of detection with the 4-methylumbelliferone ester of *p*-guanidinobenzoic acid, 6×10^{-9} M. In addition, unlike fluorescein (**1**), 4-methylumbelliferone undergoes rapid photolytic decomposition, which makes kinetic analysis extremely difficult, since the titration reaction must be monitored intermittently instead of continuously. The main advantage of the fluorescein esters **2** and **3** over FDE is that the structures of FMGB·HCl (**2**) and FDGB·2HCl (**3**) have been established, whereas the structure of FDE is still under investigation. Considering that it takes 1 equiv of FDE to titrate 1 equiv of trypsin under conditions in which FDE is limiting,[11] it now appears likely that FDE is a monoester, not a diester. The structure determination of FMGB·HCl (**2**) and FDGB·2HCl (**3**) and the spectral behavior of the compounds reported here guide our attempts to clarify the structure of FDE and to design substrate analogues for serine proteases that exhibit high enzymatic specificity as well as high sensitivity. At present, FMGB·HCl (**2**) is the best active-site titrant for serine proteases available.

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Registry No. 1, 2321-07-5; **2**, 83616-10-8; **3**, 83616-11-9; **4**, 42823-46-1; serine protease, 37259-58-8; trypsin, 9002-07-7; plasmin, 9001-90-5; urokinase, 9039-53-6; thrombin, 9002-04-4.

Biosynthetic and Structural Studies on Pheomelanin

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Abstract: Pheomelanin, the red-brown or yellow 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. A reinvestigation of the structure using radiotracer biosynthetic methodology is reported. Carbon-14 and tritium-labeled dopas and sulfur-35-labeled cysteine were used to demonstrate that, contrary to earlier reports, (1) pheomelanin is not a 1:1 copolymer of dopa and cysteine, (2) during the course of pheomelanin biosynthesis up to 50% of the dopa residues suffer decarboxylation, and (3) hydrogen peroxide, produced in situ during the course of the biosynthesis, does not play a role in modification of the pheomelanin polymer. Methods for isolation and purification of synthetic pheomelanin and the use of trichochrome F as a marker for monitoring the progress of the polymerization are reported.

Melanin pigments and their distribution in human skin are generally believed to be the most important factor in the protection of human skin from the biochemical devastation caused by chronic exposure to solar radiation.[2] Epidermal melanin pigmentation is thought to be composed of intimate mixtures of two separate but biogenetically related pigments: the black-brown eumelanins and the yellow and red-brown pheomelanins.[3] The two pigments exhibit different photochemistries, and it has been proposed that the facile photodegradation of pheomelanin coupled with concomitant production of superoxide radicals is responsible for the increased susceptibility of fair-skinned Caucasians to the detrimental effects of sunlight.[4]

Epidermal melanins consist of a polymeric chromophore covalently bound to a protein fraction. Extensive biosynthetic and degradative studies have demonstrated that the eumelanin chromophore is an amorphous, irregular polymer composed of several major monomers (see partial structure **1**).[5] Much less work has been done on the pheomelanin polymer; however, chemical degradative and biosynthetic studies indicate that the pheomelanin chromophore, a product of tyrosinase-catalyzed copolymerization of L-3,4-dihydroxyphenylalanine (dopa) and L-cysteine,[6] is mainly composed of 6,7,8,9-tetrahydro-4-hydroxythiazolo[4,5-*h*]isoquinoline-7-carboxylic acid (**2**) monomers.[7] However, the chemical 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 reinvestigate the structure of pheomelanin using radiotracer biosynthetic methodology and herein report our initial findings.

Materials and Methods

Materials. Electrophoresis grade acrylamide, ammonium persulfate, *N,N,N',N'*-tetramethylenediamine (TEMED), and sodium dodecyl sulfate ($NaDodSO_4$) were purchased from Bio-Rad Laboratories. Electrofocusing gel was purchased from Pharmacia Fine Chemicals. Ampholytes were prepared synthetically as previously described.[8] Tyrosinase (EC 1.14.18.1) and catalase (EC 1.11.1.6) were purchased from Sigma Chemical Co. and assayed by literature methods.[9,10] All other biochemicals were purchased from Sigma Chemical Co. Radioactive compounds were purchased from Amersham, organic chemicals were from Aldrich, and liquid scintillation cocktail was from Beckman. Absorbance spectra were obtained on a Varian Cary 219 spectrophotometer.

Preparation of Synthetic Pheomelanin. An adaption of an earlier method was employed for preparation of pheomelanin.[11] L-dopa (70 mg) was added to a stirred 50-mL solution of 0.1 M phosphate buffer (pH 6.8) at 37 °C. To this solution was added 10 mg of tyrosinase (1200 eu/mg), and when a pink color developed (ca. 30 s), 85.6 mg of L-cysteine (dissolved in the minimum amount of phosphate buffer) was added. The solution had turned dark brown after 3 h; however, the yield of pigment was maximized if the reaction was allowed to continue for an additional 21 h. The reaction was quenched after 24 h by adjusting the pH to 1.5

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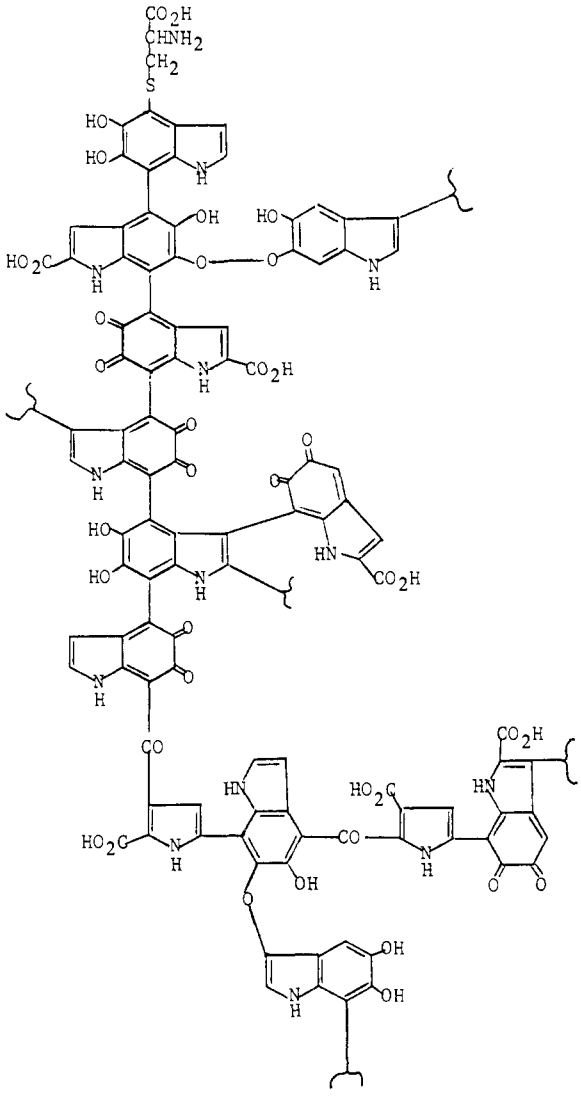

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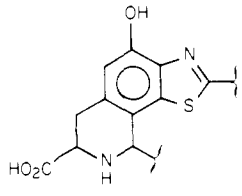

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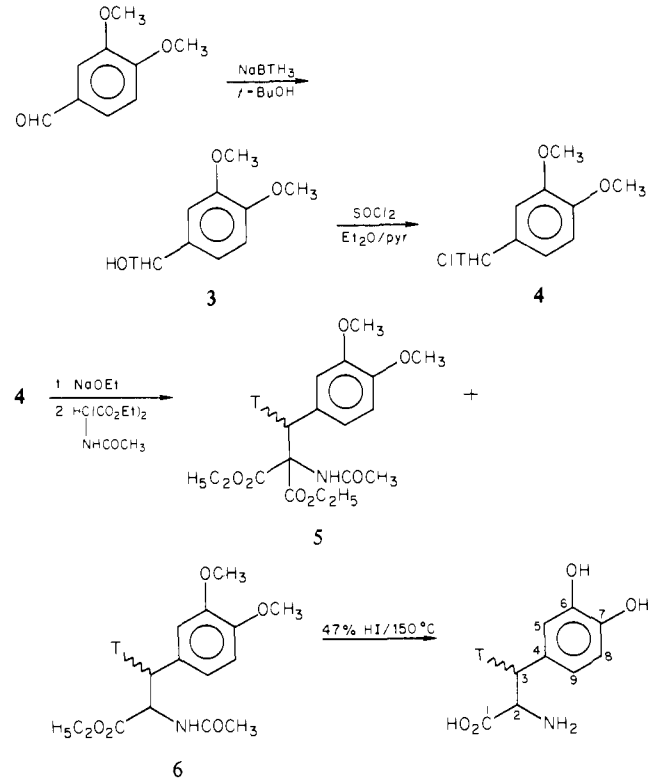
with concentrated HCl. The resultant precipitate was collected by centrifugation and washed several times with a pH 1.5 HCl solution. Finally the pigment was washed with acetone and allowed to dry. Typically, 130 mg of a brown solid was obtained.

Purification of Pheomelanin. Synthetic pheomelanin was fractionated into six major bands by preparative isoelectric focusing (IEF) on a granulated bed.[8] The deeply colored bands along with the focused ampholytes were eluted from the gel bed with 0.01 M Na_3PO_4. When necessary, the ampholytes could be removed by gel filtration over a short column of Bio-Gel P-2 (0.01 M Na_3PO_4 eluant).

Synthesis of L-[3-^{3}H]dopa (See Scheme I). 3,4-Dimethoxybenzaldehyde (5.0 g) was dissolved in *tert*-butyl alcohol (50 mL). To this was added sodium boro[^{3}H]hydride (0.6 g, specific activity 2.13 × 10^{10} dpm/mmol), and the mixture was heated at reflux for 4 h. Water (50 mL) was added to the cooled suspension, which was subsequently extracted with chloroform (4 × 40 mL). The organic layer was separated and dried ($MgSO_4$), and the solvent was removed in vacuo. The resultant oil was distilled to yield 4.2 g (84% yield) of 3,4-dimethoxy[7-^{3}H]benzyl alcohol (**3**): bp 120 °C (0.7 mm); specific activity 3.67 × 10^9 dpm/mmol.

Scheme I



A solution of 6.1 g of thionyl chloride in 25 mL of diethyl ether was added dropwise (0.3 h) with stirring to a solution of 4.2 g of **3** and 0.5 mL of pyridine in 45 mL of diethyl ether. Stirring was continued for 0.33 h, whereupon the mixture was washed with water (3 × 20 mL) and the organic layer separated and dried ($MgSO_4$). The solvent was removed in vacuo to give 3.8 g of 3,4-dimethoxy[7-^{3}H]benzyl chloride (**4**) as a crystalline residue. Recrystallization from diethyl ether afforded 3.47 g (75% yield) of analytically pure **4** (mp 46–47 °C).

Sodium (0.45 g) was reacted with 50 mL of dry ethanol[12] followed by addition of diethyl acetamidomalonate (3.4 g). This solution was then added dropwise (0.25 h), with stirring, to a solution of 3.45 g of **4** in 10 mL of dry ethanol. Stirring was continued for 1 h, and the mixture was then heated at reflux for 3 h. The resultant mixture was filtered and the ethanol removed in vacuo. The residue was washed with water and extracted with chloroform (3 × 40 mL). The organic layers were combined and dried (Na_2SO_4), and the solvent was removed in vacuo to afford 6.1 g of a crystalline mixture of two compounds, **5** and **6**. The mixture could be separated by chromatography over silica gel; however, since acid hydrolysis of the mixture yielded a single compound, separation was deemed unnecessary. L-[3-^{3}H]dopa was prepared fresh for each biosynthetic experiment. A typical preparation is as follows: hydriodic acid (5 mL of 47%) and 0.5 g of a mixture of **5** and **6** were mixed and heated in a sealed tube at 150 °C for 24 h. After cooling, the solvent was removed in vacuo and 0.3 g of unlabeled L-dopa was added. The combined solids were dissolved in the minimum amount of 1 N HCl, and L-dopa was crystallized by adjusting the pH to 6 with 1 N NaOH. Recrystallization was repeated in a like manner until a constant specific activity was obtained. Dual-labeled dopas (^{3}H and ^{14}C) were prepared in a similar manner with the exception that in addition to 0.3 g of unlabeled L-dopa, several micrograms of an appropriately labeled [^{14}C]dopa was added prior to crystallization.

Preparation of [^{35}S]Cysteine. Commercially available L-[^{35}S]cysteine hydrochloride was diluted with an appropriate amount of unlabeled L-cysteine and subsequently crystallized to constant specific activity (water/acetone) prior to use.

Isolation of Trichochrome F. Trichochrome F can be obtained during the course of pheomelanin synthesis. This trichochrome is produced in situ by lowering the pH of the enzyme reaction to 1.5 and allowing the solution to stand for 1 h. Purification of the trichochromes by literature methods involves (1) removal of pheomelanin by centrifugation, (2) lyophilization of the supernatant, (3) chromatography over Sephadex

(12) "Super-dry" ethanol was prepared as outlined in Vogel, A. I. "Textbook of Practical Organic Chemistry"; Longman Group Ltd.: London, 1956.

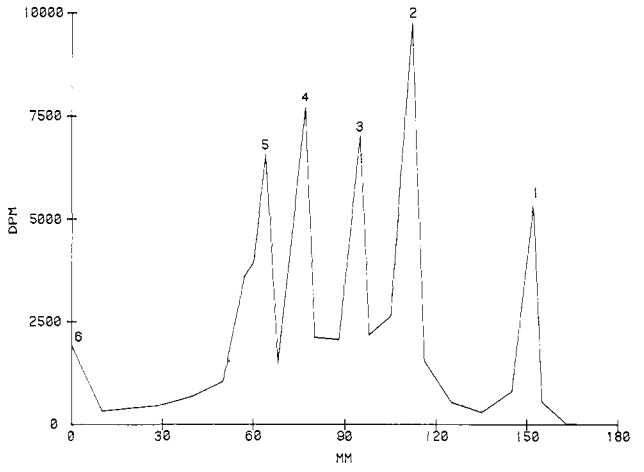
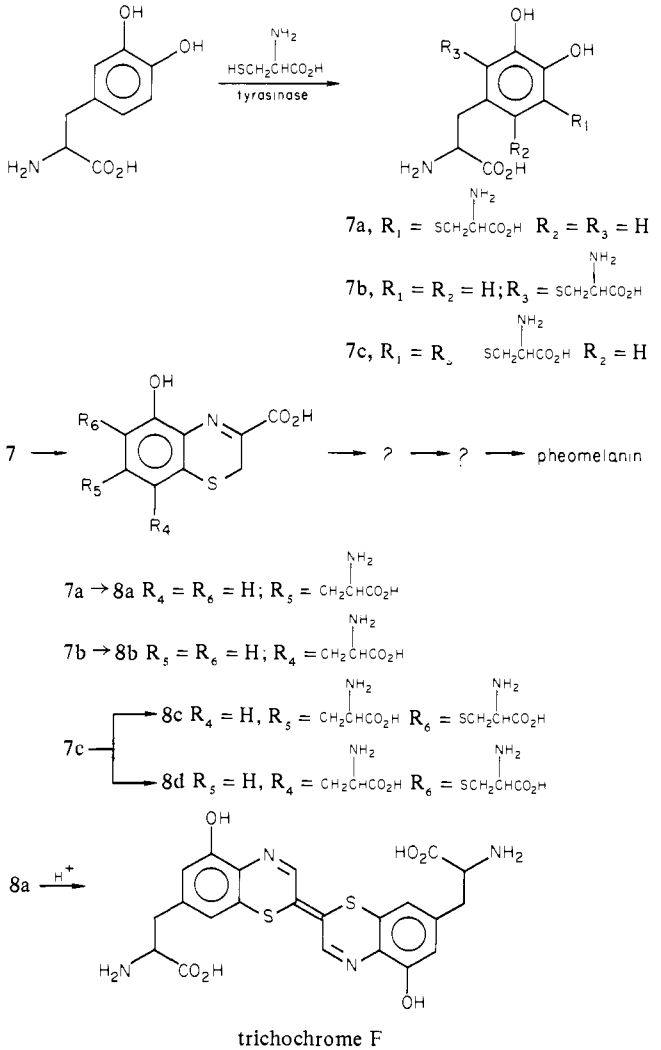


Figure 1. Typical isoelectric focusing pattern of synthetic pheomelanin. This pattern is for a [3-^{3}H,^{35}S]pheomelanin.

Scheme II



trichochrome F

LH-20 (95:5 methanol/3 N HCl eluant), and, finally, (4) cellulose thin-layer chromatography (30:70:1 2-propanol/formic acid/concentrated HCl, v/v).[13]

Results and Discussion

A polymeric chromophore that has similar, if not identical, structural properties to the naturally occurring eu- and pheomelanin chromophores may be produced in vitro by incubation of tyrosinase with the appropriate precursors.[5,6] Indeed, the pheomelanin chromophore may be produced by the action of tyrosinase on mixtures of L-dopa and L-cysteine.[6] This chromophore is not homogeneous and is readily separated by isoelectric focusing (IEF) into six major bands (see Figure 1).[11] It has been demonstrated that cysteinyldopas **7**, the initial isolable products of the reaction,[14] most probably arise from addition of cysteine to the semidione of dopa[15] (see Scheme II). Details of the next series of steps, leading to the production of benzothiazines **8**, are uncertain, and nothing is known of the succeeding steps leading to polymerization. Scheme II represents the overall picture of pheomelanin biosynthesis that existed at the outset of our experiments.

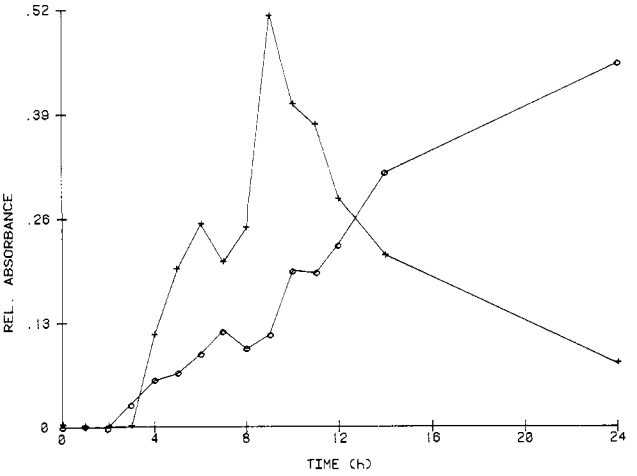


Figure 2. Time course study: buildup of trichochrome F (+); production of pheomelanin (O).

Table I. Radiochemical Analysis of Pheomelanin Bands

band no.	% retention of tritium from [3-^{3}H,2-^{14}C]dopa	% retention of dopa carboxy group from [3-^{3}H,1-^{14}C]dopa	Cys/dopa
1	47	91	3.1
2	55	53	2.2
3	76	99	2.1
4	77	55	1.7
5	72	63	1.4
6	87	64	2.1

Inconsistencies in the existing experimental and hypothetical work led us to question several aspects of the preparation and structure of pheomelanin: (1) Pheomelanin is purported to be a 1:1 copolymer of dopa and cysteine, yet its biosynthetic preparation from dopa and cysteine calls for an initial 1:2 ratio of dopa to cysteine.[6] (2) The six major pheomelanin bands observed upon isoelectric focusing exhibit different isoelectric points;[11] however, the origin of these differences is not clear. (3) Tyrosinase-mediated synthesis of eumelanin is known to result in the production of hydrogen peroxide, and, if left unscavenged, the peroxide reacts with the forming polymer resulting in extensive modification of the pigment.[5] Are similar processes operative in pheomelanin synthesis? In order to examine these points, we have employed a combination of carbon-14 and tritium-labeled dopas as well as sulfur-35-labeled cysteine to probe the structure of pheomelanin produced by the action of tyrosinase on dopa and cysteine.

In the case of eumelanin biosynthesis, hydrogen peroxide, produced in situ, was demonstrated to cause extensive oxidative degradation to the forming eumelanin chromophore.[5] Addition of catalase to the incubation substantially negated this oxidation. We reasoned that while degradation of the alanyl side chain or the benzene ring of dopa may occur during pheomelanin bio-

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Table II. Relative Abundances of the Six Major Isolable Bands Produced during the Formation of Pheomelanin

band no.	starting Cys/dopa 0.5	1	2	5
1	73		10	
2	271	67	36	3
3	148	40	10	2
4	21	6	14	
5	33	5	9	1
6	9	23	37	7

Table III. Cysteine/dopa for the Six Major Isolable Bands during the Formation of Pheomelanin

band no.	starting Cys/dopa 0.5	1	2
1	1.2	1.3	3.1
2	1.1	1.1	2.2
3	1.4	1.2	2.1
4	1.1	1.4	1.7
5	1.3	2.0	1.4
6	1.2	1.9	2.1

synthesis, it was unlikely that the C-3 atom would be lost from the polymer (i.e., the benzylic carbon of dopa). Incubations of L-[3-^{14}C,3-^{3}H]dopa and unlabeled L-cysteine with tyrosinase afforded, after IEF, six pheomelanin bands that had lost from 13 to 53% of the tritium. Similar results were obtained when L-[2-^{14}C,3-^{3}H]dopa and unlabeled L-cysteine were employed (see Table I). The loss of tritium was unaffected by the presence of catalase during biosynthesis. While we have not yet determined whether the retained tritium is associated with the same carbon atom as in the precursors, the relative retention of tritium for a given band was quite uniform from run to run and can reliably be used as an internal marker.

The overall incorporation of carbon-14 radioactivity into the six major pheomelanin bands from either L-[3-^{14}C]- or L-[2-^{14}C]dopa is on the order of 5%. At the end of the reaction (i.e., after 24 h), 90% of the carbon-14 radioactivity is present as unidentified low-molecular-weight species. A number of these species bind to pheomelanin and are coprecipitated with it when the pH is lowered. These species may be conveniently removed prior to IEF by eluting the crude pheomelanin through a short column of Biogel P-2.

Using L-[3-^{14}C,3-^{3}H]dopa, we have examined the rate of formation of the various pheomelanin bands. There is an approximate 6-h lag time prior to the appearance of any pheomelanin, and during this lag time all the dopa is converted to cysteinyldopas **7** and subsequently to benzothiazine **8** (see Scheme II). Analysis and quantification of the role of benzothiazines such as **8** are complicated by their instability. We have found it convenient to spectrophotometrically assay for the production and subsequent decay of the major benzothiazine, **8a**, by in situ conversion and subsequent purification to the well-defined trichochrome F (see Scheme II).[16] As depicted in Figure 2, the buildup of large concentrations of **8a** preceded the formation of the pheomelanin bands. Polymerization of the benzothiazines via unknown mechanisms ultimately affords the pheomelanin bands. These bands were found to be formed concurrently, not sequentially, and catalase had little effect on either their rate of formation or their relative abundances.

Up to 50% of the carboxy groups of dopa may be lost during the formation of pheomelanin. Retention of the tritium from L-[3-^{3}H]dopa was used as an internal standard (vide supra) to demonstrate that several of the pheomelanin bands suffered substantial loss of the carboxy group from L-[1-^{14}C,3-^{3}H]dopa during the course of their formation (see Table I). Similar experiments with L-[3-^{3}H]dopa and L-[^{35}S]cysteine indicated that the six major pheomelanin bands vary in their relative ratios of cysteine/dopa from about 0.8 to 3 (see Table I). Catalase had little effect on either the loss of the carboxy group from dopa or the cysteine/dopa of the major pheomelanin bands. Variability in the carboxy and cysteine content of the various bands could well explain the success of IEF as a separation technique for pheomelanin.

The relative abundance and absolute amounts of each of the six major pheomelanin bands is dependent on the initial cysteine/dopa ratio employed (see Tables II and III). In addition, if we assume that tritium retention from L-[3-^{3}H]dopa is the same for initial cysteine/dopa of 0.5, 1, and 2, the amount of cysteine- and dopa-type residues incorporated into each of the major pheomelanin bands is also dependent on the initial cysteine/dopa. Thus, depending on the experimental conditions, a given pheomelanin band may be anywhere from a 1:1 to a 3:1 copolymer of cysteine and dopa. This "extra" cysteine could arise by addition of cysteine to the growing polymers or incorporation of large amounts of 2,5-*S*,*S*'-dicysteinyldopa (**7c**) into the polymers. This latter possibility seems unlikely in view of the relatively small amount of **7c** vs. **7a** produced during the reaction.[14]

While a great deal more work is clearly necessary, our initial results provide explanations for some of the aforementioned inconsistencies and establish the ground work for further studies. Our findings indicate that (1) the pheomelanin chromophore is not a uniform polymer of fixed chemical structure but rather a mixture of a number of polymers (bands), (2) each polymer contains a wide range of chemical species (differences in ionizable groups and residues such as carboxy and amino acids are the basis for separation and characterization of the various pheomelanin bands), (3) variation of the initial cysteine/dopa will have significant effects on the quantity and composition of the resultant pheomelanin, (4) catalase has little or no effect on the course of the polymerization, (5) contrary to previous beliefs, the pheomelanin chromophore is not a 1:1 copolymer of dopa and cysteine but rather is a mixture of polymers with cysteine to dopa ratios of approximately 1.5–3, and (6) whatever the mechanisms for polymerization of benzothiazines **8**, a significant amount of the time they involve loss of the carboxy group from the alanyl side chain of cysteinyldopas **7**. 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. The currently accepted structure of pheomelanin, deduced by analysis of these artifacts, does not accurately represent the structure of the intact chromophore; more sulfur is clearly indicated. Whether or not pheomelanin contains an acid-labile moiety such as benzothiazine **8** or an acid-stable heterocycle such as the proposed benzothiazole **2** is still an open question. The answers to this and other questions concerning the structure of pheomelanin are currently under investigation.

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Registry No. 3, 83632-75-1; **4**, 83632-76-2; **5**, 83632-77-3; **6**, 83632-78-4; **7a**, 19641-92-0; **7b**, 25565-17-7; **7c**, 57954-84-4; **8a**, 83632-79-5; **8b**, 83632-80-8; **8c**, 83632-81-9; **8d**, 83632-82-0; tyrosinase, 9002-10-2; L-3,4-dihydroxyphenylalanine, 59-92-7; L-cysteine, 52-90-4; 3,4-dimethoxybenzaldehyde, 120-14-9; trichochrome F, 25942-17-0; L-[3-^{3}H]dopa, 83632-83-1.

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