Autoxidation of the Indolic Neurotoxin 5,6-Dihydroxytryptamine

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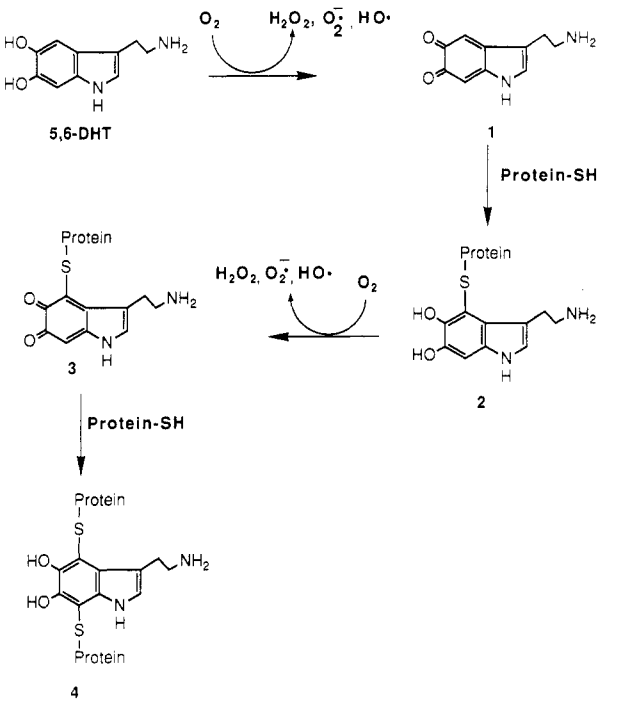
Received May 1, 1989

The autoxidation of the indolic neurotoxin 5,6-dihydroxytryptamine has been studied in pH 7.2 phosphate buffer. The major initial product of the autoxidation has been isolated and by using spectral methods its structure is shown to be 2,7′-bi(5,6-dihydroxytryptamine). Liquid chromatography–mass spectrometry has been used to identify two trihydroxytryptamines and a second dimer of 5,6-DHT as minor autoxidation products.

5,6-Dihydroxytryptamine (5,6-DHT) is a pharmacological tool used to selectively destroy 5-hydroxytryptamine (serotonin) containing neurons.[1-3] The selectivity of 5,6-DHT is almost certainly derived from its high affinity uptake by the serotonergic membrane pump. However, the molecular mechanism by which 5,6-DHT expresses its neurodegenerative effects are still in question. It is rather widely thought that the ability of 5,6-DHT to induce neuronal degeneration is related to an inherent chemical property, namely ease to autoxidation (i.e., oxidation by dissolved oxygen at physiological pH without catalysis by an enzyme). Two main theories have been advanced to explain how the autoxidation of 5,6-DHT results in its neurodegenerative properties.[4-11] The first postulates that the autoxidation reaction generates an electrophilic *o*-quinone (1) which alkylates and cross-links neuronal membrane proteins as conceptualized in Scheme I. Experiments with radioactive 5,6-DHT suggest that the autoxidation product(s) undergo extensive covalent binding with protein nucleophiles both in vitro[11] and in vivo.[9] A second theory proposes that byproducts of autoxidation of 5,6-DHT are cytotoxic reduced oxygen species such as H_2O_2, $O_2^{\bullet-}$, or $HO^{\bullet}$ which attack neuronal lipids and proteins. The work of Klemm et al.[8] indicates that substantial amounts of H_2O_2 are indeed generated during the autoxidation of 5,6-DHT. These workers have suggested that the autoxidation is autocatalytically promoted by H_2O_2 formed in the reaction. Unfortunately, adducts of the type **2–4** have never been isolated and structurally characterized even using model nucleophiles such as small thiol-containing peptides.

Autoxidation of 5,6-DHT at physiological pH ultimately results in formation of a black, extremely insoluble melanin-like polymer.[8] It has been suggested that much of the protein-bound radioactivity that is recovered as a result of autoxidation of labeled 5,6-DHT in the presence of brain tissue[12,13] derives from this polymer.

Scheme I



Neither the melanin-like polymer nor any of its precursor species formed as a result of autoxidation of 5,6-DHT has been structurally characterized. Formation of various melanins in vivo, including neuromelanin, generally proceeds through enzymatic oxidation of tyrosine, L-dihydroxyphenylalanine (DOPA), and various catecholamines to 5,6-dihydroxyindoles which are rapidly oxidatively polymerized.[14-18] However, hitherto no stage between the indolic monomer and melanin polymer ever has been characterized.

Recently, Sinhababu et al.[19] synthesized a series of methyl-substituted analogues of 5,6-DHT (**5–7**) in order to block either simultaneously or independently the 4- and

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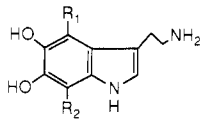
7-positions of putative quinone 1 from nucleophilic attack. This strategy was based on the assumption that the latter sites are those attacked by nucleophiles. Substitution of methyl groups had no deleterious effects on the cytotoxicity of 5,6-DHT, although it did result in a reduction in uptake affinity compared to the unsubstituted compound. In fact, 4,7-dimethyl-5,6-dihydroxytryptamine (5) was



5: $R_1 = R_2 = CH_3$
6: $R_1 = CH_3$; $R_2 = H$
7: $R_1 = H$; $R_2 = CH_3$

found to be at least 50 times more cytotoxic than 5,6-DHT. In view of the alkylation theory the latter observation is surprising because in 5 the generally accepted electrophilic sites are blocked. Compound 5 did not apparently polymerize to any detectable extent whereas 6 and 7 formed black polymers under the conditions employed.[19] The latter observation is also surprising because autoxidation of 6 and 7 should yield quinones having only one available electrophilic site such that the usual polymerization reaction should terminate at the stage of dimer. This suggests, therefore, that autoxidation of 5,6-DHT, 6, and 7 should generate an intermediate in which additional sites are activated toward nucleophilic attack.

An understanding of the major initial products of autoxidation of 5,6-DHT should, therefore, provide valuable clues concerning the nature of the reactive intermediate species and the chemical constitution of the black melanin-like ultimate reaction product.

The related indolic neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), has for many years been thought to express its neurodegenerative effects by its autoxidation to an electrophilic quinone imine intermediate which alkylates neuronal proteins[20] accompanied by formation of cytotoxic reduced oxygen species as byproducts. Recent work, however, has demonstrated that a quinone imine intermediate is probably not formed upon autoxidation of 5,7-DHT[21,22] but rather that radical intermediates are formed. Furthermore, suggestions that only the 4-position of 5,7-DHT is activated by autoxidation[23] are also incorrect. Both the 4- and 6-positions are activated in the reaction.[22] In addition, H_2O_2 is apparently not formed in significant amounts as a byproduct of the autoxidation reaction, and there is no experimental evidence to support formation of other cytotoxic reduced oxygen species.[8] Recent results suggest that the neurodegenerative properties of 5,7-DHT are in fact expressed by one of its autoxidation products, 5-hydroxytryptamine-4,7-dione,[22,24] although the mechanism of this process remains unknown. These recent insights into the autoxidation chemistry of 5,7-DHT and the lack of information concerning the autoxidation of 5,6-DHT suggested that it would be valuable to obtain information about the initial stages of the latter reaction.

Experimental Section

5,6-Dihydroxytryptamine (creatinine sulfate salt) was obtained from Sigma (St. Louis MO) and was used without further purification. High-performance liquid chromatography (HPLC) employed a Bio-Rad gradient instrument equipped with dual pumps, an Apple II_e controller, a Rheodyne Model 7125 loop injector, and an Isco Model 226 UV detector (254 nm). Analytical HPLC was carried out with a reversed-phase column (Brownlee Laboratories, RP-18, 5 μm particle size, 25 × 0.7 cm). A short guard column (Brownlee, RP-18, 5μm, OD-GU, 5 × 0.5 cm) was always employed. The mobile-phase solvents were prepared as follows: Solvent A was prepared by adding 20 mL of methanol and 10 mL of concentrated ammonium hydroxide to 970 mL of water. The pH of this solution was adjusted to 3.25 by addition of concentrated formic acid. Solvent B was prepared by adding 400 mL of methanol and 10 mL of concentrated ammonia to 590 mL of water, and then the pH was adjusted to 3.25 with formic acid. The gradient employed was as follows: 0–16 min 100% solvent A at a flow rate of 1.5 mL min^{-1}; 16–35 min, a linear gradient to 5% solvent B and a concommitant increase of flow rate to 2.5 mL min^{-1}; 35–50 min, a linear gradient to 60% solvent B and linear increase of flow rate to 3.0 mL min^{-1}. The latter mobile phase was then maintained for 6 min with a linear increase of flow rate to 3.5 mL min^{-1}. The mobile phase was then linearly returned to 100% solvent A over 4 min. The column was equilibrated at 100% solvent A for 10 min (1.5 mL min^{-1}) before another sample was injected. Typically, 2-mL injections of reaction product solutions were employed with this system.

Low- and high-resolution fast atom bomardment mass spectrometry (FAB-MS) was carried out with a VG Instruments ZAB-E spectrometer. Liquid chromatography–mass spectrometry (LC–MS) utilized a Kratos MS 25/RFA instrument equipped with a thermospray source. The source was maintained at 240–250 °C and the thermospray capillary tip at 188–190 °C. The mobile phase was 0.1 M ammonium acetate in water at a flow rate of 0.9 mL min^{-1}. All LC–MS results were obtained by injecting aliquots (0.2–2 mL) of fractions, collected from conventional HPLC separations, directly into the thermospray source using a loop injector (Rheodyne Model 7125). Reported LC–MS results are for positive ions. ^1H NMR spectra (300 MHz) were recorded on a Varian 300 XL spectrometer. The nuclear Overhauser effect difference FID's were obtained using a gated decoupling program.[25] For each measurement, 256 scans with irradiation were subtracted from those with irradiation on resonance. A decoupler amplitude up to 40.0 Hz was employed, and a flip angle of about 45° was applied. UV–visible spectra were recorded on a Hitachi 100-80 spectrophotometer.

Cyclic voltammetry was carried out with a conventional operational amplifer-based instrument and all voltammograms were corrected for iR drop. Voltammograms were obtained with a pyrolytic graphite electrode (PGE, Pfizer Minerals, Pigments and Metals Division, Easton, PA). A conventional electrochemical cell containing a platinum counter electrode and saturated calomel reference electrode (SCE) was used. All potentials are referred to the SCE at ambient temperature (22 ± 2 °C).

Autoxidation Procedure and Product Isolation. The autoxidation reaction was normally carried out by stirring 20 mL of a 2 mM solution of 5,6-DHT (0.0161g) in pH 7.2 phosphate buffer ($\mu = 0.1$[26]) at room temperature in a round-bottomed flask open to the atmosphere. The course of the autoxidation reaction was most easily followed by HPLC analysis. The maximal yields of products were obtained after 3–5 h of autoxidation. After this time samples of the autoxidation product mixtures were separated and analyzed by HPLC.

2,7'-Bi(5,6-dihydroxytryptamine) (10). This compound was eluted under HPLC peak 8. In the HPLC mobile phase (pH 3.25) 10 showed a characteristic spectrum with $\lambda_{max} = 308$ nm, $\lambda_{min} = 253$ nm. LC–MS of 10 dissolved in the HPLC mobile phase showed an intense pseudomolecular ion (MH^+) at $m/e = 383$ (100%) suggesting a molar mass of 382 g.

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Compound **10** was isolated using a preparative reversed-phase HPLC column (J. T. Baker, Bakerbond C_{18}, 25 × 2.12 cm, 10 μm). Two mobile phase systems were employed: Solvent C was 1.1% acetonitrile in water adjusted to pH 2.1 with HCl. Solvent D was 100% acetonitrile. The gradient employed was as follows: 0–12 min, a linear gradient from 2% to 12% solvent D and a linear increase of flow rate from 4.0 to 4.5 mL min^{-1}; 12–20 min, a linear gradient to 20% solvent D at a constant flow rate of 4.5 mL min^{-1}; the latter mobile phase composition was then maintained for 10 min with a linear increase of flow rate to 5.0 mL min^{-1}. The latter conditions were then held constant for 10 min. Between 40–45 min a linear gradient to 25% solvent D (5 mL min^{-1}); 45–50 min a linear gradient to 50% solvent D (5 mL min^{-1}). After 5 min under the latter conditions the mobile-phase composition was linearly returned to 2% solvent D and the flow rate to 4.0 mL min^{-1}. The column was equilibrated under these conditions for 5 min before another sample was injected. The injected volume of sample was 5.0 mL. Dimer **10** eluted at a retention time (t_R) of ca. 36 min.

The collected sample was degassed under vacuum before freezing and was then freeze-dried to give a light orange product (**10**). FAB-MS (3-nitrobenzyl alcohol matrix) gave ions at m/e (relative abundance): 383 (MH^+, 28%), 382 (M^+, 30%). Exact mass measurements on MH^+ gave 383.1739 ($C_{20}H_{23}N_4O_4$; calcd m/e = 383.1719). Thus, **10** is a dimer of 5,6-DHT. ^1H NMR (Me_2SO-d_6) δ 10.41 (s, 1 H, N(1)-H), 9.94 (d, $J_{1',2'}$ = 2.0 Hz, 1 H, N(1')-H), 9.00 (s, 1 H, OH), 8.72 (s, 1 H, OH), 8.21 (s, 1 H, OH), 8.15 (br s, ~3 H, NH_3^+), 8.04 (s, 1 H, OH), 7.84 (br s, ~3 H, NH_3^+), 6.95 (s, 1 H, C(4')-H), 6.92 (s, 1 H, C(4)-H), 6.86 (d, $J_{1',2'}$ = 2.0 Hz, 1 H, C(2')-H), 6.78 (s, 1 H, C(7)-H), 2.97 (m, 2 H, CH_2), 2.90 (m, 4 H, CH_2CH_2), 2,80 (m, 2 H, CH_2). Addition of a few drops of D_2O caused the resonances at 10.41, 9.94, 9.00, 8.72, 8.21, 8.15, 8.04, and 7.84 ppm to disappear and the doublet at 6.86 ppm to collapse into a singlet. For comparative purposes the ^1H NMR data of 5,6-DHT (Me_2SO-d_6) is presented: δ 10.30 (d, $J_{1,2}$ = 2.1 Hz, 1 H, N(1)-H), 7.60 (br s, ~4 H, NH_3^+, OH), 6.91 (d, $J_{1,2}$ = 2.1 Hz, 1 H, C(2)-H), 6.82 (s, 1 H, C(4)-H), 6.74 (s, 1 H, C(7)-H), 3.00 (m, 2 H, CH_2), 2.86 (m, 2 H, CH_2). These spectral data clearly indicate that **10** is an asymmetric dimer and that one 5,6-DHT residue is linked at C(2) while the other is linked at either C(4) or C(7). In order to resolve this ambiguity a series of nuclear Overhauser enhancement difference (NOED) experiments were carried out. The simplest NMR spectral assignments were the singlet at δ 10.41 ppm (N(1)-H) and the doublet (J = 2.0 Hz) at δ 9.94 ppm (N(1')-H). Preirradiation of the former caused a significant enhancement of the signal at δ 6.92 ppm (C(7)-H) confirming substitution at C(2). Preirradiation of the doublet at 9.94 ppm caused a significant enhancement of the signal at 6.86 ppm (C(2')-H). This observation confirmed that C(7') has no proton attached, i.e., it has been substituted with the second unit of 5,6-DHT. In order to provide additional support for these results NOED experiments were carried out with 5,6-DHT. Preirradiation of the signal at 10.30 ppm (N(1)-H) caused clear enhancements of the signals at 6.91 ppm (C(2)-H) and 6.74 ppm (C(7)-H). Thus, the NOED experiments indicate that **10** contains two 5,6-DHT residues linked 2 → 7'. Homonuclear 2D-correlated spectroscopy (COSY) was exploited to correctly assign the chemical shifts of C(4)-H (6.92 ppm) and C(7)-H (6.78 ppm) of the 5,6-DHT residue in **10** linked at its C(2)-position. The same long-range coupling was observed between C(4)-H and C(7)-H at 6.82 and 6.71 ppm, respectively, in 5,6-DHT.

Results and Discussion

Spectral changes which accompany the autoxidation of 5,6-DHT at pH 7.2 are shown in Figure 1. 5,6-DHT shows bands at λ_{max} = 295, 276(sh), and 214 nm (curve 1, Figure 1). During the initial stages of the autoxidation the band at 295 nm slowly shifts to longer wavelengths and grows. Correspondingly, the short wavelength band (214 nm) decreases. When the initial concentration of 5,6-DHT was 0.2 mM the long wavelength band reached its maximal height at λ_{max} = 304 nm after approximately 1.75 h (curve 7, Figure 1) and then began to decrease and shift to longer wavelengths (curves 8–16, Figure 1). The short wavelength band decreased only a small amount during the initial 1.25–1.5 h but then decreased more rapidly at longer times and, simultaneously, shifted to longer wavelengths. It is this apparent acceleration in the latter stages of the reaction which has led to the conclusion that the autoxidation process is autocatalytically promoted by H_2O_2 formed in the reaction.[8] After approximately 4 h, autoxidation of 0.2 mM 5,6-DHT is complete, and the only remaining spectral band (λ_{max} = 229 nm) is due to creatinine. The general increase in absorbance throughout the entire UV–visible regions of the spectrum is caused by formation of a black precipitate in the reaction solution.

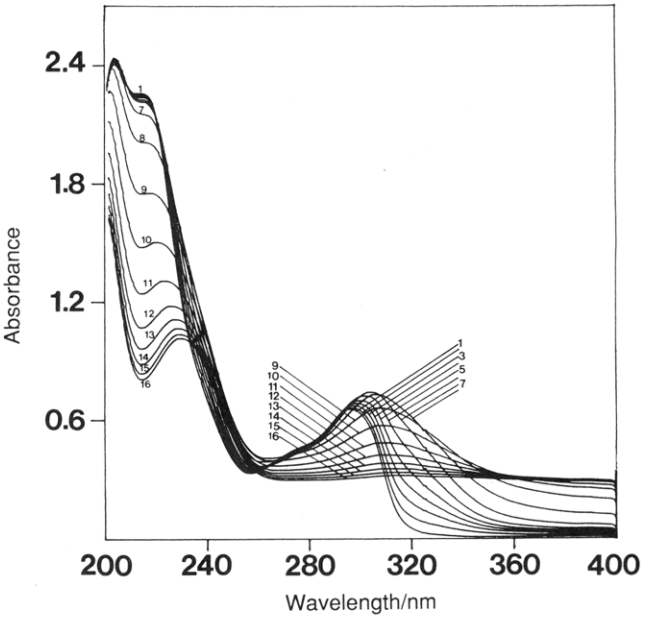


Figure 1. Spectral changes which accompany the autoxidation of 0.2 mM 5,6-dihydroxytryptamine in pH 7.21 phosphate buffer (μ = 0.1) at room temperature. Curve 1 is the spectrum of 5,6-DHT. Curves 2–16 were recorded at 15-min intervals. Spectra were recorded in a 0.5-cm pathlength cell open to the atmosphere.

The cource of the autoxidation reaction was also followed by HPLC analysis. Figure 2A shows a chromatogram of a freshly prepared solution of 5,6-DHT in pH 7.2 phosphate buffer (μ = 0.1). HPLC peak 1 is due to creatine and peak 6 to 5,6-DHT. The chromatogram shown in Figure 2B was obtained after the autoxidation of 2 mM 5,6-DHT had progressed for about 1 h. HPLC peak 8 clearly represents the major initial product along with very much smaller HPLC peaks 2 and 7. After autoxidation for approximately 2.5 h the component responsible for HPLC peak 8 continues to represent the major product species, but peaks 2 and 7 also grow and peaks 5 and 9 appear (Figure 2C). At even longer reaction times (3 h 42 min, Figure 2D) HPLC analysis reveals that most 5,6-DHT (peak 6) has disappeared and that HPLC peak 8 has also decreased in height and additional, small chromatographic peaks (3, 4, and 10) appear. After a further 1–2 h, HPLC analysis showed only a peak due to creatinine (Figure 2E). The solution at this stage contained a heavy, black precipitate.

HPLC analyses, therefore, indicate that the major and indeed the first detectable autoxidation product of 5,6-HT is responsible for chromatographic peak 8 (Figure 2B).

The product responsible for this peak has been isolated and, based upon spectral information, has been identified as the asymmetric dimer 2,7'-bi(5,6-dihydroxytryptamine) (**10**). The other products were formed in low yields and were unstable, and all attempts to isolate these compounds led to formation of multiple secondary products and, ul-

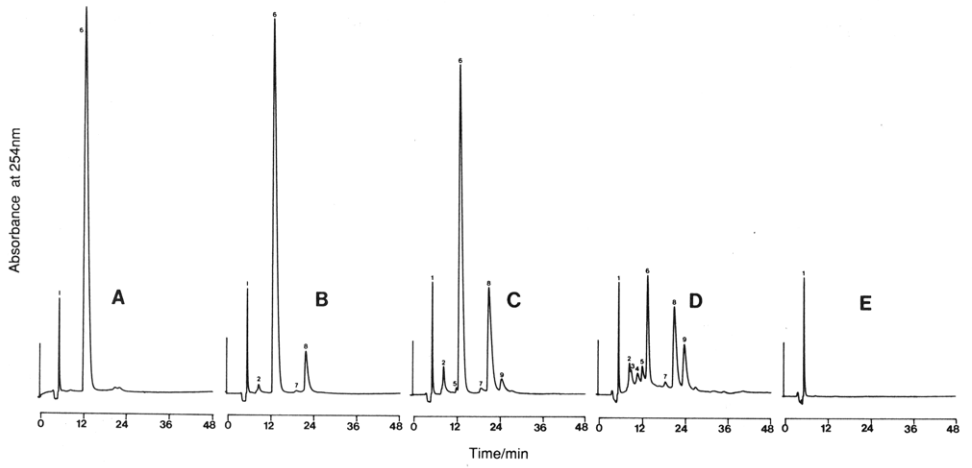


Figure 2. High-performance liquid chromatograms obtained throughout the course of autoxidation of 1.90 mM 5,6-dihydroxytryptamine in pH 7.21 phosphate buffer (μ = 0.1) at room temperature. (A) Freshly prepared solution; (B) after 70 min; (C) after 148 min; (D) after 222 min; (E) after 328 min. Chromatographic conditions are given in the Experimental Section.

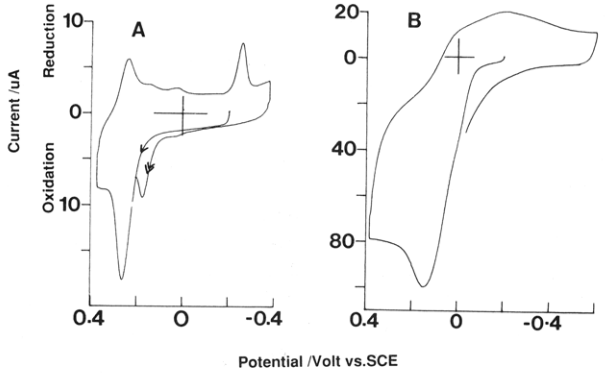


Figure 3. Cyclic voltammograms at the PGE of 5,6-dihydroxytryptamine in (A) HPLC mobile phase pH 3.25 and (B) 2 mM in pH 7.21 phosphate buffer (μ = 0.1). Sweep rate: 200 mV s^{-1}.

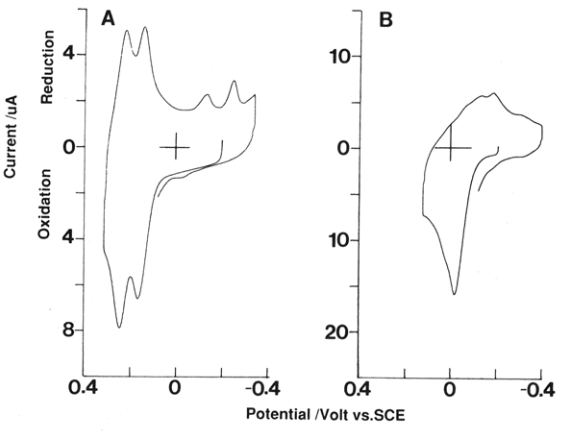


Figure 4. Cyclic voltammograms at the PGE of 2,7′-bi(5,6-dihydroxytryptamine) (**10**) in (A) HPLC mobile phase pH 3.25 and (B) in pH 7.2 phosphate buffer (μ = 0.1). Sweep rate: 200 mV s^{-1}.

timately, insoluble polymeric materials. Nevertheless, important additional insights into the autoxidation reaction and the possible identity of some of these unstable products was obtained from their spectral, electrochemical, and LC–MS behaviors.

Electrochemical, Spectral and LC–MS Studies. Cyclic voltammograms (CVs) of 5,6-DHT at a PGE are shown in Figure 3. At pH 3.25 (HPLC mobile phase) the peak potential (E_p) for oxidation of 5,6-DHT is 0.262 V. After sweep reversal a reduction peak (E_p = 0.240 V) appears. The peak separation (ΔE_p) between these peaks, 22 mV, is close to that expected for a reversible two-electron electrode reaction (29 mV). A second reduction peak occurs at E_p = −0.260 V but only appears if the primary oxidation peak is first scanned. On the second anodic sweep a new oxidation peak appears at E_p = 0.175. The latter peak appears only after the reduction peak at −0.260 V has been scanned. At pH 7.2 the primary oxidation peak of 5,6-DHT occurs at E_p = 0.150 V. The electrode reactions associated with the various peaks observed in CVs of 5,6-DHT remain to be studied. However, the low oxidation potential of 5,6-DHT at pH 7.2 is in accord with its ease of autoxidation.

The major autoxidation product of 5,6-DHT is the asymmetric dimer **10**. CVs of **10** at pH 3.25 and 7.2 are shown in Figure 4. At pH 3.25 two closely spaced oxidation peaks appear (E_p = 0.140 and 0.210V) and, after scan reversal, two reversible reduction peaks (E_p = 0.180 and 0.110 V). Two additional, apparently irreversible, reduction peaks appear at −0.130 and −0.250 V. It is interesting to note that a reduction peak is observed at the latter potential in CVs of 5,6-DHT at pH 3.25. This might indicate that **10** is formed and then further oxidized to the

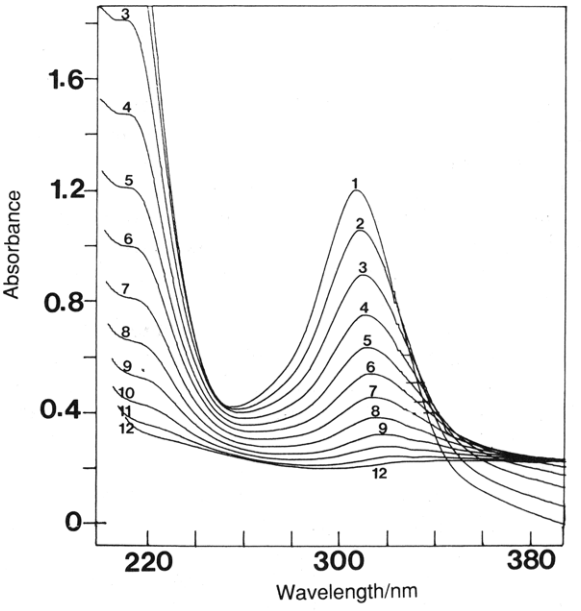


Figure 5. Spectral changes which accompany the autoxidation of 2,7′-bi(5,6-dihydroxytryptamine) (**10**) in pH 7.2 phosphate buffer (μ = 0.1) at room temperature. Curve 1 is the spectrum of **10**. Curves 2–16 were recorded at 10-min intervals.

Table I. Spectral, Electrochemical, and LC–MS Data for the Autoxidation Products of 5,6-Dihydroxytryptamine

HPLC component	pH	λ_{max}, nm		E_p, V oxdn	E_p, V redn[a]	major LC–MS peaks, m/e (relative abundance)
2	3.25[b]	304		0.17	0.07	209 (MH^+, 100), 193 (55)
	7.2[c]	304[d]		−0.02	−0.07	
4	3.25[b]	311	(1)[e]	0.11	0.09	209 (MH^+, 40), 193 (15)
			(2)[e]	0.20		
	7.2[c]	317,[d] 327 sh[f]		—[g]	—[g]	
5	3.25[b]	311	(1)[e]	0.19	0.17	
			(2)[e]	0.29		
	7.2[c]	312[d]	(1)[e]	−0.02	−0.08	
			(2)[e]	0.04		
6 (5,6-DHT)	3.25[b]	296, 276 sh[f]		0.26	0.24	385 ($[2M]H^+$, 25), 193 (MH^+, 100)
	7.2[c]	295,[d] 276 sh[f]		0.15		
7	3.25[b]	311		0.22	0.14	383 (MH^+, 65), 209 (12), 193 (100)
	7.2[c]	315[d]		—[g]	—[g]	
8	3.25[b]	307	(1)[e]	0.14	0.11	383 (MH^+, 100)
			(2)[e]	0.21	0.18	
	7.2[h]	308,[d] 212		−0.02		
9	3.25[b]	310		0.14	0.11	
	7.2[c]	312[d]		0.00	−0.07	

[a] The reduction peaks reported were reversibly or quasi-reversibly coupled to the corresponding oxidation peak. No product exhibited a reduction peak without first scanning an oxidation peak. [b] HPLC mobile phase; see Experimental Section. [c] HPLC mobile phase containing the component was adjusted to pH 7.2 by addition of phosphate buffer (μ = 1.0; pH = 10.47). [d] Absorbance centered at λ_{max} decayed with time. At all other wavelengths the absorbance systematically increased with time owing to formation of turbidity or a precipitate in the solution. [e] Two oxidation peaks appeared. [f] sh = shoulder. [g] Not obtained owing to rapid decomposition at pH 7.2. [h] In pH 7.2 phosphate buffer, μ = 0.1.

species responsible for this reduction peak as a result of electrochemical oxidation of 5,6-DHT. At pH 7.2 **10** shows a single oxidation peak at E_p = −0.02 V. On the reverse sweep only rather ill-defined reduction peaks occur. The fact that the electrochemical oxidation peak of **10** occurs at negative potentials at pH 7.2 indicates that this compound should be readily autoxidized. Spectral studies confirm this. Curve 1 in Figure 5 is the spectrum of **10** at pH 7.2 (λ_{max} = 308, 212 nm). When this solution was exposed to air both absorption bands decrease. Furthermore, the band originally at 308 nm shifts to longer wavelengths as the autoxidation proceeds (λ_{max} for curve 9 is 317 nm). The growth in absorbance noted at 350–400 nm (and at longer wavelengths) during the early stages of the autoxidation was caused by formation of an insoluble presumably polymeric material.

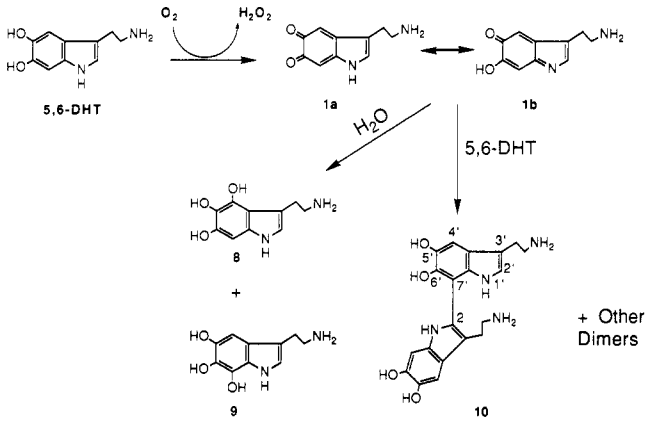
Electrochemical, spectral, and LC–MS data for each of the other autoxidation products of 5,6-DHT were obtained in the following way. 5,6-DHT (2 mM in pH 7.2 phosphate buffer, μ = 0.1) was stirred in air for approximately 4 h at room temperature. A 10-mL aliquot of the resulting solution was filtered (5 μm filter) and separated using preparative HPLC. The individual components eluted under the various peaks were collected. Attempts to purify any product except **10** were unsuccessful owing to their low yield, decomposition to numerous secondary products, and ease of autoxidation to insoluble polymers. Accordingly, CVs, spectra, and LC–MS results were first obtained with each product dissolved in the HPLC mobile phase (pH 3.25). The pH of each product solution was then adjusted to 7.2, and spectra and CVs of the resulting solutions were measured. In several instances it was not possible to obtain CVs at pH 7.2 because of the low concentration of some products and their rapid air oxidation. A summary of electrochemical, spectral, and LC–MS results is presented in Table I. At pH 7.2 all of the autoxidation products absorb at slightly longer wavelengths than 5,6-DHT. This accounts for the shift of λ_{max} to longer wavelength during the autoxidation of 5,6-DHT (Figure 1). At pH 7.2 all autoxidation products were themselves air oxidized to form insoluble presumably polymeric materials.

Reliable LC–MS data was obtained for only four autoxidation products. The products responsible for HPLC peaks 2 and 4 showed intense pseudomolecular ions (MH^+) and m/e = 209, indicating that these compounds have a molar mass of 208 g. These compounds therefore must be trihydroxytryptamines. The products responsible for HPLC peaks 7 and 8 both showed intense pseudolecular ions at m/e = 383, indicating that they must both be simple dimers having molar masses of 382 g. HPLC peak 8 is due to **10** and hence the component responsible for peak 7 must possess at least one different linkage site between the 5,6-DHT residues.

Reaction Pathways

The autoxidation reaction of 5,6-DHT appears to be a complex process. In the initial slow stage of the reaction 5,6-DHT is oxidized by molecular oxygen to generate a reactive intermediate which can react further to give the asymmetric dimer **10** as the major product along with at least one more dimer and at least two trihydroxyindoles. The spectral and electrochemical similarities between the various products eluted under HPLC peaks 2–5 and 7–9 suggests that all are structurally similar compounds. Oxidation of 5,6-DHT by molecular oxygen could yield a reactive radical intermediate which in turn could generate **10** by radical–radical or radical–substrate mechanisms. There are, however, several lines of evidence to suggest that radical mechanism are not involved. For example, a one-electron abstraction from 5,6-DHT to yield a radical intermediate might be expected to yield superoxide radical anion as a byproduct. However, no radical species has ever been detected in the autoxidation reaction.[8] It is also difficult to reconcile the formation of trihydroxytryptamines as products by attack of water on a radical intermediate. Finally, the fact that high yields of H_2O_2 are formed as a reaction byproduct[8] suggests that 5,6-DHT is autoxidized to the quinonoid intermediate **1** as shown in Scheme II. Electron-deficient quinone **1a** would be subject to nucleophilic attack by water to generate trihydroxytryptamines such as **8** and **9** although the instability of these compounds has precluded isolation and complete structural characterization. Nucleophilic attack by 5,6-DHT on **1a**/**1b** could, in principle, lead to six different carbon–carbon linked dimers. The major product

Scheme II



of the autoxidation reaction is the asymmetric 2,7′-linked dimer **10** but at least one other dimer has been detected by LC–MS experiments.

It is of some interest to note that at both pH 3.25 and pH 7.2 the potential of the first voltammetric oxidation peak of 5,6-DHT is more positive than those for any of its autoxidation products (Table I). For example, at pH 7.2 E_p for the oxidation peak of 5,6-DHT is 0.15 V whereas that for **10** under similar conditions is −0.02 V. Such information implies that the autoxidation products are inherently more easily oxidizable than 5,6-DHT. It is therefore somewhat surprising that these more easily oxidizable products accumulate in solution to some extent during the autoxidation of 5,6-DHT. All of the prepolymeric products of autoxidation of 5,6-DHT absorb at longer wavelengths than does the parent compound. This accounts for the shift of λ_{max} to longer wavelengths observed during the autoxidation of 5,6-DHT (Figure 1). Subsequent oxidation of **10** and the various other initial products to form, ultimately, insoluble polymeric materials accounts for the subsequent decrease of absorbance.

Previous workers have demonstrated that H_2O_2 is formed during the autoxidation of 5,6-DHT.[8] Preliminary results have shown that in unbuffered aqueous solution 5,6-DHT is oxidized much more slowly by H_2O_2 than by molecular oxygen.[27] The reactions of **10** and other autoxidation products of 5,6-DHT with H_2O_2 remain to be investigated in detail.

The results reported here clearly indicate that the major initial autoxidation product from 5,6-DHT is the asymmetric dimer **10**. Autoxidation of **10** would be expected to yield a dimer containing one or two electrophilic quinoidal residues, which could undergo a rather diverse range of coupling reactions to yield tetramers and ultimately melanin-like polymeric materials. Autoxidation of **10** in the presence of trihydroxyindoles such as **8** and **9** would be expected to ultimately yield polymers containing di- and trihydroxylated indolic residues.

There has been much interest over many decades in the probable mechanism of formation and structure of indolic melanin pigments.[14-18] By freezing reaction mixtures and studying fluorescence and absorption spectra Bu'Lock[17] proposed that the initial product of oxidation of 5,6-dihydroxyindole (5,6-DHI) was a 3,4′-linked dimer. This product was proposed to be formed by oxidation of 5,6-DHI to the corresponding *o*-quinone, which was rapidly attacked by 5,6-DHI. However, detailed support for dimer formation was not provided. The work reported here provides the first complete characterization of one species, i.e., **10**, intermediate between a dihydroxyindole and melanin polymer. Furthermore, evidence has been provided that other dimers and trihydroxyindoles are also formed upon autoxidation of 5,6-DHT.

The actual significance of these new discoveries regarding the autoxidation chemistry of 5,6-DHT in relation to the ways in which this compound expresses its neurodegenerative effects is not yet clear. Work is currently underway to evaluate the neurotoxicity and selectivity of **10** and other autoxidation products of 5,6-DHT in order to address this question.

Acknowledgment. This work was supported in part by Grant No. GM-32367 from the National Institutes of Health and in part by Contract No. 3506 from the Oklahoma Center for the Advancement of Science and Technology. Additional support was provided by the Research Council of the University of Oklahoma.

Registry No. **10**, 124782-14-5; 5,6-DHT, 5090-36-8.

(27) Singh, S.; Dryhurst, G. Work in progress.