position of the alanyl side chain and carbon-14 at the 2-position of the cysteinyl side chain with mushroom tyrosine afforded, after IEF, pheomelanin bands which exhibited a loss relative to the β-carbon of 14% of sulfur-35 and 19% of carbon-14, respectively (Table II). From these results, it appears that the cysteinyl side chain is labile.

After assessing the above results, comparisons were made using the 2-*S*-CDs labeled in the same relative positions as in the the 5-*S*-CDs to produce the corresponding 2-*S*-CPs. After IEF of the 2-*S*-CP, six major bands were analzyed for loss of the particular label of interest (see Table III). While losses of the various labels differed a small amount from the 5-*S*-CP experiments, they are still quite uniform from band to band and indicate that 2-*S*-CP can also be used as a model pheomelanin polymer.

Our findings indicate that (1) the CP polymers are more homogeneous than the SP polymer and, as such, will serve as good models for further structural studies, (2) during the polymerization process a small amount of the α-carbon of the alanyl side chain is lost, indicating that at least some of the units exist in the pheomelanin polymer which do not have the proposed isoquinoline or the tetrahydroisoquinoline moiety depicted in **2**, and (3) at least 14% of the cysteinyl side chains suffer degradation during polymerization.

To explain the homogeneity of the CP polymers compared with SP, we propose that since the cysteinyldopas are only one step further along the biosynthetic pathway, something must be occurring during the first step of the biosynthesis (i.e., the condensation of dopa and cysteine to form cysteinyldopas). This condensation is an intermolecuar reaction and must compete with several other intermolecular processes (see Scheme II). However, when one of the cysteinyldopas is the starting material, enzymatic oxidation to the cysteinyldopa quinone results in a reactive species which can undergo several rapid intramolecular reactions, and thus when polymerization is initiated, there are fewer types of reactive monomers available in solution and hence a more homogeneous polymer is formed.

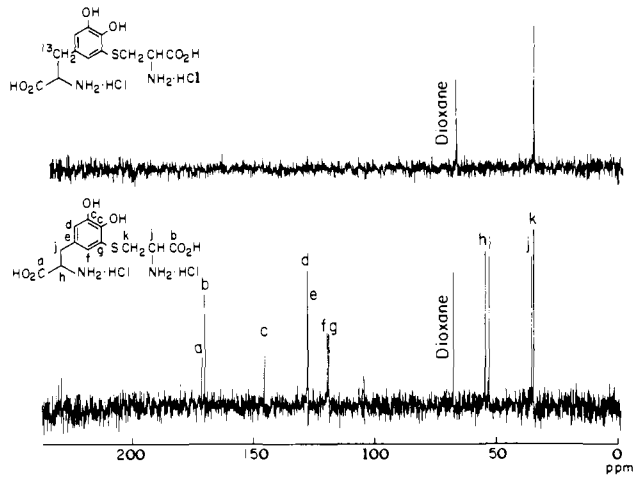
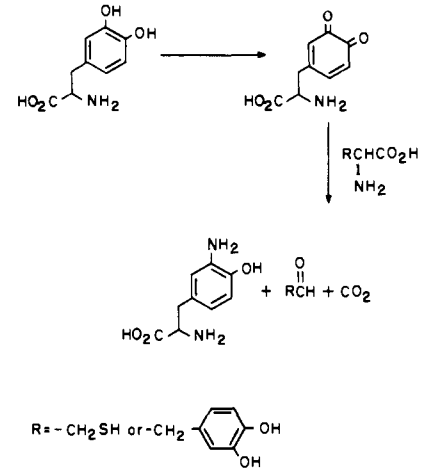


Figure 3. Carbon-13 NMR spectra of 5-*S*-CD: (top) enriched at benzylic carbon; (bottom) natural-abundance, proton-decoupled spectra.

Scheme II



Carbon-13 Studies. Further insights on the structure of the pheomelanin polymer were obtained by using specifically labeled carbon-13 NMR-active precursors. Attempts to obtain several natural abundance carbon-13 NMR signals have met with failure, and in our studies it was particularly useful for us to synthetically enrich a specific carbon atom of the dopa or cysteinyldopa precursors such that the carbon-13 spectrum was dominated by the signal for the enriched carbon (see Figure 3). With this in mind, we reasoned that by using carbon-13 NMR, information concerning the fate of this carbon during pheomelanin biosynthesis could be obtained.

We have synthesized [3-^{13}C]dopa and [β-^{13}C]-5-*S*-cysteinyldopa, both enriched with 99% carbon-13 at the indicated position. Each of these putative precursors was converted to pheomelanin by appropriate incubation with polyphenol oxidase and, if necessary, cysteine.

Pheomelanin is reported to be a stable free radical,[16] and because of this, it has been suggested that it might be difficult to obtain its NMR spectrum. The presence of free-radical species in solution causes the nuclear spins to exhibit short relaxation times and thus very broad signals, so much so that they often broaden into the base line. In order to find a solvent and conditions to minimize these effects, the ESR signal intensity of pheomelanin solutions was monitored in aqueous solutions at various pH values. It was found that the ESR signal was minuscule when 0.1 M phosphate buffer (pH 6.8) was used as the solvent.

The carbon-13 NMR spectra of pheomelanin, prepared from [3-^{13}C]dopa and unlabeled cysteine, and of 5-*S*-CP, made from

(16) Sealy, R. C.; Hyde, J. J.; Felix, C. C.; Menon, I. A.; Prota, G.; Swartz, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 2885–2889.

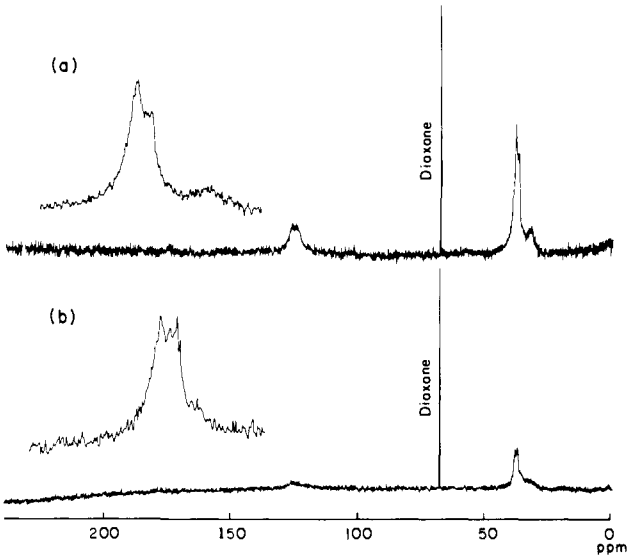


Figure 4. Carbon-13 NMR spectra of pheomelanin prepare by polyphenol oxidase polymerization of (a) **5a** and (b) [3-^{13}C]dopa and cysteine.

[β-^{13}C]-5-*S*-CD, are shown in Figure 4.[17] From these spectra it is apparent that there are two different types of carbons, (1) benzylic-type carbons indicated by the envelope of signals at ca. 35 ppm and (2) either aromatic or olefinic carbons indicated by the envelope of signals at 125 ppm. By comparison with model compounds, partial structures such as **9** and **10** could be predited to exhbit chemical shifts in the regions of 125 and 35 ppm and as such could be proposed to be possible partial monomer units in the pheomelanin polymer. While we cannot be sure that the two signals represent 100% of the tagged carbons, these results indicate that the loss of tritium documented during the biosynthesis can, in a large part, be attributed to dehydration to a structure such as **9** and not oxidation of a carbonyl, e.g., **11**, or simple exchange processes.

In comparing the NMR spectra of SP and 5-*S*-CP, it is apparent that the two pheomelanins are similar. However, it is also apparent that the spectrum of 5-*S*-CP is more defined, most likely due to a greater homogeneity of 5-*S*-CP. From these NMR studies, we conclude that (1) under suitable conditions it is possible to obtain NMR spectra of pheomelanins and to assess the type of possible units associated with a particular carbon, (2) the benzylic carbon of the alanyl side chain does not undergo extensive chemical alteration during polymerization, and (3) the loss of tritium from the β-carbon position of the alanyl side chain in the radiolabeled studies can be attributed to oxidation of the 3-position to an sp^2 carbon.

In conclusion, we have demonstrated that (1) CP polymers are more homogeneous that these from dopa and cysteine and will serve as good models for further biosynthetic studies, (2) during the biosynthesis of pheomelanin there is a significant loss of tritium from the benzylic position of 5-*S*-CD which, by carbon-13 NMR studies, corresponds to formation of an sp^2 carbon in the alanyl side chain, and (3) there appears to be a small amount of degradation of both the alanyl and cysteinyl side chains during biosynthesis, which contributes to the heterogeneity of the polymer. These results reinforce our contention that the harsh degradative methods used during the initial studies on the structure of pheomelanin led to the production of artifacts, and the structure of pheomelanin proposed by Minale et al.[6-8] does not accurately represent the structure of the intact chromophore.

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Registry No. 3, 90140-57-1; **4**, 91384-88-2; **5**, 25565-25-7; **6**, 25565-17-7; **7**, 57954-84-4; 4-bromoveratrole, 2859-78-1; polyphenol oxidase, 9002-10-2.

(17) The chemical shifts of the carbons in 5-*S*-cysteinyldopa are 171.5, 170.3, 145.6, 127.7, 127.5, 119.5, 118.9, 54.8, 53.1, 35.5, and 34.8 ppm and were assigned by comparison with chemical shifts for L-tyrosine, L-cysteine, L-dopa, and L-3-*S*-cysteinylcatecol.

Theoretical Study of the Metal Chemical Shift in Nuclear Magnetic Resonance Spectroscopy. Mn Complexes

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Abstract: The ^{55}Mn nuclear magnetic shielding constants of the Mn complexes, $Mn(CO)_5L$ (L = H, CN, CH_3, Cl), are studied by the ab initio finite perturbation SCF-MO method. The ^{55}Mn chemical shifts are determined predominantly by the 3d contributions of the paramagnetic term. This is because the Mn atom has an open 3d shell. This is in sharp contrast to the previous results for the complexes of the Cu, Zn, Ag, and Cd metals which have $d^{10}s^{1-2}p^0$ configuration. The 3d contribution of the Mn complexes arises from the d-d excitations. The chemical shift is determined by the effects of the ligand on these occupied and unoccupied d orbitals. It increases with the increases in the π-donating ability and the hardness of the ligand base. For the diamagnetic term, which is a minor part of the chemical shift, the Pascal rule like formula applies as for the 1B and 2B metal complexes.

In the previous study of this series,[1] we have studied theoretically the metal NMR chemical shifts for the Cu, Zn, Ag, and Cd complexes. We have used the ab initio finite perturbation method. The results of the calculations compared well with the experimental chemical shifts. We have clarified the mechanism of the metal chemical shift in these $d^{10}s^{1-2}p^0$ metal complexes. The paramagnetic term is a major part of the chemical shift and is due to the d and p mechanisms. The former is due to the hole produced in the valence d shell of the metal by the electron-withdrawing ligands. The latter is due to the electrons in the valence p orbitals of the metal transferred from the electron-donating ligands. For

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