Oxidation of Serotonin by Superoxide Radical: Implications to Neurodegenerative Brain Disorders

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Received October 13, 1997

Many new lines of evidence implicate both superoxide anion radical ($O_2^{\bullet-}$) and biogenic amine neurotransmitters in the pathological mechanisms that underlie neuronal damage caused by methamphetamine (MA), glutamate-mediated oxidative toxicity, ischemia-reperfusion, and other neurodegenerative brain disorders. In this investigation the oxidation of 5-hydroxytryptamine (5-HT, serotonin) by an $O_2^{\bullet-}$-generating system (xanthine/xanthine oxidase) in buffered aqueous solution at pH 7.4 has been studied. The major product of the $O_2^{\bullet-}$-mediated oxidation of 5-HT is tryptamine-4,5-dione (T-4,5-D). However, $O_2^{\bullet-}$ and H_2O_2, cogenerated by the xanthine oxidase-mediated oxidation of xanthine to uric acid, together react with trace levels of iron that contaminate buffer constituents to give a chemically ill-defined oxo-iron species. This species mediates the oxidation of 5-HT to a C(4)-centered carbocation intermediate that reacts with 5-HT to give 5,5′-dihydroxy-4,4′-bitryptamine (4,4′-D) and with uric acid to give 9-[3-(2-aminoethyl)-5-hydroxy-1*H*-indol-4-yl]-2,6,8-triketo-1*H*,3*H*,7*H*-purine (**7**) as the major products. These products differ from those formed in the HO•-mediated oxidation of 5-HT under similar conditions. When the reaction is carried out in the presence of the intraneuronal nucleophile glutathione (GSH), T-4,5-D is scavenged to give 7-(*S*-glutathionyl)-tryptamine-4,5-dione, whereas the putative carbocation intermediate is scavenged to give 4-(*S*-glutathionyl)-5-hydroxytryptamine. T-4,5-D also reacts with the sulfhydryl residues of a model protein, alcohol dehydrogenase, and inhibits its activity. Previous investigators have proposed that T-4,5-D is a serotonergic neurotoxin. This raises the possibility that T-4,5-D and perhaps other putative intraneuronal metabolites formed by the $O_2^{\bullet-}/H_2O_2$/oxo-iron-mediated oxidations of 5-HT might be endotoxins that contribute to neurodegeneration in brain regions innervated by serotonergic neurons caused by MA, ischemia-reperfusion, and other neurodegenerative brain disorders.

Introduction

Oxidative stress has been implicated with the neurodegeneration that occurs in the brain in disorders such as Alzheimer's disease (AD)[1] (*1*), Parkinson's disease (PD) (*2*), and ischemia-reperfusion (*3*) and as a consequence of neurotoxic doses of methamphetamine (MA) (*4*). Oxidative stress refers to a situation where the production of reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical (HO•), and hydrogen peroxide (H_2O_2), exceeds the protective capacities of endogenous cellular defense mechanisms (*5*).

To understand the pathologic processes underlying the neurodegeneration that occurs in these brain disorders, it is important not only to elucidate the mechanisms responsible for oxidative stress but also to characterize the actual reduced oxygen species generated and their primary in vivo targets. It is widely believed, for example, that cellular damage caused by ROS is sustained directly by lipids, proteins, and nucleic acids (*6*). However, a number of lines of evidence indicate that the roles of ROS in neurodegenerative brain disorders might be appreciably more complex. Potentially useful insights into these complexities emerge from studies of MA administration and ischemia-reperfusion using experimental animals. To illustrate, cerebral ischemia evokes a massive release of 5-hydroxytryptamine (5-HT) (*7*), norepinephrine (NE) (*8*), and dopamine (DA) (*9*) in addition to glutamate (Glu) and aspartate (Asp) (*10*). Upon reperfusion (reoxygenation) serotonergic, noradrenergic, and dopaminergic terminals degenerate (*11*) as do cells in the CA1 region of the hippocampus and in several layers of the cortex (*7*). Accumulating evidence suggests that tissue injury occurs during reperfusion and is mediated, directly or indirectly, by ROS (*3, 12*). Because transgenic mice that overexpress cytoplasmic Cu−Zn superoxide dismutase (SOD) are protected against reperfusion injury (*13*), it follows that intraneuronal $O_2^{\bullet-}$ plays a key role in the neurodegenerative mechanism(s). There are several possible sources of this $O_2^{\bullet-}$. For example, during the ischemic period ATP levels fall,

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[1] Abbreviations: Alzheimer's disease, AD; Parkinson's disease, PD; methamphetamine, MA; reactive oxygen species, ROS; superoxide anion radical, $O_2^{\bullet-}$; hydroxyl radical, HO•; 5-hydroxytryptamine, 5-HT; norepinephrine, NE; dopamine, DA; glutamate, Glu; aspartate, Asp; superoxide dismutase, SOD; xanthine oxidase, XOD; *N*-methyl-D-aspartate, NMDA; 5,6-dihydroxytryptamine, 5,6-DHT; 6-hydroxydopamine, 6-OHDA; 5-(hydroxyindolyl)-3-(ethylamino)-2-oxindole, 5-HEO; glutathione, GSH; tryptamine-4,5-dione, T-4,5-D; 7-(*S*-glutathionyl)tryptamine-4,5-dione, 7-*S*-Glu-T-4,5-D; 4-(*S*-glutathionyl)-5-hydroxytryptamine, 4-*S*-5-HT; 5,5′-dihydroxy-4,4′-bitryptamine, 4,4′-D; 9-[3-(2-aminoethyl)-5-hydroxy-1*H*-indol-4-yl]-2,6,8-triketo-1*H*,3*H*,7*H*,-purine, **7**; saturated calomel reference electrode, SCE; alcohol dehydrogenase, ALCD; potassium superoxide, KO_2; dimethyl sulfoxide, Me_2SO; bovine serum albumin, BSA; perhydroxy radical, HO_2•; blood−brain barrier, BBB.

S0893-228x(97)00185-9 CCC: $15.00
Published on Web 05/05/1998

xanthine accumulates (*14*), and a Ca^{2+} influx triggers proteolytic cleavage of xanthine dehydrogenase to xanthine oxidase (XOD) (*15*). Thus, upon reoxygenation XOD catalyzes the oxidation of xanthine to uric acid in a reaction that generates $O_2^{\bullet-}$ (and H_2O_2). Elevated extracellular Glu and Asp levels can stimulate *N*-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity (*16*) in a process that also involves intraneuronal $O_2^{\bullet-}$ production (*17*). MA also evokes a massive release of 5-HT, NE, and DA and, with appropriate dose regimens, degeneration of serotonergic and dopaminergic terminals in certain brain regions (*18*−*20*) along with a subpopulation of unidentified cell bodies in the somatosensory cortex (*21*). The neurodegeneration evoked by MA does not appear to be mediated by the drug itself or its known metabolites (*19, 20*) but, directly or indirectly, by ROS (*4, 22*). Cu−Zn SOD transgenic mice are protected against MA-induced neurodegeneration (*4*) implying again that $O_2^{\bullet-}$ is the ROS primarily involved in the neuropathological mechanisms.

However, the connection between elevated production of $O_2^{\bullet-}$ or other ROS and neuronal damage caused by ischemia-reperfusion and MA does not seem to be related simply to elevated Glu and Asp release and NMDA receptor-mediated excitotoxicity. For example, the protective effects of DA and 5-HT uptake inhibitors against MA-induced neurodegeneration is not easily reconciled with NMDA receptor-mediated excitotoxicity (*23, 24*). Furthermore, surgical procedures or pharmacological manipulations that deplete one or more biogenic amines provide significant protection against the neurodegeneration caused by ischemia-reperfusion (*25*−*27*) and MA (*21, 28*) in areas of the brain innervated by these neurotransmitter systems. Taken together, such observations implicate both the biogenic amine and excitatory amino acid neurotransmitters in addition to $O_2^{\bullet-}$ and perhaps other ROS in mechanisms that mediate the neurodegeneration caused by MA and ischemia-reperfusion. A link between these three factors can be inferred from a recent study using cultures of rat cortical and hippocampal neurons demonstrating that the uptake and intraneuronal oxidation of a biogenic amine neurotransmitter are both essential steps associated with Glu oxidative toxicity (*29*). The conclusion of this study was that intraneuronal oxidation of the biogenic amine neurotransmitter by an unknown enzyme is responsible for generation of cytotoxic levels of ROS.

However, it seems equally plausible to suggest that $O_2^{\bullet-}$, generated by NMDA receptor activation, might oxidize intraneuronal 5-HT, DA, or NE to endotoxic metabolites that mediate Glu excitotoxicity. Similarly, the neurodegeneration evoked in vivo by ischemia-reperfusion, MA, or other neurodegenerative brain disorders in which oxidative stress and biogenic amines are implicated might also involve endotoxins formed by intraneuronal oxidation of the latter neurotransmitters. Indeed, it has been reported that MA induces formation of the serotonergic neurotoxin 5,6-dihydroxytryptamine (5,6-DHT) (*30*) and the catecholaminergic neurotoxin 6-hydroxydopamine (*31*) that are responsible for mediating neurodegeneration. However, efforts to reproduce these results have been unsuccessful (*32*−*34*). Furthermore, 5,6-DHT and 6-OHDA appear to be products of only the $HO^{\bullet}$-mediated oxidation of 5-HT (*35*) and DA (*36*), respectively. The major and most stable product of the $HO^{\bullet}$-mediated oxidation of 5-HT in vitro is 5-(hydroxyindolyl)-3-(ethylamino)-2-oxindole (5-HEO) (*35*). However, neurotoxic doses of MA do not evoke elevated levels of this compound in any region of rat brain (*34*).

Taken together, the preceding information suggests that neither MA nor ischemia-reperfusion mediates abnormally high fluxes of $HO^{\bullet}$ in the brain. Rather, available evidence more strongly implicates $O_2^{\bullet-}$, the biogenic amine neurotransmitters, and, perhaps, their resulting oxidation products (metabolites) in the neurodegenerative mechanisms. Accordingly, as a first step toward exploring this idea, this article describes a study of the oxidation of 5-HT by the xanthine/XOD system employed as a source of $O_2^{\bullet-}$.

Materials and Methods

Chemicals. 5-Hydroxytryptamine hydrochloride (5-HT·HCl), xanthine, xanthine oxidase (EC 1.1.3.22), uric acid, potassium superoxide (KO_2), superoxide dismutase (SOD), catalase, ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), diethylamine, diethylenetriaminepentaacetic acid disodium salt (Na_2DTPA), nicotine adenine dinucleotide (NAD^+), semicarbazide, bovine serum albumin, alcohol dehydrogenase (EC 1.1.1.1.), mannitol, desferrioxamine mesylate (desferal), sodium octyl sulfate, and glutathione (GSH, free base) were obtained from Sigma (St. Louis, MO) and were used without further purification. Citric acid monohydrate was obtained from Mallinckrodt (St. Louis, MO). Tryptamine-4,5-dione (T-4,5-D) (*37*), 7-(*S*-glutathionyl)tryptamine-4,5-dione (7-*S*-Glu-T-4,5-D) (*38*), 4-(*S*-glutathionyl)-5-hydroxytryptamine (*39*), and 5,5′-dihydroxy-4,4′-bitryptamine (4,4′-D) (*40*) were synthesized using published procedures.

High-Performance Liquid Chromatography. Two HPLC systems equipped with glassy carbon electrochemical detectors were employed, one with the detector set in the oxidative mode (HPLC-EC_{ox}) and the other with the detector set in the reductive mode (HPLC-EC_{Red}). A third preparative HPLC system employed a UV detector (HPLC-UV).

HPLC-EC_{ox}: This system employed a Gilson (Middleton, WI) model 307 pump, a Rheodyne (Cotati, CA) 7125 injector equipped with a 5.0-μL sample loop, and a BAS (Bioanalytical Systems, West Lafeyette, IN) LC-4C amperometric detector equipped with a glassy carbon detector electrode set at +825 mV versus a silver/silver chloride (Ag/AgCl) reference electrode. The detector filter was set at 0.1 Hz. A reversed-phase column (BAS Phase II − ODS, 3 μm, 100 × 3.2 mm) and guard column (BAS Phase II − ODS, 7 μm, 15 × 3.2 mm) were employed. The mobile phase consisted of 0.085% (v/v) diethylamine, 0.63 mM Na_2EDTA, 0.258 mM sodium octyl sulfate, 0.1 M citric acid dissolved in deionized water containing 5% (v/v) HPLC grade acetonitrile (MeCN). The pH of this solution was adjusted to 2.14 with concentrated hydrochloric acid. After preparation, the mobile phase was filtered through a 0.22-μm type GVHP membrane (Millipore, Bedford, MA) and degassed by stirring under vacuum for 20 min. The flow rate was 0.6 mL min^{-1}.

HPLC-EC_{ox} was employed to detect uric acid, 4,4′-D, 4-*S*-Glu-5-HT, 5-HT, and compound **7** in reaction product solutions.

HPLC-EC_{Red}: A BAS 200B instrument was used equipped with a Rheodyne 9125 injection valve with a 5.0-μL sample loop and dual parallel thin-layer glassy carbon detector electrodes. One electrode was set at −50 mV and the other at −550 mV vs Ag/AgCl. The detector filter was set at 0.05 Hz. To remove molecular oxygen the entire system was purged with ultrahigh purity He gas for 2 h. The exhaust valve was then closed, and the system was pressurized at 4 psi with He. The mobile phase, detector oven, detector cell, and column were maintained at 35 ± 0.1 °C. The analytical and guard columns were the same as used for the HPLC-EC_{ox} system. The mobile phase was prepared using 7.5% (v/v) MeCN in deionized water but was otherwise identical to that used for the HPLC-EC_{ox} system. The flow rate was 1.0 mL min^{-1}. The background current at the

detector electrode set at −50 mV vs Ag/AgCl was typically between 1.3 and 6.0 nA, and that at the −550 mV detector electrode was between 24 and 35 nA. The detector electrodes were resurfaced when the background current (mobile phase alone flowing) exceeded the latter limits. The glassy carbon detector electrodes were resurfaced (polished) using the abrasion method recommended by the manufacturer (BAS). The electrodes were then electropolished at −2.0 V vs Ag/AgCl for 2 h with the mobile phase flowing through the cell. Typically, detector electrode resurfacing was necessary every 2−3 weeks. The BAS 200B was controlled by BAS ChromGraph software, and data were analyzed using ChromGraph Report software. HPLC-EC_{Red} was employed to detect T-4,5-D and 7-*S*-Glu-T-4,5-D because these compounds gave no response using HPLC-EC_{ox}. Quantitative determinations of T-4,5-D using HPLC-EC_{Red} were based on linear analytical calibration curves. The procedures employed to prepare solutions of T-4,5-D of known concentration by exhaustive controlled potential electro-oxidation of 5-HT in 0.01 M HCl at 740 mV vs SCE (saturated calomel electrode) have been described in detail elsewhere (*37*); λ_{max} (nm) (log ϵ_{max}, M^{-1} cm^{-1}) of T-4,5-D in 0.01 M HCl was 540 (3.21).

Preparative HPLC-UV: The equipment and chromatographic conditions employed for preparative HPLC-UV were the same as described previously (*40*). This system was employed for identification of reaction products and for isolation of **7**.

Spectroscopy. NMR spectra were recorded on a Varian (Palo Alto, CA) Inova-400 spectrometer. Electrospray mass spectra were obtained with a PE Sciex model API III instrument with the orifice voltage set at 45 V. UV−visible spectra were recorded on a Hewlett-Packard (Palo Alto, CA) 8452A diode array spectrophotometer.

Oxidation of 5-HT by $O_2^{\bullet-}$ (Xanthine/Xanthine Oxidase System). The xanthine oxidase (XOD)-mediated oxidation of xanthine to uric acid in buffered aqueous solution at pH 7.4 was used to generate $O_2^{\bullet-}$ (*47, 48*). A typical reaction solution consisted of 400 μL of 1.0 mM xanthine in pH 7.4 phosphate buffer ($\mu = 0.2$), 200 μL of 1.0 mM Na_2EDTA (in water), 600 μL of pH 7.4 phosphate buffer ($\mu = 0.2$), 740 μL of deionized water, and 40 μL of 10 mM 5-HT (in water). After this solution was thoroughly mixed the oxidation reaction was initiated by addition of 20 μL of XOD suspension [in 2.3 M $(NH_4)_2SO_4$, 10 mM pH 7.8 phosphate buffer containing 1 mM Na_2EDTA, and 1 mM sodium salicylate as supplied by the manufacturer]. Thus, the final reaction solution (2000 μL) initially contained 200 μM xanthine, 100 μM Na_2EDTA, 200 μM 5-HT, and 79 mg mL^{-1} XOD (0.087 unit mL^{-1}). When xanthine or XOD was omitted from this reaction solution 5-HT was not oxidized. Under the preceding reaction conditions but substituting the 5-HT solution with water (40 μL), it was found that the concentration of uric acid increased with time up to 8−10 min when it reached a constant level of 186.0 ± 10.9 μM (mean ± standard deviation; $n = 3$). Thus, under the experimental conditions employed xanthine was almost quantitatively oxidized to uric acid in ≤10 min. Because the generation of $O_2^{\bullet-}$ parallels uric acid formation in this reaction, a maximum incubation time of 10 min was normally employed for oxidation of 5-HT by the xanthine/XOD system. The progress of this reaction was monitored by periodic withdrawal of samples for analysis by HPLC-EC_{ox} (to measure 5-HT consumed and formation of 4,4′-D) and by HPLC-EC_{Red} (to measure T-4,5-D formed). Aliquots of the reaction solution (5 μL) were injected directly into the HPLC-EC_{Red} system. However, prior to HPLC-EC_{ox} analysis an aliquot (40 μL) of the reaction solution was transferred into 360 μL of ice-cold 0.1 M HCl. The resulting solution was vortexed and immediately injected into the HPLC-EC_{ox} system.

The preparative HPLC-UV system was employed on occasion to monitor the decrease of 5-HT concentration and the appearance of various products. However, the total reaction solution volume was then increased to 10-mL keeping the concentrations of all reactants the same as described previously. The entire reaction solution was injected via a 10 mL sample loop into the HPLC-UV system.

9-[3-(2-Aminoethyl)-5-hydroxy-1*H*-indol-4-yl]-2,6,8-triketo-1*H*,3*H*,7*H*-purine (7). Compound **7** was initially separated from other reaction products using preparative HPLC-UV. The solution that eluted under the peak corresponding to **7** was desalted using the same HPLC-UV system but with deionized water as the only mobile phase. The eluent containing **7** was collected, shell-frozen, and freeze-dried to give a white solid that had very low solubility in water or common organic solvents. Compound **7** gave a UV spectrum (pH 3.0) with $\lambda_{max} = 294$ nm. Attempts to obtain a fast atom bombardment mass spectrum or matrix-assisted laser desorption ionization mass spectrum on **7** using a variety of matrixes were unsuccessful. However, a solution of **7** freshly collected in the HPLC-UV mobile phase (5% MeCN in ammonium formate buffer, pH 4.7) and diluted in 1% acetic acid in 1:1 methanol water gave an electrospray mass spectrum with $m/z = 343$ (MH^+, 100%). ^1H NMR (400 MHz, D_2O) gave δ 7.30 (d, $J = 8.8$ Hz, 1H, C(7)-H), 7.08 (s, 1H, C(2)-H), 6.77 (d, $J = 8.8$ Hz, 1H, C(6)-H), 2.80 (m, 2H, C(β)-H_2), 2.60 (m, 2H, C(α)-H_2). Compound **7** was also synthesized by an independent electrochemical method. In a typical experiment, 5-HT·HCl (2 mg) and uric acid (2.4 mg) were dissolved in 40 mL of pH 7.4 phosphate buffer ($\mu = 0.2$) and electro-oxidized at a working electrode consisting of several plates of pyrolytic graphite using an applied potential of 195 mV vs SCE [equipment and procedures are described elsewhere (*37*)]. This applied potential was sufficient to electro-oxidize 5-HT but not uric acid. Under these conditions **7** was formed in high yield and was separated and purified as described earlier. The spectral and chromatographic properties of electrochemically synthesized **7** were identical to those of the compound formed in the xanthine/XOD-mediated oxidation of 5-HT.

Alcohol Dehydrogenase (ALCD) Activity Measurement. Typically, 250 μL of ALCD (250 μg mL^{-1} in pH 7.4 phosphate buffer) was initially incubated for a predetermined period of time (0−22 h) with 250 μL of pH 7.4 phosphate buffer ($\mu = 0.2$; control) or 250 μL of T-4,5-D (20−110 μM in pH 7.4 phosphate buffer) at 25 °C. Then, to a 250-μL aliquot of this solution was added 390 μL of phosphate buffer, 350 μL of 2 mM Na_2EDTA, 500 μL of 10 mM NAD^+, and 500 μL of 280 mM semicarbazide, all reactants being dissolved in pH 7.4 phosphate buffer. The reaction was initiated by addition of 10 μL of ethanol (96%). Activity of ALCD was based on the absorbance measured at 340 nm (NADH) after 10 min.

Results

Products of the $O_2^{\bullet-}$ (Xanthine/XOD)-Mediated Oxidation of 5-HT. Preliminary experiments established that under the reaction conditions employed a 200 μM solution of xanthine was almost quantitatively (≥93%) oxidized to uric acid in ≤10 min in the presence of XOD (0.087 unit mL^{-1}). Chromatograms of the product solution formed following incubation of 5-HT (200 μM) with xanthine (200 μM) and XOD (0.087 unit mL^{-1}) for 10 min are presented in Figure 1. HPLC-EC_{ox} (Figure 1A) showed formation of two major electro-oxidizable products, 5,5′-dihydroxy-4,4′-bitryptamine (4,4′-D) and **7**. Additional peaks in this chromatogram corresponded to unreacted 5-HT and uric acid. HPLC-EC_{Red} (detector set at −50 mV vs Ag/AgCl) showed formation of T-4,5-D as the only electrochemically reducible product (Figure 1B). Product identities were confirmed by injecting reaction solutions into the preparative HPLC-UV system and collecting solutions that eluted under the peaks corresponding to each product. T-4,5-D and 4,4′-D were identified on the basis of their UV−visible spectra and electrochemical and chromatographic properties (*37, 40*).

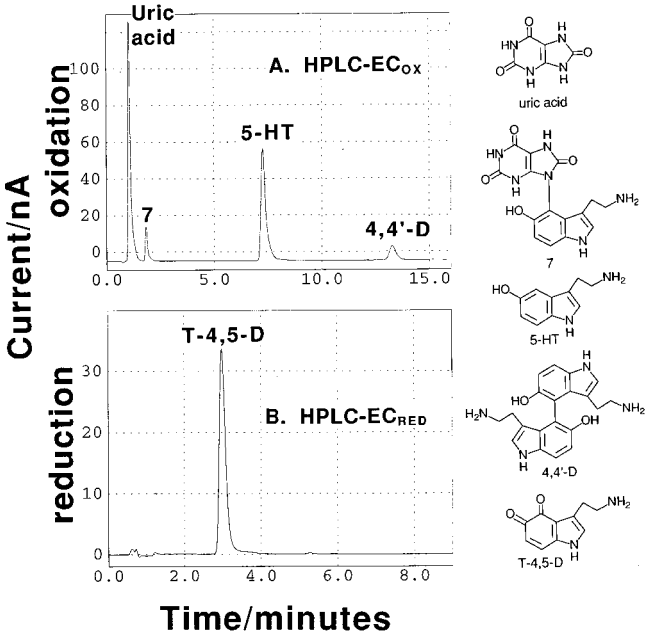


Figure 1. (A) HPLC-EC$_{ox}$ (detector potential 825 mV vs Ag/AgCl) and (B) HPLC-EC$_{Red}$ (detector potential −50 mV) chromatograms of the product solution obtained following incubation of 5-HT (200 μM) with xanthine (200 μM) and XOD (0.087 unit mL^{-1}) in pH 7.4 phosphate buffer for 10 min at 25 °C.

Procedures employed to structurally characterize **7** are presented in Materials and Methods.

The time course for oxidation of 5-HT by the xanthine/XOD system is shown in Figure 2. Thus, during the initial 8 min of the reaction the concentration of 5-HT decreased rapidly and that of T-4,5-D and 4,4′-D correspondingly increased. At longer times the consumption of 5-HT and formation of 4,4′-D became very much slower. Under the experimental conditions employed, the results of several replicate experiments (n = 9) demonstrated that after 10 min the xanthine/XOD-mediated oxidation of 141 ± 27 nmol (mean ± SD) of 5-HT generated 50 ± 4 nmol of T-4,5-D and 32 ± 3 nmol of 4,4′-D. Thus, 1 mol of 5-HT was oxidized to 0.36 mol of T-4,5-D and 0.23 mol of 4,4′-D. The remaining 5-HT oxidized was accounted for primarily as **7**. However, it was noted throughout the course of this investigation that different batches of XOD had different activities and, hence, somewhat variable rates of $O_2^{\bullet-}$ (and H_2O_2) production that in turn were reflected in the slightly variable product distributions reported in this article. Using very high detector sensitivities, HPLC-EC$_{ox}$ analysis of product solutions showed peaks for several very minor electro-oxidizable products. These peaks, however, did not correspond to 5-HEO, 5,6-DHT, or other products characteristic of the HO$^{\bullet}$-mediated oxidation of 5-HT (*35*).

T-4,5-D accumulated only during the initial 8−10 min when 5-HT was oxidized by the xanthine/XOD system (Figure 2B), i.e., during the period when $O_2^{\bullet-}$ was generated. Subsequently, T-4,5-D concentrations declined. Incubation of T-4,5-D with all of the constituents of the reaction mixture except XOD resulted in the loss (decomposition) of less than 5% of the dione after 60 min (Figure 3). However, in the presence of XOD but not xanthine, the concentration of T-4,5-D decreased with time to 74% of its initial value after 60 min (Figure 3). These results suggested that T-4,5-D might bind covalently to XOD, perhaps by addition to nucleophilic

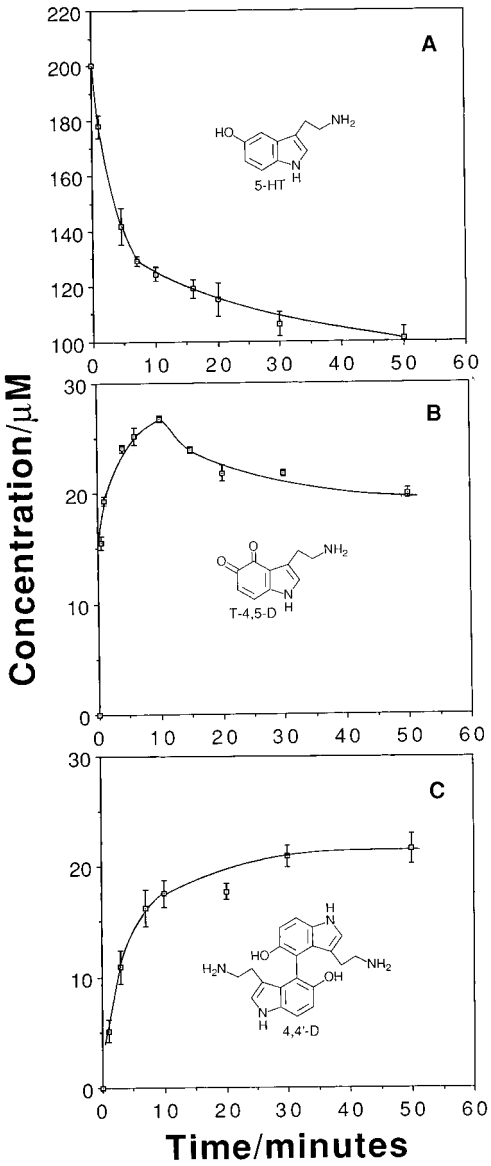


Figure 2. Time course for the oxidation of 5-HT (200 μM) by xanthine (200 μM)/XOD (0.087 unit mL^{-1}) in pH 7.4 phosphate buffer at 25 °C: (A) consumption of 5-HT; (B) formation of T-4,5-D; (C) formation of 4,4′-D. 5-HT and 4,4′-D were determined by HPLC-EC$_{ox}$; T-4,5-D was determined by HPLC-EC$_{Red}$.

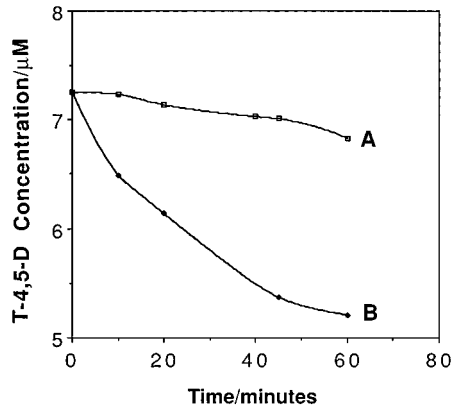


Figure 3. Stability of T-4,5-D (7.3 μM) when incubated with (A) 200 μM xanthine or (B) 0.087 unit mL^{-1} XOD in phosphate buffer at 25 °C. The concentration of T-4,5-D was measured using HPLC-EC$_{Red}$ (−50 mV).

residues of the enzyme. However, such binding did not affect the activity of XOD. To illustrate, following a 60-

Table 1. Influence of Superoxide Dismutase (SOD), Catalase, Hydroxyl Radical Scavengers, and Iron Chelators on the Oxidation of 5-HT by the Xanthine/Xanthine Oxidase System[a]

added compd (concn)		5-HT oxidized (nmol)	nmol of T-4,5-D/nmol of 5-HT oxidized	nmol of 4,4′-D/nmol of 5-HT oxidized
SOD	(0)	134	0.34	0.25
	(25 μg mL^{-1})	204	0.06	0.30
	(102 μg mL^{-1})	174	0.04	0.29
	(255 μg mL^{-1})	100	0.04	0.43
catalase	(0)	128	0.41	0.23
	(1 μg mL^{-1})	82	0.57	0.18
	(20 μg mL^{-1})	22	0.91	0.10
mannitol	(0)	122	0.39	0.29
	(10 mM)	128	0.36	0.29
formate	(10 mM)	110	0.44	0.33
desferal	0	158	0.29	0.22
	(100 μM)	142	0.43	0.28
	(200 μM)	134	0.53	0.27
	(1000 μM)	136	0.56	0.12
	(1850 μM)	102	0.77	0.10

[a] 5-HT (200 μM) was incubated in pH 7.4 phosphate buffer (μ = 0.2) at 25 °C with xanthine (200 μM) and XOD (0.087 unit mL^{-1}) in a total solution volume of 2.0 mL. Other additions are shown in the table. Ten minutes after addition of XOD, the resulting product solution was analyzed for T-4,5-D (HPLC-EC$_{Red}$) and 4,4′-D (HPLC-EC$_{ox}$).

min incubation of XOD (0.087 unit mL^{-1}, 7.9 mg of protein mL^{-1}) with T-4,5-D (50 μM) in pH 7.4 phosphate buffer at 25 °C, HPLC-EC$_{Red}$ analysis revealed that approximately 26% of the initially added dione had been lost. After addition of xanthine (200 μM), the concentration of uric acid measured (HPLC-EC$_{ox}$) after 10 min was identical to that measured when XOD was preincubated for 60 min in the absence of T-4,5-D prior to addition of xanthine. However, even in the absence of XOD, T-4,5-D slowly decomposed in pH 7.4 phosphate buffer. For example, after 24 h the concentration of a solution initially 50 μM in T-4,5-D had declined to 27.3 ± 2.8 μM. In the presence of XOD (0.087 unit mL^{-1}, 7.9 mg of protein mL^{-1}) an otherwise identical solution of T-4,5-D declined to 10.6 ± 5.6 mM after 6 h and was undetectable (HPLC-EC$_{Red}$) after 24 h.

Oxidation of 5-HT with Potassium Superoxide (KO_2). Addition of KO_2 (1.8 mg, 14.4 μmol) to a vigorously stirred solution of 5-HT (2 mM, 6 μmol) in dimethylsulfoxide (Me_2SO; 3 mL) exposed to the atmosphere caused the colorless solution to initially turn pink and then bright green. Addition of 7 mL of mobile phase A (pH 4.7), used for preparative HPLC-UV (*37*), caused this solution to become bright purple. The resulting solution was injected into the preparative HPLC-UV system. The only product formed in the reaction based on its chromatographic retention time, cyclic voltammetry, UV−visible spectrum, and subsequent analysis by HPLC-EC$_{Red}$ was T-4,5-D.

Influence of Antioxidants, Radical Scavengers, and Iron Chelators on the Oxidation of 5-HT by $O_2^{\bullet-}$ (Xanthine/XOD System). The most obvious effect of SOD on the oxidation of 5-HT by the xanthine/XOD system was a dramatic decrease in the formation of T-4,5-D along with a tendency to increased yields of 4,4′-D (Table 1). However, increasing SOD concentrations first stimulated and then inhibited oxidation of 5-HT. By contrast, catalase inhibited oxidation of 5-HT but simultaneously shifted the reaction toward formation of T-4,5-D at the expense of 4,4′-D. High concentrations of the HO$^{\bullet}$ scavengers mannitol and formate had no significant effects on the oxidation of 5-HT by the xanthine/XOD system. The powerful iron (Fe^{3+})-complexing agent desferal appeared to have relatively little influence on the extent of oxidation of 5-HT when included in the normal incubation mixture with xanthine and XOD, although with increasing concentrations it shifted the reaction toward formation of T-4,5-D at the expense of 4,4′-D. Heat-denatured catalase (74 μg mL^{-1}) or bovine serum albumin (BSA; 473 μg mL^{-1}) had no significant effects on the oxidation of 5-HT by the xanthine/XOD system in terms of reaction rate or identity and yields of products. Similarly, Na_2EDTA (0−500 μM) and Na_2DTPA (0−500 μM) had no effect on the reaction rate and products. Quantitative analytical data for **7** were not obtained. However, factors that caused an increase in the yield of 4,4′-D always evoked a parallel increase in the chromatographic peak height (HPLC-EC$_{ox}$) for **7** and a corresponding decrease in that for uric acid.

Table 2. Influence of Glutathione on the Oxidation of 5-HT by the Xanthine/Xanthine Oxidase System[a]

GSH concn (μM)	5-HT oxidized (nmol)	nmol of T-4,5-D/nmol of 5-HT oxidized	nmol of 4,4′-D/nmol of 5-HT oxidized
0	134	0.34	0.26
20	190	0.10	0.23
50	186	0.10	0.25
100	164	0.14	0.23
500	64	0.23	0.11
1000	62	0.21	0.06
5000	44	0.14	0.02

[a] 5-HT (200 μM) was incubated in pH 7.4 phosphate buffer (μ = 0.2) at 25 °C with xanthine (200 μM), XOD (0.087 unit mL^{-1}), and the indicated concentration of GSH in a total volume of 2000 μL. Ten minutes after addition of XOD, the resulting product solution was analyzed for T-4,5-D (HPLC-EC$_{Red}$) and 4,4′-D (HPLC-EC$_{ox}$).

Influence of Glutathione on the Oxidation of 5-HT by $O_2^{\bullet-}$ (Xanthine/XOD). Low concentrations of GSH (20−100 μM) appeared to potentiate the oxidation of 5-HT by the xanthine/XOD system (Table 2). However, higher concentrations (≥500 μM) inhibited the reaction, although even 5 mM GSH was unable to completely block the oxidation of 5-HT. Low concentrations of GSH inhibited formation of T-4,5-D, whereas high concentrations inhibited formation of 4,4′-D. A comparison of the chromatograms (HPLC-EC$_{Red}$, detector set at −50 mV vs Ag/AgCl) of the product solutions obtained when 5-HT was oxidized by the xanthine/XOD system in the absence (Figure 4A) and presence (Figure 4B) of 30 μM GSH indicated that the decreased yield of T-4,5-D under the latter conditions was primarily due to formation of 7-*S*-Glu-T-4,5-D. However, HPLC-EC$_{Red}$ with the detector electrode set at −550 mV vs Ag/AgCl revealed that additional unknown electrochemically reducible products (identified as Unk 1 and Unk 2 in Figure 4C) were formed when 5-HT was oxidized by xanthine/XOD in the presence of GSH. The chromatogram (HPLC-EC$_{Red}$) shown in Figure 4D was recorded 60 s after addition of GSH (30 μM) to the product solution analyzed in Figure 4A and demonstrates the rapid reaction of GSH with T-4,5-D to give 7-*S*-Glu-T-4,5-D along with minor yields of Unk 1 and Unk 2. However, incubation of 7-*S*-Glu-T-4,5-D (14 μM) with the xanthine (200 μM)/XOD (0.087 unit mL^{-1}) system at pH 7.4 for 10 min resulted in its almost

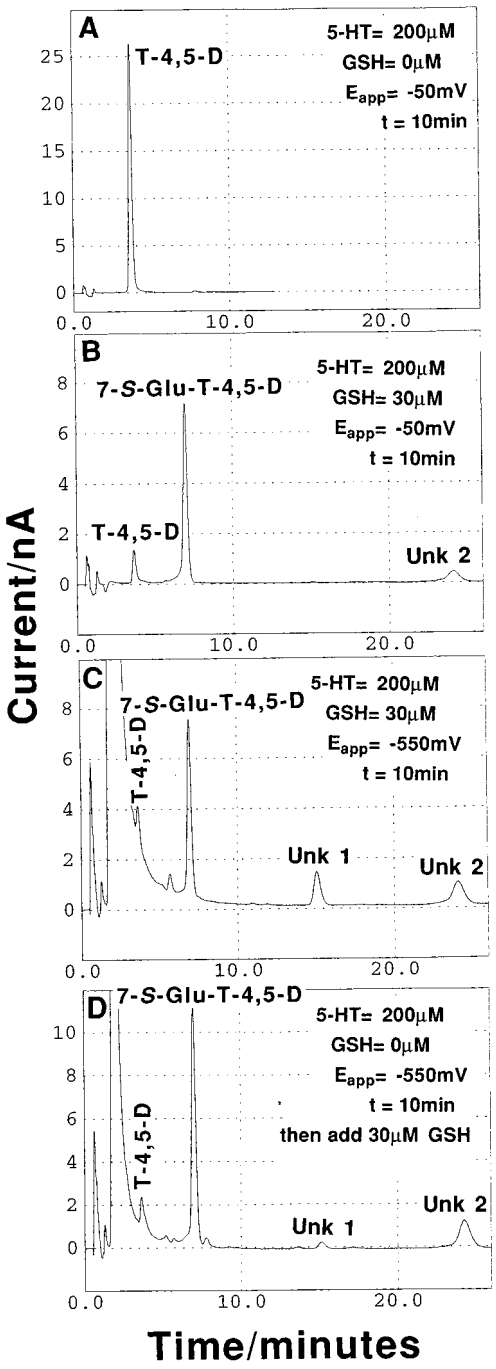


Figure 4. HPLC-EC_{Red} chromatograms of the product solutions formed after oxidation of 5-HT (200 μM) by the xanthine (200 μM)/XOD (0.087 unit mL^{-1}) system in pH 7.4 phosphate buffer ($\mu = 0.2$) for 10 min: (A) in the absence of GSH; (B and C) in the presence of GSH (30 μM); detector electrode set at (A) −50 mV and (B and C) −550 mV. In panel D GSH (30 μM) was added to the solution in panel A and analyzed after 60 s (−550 mV).

complete (>90%) transformation into Unk 1 and Unk 2 (Figure 5). Because free GSH was not present in this reaction, it appears that 7-*S*-Glu-T-4,5-D may be oxidized by the xanthine/XOD system. In the absence of free GSH or exposure to the xanthine/XOD system, solutions of 7-*S*-Glu-T-4,5-D were moderately stable in pH 7.4 phosphate buffer. To illustrate, the concentration of a 46.5 μM solution of 7-*S*-Glu-T-4,5-D declined to 41.0 ± 4.0 and 12.1 ± 6.0 μM after 1 and 14 h, respectively.

The decreased yields of 4,4′-D observed when 5-HT was oxidized by the xanthine/XOD system in the presence of

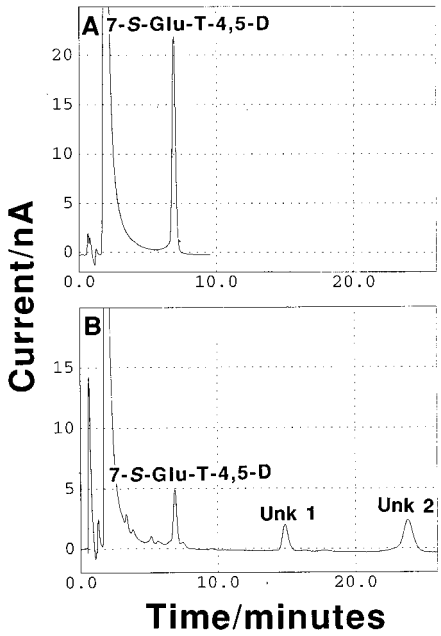


Figure 5. HPLC-EC_{Red} chromatograms of (A) 7-*S*-Glu-T-4,5-D (14 μM) and (B) the same solution 10 min after adding xanthine (200 μM) and XOD (0.087 unit mL^{-1}). Detector electrode set at −550 mV vs Ag/AgCl.

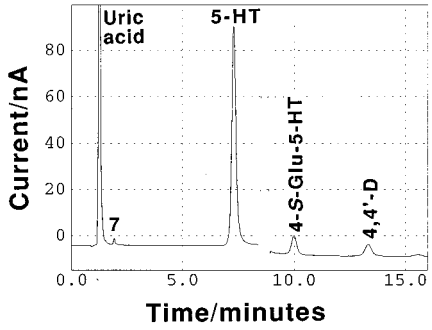


Figure 6. HPLC-EC_{ox} chromatogram of the product solution formed after oxidation of 5-HT (200 μM) by xanthine (200 μM)/XOD (0.087 unit mL^{-1}) in pH 7.4 phosphate buffer ($\mu = 0.2$) for 10 min in the presence of GSH (1000 μM).

relatively high concentrations of GSH (Table 2) were in part related to increased formation of 4-*S*-Glu-5-HT (Figure 6).

Role of Oxo-Iron Species in the Oxidation of 5-HT by $O_2^{\bullet-}$ (Xanthine/XOD). Heat-denatured catalase and BSA had no significant effect on the oxidation of 5-HT by the xanthine/XOD system. Thus, the inhibitory effect of active catalase and the shift of the reaction toward T-4,5-D formation (Table 2) imply that a major route leading to 4,4′-D requires H_2O_2 and is not related to oxygen radical-scavenging properties of the protein. Furthermore, the phosphate buffer system employed in this investigation was certainly contaminated with trace (micromolar) concentrations of iron and other transition-metal ions. Under the reaction conditions employed, iron chelated by EDTA or phosphate would initially be in the Fe(III) oxidation state which is readily reduced to Fe(II) by $O_2^{\bullet-}$ that, in turn, is capable of generating $HO^{\bullet}$ from H_2O_2 by Fenton chemistry or from H_2O_2 and $O_2^{\bullet-}$ together by the Haber−Weiss reaction (*41*). This raised the possibility that 4,4′-D (and **7**) is formed by oxidation of 5-HT by $HO^{\bullet}$ generated by Fe(II) and $O_2^{\bullet-}/H_2O_2$ that are the byproducts of the xanthine/XOD reaction (*42*). However, $HO^{\bullet}$ scavengers had no significant influence on the

Table 3. Oxidation of 5-HT when Added after Completion (10 min) of the XOD-Mediated Oxidation of Xanthine[a]

time (min)	uric acid concn (μM)	5-HT oxidized (nmol)	nmol of T-4,5-D/nmol of 5-HT oxidized	nmol of 4,4′-D//nmol of 5-HT oxidized	peak height for dimer **7**[b] (nA)
10	185				
14	165	106	0.04	0.32	12.4
30	120	270	0.03	0.25	34.6
55	98	318	0.03	0.23	40.4

[a] Xanthine (200 μM) and XOD (0.087 unit mL^{-1}) were incubated in pH 7.4 phosphate buffer at 25 °C for 10 min. The uric acid concentration was measured (HPLC-EC_{ox}) at 10 min, and simultaneously, 5-HT (200 μM) was added. The resultant product solution was analyzed at the times indicated for T-4,5-D (HPLC-EC_{Red}) and uric acid, 5-HT, 4,4′-D, and **7** (HPLC-EC_{ox}). [b] Chromatographic peak height for **7** using HPLC-EC_{ox}.

Table 4. Oxidation of 5-HT when Added after Completion (10 min) of the XOD-Mediated Oxidation of Xanthine in the Presence of Desferal[a]

time (min)	uric acid concn (μM)	5-HT oxidized (nmol)	nmol of T-4,5-D/nmol of 5-HT oxidized	nmol of 4,4′-D/nmol of 5-HT oxidized	peak height for dimer **7**[b] (nA)
10	190				
14	178	182	0.62	0.10	10.6
30	128	298	0.59	0.09	21.4
55	131	298	0.52	0.10	22.0

[a] Xanthine (200 μM), XOD (0.087 unit mL^{-1}), and desferal (1.85 mM) were incubated in pH 7.4 phosphate buffer at 25 °C for 10 min. The uric acid concentration was measured (HPLC-EC_{ox}) at 10 min, and simultaneously, 5-HT (200 μM) was added. The resultant product solution was analyzed at the times indicated for T-4,5-D (HPLC-EC_{Red}) and uric acid, 5-HT, 4,4′-D, and **7** (HPLC-EC_{ox}). [b] Chromatographic peak height for **7** using HPLC-EC_{ox}.

xanthine/XOD-mediated oxidation of 5-HT (Table 1). Furthermore, 5-HEO, the major product of the in vitro oxidation of 5-HT by HO$^{\bullet}$ (*35*), was not observed in the xanthine/XOD-mediated reaction. Conversely, 4,4′-D is not a product of the HO$^{\bullet}$-mediated oxidation of 5-HT (*35*). The fact that only the C(4)-position of 5-HT is activated (to form an intermediate that is the precursor of 4,4′-D, **7**, and 4-*S*-Glu-5-HT) in the xanthine/XOD-mediated oxidation also suggests that the reaction must involve a much more selective oxidant than HO$^{\bullet}$. Because incubations of 5-HT with XOD and H_2O_2 in pH 7.4 phosphate buffer for 10 min in the absence of xanthine, and therefore $O_2^{\bullet-}$, resulted in no significant oxidation of the neurotransmitter, it appeared that a major reaction pathway leading to 4,4′-D (and **7**) depends on $O_2^{\bullet-}$, H_2O_2, and trace concentrations of iron species. Indeed, in this respect, the complex effects of SOD, catalase, desferal (Table 1), and other reducing agents such as GSH (Table 2) are similar to those reported for iron-catalyzed lipid peroxidation reactions (*43*, *44*). In these reactions $O_2^{\bullet-}$ or other reductants serve to reduce Fe(III) to Fe(II), and the subsequent oxidation of Fe(II) by H_2O_2 together serve to generate a chemically ill-defined oxo-iron species that is a strong oxidizing agent (*43*, *44*). This oxidant requires both Fe(III) and Fe(II), and optimal oxidizing activity occurs when these species are present in a 1:1 ratio. The Fe(III)−desferal complex cannot be reduced to the Fe(II) oxidation state by $O_2^{\bullet-}$ or other species with similar reducing properties (*41*). Furthermore, desferal shifts the xanthine/XOD-mediated oxidation of 5-HT toward formation of T-4,5-D at the expense of 4,4′-D (Table 1). These results, therefore, support a role for Fe(II) and thence an oxo-iron species in a major reaction pathway that leads to 4,4′-D (and **7**) (see also later discussion for the influence of desferal on the $O_2^{\bullet-}$-mediated oxidation of 5-HT).

To explore a role for oxo-iron species in the xanthine/XOD-mediated oxidation of 5-HT, additional experiments were performed. Thus, xanthine (200 μM) and XOD (0.087 unit mL^{-1}) were incubated in pH 7.4 phosphate buffer for 10 min initially in the absence of 5-HT. At the end of this time, i.e., when the generation of $O_2^{\bullet-}$ had terminated, an aliquot (5 μL) of the solution was analyzed by HPLC-EC_{ox} to measure the concentration of uric acid. Simultaneously, 5-HT (200 μM) was added to the solution that was then periodically analyzed for T-4,5-D (HPLC-EC_{Red}) and uric acid, 5-HT, 4,4′-D, and **7** (HPLC-EC_{ox}) with the results presented in Table 3. Thus, despite the fact that the XOD-mediated oxidation of xanthine was completed, 5-HT was rather extensively oxidized. Very little T-4,5-D was formed under these conditions, whereas yields of 4,4′-D were about the same as when 5-HT was initially included in the incubation mixture with xanthine and XOD. However, it was notable that uric acid concentrations decreased appreciably with time and, correspondingly, the yields of **7** increased. Control experiments confirmed that $O_2^{\bullet-}$ was essential for these reactions. Thus, when xanthine (200 μM) and H_2O_2 (220 μM) were incubated for 10 min or XOD (0.087 unit mL^{-1}) and H_2O_2 (220 μM) were incubated for 10 min, subsequent addition of 5-HT (200 μM) resulted in no significant oxidation of the neurotransmitter. Together, the preceding results indicated that in the absence of 5-HT, the xanthine/XOD system generates an oxidant dependent on $O_2^{\bullet-}$ and H_2O_2 that is subsequently responsible for oxidation of the neurotransmitter primarily to 4,4′-D and **7**. Because of their instability in aqueous solution, neither $O_2^{\bullet-}$ nor HO$^{\bullet}$ can be this oxidant species. A plausible interpretation of these results is that in the initial absence of 5-HT more $O_2^{\bullet-}$ is available to reduce trace levels of Fe(III) to Fe(II) with the same concentrations of H_2O_2 required for oxidation of Fe(II) to an oxo-iron species; i.e., conditions are favorable for formation of the species that oxidizes 5-HT primarily to 4,4′-D and **7**. Because the concentrations of iron in the reaction solutions must be very low (although these were not measured), the oxidation of 5-HT to the latter compounds must involve catalytic regeneration of the putative oxo-iron species.

The Fe(III)−desferal complex cannot be reduced by $O_2^{\bullet-}$ (*41*). Accordingly, the putative oxo-iron oxidant activity should be suppressed in the presence of desferal, and indeed, this was observed when 5-HT is oxidized by the xanthine/XOD system in the presence of this complexing agent (Table 1). However, it is also known that desferal can react with $O_2^{\bullet-}$ to form a rather strongly oxidizing nitroxide radical having a lifetime of several minutes (*45*). To investigate the possible role of the latter nitroxide radical, the xanthine/XOD system was incubated with desferal (1.85 mM) for 10 min at 25 °C in the absence of 5-HT. After this time an aliquot of the reaction solution (5 μL) was analyzed by HPLC-EC_{ox} to measure uric acid formation, and simultaneously, 5-HT (200 μM) was added to the solution. The resulting product mixture was then analyzed at various times with the results shown in Table 4. Thus, at a stage of the reaction (≥10 min) when the XOD-mediated oxidation

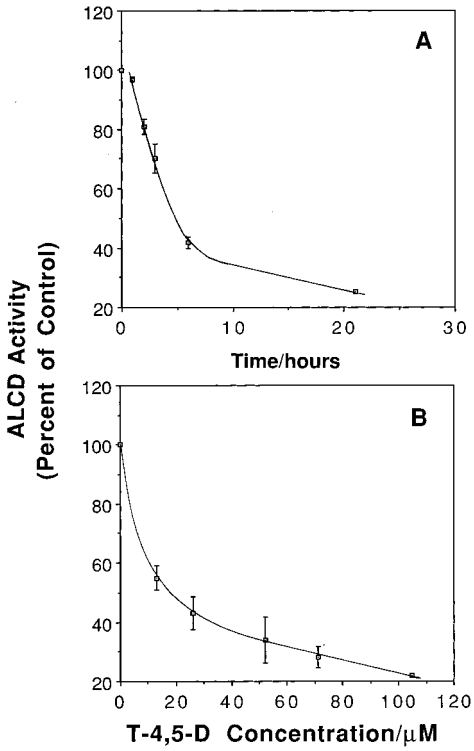


Figure 7. (A) Time-dependent and (B) concentration-dependent inhibition of ALCD by T-4,5-D. (A) ALCD (125 μg mL^{-1}) was incubated with T-4,5-D (70.9 μM) for indicated times, and activity was then assayed. (B) ALCD (125 μg mL^{-1}) and T-4,5-D at concentrations indicated in pH 7.4 phosphate buffer ($\mu = 0.2$) were incubated for 20 h, and then activity was assayed. The assay for the ALCD-mediated oxidation of ethanol is described in Materials and Methods.

of xanthine to uric acid was complete and $O_2^{\bullet-}$ generation had ceased, 5-HT was oxidized for several minutes. The major product of this reaction was T-4,5-D. Much lower yields of 4,4′-D were formed. Furthermore, the presence of desferal in the $O_2^{\bullet-}$-generating reaction prior to addition of 5-HT resulted in significantly lower uric acid consumption and hence lower yields of **7** (Table 4) than in the absence of this iron chelator (Table 3). In a control experiment, desferal (1.85 mM) and XOD (0.087 unit mL^{-1}) were incubated for 10 min in the absence of xanthine prior to addition of 5-HT (200 μM). Under these conditions $O_2^{\bullet-}$ is not generated, and subsequently, 5-HT was not oxidized. These results strongly suggest that the influence of desferal on the xanthine/XOD-mediated oxidation of 5-HT (Table 1) is due to its reaction with $O_2^{\bullet-}$ to give a nitroxide radical (*45*) that then oxidizes the neurotransmitter primarily to T-4,5-D with correspondingly lower yields of 4,4′-D and **7**.

Binding of T-4,5-D with Proteins. T-4,5-D reacted both with XOD (Figure 3) and, more rapidly, with GSH (Figure 4D). These observations suggested that reactions of T-4,5-D with protein nucleophiles, particularly sulfhydryl residues, might contribute to its putative neurotoxic properties (see later discussion). To explore the binding of T-4,5-D to proteins, its effects on a model enzyme, alcohol dehydrogenase (ALCD) that contains 24 sulfhydryl residues (*46*), was investigated. Indeed, T-4,5-D evoked a time-dependent (Figure 7A) and concentration-dependent (Figure 7B) inhibition of the ALCD-mediated oxidation of ethanol.

Discussion

In Me_2SO solution the $O_2^{\bullet-}$-mediated oxidation of 5-HT generated T-4,5-D as the only detected reaction product. Furthermore, in buffered aqueous solution at pH 7.4 SOD profoundly, although not completely, inhibited formation of T-4,5-D when 5-HT was incubated with the xanthine/XOD system as a source of $O_2^{\bullet-}$ (Table 1). Although the xanthine/XOD system generates both $O_2^{\bullet-}$ and H_2O_2 (*41*, *42*), the latter observations support the conclusion that T-4,5-D is predominantly formed from 5-HT in a reaction mediated by $O_2^{\bullet-}$. However, $O_2^{\bullet-}$ is unable to directly oxidize organic molecules with acidic protons such as 5-HT (*47*, *48*). Rather, it is the strong tendency of $O_2^{\bullet-}$ to disproportionate via abstraction of protons from such substrates that accounts for its ability to indirectly mediate oxidation reactions (*47*, *48*). Thus, it is probable that $O_2^{\bullet-}$ abstracts a proton from 5-HT to generate anion **1a**/**1b**, HO_2^-, and molecular oxygen (Scheme 1). By analogy with oxidations of related hydroxyindolamines, it is probable that **1b** is the principal electron donor to molecular oxygen giving the free-radical−superoxide complex **2** (*49*, *50*). Recombination of the superoxide residue of **2** with the incipient C(4)-centered free radical yields the hydroperoxy anion **3** that, upon protonation, generates hydroperoxide **4** that then decomposes to T-4,5-D (*48*, *49*).

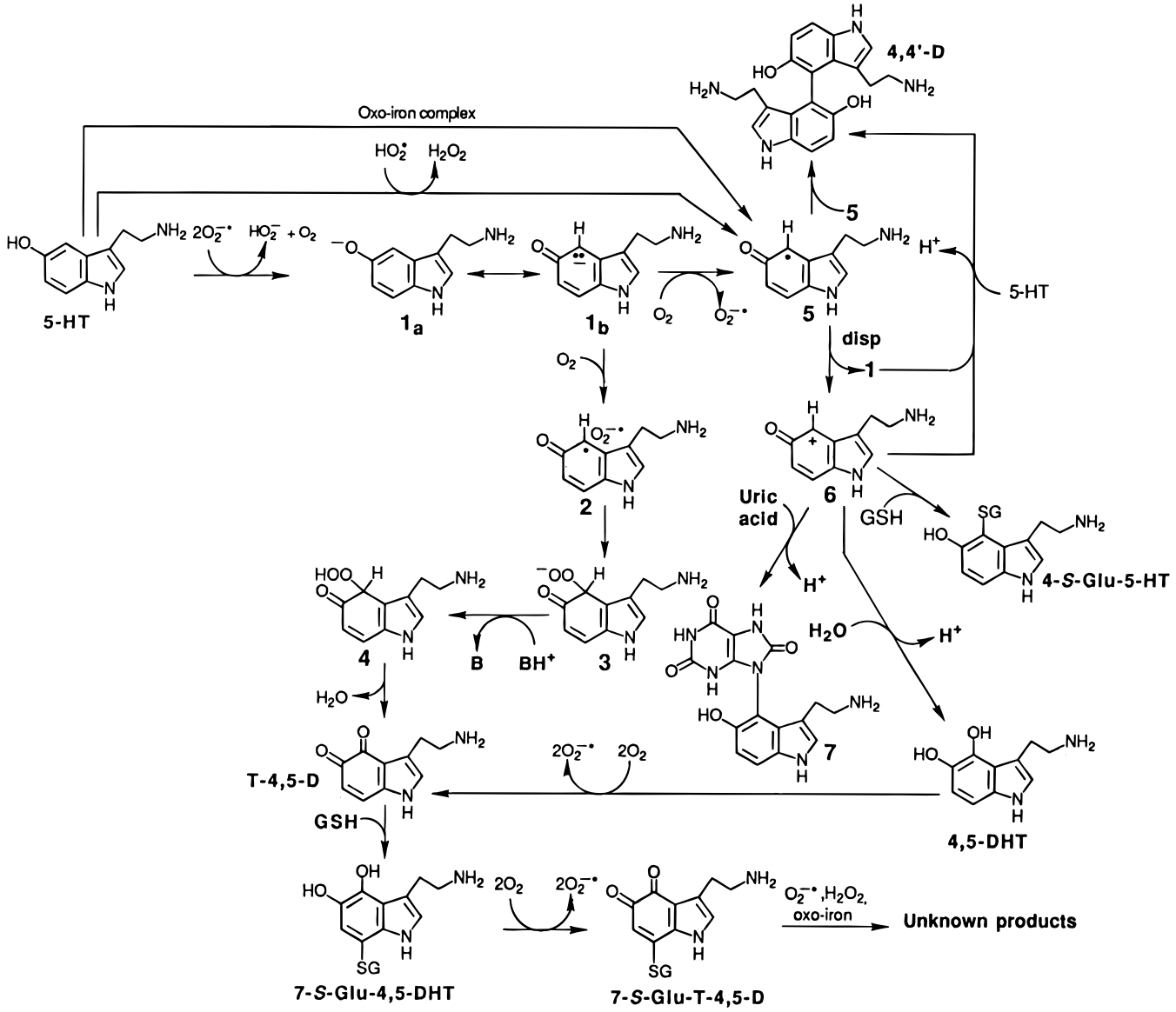
The pK_a of the perhydroxyl radical ($HO_2^{\bullet}$) in water is 4.69 (*48*), and hence its concentration at pH 7.4 is low compared to the concentration of $O_2^{\bullet-}$. However, $HO_2^{\bullet}$ is also formed by deprotonation of H_2O_2 by $O_2^{\bullet-}$ (eq 1) (*51*), both of which are byproducts of the XOD-mediated oxidation of xanthine.

$$O_2^{\bullet-} + H_2O_2 \rightarrow HO_2^{\bullet} + HO_2^- \qquad (1)$$

Thus, it is likely that significant albeit transient fluxes of $HO_2^{\bullet}$ are formed. Accordingly, it is conceivable that $HO_2^{\bullet}$ directly oxidizes (one-electron abstraction) 5-HT (or **1**) to the C(4)-centered radical **5** (Scheme 1). Dimerization of **5**, therefore, represents a plausible although minor reaction leading to 4,4′-D. Alternatively, disproportionation of **5** to carbocation **6** (and **1**) followed by nucleophilic addition of 5-HT (or **1**) could also lead to 4,4′-D. Nucleophilic addition of uric acid or water to **6** provides minor routes to **7** and 4,5-dihydroxytryptamine (4,5-DHT) that is easily autoxidized to T-4,5-D (*37*).

SOD both potentiates (low concentrations) and inhibits (high concentrations) the xanthine/XOD-mediated oxidation of 5-HT (Table 1) but shifts the reaction toward formation of 4,4′-D (and **7**) at the expense of T-4,5-D. Catalase, however, inhibits the oxidation of 5-HT but shifts the reaction that does occur toward T-4,5-D at the expense of 4,4′-D (Table 1). These effects appear to reflect the influence of $O_2^{\bullet-}$ and H_2O_2 on formation of an oxo-iron species that has also been implicated in lipid peroxidation reactions (*43*, *44*). Indeed, the experiments described in connection with Table 3 strongly implicate $O_2^{\bullet-}$ and H_2O_2 together in formation of such an oxo-iron species that, after exhaustion of the XOD-mediated oxidation of xanthine, oxidizes 5-HT predominantly to 4,4′-D and **7**. The chemically ill-defined oxo-iron species therefore selectively activates the C(4)-position of 5-HT. It is proposed therefore that the oxo-iron complex abstracts an electron from 5-HT to give radical **5** and thence carbocation **6**, the precursors of 4,4′-D and **7** (Scheme 1).

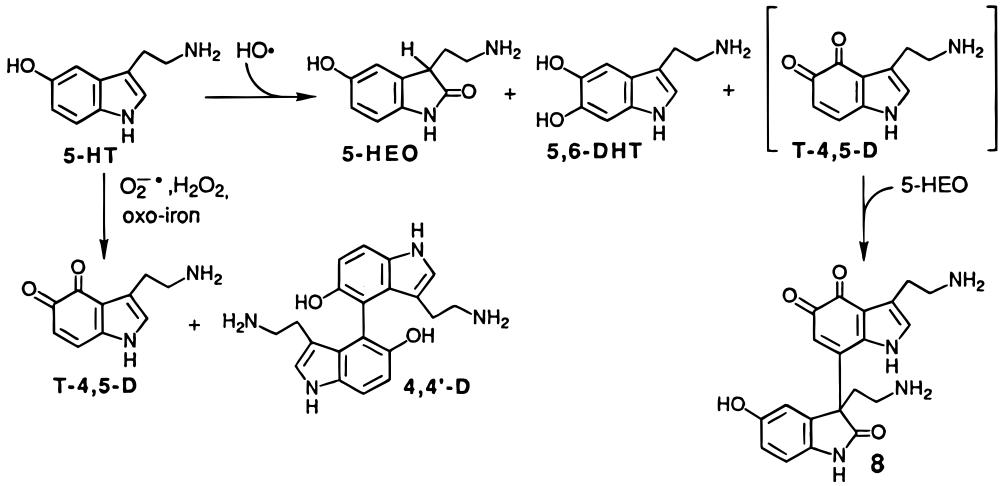
Scheme 1



At relatively low concentrations, GSH potentiates oxidation of 5-HT by the xanthine/XOD system, but at higher concentrations it inhibits the reaction (Table 2). These effects are probably related to the ability of GSH to influence the Fe(III)/Fe(II) concentration ratio and hence the reaction mediated by oxo-iron species. Decreased yields of T-4,5-D are, in part, accounted for by its reaction with GSH to give 7-*S*-Glu-4,5-DHT that is readily autoxidized to 7-*S*-Glu-T-4,5-D (Scheme 1). However, that latter conjugate can be further oxidized to give unknown products (Figure 5). Decreased yields of 4,4′-D result, at least in part, from nucleophilic addition of GSH on putative carbocation **6** to give 4-*S*-Glu-5-HT (Scheme 1), a reaction favored by high concentrations of the tripeptide (Table 2).

The most important conclusion to be drawn from this investigation is that oxidation of 5-HT in aqueous solution at pH 7.4 by the xanthine/XOD system generates two major products, T-4,5-D and 4,4′-D. T-4,5-D is primarily formed by an oxidation reaction driven by $O_2^{\bullet-}$. By contrast 4,4′-D is primarily formed by oxidation of 5-HT by an unknown oxo-iron species present in very low concentrations and dependent for its formation on simultaneous generation of $O_2^{\bullet-}$ and H_2O_2. The HO•-mediated oxidation of 5-HT differs significantly from the xanthine/XOD-mediated reaction forming 5-HEO, 5,6-DHT, and **8** (Scheme 2) as major reaction products (*35*). Although T-4,5-D is formed in the HO•-mediated oxidation of 5-HT, it reacts rapidly with the carbanion of 5-HEO to give **8**, and hence the dione does not appear as a free product (*35*). In the presence of free GSH, a major constituent of nerve terminals and axons (*52*), both the $O_2^{\bullet-}$- and HO•-mediated oxidations of 5-HT form 7-*S*-Glu-T-4,5-D as a result of reaction of the tripeptide with T-4,5-D. However, the xanthine/XOD-mediated reaction also forms 4-*S*-Glu-5-HT, a product not observed in the HO•-mediated oxidation of 5-HT. In principle, therefore, 5-HEO and 5,6-DHT should be formed in regions of the brain innervated by serotonergic neurons under pathological conditions where HO• is generated. While there are reports that 5,6-DHT is formed in rat brain following a neurotoxic dose of MA and has, therefore, been proposed to mediate serotonergic terminal degeneration (*30*), efforts to confirm this or to detect 5-HEO or 6-OHDA have been unsuccessful (*20, 32*−*34*). The protection provided by elevated levels of cytoplasmic Cu−Zn SOD against the

Scheme 2



neurodegeneration evoked by MA (*4*) and ischemia-reperfusion (*13*) points to $O_2^{\bullet-}$ rather than HO• playing a role in the neurotoxic mechanisms. Both an experimentally induced ischemic insult (*7*−*9*) and MA (*16*−*18*) evoke a massive release of 5-HT, other biogenic amines, Glu, and Asp and generation of $O_2^{\bullet-}$, probably in the neuronal cytoplasm (*4, 13*). Such $O_2^{\bullet-}$ might be generated by interaction of released Glu and Asp (*10*) with NMDA receptors (*16, 17*) or by activation of the xanthine/XOD system (*14, 15*). Subsequent reuptake of 5-HT, DA, and NE into their parent and indeed other neurons (*29*) would therefore necessarily expose elevated cytoplasmic levels of these neurotransmitters to $O_2^{\bullet-}$ and its dismutation product H_2O_2. Furthermore, the normally very low cytoplasmic levels of low-molecular-weight iron would be augmented by its mobilization from ferritin by $O_2^{\bullet-}$ (*53*). Thus, both MA and ischemia-reperfusion might result in intraneuronal conditions in which $O_2^{\bullet-}$, H_2O_2, and trace levels of oxo-iron species are simultaneously present. This raises the possibility that intraneuronal oxidation of elevated cytoplasmic levels of the biogenic amine neurotransmitters by the $O_2^{\bullet-}/H_2O_2$/oxo-iron system might generate significant levels of endotoxic metabolites that contribute to neurodegenerative processes. Preliminary experiments have demonstrated that all the biogenic amines are oxidized by the xanthine/XOD system, although 5-HT is the most vulnerable to this reaction. To illustrate, incubation of xanthine (200 μM) and XOD (0.087 unit mL^{-1}) in pH 7.4 phosphate buffer with these neurotransmitters (200 μM) for 10 min resulted in the oxidation of 138 ± 5, 44 ± 4, and 37 ± 5 nmol of 5-HT, DA, and NE, respectively.

Several reports suggest T-4,5-D might be a serotonergic neurotoxin and that unusual unidentified oxidized forms of 5-HT are present in the cerebrospinal fluid of AD patients (*54*−*56*). Furthermore, an in vitro oxidation product of DA speculated to form in the cytoplasm of dopaminergic cell bodies in the substantia nigra of PD patients has recently been demonstrated to evoke irreversible inhibition of mitochondrial respiration at the complex I level (*57*). Thus, the massive release of 5-HT, DA, and NE followed by subsequent reuptake into their parent and other proximate neurons and cytoplasmic oxidation to endotoxic metabolites might explain why monoamine uptake inhibitors (*23, 24*) or prior manipulations that deplete these neurotransmitters provide protection against the neurodegenerative consequences of MA (*21, 28*), ischemia-reperfusion (*25*−*27*), and Glu-mediated oxidative toxicity (*29*). It is also of possible relevance that blood platelets, the major cellular structure within an acute cerebral thrombus (*58*), store high concentrations of 5-HT (*59*). Furthermore, not only is the integrity of the blood−brain barrier (BBB) disrupted at the site of cerebral ischemia and becomes permeable to biogenic amines (*60*), but also platelets secrete a number of substances at this site. These include 5-HT and a factor that stimulates glial cell proliferation (*61, 62*) and activation during postischemic injury (*63*) with resultant $O_2^{\bullet-}$ generation (*64*). Thus, at the site of ischemic injury, 5-HT is released into brain parenchyma from both sides of the BBB and activated microglia and intraneuronal mechanisms described earlier generate $O_2^{\bullet-}$, i.e., conditions that should oxidize 5-HT to T-4,5-D and 4,4′-D. Indeed, recent studies have demonstrated that activated macrophages and brain microglia oxidize 5-HT to 4,4′-D (*65, 66*) which strongly suggests that the $O_2^{\bullet-}/H_2O_2$/oxo-iron system is the oxidant and, more importantly, that the putative neurotoxin T-4,5-D should also be a major reaction product. Because of its reactivity, however, T-4,5-D will undoubtedly be very difficult to detect in the free state in brain tissue. However, the facile reaction of T-4,5-D with the sulfhydryl residues of proteins and GSH might indicate that the putative neurotoxic properties of this dione might be related to this property. Indeed, modification of the sulfhydryl residues of brain guanine nucleotide-binding regulatory proteins has been proposed to underlie the serotonergic neurotoxicity of T-4,5-D (*67*). Interestingly, the activities of phospholipase C (*68*), the α-ketoglutarate dehydrogenase complex (*69*), and protein kinase C (*70*), enzymes with vulnerable sulfhydryl residues, have all been found to be significantly reduced in AD brains.

In summary, the xanthine/XOD system generates T-4,5-D, by oxidation of 5-HT in a reaction mediated by $O_2^{\bullet-}$, and 4,4′-D, in a reaction that appears to be mediated catalytically by an ill-defined oxo-iron species. Strong evidence implicates 5-HT (and other biogenic amine neurotransmitters) and $O_2^{\bullet-}$ in pathological mechanisms that underlie the neurodegeneration caused by MA, ischemia-reperfusion, and other neurodegenerative brain disorders. Accordingly, it is conceivable that T-4,5-D and perhaps other products of the $O_2^{\bullet-}/H_2O_2$/oxo-iron oxidation of 5-HT might be endotoxins that contribute to the neurodegeneration in these brain disorders.

Acknowledgment. This work was supported by a grant from the National Institutes of Health (GM-32367) and additional support provided by the Vice President for Research and Research Council at the University of Oklahoma.

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TX970185W