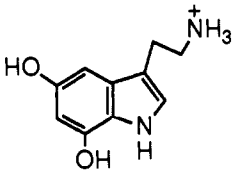
Chemical and Enzyme-Mediated Oxidation of the Serotonergic Neurotoxin 5,7-Dihydroxytryptamine: Mechanistic Insights

Tahereh Tabatabaie and Glenn Dryhurst*

Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Oklahoma 73019. *Received October 2, 1991*

The oxidation chemistry and biochemistry of the serotonergic neurotoxin 5,7-dihydroxytryptamine (1) has been studied under anaerobic and aerobic conditions in aqueous solution at physiological pH. Under anaerobic conditions, one-electron oxidants (ferricytochrome *c*, peroxidase/H_2O_2, ceruloplasmin, Cu^{2+}) generate a radical intermediate. Dimerization of the C(6)-centered resonance form of this radical followed by secondary oxidations yields 3-(2-aminoethyl)-6-[3-(2-aminoethyl)-1,7-dihydro-5-hydroxy-7-oxo-6*H*-indol-6-ylidene]-1-*H*-indole-5,7(4*H*,6*H*)-dione. Under aerobic conditions, molecular O_2 attacks the C(4)-centered 1 radical to yield a hydroperoxy radical which decomposes to 5-hydroxytryptamine-4,7-dione (2). Autoxidation of 1 proceeds by primary attack by molecular O_2 on a C(4)-centered carbanion to form a superoxide–radical complex. This rearranges to a C(4)-centered hydroperoxide which decomposes to 2. A C(6)-centered carbanion of 1 combines with 2 to give, ultimately, 6,6'-bi-5-hydroxytryptamine-4,7-dione (3). Trace concentrations of transition metal ions (Fe^{3+}, Fe^{2+}, Cu^{2+}, Mn^{2+}) catalyze the autoxidation of 1 by catalytic cycles in which a hydroperoxide intermediate plays key roles. A byproduct of the transition metal-catalyzed oxidation of 1 is superoxide, $O_2^{\bullet-}$. Because of its enormous basicity $O_2^{\bullet-}$ facilitates deprotonation of 1. The C(4)-centered carbanion so produced is oxidized by molecular O_2 or by the hydroperoxy radical ($HO_2^{\bullet}$) to give radical intermediates and thence 2 and 3. Mechanistic pathways leading to the various products of oxidation of 1 are proposed and the potential roles of oxidation reactions of the indolamine are related to its neurodegenerative properties.

5,7-Dihydroxytryptamine (1) is used to chemically lesion central serotonergic neurons.[1,2] The selectivity of 1 derives



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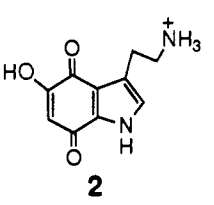
from its high-affinity uptake by serotonergic neurons. The molecular mechanism(s) by which this drug expresses its neurodegenerative effect, however, is (are) unknown. It has been speculated that the neurotoxicity of 1 derives from its intraneuronal autoxidation to an electrophilic quinone imine intermediate which alkylates thiol residues of neuronal proteins[3] with resulting compromise of their function leading, ultimately, to cell death. Another suggestion is that byproducts of the autoxidation reaction are cytotoxic reduced oxygen species ($O_2^{\bullet-}$, $HO^{\bullet}$, H_2O_2).[2,4-6] It has also been speculated that the putative quinone imine intermediate expresses the neurotoxic effects of 1 by interacting with the electron-transport chain.[7] However, there is little direct experimental evidence to support many aspects of these various theories. The autoxidation of 1 is first order with respect to both molecular oxygen and the indolamine.[7,8] This observation is inconsistent with the transformation of 1 into a quinone imine and suggests a mechanism which involves incorporation of O_2 into the indolic nucleus. There have been reports that small amounts of H_2O_2 are formed during the autoxidation of 1[9] although this observation has not been confirmed by other workers.[7] Experiments aimed at detecting other reduced oxygen species such as $O_2^{\bullet-}$ and $HO^{\bullet}$ as byproducts of autoxidation of 1 have not been carried out. However, radical scavengers appear to provide some protection to peripheral nerves from damage by 1[9] although it is not known whether these scavengers provide similar protection to central serotonergic neurons.

In the event that the neurodegenerative properties of 1 do derive from its in vivo oxidation it is clearly of importance to understand the chemical fate of both the indolamine and oxygen in order to gain insights into the mechanisms by which this compound expresses its neurotoxicity. Recently, we reported[10] that autoxidation of 1 at physiological pH gives two major products, 5-hydroxytryptamine-4,7-dione (2) and 6,6-bi-5-hydroxytryptamine-4,7-dione (3). Based primarily on the identities of these products, a tentative mechanism for the autoxidation reaction was suggested. The fate of molecular O_2 was not elucidated. However, previous investigators have reported that superoxide dismutase greatly inhibits



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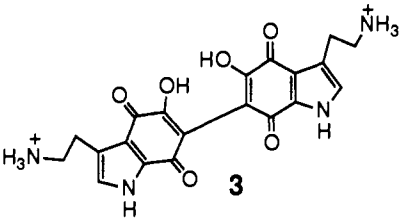

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the rate of oxygen consumption by 1, whereas catalase causes a small but significant enhancement.[7] Such observations might suggest roles for $O_2^{\bullet-}$ and H_2O_2 or other peroxy species in the autoxidation reaction.

Cohen and Heikkila[9] first reported that rat brain mitochondria catalyze the oxidation of 1 by molecular oxygen. Klemm et al.[7] confirmed this observation and provided evidence that rules out any significant involvement of monoamine oxidase in the catalytic effect of mitochondria. Ferricytochrome *c* also promotes a rapid, short-lasting increase in oxygen consumption by 1, suggesting that electron transport from the indolamine to ferricytochrome *c* might occur within mitochondria in the terminal cytochrome *c* oxidase segment of the respiratory chain. The products of the ferricytochrome *c* and mitochondria promoted oxidations of 1 were not determined.

The aim of the work described in the present communication was to describe in more detail the autoxidation of 1. The fate of molecular oxygen has been studied in order to provide additional insights into the mechanism of the autoxidation reaction. Once sequestered within a serotonergic neuron, 1 must be potentially exposed to a variety of enzyme systems and transition metal ions which could play functional roles in catalyzing the oxidation of the drug. Accordingly, interactions of 1 with a number of oxidative enzyme systems and with transition metal ions have been studied.

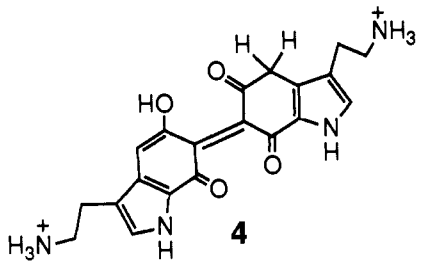
Results

Unless otherwise stated, the oxidation reactions described below employed 1.0 mM 1 in pH 7.4 phosphate buffer having an ionic strength (μ) of 0.1 at 37 °C.

Autoxidation of 1. Spectral changes which accompany the autoxidation of 1 are shown in Figure 1A. As the reaction progresses the characteristic bands of 1 (λ_{max}: 344, 250, sh, 214 nm) decrease and new bands grow in at 525, 296, and 231 nm. The initially colorless solution of 1 turns pink-purple as the autoxidation progresses. The initial rate of oxygen consumption, measured with a Clark-type oxygen electrode, was 34.1 ± 2.8 nmol of O_2/min (Figure 1B). Complete oxidation of 1 in a vigorously stirred solution exposed to the atmosphere required 6.5 ± 0.5 h.

In order to obtain evidence for formation of radical species in the autoxidation of 1, the reaction was carried out in the presence of the spin trapping agent 5,5-dimethyl-1-pyrroline 1-oxide (DMPO; 40 mM). The reaction was accelerated by bubbling O_2 gas through the solution. After several minutes the ESR spectrum showed features of spin adducts of both a carbon-centered radical and the hydroxyl radical. The hydroxyl radical–DMPO spin adduct showed a characteristic 1:2:2:1 quartet with a hyperfine splitting constant (hfsc) of 14.84 G. The carbon-centered spin adduct exhibited a six-line pattern consisting of a doublet of triplets with a β-hydrogen hfsc (α_β^H) of 29.2 G and a nitroxide hfsc (α_N) of 17.3 G.

HPLC analysis of autoxidized 1 revealed that two major products are formed, 2 and 3 (Figure 1C).[10,11] A very minor product of the autoxidation reaction is 1*H*-indole-5,7(4*H*,6*H*)-dione, 3-(2-aminoethyl)-6-[3-(2-aminoethyl)-1,7-dihydro-5-hydroxy-7-oxo-6*H*-indol-6-ylidene] (4) (see Experimental Section for spectroscopic evidence for the structure of this compound). Compounds responsible for the other minor chromatographic peaks shown in Figure 1C remain to be identified. After complete autoxidation of 1 (1.07 mM), compounds 2 and 3 were formed in equimolar yields (0.36 ± 0.02 mM); i.e., twice as much 1 is converted to dimer 3 as is converted to monomer 2.



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Influence of Catalase and H_2O_2 on the Autoxidation Reaction. In the presence of 22.5, 45.0, and 82.5 μg/mL of catalase the initial rate of oxygen consumption by 1 decreased to 26.2 ± 0.4, 23.3 ± 1.1, and 20.2 ± 0.6 nmol of O_2/min, respectively (compared to 34.1 ± 2.8 nmol of O_2/min in the absence of catalase). At a catalase concentration of ≥110 μg/mL the initial rate of oxygen consumption became constant at 16.6 ± 2.1 nmol of O_2/min. Complete oxidation of 1 in the presence of catalase (160 μg/mL) required 10 ± 0.5 h, i.e., approximately 50% longer than in the absence of the enzyme. HPLC analysis of the resulting product solution indicated that the major products were 2 and 3; dimer 4 was a minor product. The yields of 2 and 3 were approximately the same as was observed in the simple autoxidation reaction. Addition of a solution of catalase (2 mg/200 μL of water) to the reaction compartment of the oxygen electrode assembly after autoxidation of 1 (3.0 mL) had proceeded for times ranging from 15 to 30 min resulted in the return of between 5.6 and 8.1% of the consumed oxygen to the solution. Thus, H_2O_2 is formed as a minor byproduct of the autoxidation reaction. However, addition of H_2O_2 (50–100 μM) to an autoxidizing solution of 1 did not significantly increase the initial rate of oxygen consumption. In the presence of an equimolar concentration of H_2O_2 (1.0 mM), the initial oxygen consumption increased only slightly to 45 ± 1 nmol of O_2/min. Under the latter conditions the total time required for complete oxidation of 1 decreased to 5 ± 0.5 h, but the ultimate products and their yields were virtually identical to those observed in the simple autoxidation reaction, i.e., equimolar 2 and 3 and a trace of 4. Under anaerobic conditions the oxidation of 1 (1.0 mM) in the presence of H_2O_2 (e.g., 100 μM) was at least 50 times slower than the autoxidation reaction. HPLC analysis of the reaction solution after 6 h revealed that very small amounts of 3 and a partially characterized dimer 6 (Scheme I) were formed. These results indicate that H_2O_2 plays little or no functional role in the overall autoxidation chemistry of 1.

Influence of Superoxide Dismutase and $O_2^{\bullet-}$ on the Autoxidation of 1. In the presence of 0, 40, 67, 100, 200, and 333 units/mL of superoxide dismutase (SOD), the initial rates of oxygen consumption by 1 were 34.1 ± 2.8, 26.7 ± 1.2, 23.0 ± 0.9, 20.8 ± 0.6, 19.3 ± 1.1, and 18.2 ± 0.4 nmol of O_2/min, respectively. Higher concentrations of SOD caused no further decrease in the initial rate of oxygen consumption. Both HPLC analyses and UV spectral data confirmed the inhibitory effects of SOD during the initial 1–2 h of the autoxidation reaction. However, the total time required for complete oxidation of 1 in the absence or presence of SOD (420 units/mL) were virtually identical (6.5 ± 0.5 h). Denatured SOD (420 units/mL; boiled in water for 15 min) caused an increase

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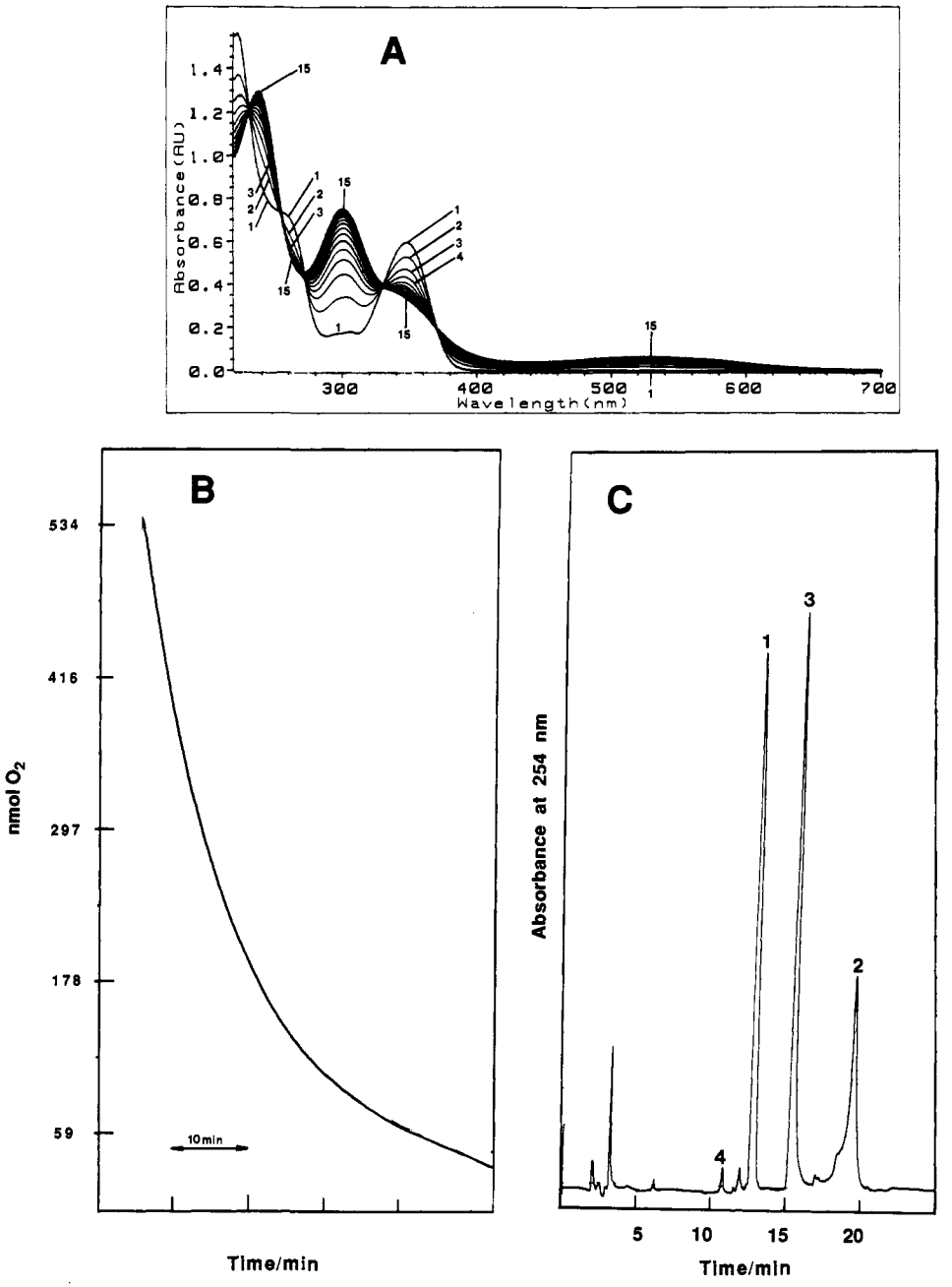

Figure 1. (A) Spectral changes observed during the autoxidation of 0.2 mM 5,7-dihydroxytryptamine (1) in pH 7.4 phosphate buffer (μ = 0.1) at 25 °C. Curve 1 is the spectrum of 1; curves 2–15 were recorded at 30-min intervals. (B) Oxygen electrode tracing for 1.0 mM 1 in an initially air-saturated pH 7.4 phosphate buffer (μ = 0.1) at 37 °C. (C) HPLC chromatogram of the product solution obtained following partial autoxidation of 1.0 mM 1 in pH 7.4 phosphate buffer (μ = 0.1) for 120 min at 37 °C. Chromatographic method II was employed (see Experimental Section).

in the initial rate of oxygen consumption to 340 ± 28 nmol of O_2/min and decreased the total reaction time to 2 ± 0.25 h. Thus, it may be concluded that under the conditions of the autoxidation reaction SOD is slowly denatured with the result that the initial inhibitory effect of the enzyme is compensated, in the latter stages of the reaction, by the catalytic influence of its denatured form. Although Cu/Zn–SOD is known to be inactivated by high concentrations of H_2O_2,[11] the latter compound is only a minor byproduct of the autoxidation of 1 and is formed in only micromolar concentrations. The initial rate of oxygen consumption by 1 in the presence of SOD (1000 units/mL) and catalase (100 μg/mL) was 13.2 ± 0.3 nmol of O_2/min.

The inhibitory influence of SOD on the autoxidation of 1 suggested that superoxide radical anion, $O_2^{\bullet-}$, is formed as a byproduct and plays a key role in the overall reaction. In order to explore this possibility, the influence of $O_2^{\bullet-}$ on the oxidation of 1 was first studied in dimethyl sulfoxide (Me_2SO), a solvent in which $O_2^{\bullet-}$ is a stable species in the absence of water (a proton source).[13] Potassium superoxide (KO_2), a convenient source of $O_2^{\bullet-}$ in Me_2SO,[14] was added to 1 (1.0 mM) dissolved in the latter solvent through which a stream of dry N_2 was continuously bubbled. The initially colorless solution of 1 instantly turned pink and during the course of 120–150 min became deep red. HPLC analysis revealed that 1 was oxidized to a mixture of 2 and 4. Control experiments indicated that 1 was indefinitely stable in Me_2SO in the absence of O_2 or $O_2^{\bullet-}$. Addition of

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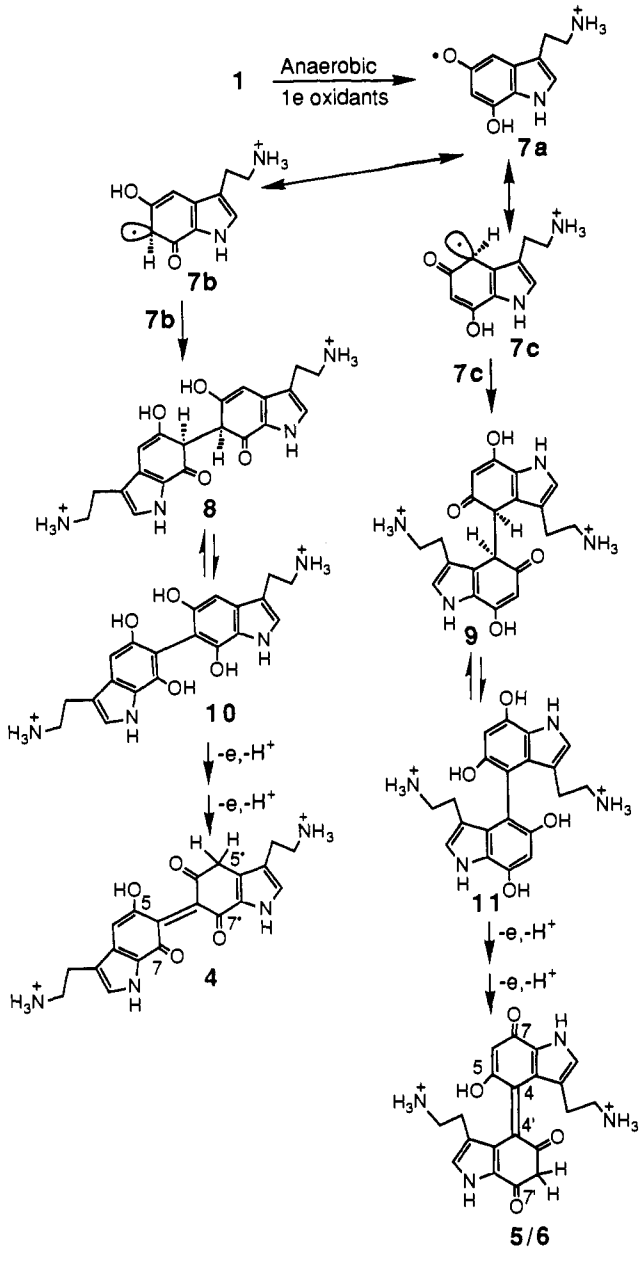
(14) Rosen, G. M.; Finkelstein, E.; Raukman, E. J. A Method for the Detection of Superoxide in Biological Systems. *Arch. Biochem. Biophys.* 1982, *215*, 367–368.

Scheme I



KO_2 (2–3 mM) to 1 (1.0 mM) in aqueous solution (pH 7.4 phosphate buffer) through which N_2 was bubbled vigorously caused the initially colorless solution to instantly turn an intense pink color. HPLC analysis showed that 2 and 4 were the major products; dimer 3 was a minor product.

Influence of Transition Metal Ions on the Autoxidation of 1. The effects of various transition metal ions and transition metal ion complexing agents on the initial rate of oxygen consumption during the autoxidation of 1 are summarized in Table I. The catalytic effects of these ions on the autoxidation of 1 decrease in the order $Cu^{2+} > Fe^{2+} \gg Mn^{2+} > Fe^{3+}$. However, the catalytic effect caused by Fe^{2+} persisted for only about 30 s and then the rate of oxygen consumption slowed to a value similar to that evoked by an equal concentration of Fe^{3+}.

Ethylenediaminetetraacetic acid (EDTA) caused a small acceleration of the autoxidation of 1 and, similarly, slightly potentiated the catalytic effects of Fe^{2+} and Fe^{3+}. However, EDTA slightly inhibited the catalytic effects of Cu^{2+} and Mn^{2+}. These results are in accord with the previously observed potentiating influence of EDTA on the catalytic effects of iron on many autoxidation reactions.[15] Diethylenetriaminepentaacetic acid (DTPA), a very powerful complexing agent for transition metal ions,[16] inhibited the autoxidation of 1 in the absence of added transition-metal ions, and greatly inhibited the catalytic effects evoked by added Cu^{2+}, Fe^{2+}, Fe^{3+}, and Mn^{2+} (Table I). These observations indicate that trace concentrations of transition metal ions, particularly Fe^{2+}/Fe^{3+}, which always contaminate the buffer salts employed, exert a catalytic influence on the autoxidation of 1.

Table I. Effects of Transition Metal Ions and Complexing Agents on the Initial Rate of Oxygen Consumption during the Autoxidation of 1[a]

added compound	initial rate of oxygen consumption,[b] nmol of O_2/min
0	34.1 ± 2.8
Cu^{2+}[c] (1.0 μM)	638 ± 34
Cu^{2+} (10 μM)	1490 ± 51
Cu^{2+} (20 μM)	2244 ± 47
Cu^{2+} (40 μM)	2367 ± 67
Cu^{2+} (10 μM) + EDTA[d] (1.0 mM)	45.6 ± 1.6
Cu^{2+} (10 μM) + DTPA[e] (1.0 mM)	32.1 ± 2.5
Fe^{2+}[f] (10 μM)	252.0 ± 4
Fe^{2+} (50 μM)	483 ± 5
Fe^{2+} (100 μM)	628 ± 9
Fe^{2+} (200 μM)	801 ± 15
Fe^{3+}[g] (10 μM)	50 ± 1
Fe^{3+} (50 μM)	68 ± 2
Fe^{3+} (100 μM)	86 ± 2.5
Fe^{3+} (200 μM)	125 ± 2
Mn^{2+}[h] (10 μM)	61 ± 1
Mn^{2+} (50 μM)	70 ± 2
Mn^{2+} (100 μM)	87 ± 2
DTPA[e] (0.5 mM)	24.0 ± 0.3
DTPA (1.0 mM)	24.2 ± 0.8
DTPA (10 mM)	23.6 ± 0.8
EDTA[d] (1.0 mM)	39.6 ± 2
Fe^{2+} (100 μM) + DTPA (10 mM)	58.4 ± 1.8
Fe^{2+} (100 μM) + EDTA (10 mM)	712 ± 12
Fe^{3+} (100 μM) + DTPA (10 mM)	39.2 ± 1.3
Fe^{3+} (100 μM) + EDTA (10 mM)	97 ± 4
Mn^{2+} (100 μM) + DTPA (10 mM)	36.7 ± 0.8
Mn^{2+} (100 μM) + EDTA (10 mM)	51. 3 ± 1.3

[a] 1.0 mM 1 dissolved in 3.0 mL of air-saturated pH 7.4 phosphate buffer (μ = 0.1) at 37 °C. [b] Measured with a Clark-type oxygen electrode assembly; initial rates are given as mean ($n \geq 2$) ± standard deviation. [c] Added as $CuSO_4$. [d] Ethylenediaminetetraacetic acid. [e] Diethylenetriaminepentaacetic acid. [f] Added as $FeSO_4$. [g] Added as $FeCl_3$. [h] Added as $MnSO_4$.

Complete oxidation of 1 in the presence of 50 and 200 μM Cu^{2+} under aerobic conditions required 2 ± 0.5 h and 45 ± 10 min, respectively. HPLC analysis revealed that more than 90% of 1 was converted to 2 and 3. However, the yield of monomer 2 increased about 20% relative to that of dimer 3 compared to the simple autoxidation. A small amount of 4 and several other very minor unidentified products were also formed. Under anaerobic conditions Cu^{2+} directly oxidized 1. The major product of this reaction was oxidized dimer 4; smaller amounts of 5, 6 (see later discussion), 2, and 3 were also formed. More than 10 other minor unidentified products were also formed.

Complete oxidation of 1 (1.0 mM) under aerobic conditions in the presence of 50 μM Fe^{2+}, Fe^{3+}, or Mn^{2+} required 4.5 ± 0.5, 5.0 ± 0.25, and 3.5 ± 0.5 h, respectively. The products and their yields were virtually identical to

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Table II. Influence of Catalase and Superoxide Dismutase on the Transition Metal Ion Mediated Autoxidations of 1[a]

added compound (concentration)	initial rate of oxygen consumption, nmol of O_2/min[b]
Cu^{2+}[c] (10 μM)	1561 ± 13[d]
Cu^{2+} (10 μM) + catalase (100 μg/mL)	1524 ± 12
Cu^{2+} (10 μM) + SOD (333 units/mL)	1583 ± 9
Fe^{2+}[e] (100 μm)	628 ± 4
Fe^{2+} (100 μM) + catalase (100 μg/mL)	328 ± 16
Fe^{2+} (100 μM) + SOD (333 units/mL)	645 ± 22
Fe^{3+}[f] (100 μM)	85.2 ± 1
Fe^{3+} (100 μM) + catalase (100 μg/mL)	51.0 ± 4
Fe^{3+} (100 μM) + SOD (333 units/mL)	48.5 ± 1
Mn^{2+}[g] (100 μM)	87.1 ± 2
Mn^{2+} (100 μM) + catalase (100 μg/mL)	54.5 ± 2
Mn^{2+} (100 mM) + SOD (333 units/mL)	81.2 ± 3

[a] 3.0 mL of 1.0 mM 1 in pH 7.4 phosphate buffer (μ = 0.1) at 37 °C. [b] Measured with a Clark-type oxygen electrode assembly. [c] Added as $CuSO_4$. [d] Mean ($n \geq 2$) ± standard deviation. [e] Added as $FeSO_4$. [f] Added as $FeCl_3$. [g] Added as $MnSO_4$.

those formed in the simple autoxidation reaction, i.e., equimolar **2** and **3** and a minor amount of **4**. In the absence of molecular O_2, Fe^{2+}, Fe^{3+}, and Mn^{2+} were unable to oxidize **1**.

Catalase caused only a minor decrease in the initial rate of oxygen consumption in the Cu^{2+}-catalyzed autoxidation of **1** but significant decreases in the Fe^{2+}-, Fe^{3+}-, and Mn^{2+}-catalyzed reactions (Table II). SOD caused only relatively minor effects on the initial rates of oxygen consumption in the transition metal ion-catalyzed reactions (Table II).

In the absence of added transition-metal ions the initial rate of oxygen consumption by **1** in the presence of DTPA (1.0 mM) and SOD (333 units/mL) was 9.24 ± 0.74 nmol of O_2/min. In the presence of DTPA (1.0 mM), SOD (333 units/mL), and catalase (100 μg/mL), the initial rate of oxygen consumption decreased to 8.3 ± 0.9 nmol of O_2/min. However, under both of the latter conditions there was no detectable oxygen consumption for ca. 3.5 min. Only after this initial lag period was the reported oxygen consumption rate observed.

Role of Hydroxyl Radical in the Autoxidation of 1. The catalytic effects of transition metal ions on the autoxidation of **1** and formation of H_2O_2 as a minor byproduct suggested the possibility that the hydroxyl radical (HO•) might play a role in the overall reaction.[17] However, HO• radical scavenges such as mannitol (0.25, 1.0, 10.0, 50.0 mM) had no effect on the initial rate of oxygen consumption in the autoxidation of **1**. Furthermore, the time required for complete autoxidation of the indolamine and the products formed were unaffected by the presence of mannitol. The HO•-generating system Fe^{2+} (200 μM), EDTA (240 μM), and ascorbic acid (Na salt, 1.0 mM)[18] in pH 7.4 phosphate buffer at 37 °C resulted in complete oxidation of **1** (1.0 mM) in <3 h. HPLC analysis of the bright yellow product solution showed that none of the major products of the autoxidation or transition metal ion-catalyzed oxidations of **1** were formed. On the basis of these results, and the observation that added H_2O_2 has very little effect on the autoxidation of **1**, it was concluded that HO• plays no significant role in the autoxidation reaction.

Peroxidase-Mediated Oxidations of 1. Aerobic oxidation of **1** in the presence of peroxidase and an equimolar concentration of H_2O_2 was a fast reaction. Initial oxygen consumption rates were very high (Table III). Complete oxidations of **1** (1.0 mM) in the presence of H_2O_2 (1.0 mM) and 3, 6, and 12 μg/mL of peroxidase required 40, 30, and <2 min, respectively. Such oxidations, carried out with the reaction solutions exposed to air, resulted in the formation of five major products: **2**, **3**, **4**, **5**, and **6** (Figure 2A).

Under anaerobic conditions the peroxidase/H_2O_2 oxidations of **1** were still fast. For example, in the presence of 12 μg/mL peroxidase and equimolar **1** and H_2O_2 (1.0 mM), oxidation of the indolamine was complete in 30 ± 3 min. However, under such anaerobic conditions oxidized dimers **4**–**6** were formed as major products (Figure 2B).

A freshly collected sample of **5** dissolved in the HPLC mobile phase (pH 3.8) was bright yellow and, within about 60 min, converted into an equimolar mixture of **5** and **6**. Similarly, a solution of **6**, also yellow, converted into an equimolar mixture of **5** and **6**. Attempts to isolate pure samples of **5** and **6** suitable for spectroscopic structure elucidation were unsuccessful. However, LC-MS on freshly chromatographed solutions of **5** and **6** both exhibited in-

Table III. Influence of Various Enzymes on the Initial Rate of Oxygen Consumption by 1[a]

enzyme system (concentration)	pH	initial rate of oxygen consumption, nmol O_2/min[b]
0	7.4	34.1 ± 2.8[c]
ceruloplasmin[d] (10.5 units/mL)	7.4	71.4 ± 1
ceruloplasmin[d] (21.0 units/mL)	7.4	129 ± 4
ceruloplasmin[d] (42.0 units/mL)	7.4	240 ± 5
ceruloplasmin[d] (10.5 units/mL)	6.4	453 ± 6
tyrosinase[e] (4.5 units/mL)	7.4	85 ± 1
tyrosinase[e] (9 units/mL)	7.4	126 ± 3
tyrosinase[e] (18 units/mL)	7.4	192 ± 3
tyrosinase[e] (36 units/mL)	7.4	304 ± 3
peroxidase (0.22 unit/mL) + H_2O_2 (1.0 mM)	7.4	787 ± 15
peroxidase (0.45 unit/mL) + H_2O_2 (1.0 mM)	7.4	1143 ± 23
peroxidase (0.90 unit/mL) + H_2O_2 (1.0 mM)	7.4	1570 ± 33
peroxidase[f] (5 units/mL)	7.4	79 ± 1
peroxidase[f] (10 units/mL)	7.4	89 ± 2
peroxidase[f] (15 units/mL)	7.4	102 ± 2

[a] 3.0 mL of 1.0 mM 1 in air-saturated phosphate buffer (μ = 0.1) at the indicated pH. [b] Measured with a Clark-type oxygen electrode assembly. [c] Mean ($n \geq 3$) ± standard deviation. [d] Bovine ceruloplasmin. [e] From mushroom. [f] No H_2O_2 was added to the solution.

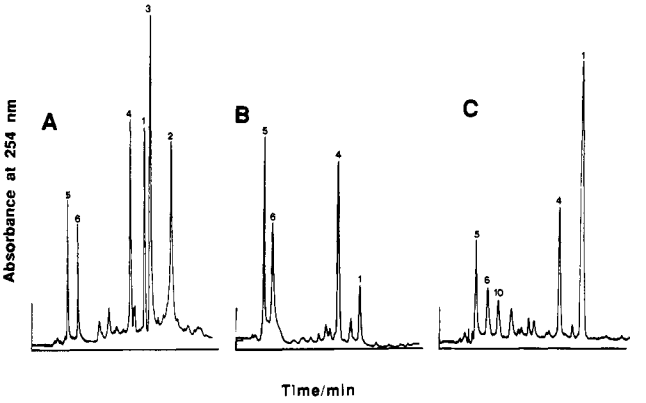


Figure 2. HPLC chromatograms of the product mixtures formed upon oxidation of 1.0 mM **1** in pH 7.4 phosphate buffer (μ = 0.1) at 37 °C: (A) in the presence of peroxidase (3 μg/mL) and H_2O_2 (1.0 mM) under aerobic conditions for 5 min; (B) in the presence of peroxidase (12 μg/mL) and H_2O_2 (1.0 mM) under anaerobic conditions for 2 min; (C) in the presence of peroxidase (42 μg/mL) and H_2O_2 (0.3 mM) under anaerobic conditions for 6 h. Chromatographic method II was employed (see Experimental Section).

(17) Walling, C. Fenton's Reagent Revisited. *Acc. Chem. Res.* **1975**, *8*, 125–131.
(18) Slivka, A.; Cohen, G. Hydroxyl Radical Attack on Dopamine. *J. Biol. Chem.* **1985**, *260*, 15466–15472.

tense pseudomolecular ions (MH^+) at m/e = 381. LC-MS (and FAB-MS) on 4 also gave a pseudomolecular ion at m/e = 381. The UV–visible spectra of freshly prepared solutions of 5, 6, and 4 at pH 3.8 (all bright yellow) exhibited many similarities.

Anaerobic oxidation of 1 in the presence of peroxidase, but with appreciably less than equimolar concentrations of H_2O_2, resulted in the formation of one key additional product, 10 (Figure 2C). A freshly collected solution of 10 in the HPLC mobile phase (pH 3.8) was colorless but slowly turned yellow when exposed to air. The same color change occurred much more rapidly when the solution was adjusted to pH 7.4. HPLC analysis, LC-MS, and UV–visible spectrophotometry revealed that under these conditions 10 quantitatively transformed into oxidized dimer 4. LC-MS on a freshly chromatographed solution of 10 showed a pseudomolecular ion (MH^+, 12%) at m/e = 383. Thus, 10 (molar mass 382 g) is a simple dimer of 1 which is readily air oxidized to 4 (molar mass 380 g). Based upon the known structure of 4, it may therefore be concluded that 10 is 6,6′-bi-5,7-dihydroxytryptamine (Scheme I).

In the absence of added H_2O_2, peroxidase catalyzes the oxidation of 1 as indicated by the increase in the initial rate of oxygen consumption (Table III). Complete oxidation of 1 in the presence of peroxidase (150 μg/mL) required 3.5 ± 0.5 h. The major products of this reaction were 2 and 3 along with a minor amount of oxidized dimer 4. Incubation of 1 with peroxidase (50 μg/mL) and catalase (100 μg/mL) resulted in an initial oxygen consumption rate of 92 ± 6 nmol of O_2/min. Comparison of this value with that for the initial oxygen consumption rate in the absence of catalase, 102 ± 2 nmol of O_2/min, and for the simple autoxidation, 34.1 ± 2.8 nmol of O_2/min, clearly indicates that peroxidase exerts a catalytic effect on the oxidation of 1 in the absence of H_2O_2. HPLC analyses revealed that autoxidation of 1 in the presence of peroxidase and catalase gave only 2 and 3 as products; in the absence of catalase a small amount of 4 was formed. Thus, formation of 4 in the presence of peroxidase requires H_2O_2, a minor byproduct of the autoxidation reaction.

Ceruloplasmin-Mediated Oxidation of 1. Human and bovine ceruloplasmin catalyzed the oxidation of 1 as indicated by the increase in the initial rate of oxygen consumption (Table III). The optimum pH for bovine ceruloplasmin-mediated oxidations of 1 was 6.4 (Table III); with human ceruloplasmin the optimum pH was 5.9–6.0. Complete aerobic oxidation of 1 in the presence of ceruloplasmin (bovine; 60 units/mL) required ca. 2 h. The four major products were 2–5. Under anaerobic conditions ceruloplasmin (bovine; 60 units/mL) caused a very slow oxidation of 1 (the initial rate estimated by HPLC analyses was ca. 100 times slower than the aerobic oxidation) to give 4 as the major product; 5 and 6 were minor products. SOD and catalase had no effects on the rates or products of ceruloplasmin-catalyzed oxidations of 1.

Tyrosinase-Mediated Oxidation of 1. Tyrosinase catalyzed the oxidation of 1 by molecular O_2 as demonstrated by its influence on the initial rate of oxygen consumption (Table III). Complete oxidation of 1 in the presence of tyrosinase (200 units/mL) required ca. 60 min and 2–4 were the major products. SOD and catalase had no effect on the rate or products formed in the tyrosinase-mediated oxidation of 1. In the absence of molecular O_2, tyrosinase did not oxidize 1.

Ferricytochrome *c* Mediated Oxidation of 1. The initial rates of oxygen consumption by 1 in the presence of 100 and 200 μg/mL of ferricytochrome *c* (Fe^{3+} cyt *c*) were 91 ± 2 and 190 ± 1 nmol of O_2/min, respectively. This increased rate of oxygen consumption compared to that measured for the simple autoxidation (34 ± 3 nmol of O_2/min) persisted for about 30 s and then abruptly decreased to 42 ± 2.4 nmol of O_2/min. Complete oxidation of 1 in the presence of Fe^{3+} cyt *c* (0.25 mM) required 3.5 ± 0.5 h. The major products of the reaction were 2 and 3 although significant yields of 4–6 were formed. SOD and catalase had no effect on either the rate or the products of the aerobic Fe^{3+} cyt *c*-catalyzed oxidation of 1.

Under anaerobic conditions the major products of the Fe^{3+} cyt *c* oxidation of 5,7-DHT were dimers 4–6; a minor amount of 2 was formed and more than 10 minor unidentified products.

Mitochondria-Mediated Oxidation of 1. Rat brain mitochondria (66 and 133 μg/mL) were incubated with 1 (1.0 mM in a medium consisting of 0.3 M mannitol, 0.01 M Tris-HCl, 0.01 M potassium phosphate, 0.01 M KCl, and 0.003 M Mg_2Cl_2, pH 7.4; 37 °C). The initial rates of oxygen consumption measured were 40.9 ± 1.2 and 61.2 ± 1.8 nmol of O_2/min, respectively. In the same medium, but in the absence of mitochondria, the initial rate of oxygen consumption was 17.6 ± 0.4 nmol of O_2/min. The major products of the mitochondria-promoted oxidations of 1 were 2 and 3 although a number of minor unidentified products were also formed.

Discussion

Under anaerobic conditions peroxidase/H_2O_2,[19,20] Fe^{3+} cyt *c*,[21] Cu^{2+},[22] and ceruloplasmin[23] function as one-electron oxidants. Under anaerobic conditions each of these oxidants oxidize 1 to form 4 as the major product. In order to account for this product, it is proposed that the initial step in the oxidation reaction is a one-electron abstraction from 1 to yield the radical represented by resonance structures 7a–c in Scheme I. Dimerization of the C(6)-centered radical 7b yields 6,6′-bi-5,7-dihydroxytryptamine (10) as conceptualized in Scheme I. The latter dimer (molar mass 382 g) was detected as a product of the anaerobic oxidation of 1 by peroxidase in the presence of less than equimolar concentrations of H_2O_2 and by anaerobic oxidation of the indolamine by Cu^{2+}. Under anaerobic conditions dimer 10 is almost instantaneously oxidized to the 6,6′-linked dimer 4 (molar mass 380 g) at pH 7.4 by peroxidase/H_2O_2, ceruloplasmin, Fe^{3+} cyt *c*, and Cu^{2+}. These oxidations presumably occur by two successive one-electron abstraction reactions. Dimer 10 is also readily oxidized by molecular oxygen to dimer 4 although the precise mechanistic pathway remains to be elucidated. In the anaerobic peroxidase/H_2O_2, Fe^{3+} cyt *c*, and ceruloplasmin oxidations of 1 two additional oxidized dimers (molar mass 380 g) were formed, i.e., 5 and 6. Because 5 and 6 are interconvertible and have similar UV–visible spectra (Figure 3B,C), it has been tentatively concluded that they are probably tautomers. While 5 and 6 have not been isolated there is considerable evidence for the formation of the C(4)-centered radical 7c upon oxidation of 1 (see later discussion). Accordingly, it is tentatively

(19) Saunders, B. C.; Holmes-Siedle, A. G.; Stark, B. P. *Peroxidase*; Butterworths: Washington, DC, 1964.
(20) Walsh, C. *Enzymatic Reaction Mechanisms*; W. H. Freeman and Co.: Boston, 1979; pp 491–494.
(21) Chance, B.; Williams, G. R. The Respiratory Chain and Oxidative Phosphorylation. *Adv. Enzymol.* **1956**, *17*, 65–130.
(22) Gutteridge, J. M. C.; Halliwell, B.; Rowley, D. R. The Involvement of Iron, Copper, and Oxygen Radicals in Human Diseases. *Life Chem. Rep.* **1985**, *3*, 232–243.
(23) Frieden, E.; Hsieh, H. S. Ceruloplasmin: The Copper Transport Protein with Essential Oxidase Activity. *Adv. Enzymol.* **1976**, *44*, 187–231.

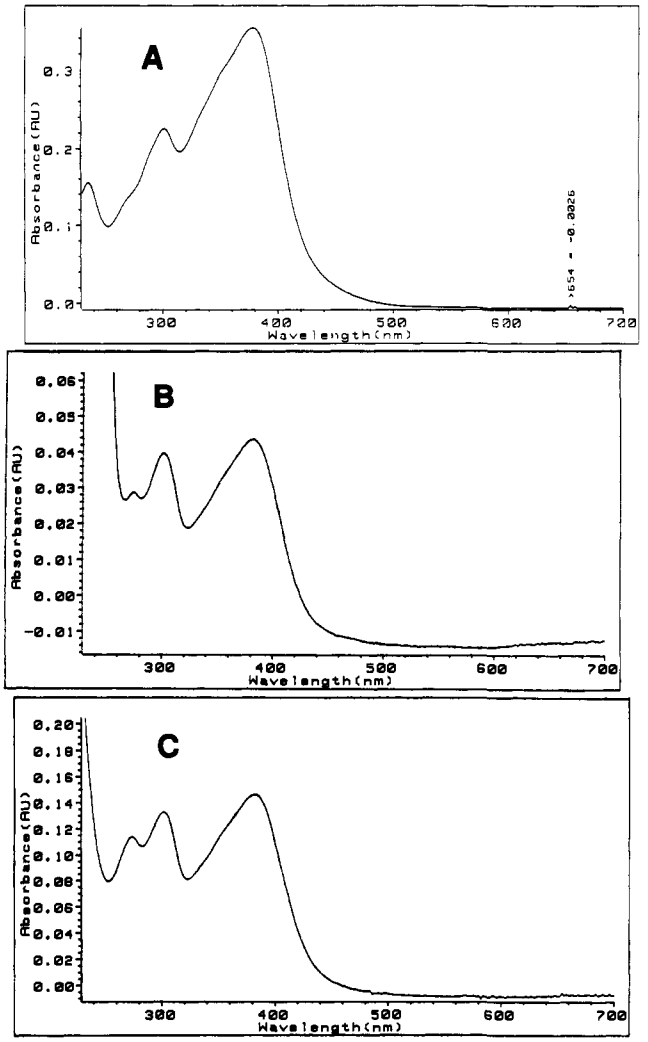


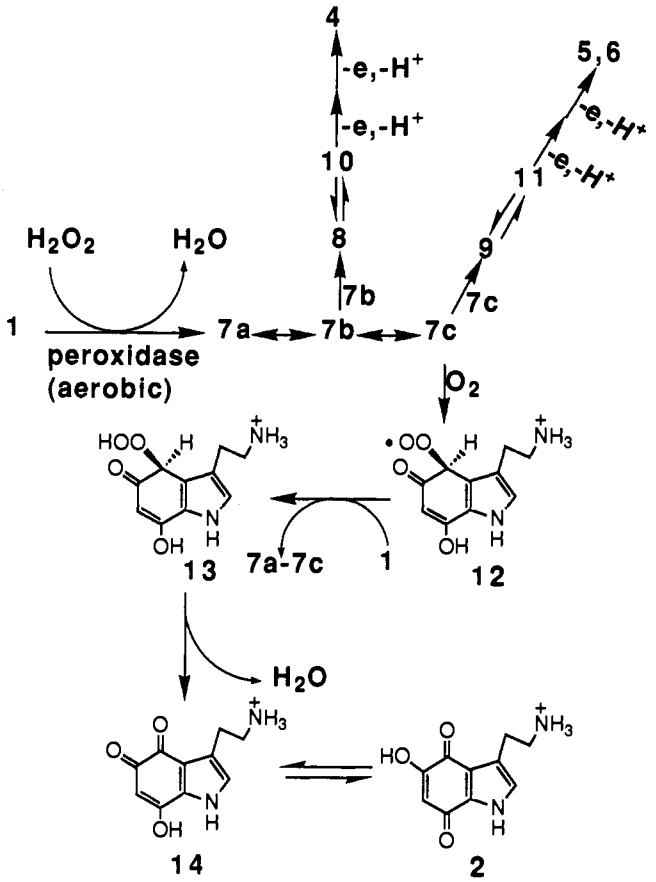
Figure 3. UV–visible spectra of (A) 3-(2-aminoethyl)-6-[3-(2-aminoethyl)-1,7-dihydro-5-hydroxy-7-oxo-6*H*-indol-6-ylidene]-1*H*-indole-5,7(4*H*,6*H*)-dione (4), and the tautomers of 3-(2-aminoethyl)-4-[3-(2-aminoethyl)-1,7-dihydro-5-hydroxy-7-oxo-4*H*-indol-4-ylidene]-1*H*-indole-5,7(4*H*,6*H*)-dione **5** (B), and **6** (C) at pH 3.8.

proposed that **5** and **6** are tautomers of 3-(2-aminoethyl)-6-[3-(2-aminoethyl)-1,7-dihydro-5-hydroxy-7-oxo-4*H*-indol-4-ylidene]-1*H*-indole-5,7(4*H*,6*H*)-dione, formed by the route outlined in Scheme I.

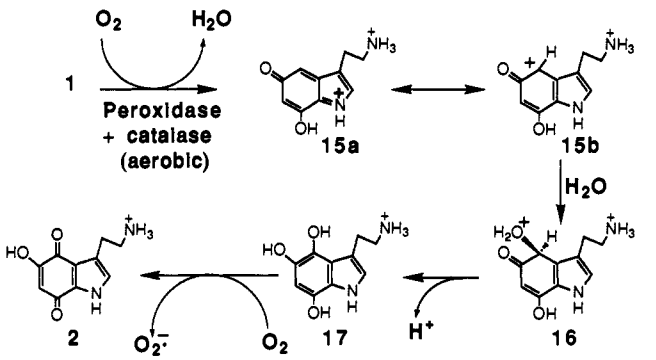
Under aerobic conditions the peroxidase/H_2O_2 oxidation of **1** gives **2** and **3** in addition to dimers **4**–**6** (Figure 2A). Both **2** and **3** contain additional oxygen atoms at the C-(4)-position of the indolic nucleus and, hence, molecular oxygen must attack a key oxidative intermediate derived from **1** at the latter position. By analogy with reaction sequences known to occur in a number of radical-chain oxidation processes,[24] it is proposed that **2** is formed by a reaction initiated by attack of molecular O_2 on the C-(4)-centered radical **7c** to give peroxy radical **12** (Scheme II). Reaction between **1** and **12** then yields hydroperoxide **13** and regenerates radical **7**. Base-catalyzed decomposition of secondary hydroperoxide **13** yields *o*-quinone **14** which tautomerizes to the more stable *p*-quinone **2**. Experiments show that under aerobic or anaerobic conditions **2** is not oxidized by peroxidase/H_2O_2 to dimer **3**. Furthermore, peroxidase/H_2O_2, ceruloplasmin, and Cu^{2+} under aerobic or anaerobic conditions oxidize **10** quantitatively

(24) Howard, J. A. Homogeneous Liquid-Phase Autoxidations. In *Free Radicals*; Kochi, J. K., Ed.; John Wiley and Sons: New York, 1973; Vol. 2, Chapter 12.

Scheme II



Scheme III



to **4**; i.e., dimer **10** is not a precursor of dimer **3**.

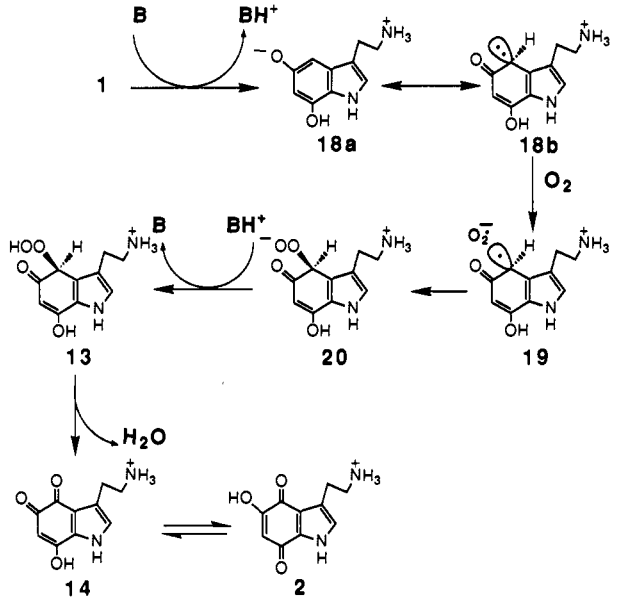
Addition of peroxidase, but not H_2O_2, to an autoxidizing solution of 5,7-DHT results in formation of **2** and **3** as the major products and a very small amount of dimer **4**. In the autoxidation reaction H_2O_2 is formed as a minor by-product and peroxidase utilizes this as an oxidant to generate radical **7a**–**c**. Radical **7c** is attacked by molecular oxygen to form, ultimately, **2** (Scheme II), hence accounting (in part) for the increased rate of O_2 consumption observed when peroxidase is present in the autoxidation reaction mixture (Table III). Radical **7b** dimerizes to give **10** which is oxidized by molecular oxygen or by peroxidase/H_2O_2 to dimer **4** (Scheme I). Autoxidation of **1** in the presence of peroxidase and the H_2O_2-destroying enzyme catalase yields only **2** and **3** as products; no traces of **4**–**6** could be detected. Furthermore, the rate of oxygen consumption under the latter conditions was about 3 times faster than for the simple autoxidation reaction. Clearly, therefore, peroxidase can function as an oxidase enzyme in the absence of H_2O_2. The above results also provide important support for the conclusion that **4** must be

formed by a radical pathway which can be driven by peroxidase/H_2O_2. The oxidase activity of peroxidase/O_2 no doubt proceeds by an initial oxidation (2e) of 1 to putative quinonoid **15a**/**15b** (Scheme III). Carbocation **15b**, a highly electrophilic intermediate, is attacked by water to yield 4,5,7-trihydroxytryptamine (17). The latter compound is very easily oxidized ($E^{\circ\prime}$ at pH 7.4 for the 17/2 couple is −0.51 V vs SCE as determined by cyclic voltammetry at a pyrolytic graphite electrode) by molecular oxygen to give 2. The enhanced rate of oxygen consumption by 1 when the autoxidation is carried out in the presence of peroxidase and catalase derives from at least two reactions: (1) the initial reaction between the indolamine and O_2 catalyzed by peroxidase as an oxidase enzyme to give **15a**/**15b** and (2) the autoxidation of the trihydroxyindolamine 17 to give 2 (Scheme III).

The one-electron oxidizing systems studied, i.e., peroxidase/H_2O_2, ceruloplasmin, Fe^{3+} cyt *c*, and Cu^{2+}, drive the same overall chemical reactions. Under anaerobic conditions all of these oxidizing systems yield 4 as the major product along with smaller yields of 5 and 6 by the routes outlined in Scheme I. In the presence of molecular oxygen, however, 2 and 3 become the major products. Monomer 2 is formed as a result of the attack by molecular oxygen on radical **7c** (Scheme II). Dimer 4 and, usually, dimers 5 and 6 are formed as only minor products. Accordingly, it may be concluded that the key intermediate in all of these reactions is radical 7 and that the sequences outlined in Schemes I and II represent the primary pathways leading to the ultimate products. Formation of very minor amounts of 2 and 3 as a result of the anaerobic oxidations of 1 by Fe^{3+} cyt *c* and Cu^{2+} is almost certainly due to traces of oxygen entering the reaction vessels during the relatively long reaction times involved. The aerobic reaction sequences outlined in Scheme II leading to 2 (and subsequently dimer 3; see later discussion) require no roles for $O_2^{\bullet-}$. That such roles are not required has been verified from the observations that SOD has no effect on either the rates of the peroxidase/H_2O_2, Fe^{3+} cyt *c*, ceruloplasmin, and Cu^{2+} oxidations of 1 or the products formed and their yields. Similarly, catalase has no effect on either the rate of the Fe^{3+} cyt *c*, ceruloplasmin, or Cu^{2+} oxidations of 1 or on the products formed, indicating that, as predicted in Scheme II, H_2O_2 or, perhaps, organic peroxides such as 13 play no functional roles in these reactions.

The rate of autoxidation of 1 is significantly decreased in the presence of both SOD and DTPA, indicating that $O_2^{\bullet-}$ and trace transition metal ions probably play roles in the overall reaction. Additions of $O_2^{\bullet-}$, and Fe^{2+}, Fe^{3+}, and Mn^{2+} all act to catalyze the autoxidation of 1, providing support for the conclusion that such species are involved in the autoxidation reaction. Catalase has an inhibitory effect on the simple autoxidation of 1 and on the Fe^{2+}-, Fe^{3+}-, and Mn^{2+}-catalyzed oxidations. However, addition of H_2O_2 has very little effect on the rate of the autoxidation reaction. Accordingly, it must be concluded that catalase expresses its inhibitory effect by mechanisms other than by the simple destruction of H_2O_2. The autoxidation of 1 in the presence of SOD (333 units/mL), catalase (100 μg/mL), and DTPA (1.0 mM) is slow (8.3 ± 0.9 nmol of O_2/min) but clearly in the absence of the catalytic influences of $O_2^{\bullet-}$ and transition metal ions proceeds to form 2 and 3 but not 4. Under such circumstances the largely uncatalyzed oxidation of 1 by molecular oxygen occurs. Schlossberger[25] has reported that 1 exhibits phenol–keto

Scheme IV



tautomerism. By comparing the pH-dependent UV spectra of 1 and various methylated analogues, Sinhababu and Borchardt[8] have concluded that the predominant keto tautomer involves ketonization of the 5-OH group with proton transfer to the C(4)-position. In addition, because colored products were not formed upon autoxidation of 4-methyl-1, the latter workers concluded that the primary site of O_2 incorporation into 1 is the C(4) position. In the absence of transition metal ions such as Cu^{2+}, which can directly oxidize 1, or $O_2^{\bullet-}$, which also facilitates the oxidation of the indolamine although by an indirect route (vide infra), it is clear that a mechanism must exist for the direct oxidation of 1 by molecular oxygen. Cyclic voltammograms of 1 at pH 7.4 show an oxidation peak having a peak potential (Ep) at +0.19 V vs SCE (sweep rate 200 mV s^{-1}).[10] However, even at sweep rates as high as 100 V s^{-1}, a reduction peak reversibly coupled to this oxidation peak cannot be observed. As a result, it is not possible to estimate a reliable $E^{\circ\prime}$ value even for the 1/15 couple. Nevertheless, assuming that the above Ep is, very approximately, close to $E^{\circ\prime}$, it is unlikely that the initial step in the simple, uncatalyzed autoxidation reaction involves a direct one-electron transfer from 1 to O_2 to form $O_2^{\bullet-}$ in view of the fact that $E^{\circ\prime}$ for the $O_2/O_2^{\bullet-}$ couple in aqueous solution at pH 7.2 is −0.33 V vs NHE (−0.572 V vs SCE).[26] Dioxygen can also undergo a two-electron reduction to H_2O_2. $E^{\circ\prime}$ for the O_2/H_2O_2 couple in aqueous solution at pH 7.0 is +0.36 V vs NHE (+0.118 V vs SCE).[27] However, because H_2O_2 is such a minor byproduct of the autoxidation reaction of 1, it is unlikely that this reaction is responsible for oxidation of the indolamine. By comparison with the pK_a of resorcinol, a structurally similar *m*-dihydroxybenzene derivative, the pK_a of 1 must be close to 9.4.[28,29] Thus, at pH 7.4 ~1% of 1 must exist in so-

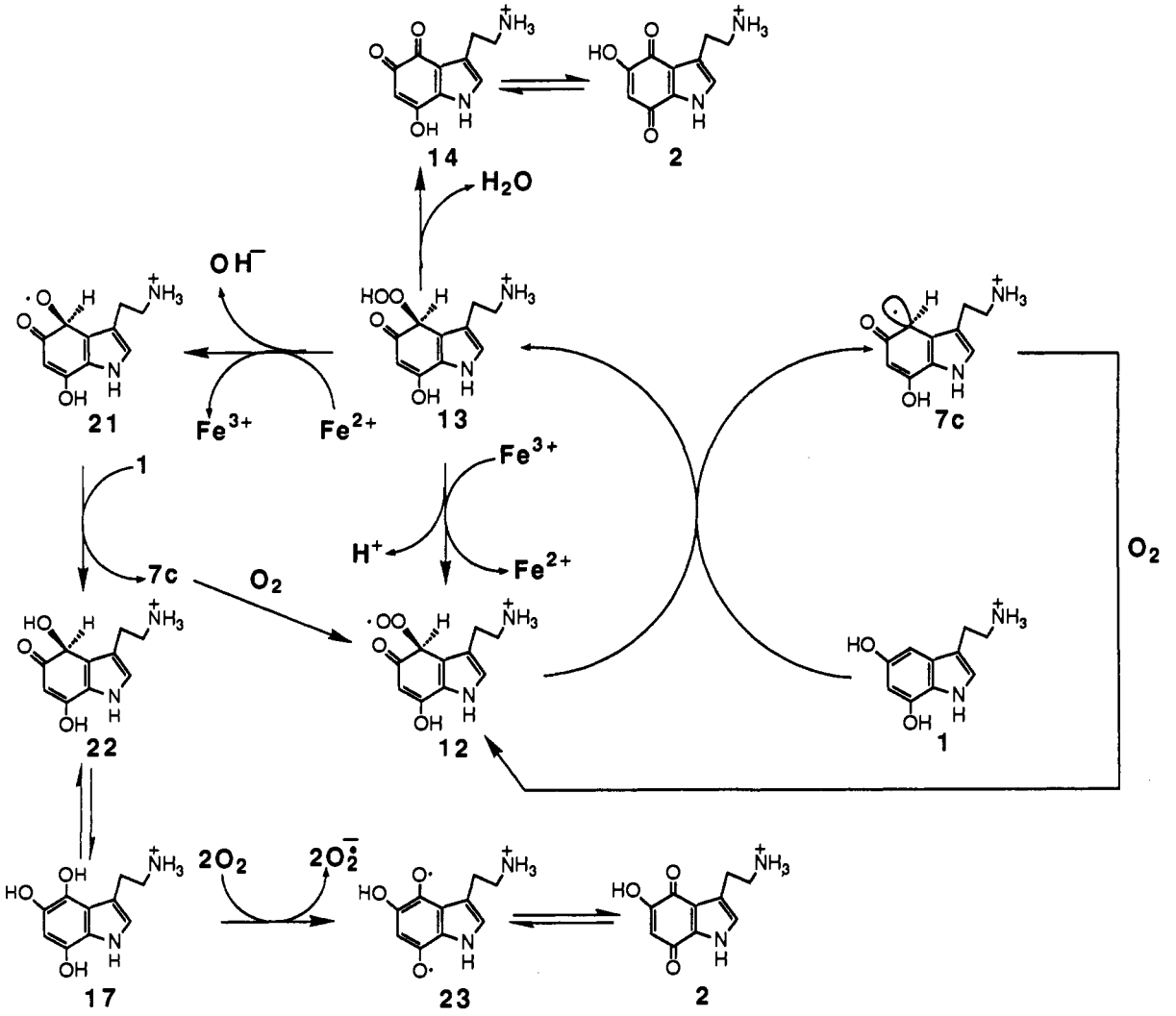
(25) Schlossberger, H. S. Synthesis and Chemical Properties of Some Indole Derivatives. *Ann. N.Y. Acad. Sci.* 1978, *305*, 25–35.

(26) Ilan, Y. A.; Meisel, D.; Czapski, G. Redox Potential of the Molecular Oxygen–Oxygen Radical $O_2^{\bullet-}$ System in Aqueous Media. *Isr. J. Chem.* 1974, *12*, 891–895.

(27) Sawyer, D. T. The Redox Thermodynamics for Dioxygen Species (O_2, $O_2^{\bullet-}$, HOO·, HOOH, and HOO^-) and Monooxygen Species (O, $O_2^{\bullet-}$, ·OH, and ^-OH) in Water and Aprotic Solvents. In *Oxygen Radicals in Biology and Medicine*; Simic, M. G., Taylor, K. A., Ward, J. F., Eds.; Plenum: New York, 1989; pp 11–20.

(28) Fieser, L. F.; Fieser, M. *Organic Chemistry*, 3rd ed.; Reinhold Publishing Corp.: New York, 1956; p 624.

Scheme V



lution as an indolic monoanion. Because of phenol–keto tautomerism of 1[25] the phenoxy anion **18a** exists in equilibrium with the C(4)-centered carbanion **18b** (Scheme IV). In accord with previous suggestions[8,10] **18b** is the primary electron donor to molecular oxygen to form the free radical superoxide complex.[19] Recombination of the superoxide residue of **19** with the incipient C(4)-radical yields the hydroperoxy anion **20**,[30,31] which upon protonation generates hydroperoxide **13** and thence *p*-quinone **2** (Scheme IV).[10] The pathway leading to **2** outlined in Scheme IV represents the course of the autoxidation reaction in the absence of catalytic influences deriving from $O_2^{\cdot-}$ and trace transition metal ions. Under such conditions the rate of autoxidation of **1** should be strongly dependent upon pH, i.e., on the extent of formation of C(4)-centered carbanion **18b**. Experimentally the initial rates of oxygen consumption measured for the autoxidation of **1** (1.0 mM; phosphate buffer, μ = 0.1; 37 °C) in the presence of SOD (333 units/mL), catalase (100 μg/mL), and DTPA (1.0 mM) at pH 5.3, 7.4, and 8.5 were 0.81 ± 0.07, 8.3 ± 0.9, and 16.1 ± 1.5 nmol of O_2/min, respectively. The products of these reactions were **2** and **3** in equimolar yield.

The inhibitory effects of DTPA on the rate of autoxidation of **1** (Table I) and the catalytic effects of transition metal ions clearly indicate a role for the latter species in the overall reaction. However, Cu^{2+} differs from the other transition metal ions studied because it can directly oxidize **1** to radical **7**, which, under anaerobic conditions, leads to formation of **4**–**6** (Scheme I) or, under aerobic conditions, to a mixture of **2**–**6**, partially by the routes conceptualized in Scheme II. The other transition metal ions studied, Fe^{2+}, Fe^{3+}, Mn^{2+}, are incapable of directly oxidizing **1** and, hence, must express their catalytic effects by a more indirect route. There is abundant evidence that such metals react with hydroperoxy compounds analogous to **13** which are formed in many autoxidation reactions.[24,32,33] The actual reaction pathway catalyzed by a transition metal ion depends on its oxidation state. For example, Fe^{3+} reacts with putative hydroperoxy intermediate **13** to yield peroxy radical **12** with concomitant formation of Fe^{2+} (Scheme V). Oxidation of **1** by peroxy

(29) In a previous paper[10] it was reported that the pK_a of 5,7-DHT is 4.8. However, the potentiometric method used to compute this value employed the creatinine salt of 5,7-DHT. The measured pK_a, in fact, was that of creatinine.

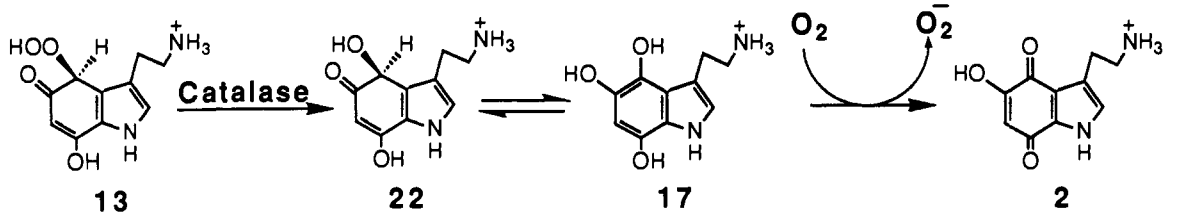
(30) Haynes, R. K.; Musso, H. Detection of Free Radicals in the Autoxidation of Orcinol. *Chem. Ber.* **1974**, *107*, 3723–3732.

(31) Haynes, R. K.; Musso, H. Oxidation of Orcinol with Potassium Hexacyano Ferrate(III) in a Flow System. *Chem. Ber.* **1974**, *107*, 3733–3748.

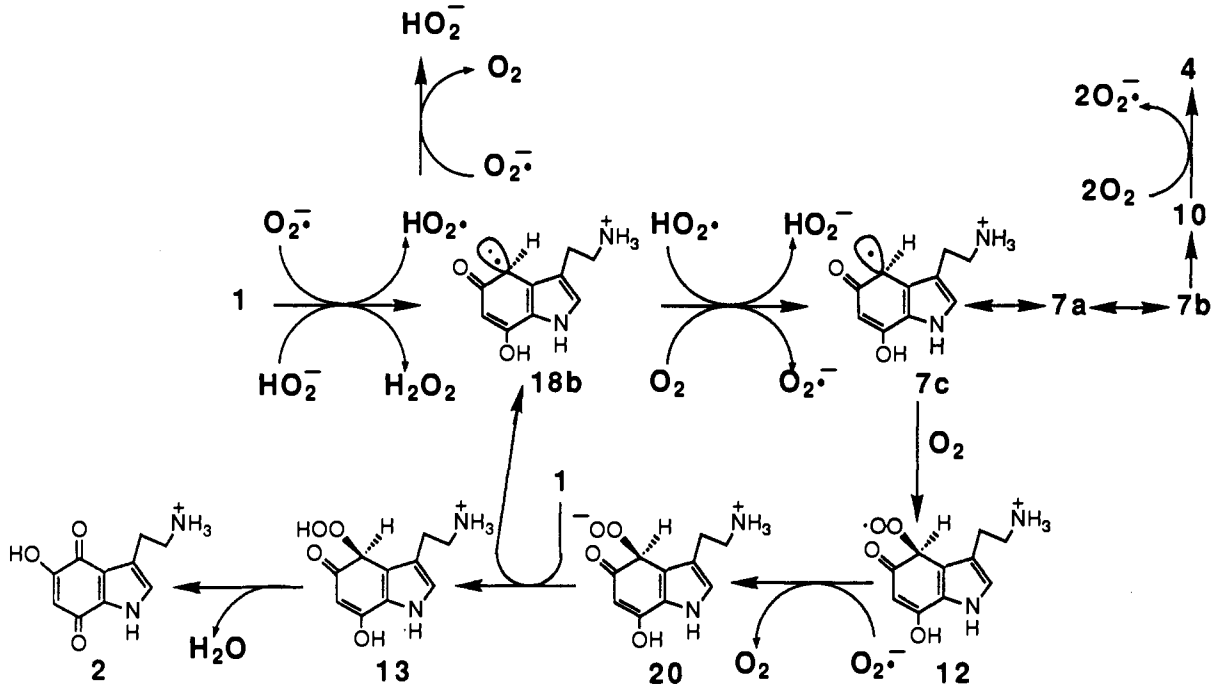
(32) Nonhebel, D. C.; Tedder, J. M.; Walton, J. C. Autoxidation. In *Radicals*; Nonhebel, D. C., Tedder, J. M., Walton, J. C., Eds.; Cambridge University Press: New York, 1979; Chapter 14.

(33) Sheldon, R. A.; Kochi, J. K. Introduction to Metal-Catalyzed Oxidations. In *Metal Catalyzed Oxidation of Organic Compounds*; Sheldon, R. A., Kochi, J. K., Eds.; Academic Press: New York, 1981; pp 1–68.

Scheme VI



Scheme VII



radical 12 then yields radical 7 and regenerates hydroperoxide 13. Direct attack by molecular oxygen on the C(4)-centered radical 7c then generates peroxy radical 12 and, hence, a catalytic cycle is established. The catalytic effect of Fe^{2+} probably proceeds by reduction of hydroperoxide 13 to oxy radical 21 with concomitant formation of Fe^{3+} (Scheme V). Again, by analogy with similar autoxidation reactions,[24,32,33] oxy radical 21 oxidizes 1 to, predominantly, radical 7c with formation of 4,5,7-trihydroxytryptamine (17) (Scheme V). Attack of molecular oxygen on 7c then generates the key peroxy radical 12 so that a second catalytic cycle is established. The trihydroxyindolamine 17 is very easily oxidized by molecular oxygen to form 2 (Scheme V). Experimentally, Fe^{2+} evokes a much stronger catalytic effect on the autoxidation of 1 than Fe^{3+} (Table I), indicating that the reaction between hydroperoxide 13 and Fe^{2+} is more facile than that with Fe^{3+}. However, the initial catalytic influence of Fe^{2+} rapidly declines to that caused by Fe^{3+}. This effect is expected on the basis of the reaction pathways proposed in Scheme V; i.e., once Fe^{2+} is consumed in converting hydroperoxide 13 to oxy radical 21 further catalytic effects of Fe^{2+} are dependent upon the rate of the Fe^{3+}-catalyzed reaction which generates Fe^{2+}.

Catalase causes a profound inhibitory effect on the rate of autoxidation of 1 which is not related to its destruction of H_2O_2. However, catalase is known to catalyze the decomposition of organic peroxides.[34] Accordingly, it is proposed that catalase converts the key hydroperoxy intermediate 13 into 4,5,7-trihydroxytryptamine (17), which is rapidly autoxidized to 2 (Scheme VI). Decomposition of 13 by catalase inhibits/terminates the catalytic cycles driven by trace transition metal ions (Scheme V) and, hence, decreases the rate of autoxidation of 1.

The rate of autoxidation of 1 is inhibited by SOD. Furthermore, addition of $O_2^{\bullet-}$ to 1 dissolved in Me_2SO or in aqueous solution even under anaerobic conditions results in the rapid oxidation of the indolamine predominantly to 2 and 4. These observations support a key role for $O_2^{\bullet-}$ in the overall autoxidation of 1. ESR experiments using DMPO as a spin trapping agent[35] reveal that during the autoxidation of 1 the DMPO–hydroxyl radical spin-adduct is formed. Similarly, the same adduct is formed during the autoxidation of 4,5,7-trihydroxytryptamine (17) at physiological pH (see Experimental Section). However, $HO^{\bullet}$ scavengers such as mannitol have no effect on either the rate or the products of the autoxidation of 1. Furthermore, while an $HO^{\bullet}$ generating system rapidly oxidized 1, none of the resulting products corresponded to those formed in the autoxidation reaction. In addition, there is no known example of direct formation of $HO^{\bullet}$ in any autoxidation reaction; i.e., $HO^{\bullet}$ forms only as a result of the Fenton reaction (from H_2O_2) or the Haber-Weiss reaction (from $O_2^{\bullet-}$).[17] Thus, it may be concluded that the DMPO–hydroxyl radical adduct trapped during the autoxidation of 1 and 17 in fact results from the rapid de-

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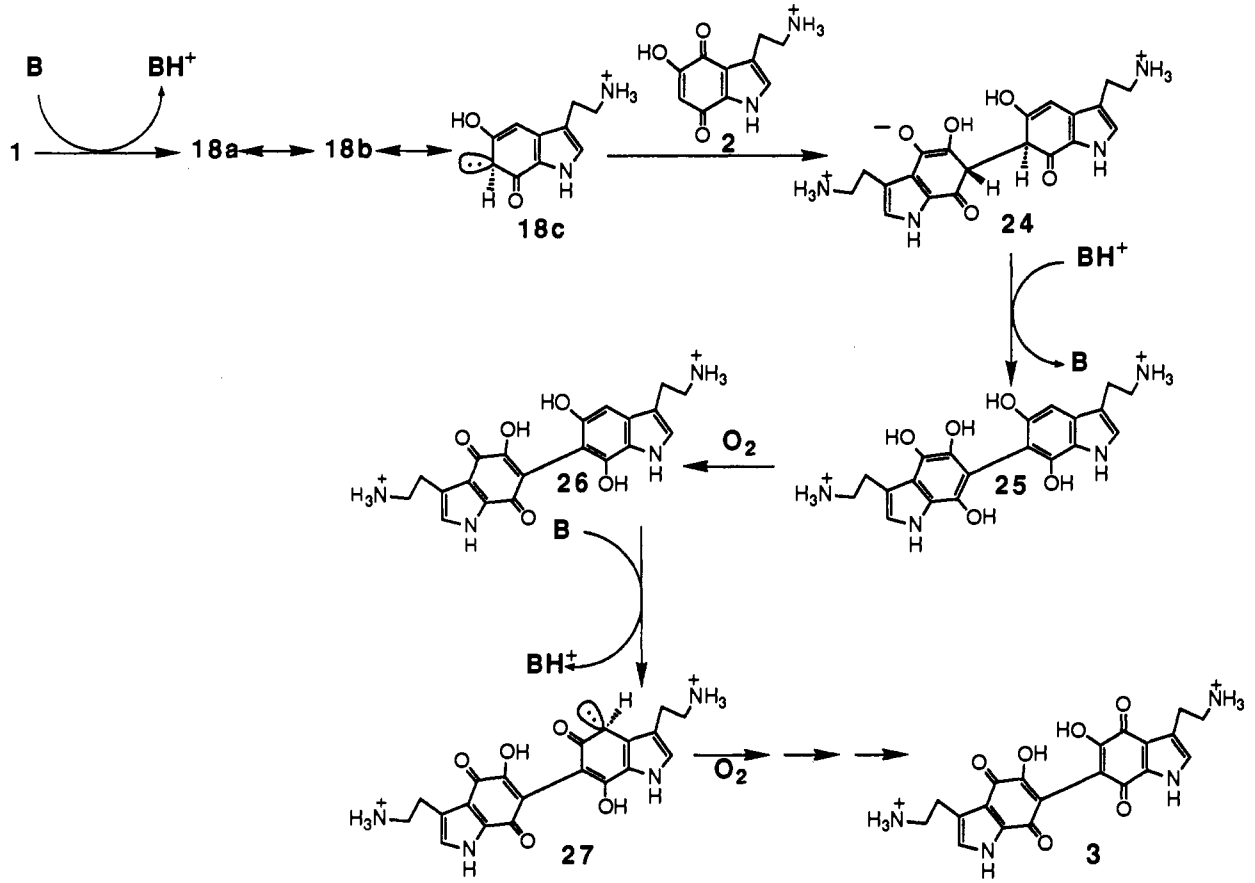
Scheme VIII



composition of the DMPO–$O_2^{\bullet-}$ adduct.[36,37] The latter adduct has a half-life of only 35 s in aqueous solution at pH 8.[38]

It is extremely unlikely that $O_2^{\bullet-}$ directly oxidizes 1 because it is a very poor oxidizing agent.[39] However, $O_2^{\bullet-}$ is a very powerful Brønsted base with a basicity equivalent to that of the conjugate base of an acid having a pK_a of ca. 23.[40,41] Accordingly, it is possible to conclude that $O_2^{\bullet-}$ deprotonates 1 to form carbanion **18b** in a reaction that forms the hydroperoxy radical ($HO_2^{\bullet}$) and, thence, HO_2^- and O_2 as byproducts (Scheme VII). Carbanion **18b** is then attacked by molecular oxygen to yield **2** (Scheme IV). This route probably represents the major pathway mediated by $O_2^{\bullet-}$ in the simple autoxidation of 1. However, in Me_2SO or aqueous solution it has been demonstrated that $O_2^{\bullet-}$ also drives a reaction leading to dimer **4**, normally a very minor product of the autoxidation of 1. This observation indicates that $O_2^{\bullet-}$ also mediates a reaction pathway which generates radical **7b**, the precursor of **4** (Scheme I). Thus, in the presence of $O_2^{\bullet-}$ carbanion **18b** is attacked directly by molecular oxygen or by $HO_2^{\bullet}$[42] to generate radical **7** (Scheme VII). Because of the enormous basicity of $O_2^{\bullet-}$, formation of carbanion **18b** must be extensive and, furthermore, $E^{\circ\prime}$ for the **18b** ⇌ **7** reaction is shifted to such negative values that O_2 is capable of acting as an effective one-electron oxidant. Dimerization of **7b** yields **10**, which is further oxidized to **4**. Direct attack by O_2 on **7c** yields the organic peroxy radical **12** and, hence, **2** as outlined in Scheme VII.

In a previous report[10] it was suggested that the precursor of **3** was the simple 6→6′ linked dimer **10**. However, work reported here indicates that **10** is quantitatively autoxidized to dimer **4**. The same reaction is driven by ceruloplasmin, Cu^{2+}, Fe^{3+} cyt *c*, and peroxidase/H_2O_2. Accordingly, **3** must be produced by a different reaction pathway. At physiological pH under aerobic conditions monomer **2** is only very slowly converted to dimer **3** (at 37 °C a 50% conversion of **2** to **3** requires about 20 days[10]). Thus, on the time scale of the autoxidation reaction or the various catalytically assisted oxidations of 1 the reaction responsible for this transformation could not possibly lead to formation of significant yields of **3**, the major ultimate autoxidation product. Experiments have revealed that $O_2^{\bullet-}$ causes a rapid conversion of **2** (0.5 mM, pH 7.4 phosphate buffer) to **3**. However, SOD has no effect on the yield of **3** in the autoxidation of 1, indicating that $O_2^{\bullet-}$ plays no

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significant role in the reaction leading to 3 in the autoxidation reaction.

A plausible route leading ultimately to 3 involves an initial nucleophilic attack (Michael addition) by the C(6)-centered carbanion 18c, on the C(5) = C(6) double bond of 2 to form dimer 25 which contains residues of 4,5,7-trihydroxytryptamine and 1 (Scheme VIII). NMR studies of 1 in D_2O indicate that both the C(4)- and C(6)-protons are exchangeable and that the latter proton exchanges twice as rapidly as the former.[8] This indicates that ketonization of both the 5-OH and 7-OH groups occurs with proton transfer to the C(6)-position. Hence, upon deprotonation, the C(6)-carbanion 18c is expected to be formed. Further oxidation of the two indolic residues of 25 then leads to 3 (Scheme VIII). Formation of dimer 25 does not require molecular oxygen and, indeed, incubation of 2 (0.4–0.7 mM) with 1 (1.0 mM, pH 7.4 phosphate buffer, 37 °C) under anaerobic conditions followed by HPLC analysis (HPLC method I) resulted in detection of a compound having a retention time (t_R) of 11.8 min. The t_R values for 2 and 1 were 16.5 and 6.1 min, respectively. In the HPLC mobile phase (pH 3.8) the compound with t_R = 11.8 min exhibited a spectrum with two closely-spaced absorption bands at λ_{max} = 278 and 288 nm, typical of dimers of 1.[43] Such a spectrum would be expected for putative dimer 25. That 25 is indeed the dimer formed is supported by the observation that upon exposure to air it was rapidly oxidized to 3. At pH 7.4 this reaction was virtually instantaneous. The very facile air oxidation of 25 precluded its isolation and spectroscopic structure elucidation. However, additional evidence in support of the conclusion that dimer 3 is formed in a reaction which involves an initial reaction between monomer 2 and 1 was provided by incubating 1 and monomer 2 under aerobic conditions. Under such reaction conditions the yield of dimer 3 increased significantly relative to that observed during the autoxidation of 1 under otherwise identical conditions. Putative dimer 26, which consists of residues of 2 and 1, has not been isolated. However, it appears reasonable to expect that autoxidation of the 1 residue of 26 follows the same general pathways proposed in Schemes IV, V, and VIII for the free base.

Autoxidations of 1 (1.0 mM, pH 7.4, 37 °C) in the presence of SOD (333 units/mL), catalase (100 μg/mL), and DTPA (1.0 mM), i.e., conditions designed to eliminate catalytic effects deriving from $O_2^{\bullet-}$, hydroperoxide 13, and trace transition metal ions and, therefore, roles for radical intermediates such as 7, 12, and 21, were quite slow and required 17.5 ± 0.5 h to reach completion. However, as predicted from Schemes IV and VIII, only 2 and 3 were formed as products, and no trace of dimer 4 was observed. Similarly, autoxidation of 1 in the presence of monomer 2 and SOD, catalase, and DTPA resulted only in an increased yield of dimer 3 as would be predicted from the reaction pathway proposed in Scheme VIII.

The tyrosinase/O_2 oxidation of 1 yields 2–4 as the major products. While no efforts have been made to investigate the mechanistic details of the reactions mediated by tyrosinase, formation of significant yields of 4 implicate radical 7 as a key intermediate. The major products of the mitochondria/O_2 oxidation of 1 were 2 and 3.

Conclusions

The results presented above indicate that in the presence of SOD, catalase, and DTPA, i.e., conditions designed to eliminate catalytic effects deriving from $O_2^{\bullet-}$, trace transition metal ions, and organic peroxy intermediates, the autoxidation of 1 is a rather slow reaction. The products of this uncatalyzed autoxidation are monomer 2 and dimer 3 in approximately equimolar yields. The key step in the reaction is attack of molecular oxygen on the C(4)-centered carbanion 18b to form the putative superoxide radical complex 19 that reacts further to give monomer 2 as outlined in Scheme IV. The C(6)-centered carbanion 18c combines with monomer 2 in a Michael addition reaction as the initial step leading to dimer 3 (Scheme VIII).

In the absence of protective effects of SOD, catalase, and DTPA, the autoxidation of 1 becomes appreciably more rapid. Trace concentrations of transition metal ions such as Fe^{3+}, Fe^{2+}, Cu^{2+}, and Mn^{2+} catalyze the autoxidation reaction by routes outlined in Scheme V in which the hydroperoxide 13 is a key intermediate in at least two catalytic cycles. The C(4)-centered radical 7c, peroxy radical 12, and oxy radical 21 play important roles in these catalytic cycles. Autoxidation of 1 in the presence of DMPO results in formation of a carbon-centered spin adduct which can be detected by ESR spectroscopy. The measured hfsc due to nitroxide (α_N, 17.3 G) and that of the β-hydrogen (α_β^H, 29.2 G) are both large[35] and suggest that the carbon-centered radical trapped as a DMPO spin adduct is a rather bulky species[42] and, therefore, is probably 7c. Superoxide radical anion is formed as a byproduct of autoxidation of 17 and, perhaps, as a result of autoxidation of other trihydroxyindolamines such as 25. The catalytic effects evoked by $O_2^{\bullet-}$ derive primarily from the basicity of this species which facilitates formation of carbanion 18b, which can be further oxidized to C(4)-centered radical 7c as shown in Scheme VII. That radical 7 is formed in both the $O_2^{\bullet-}$- and trace transition metal ion-catalyzed reactions of 1 is further supported by formation of dimer 4. Hydrogen peroxide is formed as only a very minor byproduct of the autoxidation of 1. Several reactions leading to H_2O_2 formation are proposed in Scheme VII. Dismutation of $O_2^{\bullet-}$ would also be expected to lead to H_2O_2. However, under the conditions studied neither H_2O_2 nor HO•, derived from Fenton chemistry,[17] appear to play any significant role in the autoxidation of 1.

Under anaerobic conditions, peroxidase/H_2O_2, ceruloplasmin, Fe^{3+} cyt c, and Cu^{2+} all oxidize 1 in a simple one-electron abstraction reaction to give the radical 7. The C(6)-centered radical 7b is the precursor of oxidized dimer 4 whereas the C(4)-centered radical 7c is the precursor of tautomers 5/6 (Scheme I). In the presence of molecular oxygen, however, C(4)-centered radical 7c appears to be the predominant radical intermediate, being directly attacked by molecular oxygen, leading ultimately to 2 (Scheme II) and 3 (Scheme VIII). Interestingly, peroxidase/O_2 in the absence of H_2O_2 can also function as an oxidase enzyme in which it catalyzes the two-electron oxidation of 5,7-DHT to quinonoid intermediate 15a/15b, which can react further to yield 2 (Scheme III) and thence 3 (Scheme VIII). Other oxidative enzyme systems such as tyrosinase and rat brain mitochondria also catalyze the oxidation of 1 primarily to 2 and 3 although the precise mechanistic pathways have not been investigated.

The selective destruction of serotonergic neurons by 1 almost certainly derives from the high-affinity uptake of the drug. Essentially all previous investigators have speculated that, once inside a target neuron, 1 undergoes oxidation either by molecular oxygen (autoxidation) or perhaps in an enzyme-mediated reaction, to produce cytotoxic products (i.e., electrophilic intermediates which

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alkylate cellular proteins[3]) or byproducts (H_2O_2, $O_2^{\cdot-}$, $HO^{\cdot}$).[2,4-6] Evidence for intraneuronal oxidation, however, is scientifically weak in the sense that no products or marker species indicative of in vivo oxidations of 1 have been detected. Nevertheless, assuming that intraneuronal oxidation of 1 does indeed occur, the present results provide important guidance to an ultimate understanding of the neurodegenerative actions of the drug. For example, in the absence of any catalytic effects, molecular oxygen can slowly convert 1 into 2 and 3. We have already demonstrated that 2 is toxic when centrally administered to the mouse and has an LD_{50} value (ca. 21 μg)[11] which is significantly lower than that of 5,7-DHT (55 μg).[44] Dimer 3 is also a potent toxin when centrally administered to the mouse ($LD_{50} \approx 25$ μg; work in progress). Accordingly, 2 and/or 3 might be the ultimate toxins derived from 1 as a result of intraneuronal oxidation. Indeed, it has recently been speculated that intraneuronally formed 2 might undergo redox cycling reactions to generate cytotoxic reduced oxygen species.[45] The results reported here indicate that autoxidation of 1 does indeed generate $O_2^{\cdot-}$ and H_2O_2. Under appropriate intraneuronal conditions that latter reduced oxygen species have the potential to generate hydroxyl radicals as a result of well-known Fenton chemistry[17] which could inflict cellular damage.[46]

Central administration of 1 to the rat evokes a rapid behavioral response.[47] This observation might suggest, if oxidation of 1 is indeed the intraneuronal pathway leading to toxic products or byproducts, that the reaction must be much more rapid than simple autoxidation; i.e., the reaction must be catalyzed. In this study, it has been demonstrated that a number of enzyme systems and rat brain mitochondria mediate the aerobic oxidation of 1 predominantly to the toxins 2 and 3. There can be little doubt that many of these oxidations proceed via radical 7 as the proximate oxidation product and generate other radical species such as peroxy radical 12. Previous investigators have noted that radical scavengers protect peripheral nerves from damage by 1.[9] Thus, it is conceivable that short-lived radicals formed in the catalytic oxidations of 1 might also play a functional role in the neurodegenerative actions of the drug.

Experimental Section

5,7-Dihydroxytryptamine (creatinine sulfate salt), tyrosinase (mushroom, EC 1.14.18.1), peroxidase (horseradish, type VI, EC 1.11.1.7), catalase (bovine liver, EC 1.11.1.6), superoxide dismutase (bovine liver, suspension in 3.8 M ammonium sulfate solution pH 7.0, EC 1.15.1.1), ferricytochrome c (bovine heart, type V-A), and ceruloplasmin (human and bovine in pH 7.0 0.025 M sodium chloride/0.05 M sodium acetate) were obtained from Sigma (St. Louis, MO) and were used without further purification. Rat brain mitochondria were isolated by the method of Clark and Nicklas[48] and assayed by the procedure of Chance and Williams.[21] Phosphate buffers of known ionic strength were prepared according to Christian and Purdy.[49]

High-performance liquid chromatography (HPLC) employed two systems. For analytical studies a Gilson (Middleton, WI) System 42 gradient instrument equipped with dual Model 302 pumps (5-mL pump heads), an Apple IIe controller, a Rheodyne (Cotati, CA) Model 7125 loop injector, and a Waters (Milford, MA) Model 440 UV detector (254 nm) was employed. For such studies a reversed-phase column (Brownlee Laboratories, San Jose, CA, RP-18, 5-μm particle size, 250 × 7 mm) and a short guard column (Brownlee, RP-18, 5 μm, OD-GU 50 × 5 mm) was employed. For preparative HPLC a Gilson system equipped with two Model 305 pumps (25-mL pump heads) for solvent delivery, and one Model 302 pump (10-mL pump head) for sample injection were used with an IBM PS/2 computer, and Gilson Holochrome UV detector (254 nm) was used. A preparative reversed-phase column (J. T. Baker, Phillipsburg, NJ, Bakerbond Octadecyl C_{18}, 10 μm, 21.2 × 250 mm) was used. Three different HPLC methods were employed. HPLC method I employed the semipreparative HPLC column and a binary gradient solvent system. Solvent A was prepared by dissolving 1.1 mL of triethylamine in 1 L of deionized water and adjusting the pH to 3.30 with glacial acetic acid. Solvent B was HPLC grade acetonitrile (MeCN). The gradient program was as follows: 0–3 min, 100% solvent A; 3–22 min, linear gradient to 12% solvent B. The flow rate was constant at 3.0 mL/min. HPLC method II also employed the semipreparative reversed-phase column and a binary gradient solvent system. Solvent C was prepared by adding 8.0 mL of HPLC grade methanol (MeOH) and 7.0 mL of concentrated ammonium hydroxide to 1 L of water. The pH was then adjusted to 3.8 with concentrated formic acid. Solvent D was MeOH. The following gradient was employed: 0–60 min, linear gradient from 100% solvent C to 50% solvent D; flow rate 2.5 mL/min. HPLC method III employed the preparative reversed-phase column and a binary gradient solvent system. Solvent E was prepared by adding 0.5 mL of concentrated HCl to 1 L of water. Solvent F was HPLC grade MeOH. The following gradient was employed: 0–60 min, linear gradient from 100% solvent E to 50% solvent F and a corresponding linear increase of flow rate from 5 mL/min to 10 mL/min. Typically, 10-mL sample injections were employed with HPLC method III.

Oxygen consumption rates were measured with a Clark-type oxygen electrode assembly (Model 5300, Yellow Springs Instrument Co., Yellow Springs, OH).

^1H- and ^{13}C-NMR spectra were recorded on a Varian (Palo Alto, CA) 300XL spectrometer. Fast atom bombardment-mass spectra (FAB-MS) were obtained with a VG Instruments (Manchester, England) Model ZAB-E spectrometer. Liquid chromatography–mass spectrometry (LC-MS) was performed on a Kratos MS 25/RFA instrument equipped with a thermospray source. The solvent system employed was 0.1 M ammonium acetate adjusted to pH 5.0 with acetic acid. Samples for LC-MS were collected by conventional HPLC and then injected into the thermospray source using a Rheodyne 7125 loop injector. Typically, 1- or 2-mL samples were injected. UV-visible spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer.

ESR experiments employed a Bruker Model ESP 300E spectrometer. A solution of 1 (1.0 mM in pH 7.4 phosphate buffer, $\mu = 0.1$) containing 5,5-dimethyl-1-pyrroline 1-oxide (DMPO; gift from Drs. E. G. Janzen and Y. Kotake, Oklahoma Medical Research Foundation) was bubbled with O_2 gas. After several minutes a sample of the resulting solution was transferred to a flat cell and the ESR spectrum recorded. In order to study the autoxidation of 4,5,7-trihydroxytryptamine (17) by ESR spectroscopy, the following procedure was employed. Compound 17 was prepared by dissolving 2 in pH 7.4 phosphate buffer ($\mu = 0.1$) to give a final concentration of 0.2–0.5 mM. In several instances the phosphate buffer was stirred with Chelex 100 (Bio-Rad Laboratories) for 5–6 h before use to remove traces of transition metal ions. The solution of 2 was reduced by controlled potential electrolysis at pyrolytic graphite electrodes at −0.6 V vs SCE. The reduction was carried out with a vigorous stream of N_2 gas bubbling through the solution in the working electrode com-

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partment. Completion of the electro-reduction was marked by the complete disappearance of the purple color of **2**. An aliquot of the reduced solution was then mixed with DMPO to give a final concentration of 10–40 mM of the spin-trapping agent. These solutions were continuously purged with N_2 gas. A sample of the resulting solution was then transferred to an ESR flat cell using a syringe. Both the syringe and flat cell were thoroughly purged with N_2 before use. After the flat cell was placed in the cavity of the ESR spectrometer, the solution was exposed to the air and rapidly turned purple. Control solutions were prepared in exactly the same fashion except that **1** or **2**, as appropriate, was not present. None of the control solutions gave any ESR spectrum.

Isolation and Characterization of 3-(2-Aminoethyl)-6-[3-(2-aminoethyl)-1,7-dihydro-5-hydroxy-7-oxo-6*H*-indol-6-ylidene]-1*H*-indole-5,7(4*H*,6*H*)-dione (4). The most convenient method to prepare sufficient quantities of **4** was to oxidize **1** (1.5 mM in 60 mL of phosphate buffer, pH 7.4, $\mu = 0.1$) in the presence of peroxidase (0.08 mg/mL) and H_2O_2 (0.5 mM) at ambient temperature for ca. 5 h. Repetitive 10-mL injections of the resulting product solution using HPLC method III were employed to separate **4** (t_R = 15.2 min) from the other products (primarily **2**, **3**, **5**, and **6**). The eluent containing **4** was immediately collected in a flask maintained at −80 °C (dry ice bath). The combined solutions containing **4** were freeze-dried to give an orange solid. In pH 7.4 phosphate buffer the bright yellow solution of **4** exhibited a characteristic spectrum with λ_{max}, nm (log ϵ_{max}): 378 (4.08), 300 (3.98), 232 sh (4.07). FAB-MS (thioglycerol matrix) showed m/e = 381 (MH^+, 100%). Accurate mass measurements on MH^+ gave m/e = 381.1550 ($C_{20}H_{21}N_4O_4$; calcd m/e = 381.1563). ^1H NMR (Me_2SO-d_6): δ 12.60 (d, $J_{1,2}$ = 2.1 Hz, 1 H, N(1)-H), 12.12 (d, $J_{1',2'}$ = 2.1 Hz, 1 H, N(1')-H), 8.10 (br s, ca. 4 H, NH_3^+ and OH), 7.83 (br s, ca. 3 H, NH_3^+), 7.34 (d, $J_{1,2}$ = 2.7 Hz, 1 H, C(2)-H), 7.14 (d, $J_{1',2'}$ = 2.1 Hz, 1 H, C(2')-H), 5.67 (s, 1 H, C(4)-H), 2.95 (m, 4 H, CH_2CH_2), 2.65 (m, 4 H, CH_2CH_2), 2.53 (s, 2 H, C(4')-H). Addition of D_2O caused the resonances at δ 12.6, 12.12, 8.10, and 7.83 to disappear and the doublets at δ 7.34 (C(2)-H) and 7.14 (C(2')-H) to collapse into singlets. ^{13}C NMR using the attached proton test (APT) experiment showed positive resonances (carbons with even or zero attached protons) at δ 183.40 (C(5')=0), 176.9 (C(7')=0), 172.7 (C(7)=0), 139.2 (C-5), 129.7, 129.2, 125.2, 119.3, 117.6, 117.4, 116.9, 114.9, 49.8 (C-4'), and 24.6, and negative resonances (carbons with an odd number of attached protons) at δ 126.9 (C-2), 123.7 (C-2'), and 97.8 (C-4).

Spectroscopic evidence in support of the structures of **2** and **3** has been presented elsewhere.[10,11]

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Registry No. **1**, 31363-74-3; **2**, 100513-78-8; **3**, 124620-49-1; **4**, 141272-05-1; catalase, 9001-05-2; superoxide dismutase, 9054-89-1; cerulieplasmin, 9031-37-2; tyresinase, 9002-10-2; peroxidase, 9003-99-0.

Novel Functional M_1 Selective Muscarinic Agonists. Synthesis and Structure–Activity Relationships of 3-(1,2,5-Thiadiazolyl)-1,2,5,6-tetrahydro-1-methylpyridines

Per Sauerberg,*,† Preben H. Olesen,† Susanne Nielsen,† Svend Treppendahl,† Malcolm J. Sheardown,† Tage Honoré,† Charles H. Mitch,‡ John S. Ward,‡ Andrew J. Pike,‡ Frank P. Bymaster,‡ Barry D. Sawyer,‡ and Harlan E. Shannon‡

Novo Nordisk CNS Division, Novo Nordisk Park, DK-2760 Måløv, Denmark, and The Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, Indiana 46285. *Received December 19, 1991*

A series of novel 3-(3-substituted-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridines (substituted-TZTP; **5a–l**, **7a–h**, **8**, **9c–n**, **11**, **13j**) were synthesized and tested for central muscarinic cholinergic receptor affinity by using [^{3}H]-oxotremorine-M (Oxo-M) and [^{3}H]-pirenzepine (Pz) as ligands. The potency and efficacy of the compounds for the pharmacological defined M_1 and M_2 muscarinic receptors were determined on isolated electrically stimulated rabbit vas deferens and on spontaneously beating isolated guinea pig atria, respectively. Selected compounds were also tested for M_3 activity in the isolated guinea pig ileum. The C_{1-8} alkoxy-TZTP **5a–l** analogues all displaced [^{3}H]-Oxo-M and [^{3}H]-Pz with low nanomolar affinity. Depicting chain length against Oxo-M binding and against Pz binding the unbranched C_{1-8} alkoxy-TZTP (**5a–h**) derivatives produced U-shaped curves with butoxy- (**5d**) and (pentyloxy)-TZTP (**5e**) as the optimum chain length, respectively. This U-shaped curve was also seen in the ability of the compounds **5a–h** to inhibit the twitch height in the vas deferens preparation. The (pentyloxy)- (**5e**) and the (hexyloxy)-TZTP (**5f**) analogues produced an over 90% inhibition of the twitch height with IC_{50} values in the low picomolar range. In both the atria and in the ileum preparations **5f** had low efficacy and potency. With the (alkylthio)-TZTP (**7a–h**) analogues the structure–activity relationship was similar to the one observed with the alkoxy (**5a–h**) analogues, but generally **7a–h** had higher receptor affinity and was more potent than the corresponding **5a–h**. However, the C_{3-8} alkyl-TZTP (**9c,e,g,h**) analogues had 10–100 times lower affinity for the central muscarinic receptors than the corresponding alkoxy and alkylthio derivatives, and their efficacy in the vas deferens preparation was too low to obtain IC_{50} values. The unsubstituted TZTP (**11**) compound was a potent but nonselective muscarinic agonist. The two 3-(3-butoxy/(hexyloxy)-1,2,5-oxadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridines (butoxy/(hexyloxy)-OZTP; **19a/b**) were also synthesized and tested. Both **19a** and **19b** had much lower affinity for the central muscarinic receptors than **5d** and **5f**, and the efficacy of **19a,b** was too low to give IC_{50} values in the vas deferens preparation. Therefore, the C_{5-6} (alkyloxy)/(alkylthio)-TZTP's represent a unique series of potent functional M_1 selective muscarinic agonists.

The deficits in central cholinergic transmission, which occur in patients with Alzheimer's disease,[1] have increased the attention on muscarinic pharmacology. Neurochemical examination of brain material from Alzheimer's patients has demonstrated loss of the presynaptic marker enzyme, choline acetyltransferase and of muscarinic receptors of

† Novo Nordisk.
‡ Eli Lilly and Company.

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