Biochemical Pharmacology, Vol. 46, No. 9, pp. 1637–1652, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

7-*S*-GLUTATHIONYL-TRYPTAMINE-4,5-DIONE: A POSSIBLE ABERRANT METABOLITE OF SEROTONIN

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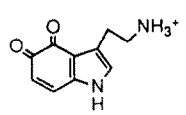
(*Received* 5 *December* 1992; *accepted* 21 *June* 1993)

Abstract—Tryptamine-4,5-dione (Compound **1**) is an *in vitro* oxidation product of 5-hydroxytryptamine (5-HT). Recent evidence has suggested that aberrant oxidations of 5-HT occur in the central nervous system of individuals with Alzheimer's disease (AD). In the event that Compound **1** is formed as a result of oxidation of 5-HT within serotonergic nerve terminals or axons, it would be expected to be rapidly conjugated by intraneuronal glutathione (GSH) to give 7-*S*-glutathionyl-tryptamine-4,5-dione (Compound **2**). When injected into the brains of laboratory mice, Compound **2** was lethal (LD_{50} = 21 μg) and evoked hyperactivity for the first 30 min following drug administration. Particularly during this hyperactive phase Compound **2** caused a statistically significant decrease in whole brain levels of norepinephrine and 5-HT. Levels of dopamine were also decreased while whole brain concentrations of its metabolites, 3,4-dihydroxyphenylacetic acid and homovanillic acid, were increased significantly. In the presence of GSH, NADPH and ascorbic acid, Compound **2** redox cycled in reactions that catalyzed the oxidation of these cellular reductants by molecular oxygen and formed H_2O_2 as a byproduct. Compound **2** also reacted with molar excesses of GSH to form more structurally complex glutathionyl conjugates. Several of these conjugates have been isolated and their structures determined using spectroscopic methods. It is conceivable that one or more of these conjugates might serve as analytical markers in a search for evidence in support of the hypothesis that aberrant oxidations of 5-HT occur in the Alzheimer brain. The redox cycling properties of Compound **2** and its facile reactions with cellular nucleophiles such as GSH may represent mechanisms that contribute to the toxicity of this drug.

Abnormalities in central serotonergic neuronal systems have been documented extensively in dementia of the Alzheimer type. For example, levels of the indolic neurotransmitter 5-hydroxytryptamine (5-HT†; serotonin) are lowered significantly in many regions of the Alzheimer brain [1–3]. Analyses of biopsy samples from the brains of patients with Alzheimer's disease (AD) have revealed that 5-HT and its major metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA), are significantly below normal levels [4]. Using HPLC with multi-electrode coulometric detection, Volicer *et al.* [5] found that the levels of 5-HT in cerebrospinal fluid (CSF) of AD patients were lower than in that of age-matched controls. Furthermore, evidence was presented for the existence of unknown but oxidized forms of 5-HT and 5-hydroxytryptophan (5-HTPP) in the CSF of AD patients [5, 6]. These unknown oxidative metabolites of 5-HT and 5-HTPP were not present in the CSF of control patients. Taken together these observations suggest that in the Alzheimer brain aberrant oxidative transformations of 5-HT, 5-HTPP and perhaps other endogenous indoles occur. Furthermore, they raise the possibility that these oxidation reactions and/or the resulting products may play roles in the neuronal degeneration or other biochemical deficits that characterize AD.

Until recently, little was known about the oxidation



Compound 1

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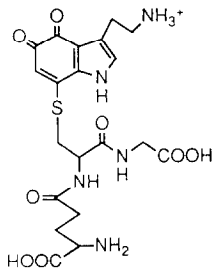
† Abbreviations: ACh, acetylcholine; AD, Alzheimer's disease; Ch, choline; Compound **1**, tryptamine-4,5-dione; Compound **2**, 7-*S*-glutathionyl-tryptamine-4,5-dione; Compound **3**, 7-*S*-glutathionyl-tryptamine-4,5-diol; Compound **4**, glycine, N-[3-(2-aminoethyl)-1,4-dihydro-6-hydroxy-7-mercapto-4-oxo-5*H*-indol-5-ylidene]-L-γ-glutamyl-L-cysteinyl-,(1→2′)-sulfide with L-γ-glutamyl-L-cysteinylglycine; Compound **4R**, glycine, *N*-[3-(2-aminoethyl)-4,6-dihydroxy-7-mercapto-1*H*-indol-5-yl]-L-γ-glutamyl-L-cysteinyl-,(1→2′)-sulfide with L-γ-glutamyl-L-cysteinylglycine; Compound **5**, 7-*S*-glutathionyl-6-hydroxy-tryptamine-4,5-dione; Compound **6**, 6,7-di-(*S*-glutathionyl)-tryptamine-4,5-dione; Compound **7**, 3a,7-di-(*S*-glutathionyl)-tryptamine-4,5-dione; COSY, two-dimensional (2D) correlated NMR spectroscopy; CSF, cerebrospinal fluid; **7,7′-D**, 7,7′-bi-(5-hydroxytryptamine-4-one); DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; $E^{o\prime}$, formal potential; E_p, peak potential; FAB-MS, fast atom bombardment-mass spectrometry; GSH, glutathione; GSSG, oxidized glutathione; HCOOH, formic acid; 6-OHDA, 6-hydroxydopamine; HVA, homovanillic acid; 5-HIAA, 5-hydroxyindole-3-acetic acid; 5-HT, 5-hydroxytryptamine; 5-HTPP, 5-hydroxytryptophan; HO·, hydroxyl radical; i.c.v., intracerebroventricular; LD_{50}, dose at which 50% of treated animals die within 1 hr; MeCN, acetonitrile; NE, norepinephrine; NHE, normal hydrogen electrode; NH_4OH, ammonium hydroxide; O_2^-, superoxide anion radical; PGE, pyrolytic graphite electrode; SCE, saturated calomel electrode; SOD, superoxide dismutase; TFA, trifluoroacetic acid; t_R, retention time; and μ, ionic strength.

chemistry and biochemistry of 5-HT and other indoles endogenous to the human CNS. Recently, however, reports from this laboratory have described the electrochemically-driven [7–12] and enzyme-mediated [13] oxidation chemistry of 5-HT. A product of both the electrochemical and several enzymatic oxidations of 5-HT was tryptamine-4,5-dione (Compound **1**). At physiological pH, Compound **1** was a product of these reactions formed as a result of both the direct oxidation of 5-HT and secondary oxidations of various oligomeric products [12, 13]. Volicer and coworkers [14, 15] have searched, unsuccessfully, for Compound **1** as an aberrant oxidation product of 5-HT in the CSF of AD patients. However, *in vitro* experiments with rat brain parts have suggested that Compound **1** is neurotoxic [16] and, in some respects, similar in action to the serotonergic neuroxotin 5,6-dihydroxytryptamine [17]. Intracerebroventricular (i.c.v.) injections of what was believed to be Compound **1** into rat brain caused a dose-dependent toxicity and nerve terminal damage to some brain structures that are degenerated in the human brain in AD [16]. However, the methods employed to prepare solutions of Compound **1** in these studies probably led to its facile conversion to secondary products [18]. Thus, neurotoxic and neurodegenerative properties attributed to Compound **1** may result from more structurally complex compounds spontaneously formed from the dione. Nevertheless, it seems clear that Compound **1** or compounds deriving from this oxidation products of 5-HT possess intriguing neurobiological properties.



Compound 2

That Compound **1** is, in fact, formed in the Alzheimer brain as a result of aberrant oxidations of 5-HT remains to be confirmed. However, a search for this substance in Alzheimer brain tissue or the CSF of AD patients is not likely to be successful. This is so because the dione is a highly electron-deficient species which would be expected to react avidly with cellular nucleophiles, particularly the intraneuronal tripeptide glutathione (GSH). GSH probably occurs at millimolar concentration levels within nerve terminals and axons [19, 20]. An important biological function of GSH is to provide protection to key cellular components by scavenging electrophiles such as Compound **1** [21]. We recently demonstrated that at physiological pH Compound **1** reacted with GSH in the absence of any enzyme catalysis to give 7-*S*-glutathionyl-tryptamine-4,5-dione (Compound **2**) as the major initial product [22]. However, it was noted that incubations of Compound **1** with molar excesses of GSH gave not only Compound **2** but several additional unidentified products. Thus, in the event that 5-HT is oxidatively transformed into Compound **1** within serotonergic terminals in the Alzheimer brain, it would be expected to be initially conjugated by GSH to give Compound **2** followed by further reactions to give additional products. Accordingly, it seemed possible that Compound **2** or perhaps other compounds derived from this conjugate might serve as analytical markers for aberrant intraneuronal oxidation of 5-HT in the Alzheimer brain. The formal potential ($E^{0\prime}$) for Compound **2** at pH 7.0 suggested that this conjugate might also be capable of redox cycling reactions in the presence of physiological reductants and molecular oxygen [22]. In view of the fact that Compound **2** retains many of the structural features of Compound **1** and that it might be capable of promoting intraneuronal redox cycling reactions, it seemed plausible to anticipate that this glutathionyl conjugate might also possess toxic and perhaps other neuropharmacological properties. The work reported in this paper was therefore designed to experimentally establish whether Compound **2** could potentially participate in redox cycling reactions under physiological conditions. The reactions between Compound **2** and GSH have been studied and the major products have been isolated and structurally characterized. Finally, it was established that Compound **2** is toxic (lethal) when administered into the brains of laboratory mice. Preliminary results are presented on the effects of centrally administered Compound **2** on whole mouse brain levels of the biogenic amine neurotransmitters, acetylcholine (ACh) and related metabolites, i.e. the transmitter systems primarily disrupted in AD.

MATERIALS AND METHODS

Materials

5-Hydroxytryptamine (hydrochloride salt, 5HT·HCl), glutathione (GSH, free base), trifluoroacetic acid (TFA), catalase (bovine liver, EC 1.11.1.6), superoxide dismutase (bovine, suspension in 3.8 M ammonium sulfate solution, pH 7.0; EC 1.15.1.1), NADPH, diethylenetriaminepentaacetic acid (DPTA), mannitol, and ascorbic acid were obtained from Sigma (St. Louis, MO). All chemicals and enzymes were of the highest purity available and were used without any further purification. Compound **1** was prepared according to procedures described elsewhere [18]. It has not been possible using these procedures to obtain a pure, solid sample of Compound **1**. HPLC analysis of the sample employed revealed that it consisted of 40% of Compound **1** and 60% of 7,7′-bi-(5-hydroxytryptamine-4-one) (**7,7′-D**).

The procedure used for synthesis of Compound **2** was a modification of that described previously [22]. A solution of 150 μg of 5-HT·HCl in 35 mL of 0.01 M aqueous HCl (20 μM 5-HT) was electrochemically oxidized at pyrolytic graphite electrodes at +0.7 V for 20 min. Under these conditions 5-HT was almost quantitatively oxidized

to Compound **1**. Another 150 μg of 5-HT·HCl was then added to the solution in the working electrode compartment and the electro-oxidation was continued for another 20 min. This process was repeated five times so that the total concentration of Compound **1** formed was *ca.* 100 μM. The product solution containing Compound **1** was bright purple. In these controlled potential electrolysis experiments the counter electrode compartment of the electrochemical cell contained 0.01 M HCl. Following each 20-min period of electrolysis, the conversion of 5-HT to Compound **1** was determined by HPLC analysis (Method I). The solution containing *ca.* 100 μM Compound **1** (35 mL) was adjusted to pH 7.0 ± 0.1 by addition of 20% aqueous ammonium hydroxide solution. The reaction of Compound **1** with GSH to form Compound **2** and the procedures for purification and isolation of the latter compound have been described in detail elsewhere [22]. Phosphate buffers of known ionic strength were prepared according to Christian and Purdy [23].

Procedures and equipment

Voltammetry employed a pyrolytic graphite electrode (PGE; Pfizer Minerals, Pigments and Metals Division, Easton, PA) having an approximate surface area of 3 mm^2. The PGE was resurfaced before recording each voltammogram using established procedures [24]. Cyclic voltammetry employed a BAS model 100A Electrochemical Analyzer (Bioanalytical Systems, West Lafeyette, IN). All voltammograms were corrected for iR drop. Controlled potential electrolyses employed a Brinkmann Instruments (Westbury, NY) model LT73 potentiostat. A three-compartment electrochemical cell was used for controlled potential electrolyses in which the working, counter and reference electrode compartments were separated with a Nafion membrane (Type 117, DuPont Co., Wilmington, DE). The working electrode compartment had a capacity of 45 mL. The working electrode consisted of several plates of pyrolytic graphite having a total surface area of *ca.* 100 cm^2. The counter electrode was platinum gauze and the reference electrode a saturated calomel electrode (SCE; Fisher Scientific, Springfield, NJ). All controlled potential electrolyses were carried out with solutions that were stirred with a Teflon-coated magnetic stirring bar and with N_2 bubbling vigorously through the solution. Unless otherwise noted, potentials have been referred to the SCE at ambient temperature (22 ± 2°).

HPLC employed a Gilson (Middleton, WI) System 42 binary gradient system equipped with a Gilson model 112 UV detector set at 254 nm and a Rheodyne model 7125 loop injector. Analytical HPLC employed a reversed phase column (Phase Sep, Clwyd, U.K., Spheri S5 ODS2, 25 × 1.0 cm) protected by a short guard column (Brownlee Laboratories, Santa Clara, CA, RP-18, 5 mm ODGU, 4.0 × 0.5 cm). HPLC Method I employed the latter columns and two mobile phase solvents. Solvent A was prepared by adding 10.0 mL of 88% formic acid (HCOOH) and 20.0 mL of HPLC grade acetonitrile (MeCN) to 970 mL of deionized water. The pH of this solution was adjusted to 3.5 with concentrated ammonium hydroxide (NH_4OH). Solvent B was prepared by adding 10 mL of HCOOH and 400 mL of MeCN to 590 mL of water; the pH was adjusted to 3.5 with NH_4OH. The gradient profile was as follows: 0–5 min, 100% solvent A; 5–6 min, linear gradient to 5% solvent B; 6–25 min, linear gradient to 33% solvent B; 25–30 min, linear gradient to 100% solvent B; 30–39 min, 100% solvent B. The flow rate throughout this gradient was 4.0 mL/min.

Preparative HPLC employed a reversed phase column (J. T. Baker, Phillipsburg, NJ, Bakerbond RP-18, 10 mm, 25 × 2.1 cm) protected with a short guard column (5 × 1 cm) packed with the same material. HPLC Method II employed this preparative column and two mobile phase solvents. Solvent C was prepared by adding 20.0 mL of MeCN to 980 mL of water; the pH was then adjusted to 2.5 with concentrated TFA. Solvent D was prepared by adding 400 mL of MeCN to 600 mL of water; the pH was adjusted to 2.5 with TFA. The following gradient was employed: 0–5 min, 100% solvent C; 5–10 min, linear gradient to 15% solvent D; 10–12 min, 15% solvent D; 12–22 min, linear gradient to 35% solvent D; 22–25 min, 35% solvent D; 25–35 min, linear gradient to 60% solvent D. The flow rate throughout this program was 9.0 mL/min. HPLC Method III employed the preparative reversed phase column, solvents C and D but a modified gradient program. Thus, 0–40 min, linear gradient from 100% solvent C to 40% solvent D; 40–45 min, linear gradient to 100% solvent D. The flow rate was constant at 9.0 mL/min.

Initial rates of oxygen consumption 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 either a Varian XL-300 or Varian XL-500 spectrometer. Fast atom bombardment-mass spectrometry (FAB-MS) employed a VG Instruments (Manchester, U.K.) ZAB-E mass spectrometer. Ultraviolet-visible spectra were recorded on a Hewlett–Packard model 8452A diode array spectrophotometer using 0.5-cm pathlength quartz cuvettes.

Animals used were outbred adult male mice of the Hsd:ICR albino strain (Harlan Sprague–Dawley, Madison, WI) weighing 30 ± 5 g. Animals were housed 10 per cage, allowed access to Purina rat chow and water *ad lib.* and maintained on a 12-hr light/dark cycle with lights on at 7:00 a.m. Animals were never used in experiments until at least 7 days following receipt from the supplier. Compound **2** was dissolved in a vehicle consisting of isotonic saline (0.9% NaCl in deionized water) containing 1 mg/mL of ascorbic acid. Mice were first anesthetized with ether (55–60 sec). The injection volume was always 5 μL. Injections were made freehand, perpendicular to the scalp and were in the vicinity of the right lateral ventricle to a depth of 3 mm. The procedures employed were similar to those described elsewhere [25]. The LD_{50} value, used as a measure of toxicity and defined as the dose of injected Compound **2** at which 50% of the treated animals died within 1 hr, was determined by injecting doses ranging from 10 to 30 μg and using the statistical methods of Dixon [26, 27]. Control animals were treated with 5 μL of vehicle alone.

To assess neurotransmitter/metabolite effects, experimental animals were treated with 5 μL of vehicle containing 21 μg of Compound **2** (free base). After an appropriate time lapse (30 min to 1 week) animals were killed by exposure to 250 msec of 7.0 kW microwave radiation (NJE-2603-10 kW Microwave Irradiator, New Japan Radio Corp., Tokyo, Japan) concentrated on the head [28]. After sacrifice, the brain was rapidly removed from the skull cavity, weighed, and homogenized. The homogenization solution (1.00 mL/brain) was prepared to contain 0.5 M acetic acid, 0.5 M sodium acetate, 0.4 M $NaClO_4$, 4.70 nmol/mL guaiacol, and 40.0 nmol/L acetylthiocholine. The latter two compounds were used as internal standards in subsequent liquid chromatographic analyses. Homogenization was accomplished with a Kontes ground-glass Dual apparatus using 20 up/down strokes with the pestle attached to a Fisher Dynamix motor at a setting of 10. The homogenate was centrifuged using a Beckman L8-80 centrifuge (50,000 *g*) at 4° for 1 hr. The supernate was filtered through a 0.45 μm BAS polyacetate filter with the help of low-speed centrifugation and captured in a 1.5-mL polypropylene tube. The filtrate was stored at −80° in the polypropylene tube until analysis by HPLC with electrochemical detection. Aliquots (20.0 μL) of this filtrate were used to measure the concentrations of ACh and choline (Ch) in whole mouse brain using a modification of the HPLC method with electrochemical detection (LCEC) described by Eva *et al.* [29]. The concentrations of norepinephrine (NE), dopamine (DA), 5-HT and related metabolites in whole mouse brain were measured in 5.0-μL aliquots of the filtrate using a modification of the LCEC method of Lin *et al.* [30]. The exact experimental procedures used for the measurements of levels of ACh, Ch and the biogenic amines and their metabolites have been described in detail recently [31].

To determine whether Compound **2** was formed from Compound **1** *in vivo*, experimental animals were treated with 5 μL of a solution containing 97 μg of Compound **1** (contaminated with 145 μg of **7,7'D**) [18] dissolved in isotonic saline. Injections were made into the vicinity of the right lateral ventricle as described previously. Control animals received 5 μL of vehicle alone. After 15 min animals were decapitated with a guillotine and the brain was rapidly removed. The brain was weighed and rapidly homogenized (1 mg of brain tissue/5 μL of the homogenization medium) using a Polytron at a setting of 5 for approximately 20 sec. The homogenization solution was prepared by dissolving 13.8 g sodium dihydrogen phosphate, 372 mg of the disodium salt of EDTA, 216 mg of sodium octyl sulfate and 0.22 mg of N_ω-methyl-5-hydroxytryptamine (internal standard) in 700 mL of deionized water. The pH of the resulting solution was then adjusted to 2.99 with a saturated solution of citric acid. Then, 300 mL of HPLC grade acetonitrile was added and the solution was stirred thoroughly before use. The brain homogenate was then centrifuged at 4° and 50,000 *g* for 45 min. The supernatant was filtered through a 0.2-μm filter (BAS; MF-5658) using low-speed centrifugation. The filtrate was captured in a 1.5-mL polypropylene tube and stored at −80° until analysis. The filtrate was analyzed using an isocratic HPLC method with electrochemical detection. This was accomplished with a BAS 480 chromatographic system consisting of a PM-80 pump and a single channel LC4C electrochemical detector equipped with a glassy carbon electrode set at 0.90 V versus a Ag/AgCl reference electrode. A reversed phase column was used (BAS, Phase-II ODS, 3 μm, 100 × 3.2 mm) protected by a short guard column (BAS, Phase-II, ODS, 7 mm, 15 × 3.2 mm) The mobile phase was prepared by dissolving 1.7 mL diethylamine, 37.22 mg EDTA (disodium salt), 118.4 mg octadecyl sulfate (sodium salt) and 42 g citric acid in 1865 mL of deionized water. The pH of the resulting solution was then adjusted to 2.30 with 10 M sodium hydroxide solution. This solution was then filtered through a 0.45 μM Millipore HA filter. Then, 150 mL of HPLC grade MeCN was added to the filtrate. The resulting solution was mixed thoroughly and degassed under vacuum for 30 min. The flow rate of this mobile phase was 0.6 mL/min and the electrochemical detector was set at 20 nA full scale. Normally, the reversed phase column was equilibrated for 24 hr under the latter conditions before analysis of brain homogenates was performed. Compound **1** eluted at a retention time (t_R) of 8.3 min; t_R for Compound **2** was 24.7 min. Experiments with pure examples of Compound **2** indicated that the detection limit for this compound was *ca.* 50 ng in 5 μL of homogenization solution. Aliquots (5 μL) of the filtered brain homogenates of both experimental and control analyses were analyzed.

Isolation and structural characterization of the reaction products formed between Compound **2** *and excess GSH*

The procedures employed to synthesize, purify, and isolate Compounds **4**, **4R**, **6** and **7**, which result from the reactions between Compound **2** and molar excesses of GSH, are described below. Full chemical names have been given for each compound. For simplicity, however, assignments of proton resonances in ^1H NMR spectra employed the atom numbering systems shown in subsequent reaction schemes.

Glycine, N-[3-(2-*aminoethyl*)-1,4-*dihydro*-6-*hydroxy*-7-*mercapto*-4-*oxo*-5H-*indol*-5-*ylidene*]-L-γ-*glutamyl*-L-*cysteinyl*-,(1→2')-*sulfide with* L-γ-*glutamyl*-L-*cysteinylglycine* (*Compound* **4**) *and glycine,* N-[3-(2-*aminoethyl*)-4,6-*dihydroxy*-7-*mercapto*-1H-*indol*-5-*yl*]-L-γ-*glutamyl*-L-*cysteinyl*-, (1→2')-*sulfide with* L-γ-*glutamyl*-L-*cysteinylglycine* (*Compound* **4R**). A *ca.* 0.3 mM solution of Compound **1** (40 mL in 0.01 M HCl) was prepared by repetitive electro-oxidations of 5-HT as described previously. The pH of this solution was adjusted to 7.2 by careful addition of 20% aqueous ammonium hydroxide. GSH (74 mg; 0.24 mmol; 6.0 mM) was then added to the stirred solution. The pH of the resulting solution (*ca.* 4.6) was adjusted to 5.8 ± 0.2 with 20% ammonium hydroxide. At higher pH values, the yield of Compound **4** became markedly smaller. The reaction solution was stirred for 5 hr at

room temperature after which time repetitive 10-mL injections into the preparative HPLC system using Method IV were employed to separate Compound **4** which eluted at t_R = 25.0 min. The collected eluent containing Compound **4** was freeze-dried. The resulting solid was dissolved in 6 mL of deionized water and was purified by repetitive injection of 2-mL aliquots using HPLC Method III. The eluent at t_R = 25.0 min was collected and freeze-dried to give a deep blue solid. Compound **4** was quite stable in the HPLC mobile phase (pH 2.5) and exhibited a characteristic spectrum with λ_{max} = 584, 359, 280 nm. However, in pH 7.4 phosphate buffer Compound **4** transformed quantitatively into Compound **5** over the course of about 1 hr (see later discussion). FAB-MS on Compound **4** (thioglycerol/glycerol matrix) gave *m/e* = 825 ($MH_2 \cdot Na^+$, 2.5%), 803 ($MH_2 \cdot H^+$, 12%). Accurate mass measurements on the pseudo-molecular ion of the reduced form of Compound **4** ($MH_2 \cdot H^+$) gave *m/e* = 803.2333 ($C_{30}H_{43}N_8O_{14}S_2$; calcd. *m/e* = 803.2340). The ease of electrochemical reduction of Compound **4** (see later discussion) was in accord with the observation that the FAB-MS spectrum corresponded to the reduced form of this compound. Thus, Compound **4** had a molar mass of 800 g and a molecular formula $C_{30}H_{40}N_8O_{14}S_2$. This indicated that Compound **4** consisted of one indolic residue and two GSH residues. ^{1}H NMR (D_2O) δ 6.94(s, 1H, C(2)-H), 4.57(dd, J = 14.2 Hz, J = 9.1 Hz, 1H, C(d')-H), 4.55(dd, J = 14.2 Hz, J = 8.6 Hz, 1H, C(d)-H), 3.82(s, 2H, C(f')-H_2), 3.80(m, 1H, C(a)-H), 3.78(s, 2H, C(f')-H_2), 3.74(m, 1H, C(a')-H), 3.71(m, 1H, C(e_1')-H), 3.45(dd, J = 8.6 Hz, J = 14.2 Hz, C(e_2')-H), 3.39(m, 1H, C(e_1)-H), 3.27–3.18(m, 3H, C(β)-H_2 and C(e_2)-H), 3.02(t, J = 7.2 Hz, 2H, C(α)-H_2), 2.52(t, J = 7.4 Hz, 2H, C(c)-H_2), 2.42(m, 2H, C(c')-H_2), 2.10(m, 2H, C(b)-H_2), 2.00(m, 2H, C(b')-H_2). Homonuclear decoupling and two-dimensional (2D) correlated spectroscopy (COSY) experiments revealed couplings between the following resonances (δ, ppm): 3.71(C(e_1')) with 3.45(C(e_2')-H); 3.71(C(e_1')-H) and 3.45(C(e_2')-H) with 4.57(C(d')-H); 3.39(C(e_1-H) with 3.27–3.18(C(e_2)-H); 3.39(C(e_1)-H) and 3.27–3.18(C(e_2)-H) with 4.55(C(d)-H); 3.80(C(a)-H) and 2.52(C(c)-H_2) with 2.10(C(b)-H_2); 3.18–3.27(C(β)-H_2) with 3.02(C(α)-H_2); 3.74(C(a')-H) and 2.42(C(c')-H_2) with 2.00(C(b')-H_2).

When a much larger excess of GSH (370 mg, 1.20 mmol, 30 mM) was added to Compound **1** (40 mL of 0.3 mM in 0.01 M HCl), then after stirring for 5 hr an additional major product peak at t_R = 20.6 min was observed in preparative HPLC (Method IV). The compound eluting under this peak was collected and freeze-dried to give a white solid. The UV-visible spectrum of this product dissolved in the HPLC mobile phase (pH 2.5) showed λ_{max} = 336 and 298 nm. A cyclic voltammogram of this compound dissolved in the HPLC mobile phase exhibited an oxidation peak at peak potential (E_p) = +120 mV and, after scan reversal, a reversible reduction peak at E_p = +110 mV. This cyclic voltammogram was identical to that of Compound **4**. When exposed to the atmosphere, the colorless solution of this product gradually turned blue. The spectrum and HPLC t_R value of the product of this auto-oxidation were identical to those of Compound **4**. Accordingly, the second product formed by reaction of Compound **2** with a very large molar excess of GSH was the reduced form of Compound **4**, i.e. Compound $\mathbf{4}_R$. ^{1}H NMR (D_2O) δ 7.23(s, 1H, C(2)-H), 4.28(dd, J = 4.5 Hz, J = 4.5 Hz, 1H, C(d)-H), 4.13(dd, J = 4.2 Hz, J = 4.2 Hz, 1H, C(d')-H), 3.72(m, 2H, C(a)-H and C(a')-H), 3.64(s, 2H, C(f)-H_2), 3.63(s, 2H, C(f')-H_2), 3.41–3.18(m, 8H, C(β)-H_2, C(e)-H_2, C(e')-H_2, C(e')-H_2, C(α)-H_2), 2.42(t, J = 7.2 Hz, 2H, C(c)-H_2), 2.33(m, 2H, C(c')-H_2), 2.08–1.98(m, 4H, C(b)-H_2 and C(b')-H_2). These assignments were based upon homonuclear decoupling and 2D COSY experiments. The single aromatic proton resonances in the spectra of Compounds **4** and **4R** occurred as singlets at δ 6.94 and 7.23, respectively. In the case of Compound **2**, two aromatic proton resonances appeared as singlets at δ 6.80(C(2)-H) and 5.82(C(6)-H). Accordingly, the single aromatic proton signal observed in the spectra of Compounds **4** and **4R** corresponded to C(2)-H.

6,7-*Di*-(S-*Glutathionyl*)-*tryptamine*-4,5-*dione* (*Compound* **6**). A 0.3 mM solution of Compound **1** (40 mL in 0.01 HCl) was adjusted to pH 7.1 ± 0.1 with pH 7.3 phosphate buffer (ionic strength (μ) = 1.0). Then 74 mg of GSH (0.24 mmol, 6.0 mM) were added to the stirred solution, which was maintained for 14–18 hr at room temperature. Repetitive 10-mL injections of the resulting solution into the preparative HPLC system using Method III were made. Compound **6** eluted under the chromatographic peak at t_R = 35 min, which was collected and freeze-dried to give a purple solid. In pH 7.4 phosphate buffer, Compound **6** exhibited λ_{max} = 530, 343 sh, 314 and 244 nm. FAB-MS (thioglycerol/glycerol/Me_2SO matrix) gave *m/e* = 801.2154(MH^+, 30%; $C_{30}H_{41}N_8O_{14}S_2$; calcd. *m/e* = 801.2184). Accordingly, Compound **6** had a molar mass of 800 g and a molecular formula $C_{38}H_{40}N_8O_{14}S_2$. ^{1}H NMR (D_2O) δ 6.92(s, 1H, C(2)-H), 4.69–4.57(m, 2H, C(d)-H and C(d')-H), 3.91(t, J = 4.5 Hz, 1H, C(a)-H), 3.90(s, 2H, C(f)-H_2), 3.78(s, 2H, C(f')-H_2), 3.77(t, J = 6.3 Hz, 1H, C(a')-H), 3.23(m, 2H, C(β)-H_2), 3.21–3.10(m, 2H, C(e_1)-H and C(e_1')-H), 3.02(m, 2H, C(α)-H_2), 2.91–2.83(m, 2H, C(e_2)-H and C(e_2')-H), 2.52–2.46(m, 4H, C(c)-H_2 and C(c')-H_2), 2.17–2.10(m, 4H, C(b)-H_2 and C(b')-H_2). Homonuclear decoupling and COSY experiments confirmed the latter assignments. ^{13}C NMR (D_2O) showed 10 resonances corresponding to carbonyl carbons at δ 183.37, 182.81, 179.67, 179.26, 179.18, 177.75, 177.65, 177.14, 177.05, and 176.94, six aromatic carbon resonances at δ 154.72, 138.88, 134.14, 127.96, 124.90 and 123.33, and fourteen aliphatic carbon resonances at δ 57.94, 57.90, 57.08, 56.82, 45.84, 43.71, 43.24, 42.86, 35.87, 35.61, 30.66, 30.64, 30.38 and 27.88.

3*a*,7-*di*-(S-*Glutathionyl*)-*tryptamine*-4,5-*dione* (*Compound* **7**). A 0.1 mM solution of Compound **1** (35 mL in 0.01 M HCl) was adjusted to pH 7.0 ± 0.2 by addition of 20% ammonium hydroxide. GSH (42 mg, 0.13 mmol, 3.9 mM) was added to the resulting solution, which was then stirred for 1–1.5 hr at room temperature. Following filtration (0.45 μm Type HA filter, Millipore, Bedford, MA),

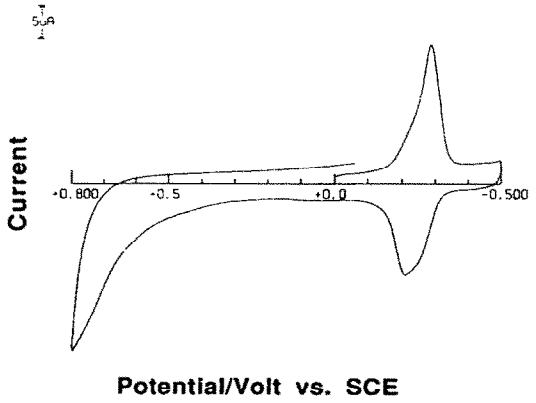


Fig. 1. Cyclic voltammogram at the PGE of 0.1 mM 7-*S*-glutathionyl-tryptamine-4,5-dione (Compound **2**) in pH 7.4 phosphate buffer ($\mu = 0.1$). Sweep rate: 200 mV sec^{-1}. [Oxidation current downward; reduction current upward.]

the entire solution was pumped through an HPLC pump into the preparative HPLC system and, using method III, Compound **7** eluted at $t_R = 33.7$ min. After freeze-drying the combined eluent containing Compound **7** the resulting solid was dissolved in 10 mL of deionized water and further purified using HPLC Method III by repetitive 2.0-mL injections. Compound **7** eluted at $t_R = 42$ min. The orange solution eluted under this peak was freeze-dried to give an orange solid. In pH 7.4 phosphate buffer ($\mu = 0.1$), Compound **7** exhibited λ_{max}, nm (log ε_{max}, $M^{-1}cm^{-1}$), 502(3.56), 382(4.27), 260sh(4.01), 232(4.22), 204(4.50). FAB-MS on Compound **7** (thioglycerol/glycerol matrix) gave m/e = 823(MNa^+, 20%), 801(MH^+, 80%), 800(M^+, 100%). Accurate mass measurements on MH^+ gave m/e = 801.2246($C_{30}H_{41}N_8O_{14}S_2$; calcd. m/e = 801,2184). Thus, Compound **7** had a molar mass of 800 g, a molecular formula $C_{30}H_{40}N_8O_{14}S_2$ and, therefore, consisted of one residue of Compound **1** and two residues of GSH. ^1H NMR (D_2O) δ 6.85(s, 1H, C(2)-H), 6.55(s, 1H, C(6)-H), 4.89(dd, J = 8.9 Hz, J = 5.0 Hz, 1H, C(d)-H), 4.63(dd, J = 7.8 Hz, J = 4.8 Hz, 1H, C(d')-H), 3.95(s), 2H, C(f)-H_2), 3.77(s, 2H, C(f')-H_2), 3.75–3.71(m, 2H, C(a)-H and C(a')-H), 3.65–3.52(m, 4H, C(e)-H_2 and C(e')-H_2), 3.26(t, J = 6.6 Hz, 2H, C(β)-H_2), 3.04(t, J = 6.6 Hz, 2H, C(α)-H_2), 2.49(t, J = 7.3 Hz, 2H, C(c)-H_2), 2.42(t, J = 7.9 Hz, 2H, C(c')-H_2), 2.11–2.01(m, 4H, C(b)-H_2 and C(b')-H_2).

RESULTS

Redox properties of 7-S-*glutathionyl-tryptamine*-4,5-*dione (Compound* 2*)*

A cyclic voltammogram of Compound **2** at pH 7.4 (Fig. 1) showed a reduction peak at a peak potential ($E_{p\,Red}$) of −286 mV corresponding to the $2e$-$2H^+$ reduction of Compound **2** to 7-*S*-glutathionyl-tryptamine-4,5-diol (Compound **3**) [22]. Following scan reversal, a quasi-reversible oxidation peak at $E_{p_{ox}} = -210$ mV appeared corresponding to the $2e$–$2H^+$ oxidation of Compound **3** to Compound **2**. The $E^{o\prime}$ for the Compound **2**/Compound **3** couple at pH 7.4 determined from the relationship

$$E^{o\prime} = \frac{E_{p\,Red} + E_{p_{ox}}}{2}$$

was, therefore, −248 mV vs SCE (−6 mV vs NHE). The $E^{o\prime}$ values for cellular reductants have been estimated to be *ca.* −400 mV vs NHE at physiological pH [32] and, accordingly, Compound **2** was expected to be readily reducible to Compound **3** by such

Table 1. Effects of 7-*S*-glutathionyl-tryptamine-4,5-dione (Compound **2**) on the initial rate of oxygen consumption by physiological reductants*

Concn of Compound **2** (μM)	Reductant	Concn of reductant (mM)	Initial rate of oxygen consumption† (nmol O_2/min)
0	Glutathione	2.0	1.9 ± 0.1
10		2.0	6.3 ± 0.1
100		2.0	36.7 ± 0.8
0	Ascorbic acid	2.0	4.8 ± 0.1
100		2.9	1934 ± 35
0	NADPH	2.0	0.55 ± 0.02
100		2.0	73 ± 1
0	Cysteine	2.0	2.6 ± 0.2
100		2.0	14.2 ± 1.0
0	GSH + ascorbate	2.0 (all)	20.5 ± 1.3
100	NADPH + cysteine	2.0 (all)	970 ± 25
0	Mouse brain homogenate‡		24 ± 4
100			650 ± 25

* In pH 7.4 phosphate buffer ($\mu = 0.1$) at 37°.

† Measurements were made with a Clark-type oxygen electrode assembly. Values are means ± SD for at least three replicate measurements.

‡ See Materials and Methods for method of preparation.

Table 2. Effects of catalase, superoxide dismutase, transition metal ions, complexing agents and hydroxyl radical scavengers on the initial rates of oxygen consumption by physiological reductants in the presence of 7-*S*-glutathionyl-tryptamine-4,5-dione (Compound **2**)

Redox cycling system*	Added compound	Initial rate of oxygen consumption† (nmol O_2/min)
Compound **2** (100 μM)/ GSH (2.0 mM)	0	36.7 ± 0.8
	Catalase (1 mg)	18.5 ± 0.1
	SOD (0.32 mg)‡	43.1 ± 1.2
	Denatured SOD (0.32 mg)	40.1 ± 0.6
	Fe^{3+} (1.0 μM)§	34.4 ± 1.6
	Fe^{3+} (100 μM)	37.7 ± 1.5
	Cu^{2+} (1.0 μM)‖	48.8 ± 1.0
	Cu^{2+} (100 μM)	55.0 ± 3.5
	DTPA (500 μM)	33.0 ± 0.8
	Ethanol (10 mM)	34.8 ± 1.0
	Mannitol (100 mM)	34.8 ± 1.4
	DMSO (100 mM)	34.0 ± 1.5
Compound **2** (100 μM)/ Ascorbic acid (2.0 mM)	0	1934 ± 35
	Catalase (1 mg)	1307 ± 41
	SOD (0.32 mg)‡	1733 ± 74
	Fe^{3+} (1.0 μM)	1909 ± 9
	Fe^{3+} (100 μM)	1987 ± 38
	Cu^{2+} (1.0 μM)	1993 ± 59
	Cu^{2+} (3.0 μM)	2285 ± 96
	DTPA (500 μM)	1883 ± 53
	Ethanol (100 mM)	1527 ± 198
	Mannitol (100 mM)	1711 ± 60
Compound **2** (100 μM) NADPH (2.0 μM)	0	73.0 ± 1.0
	Catalase (1 mg)	38.4 ± 1.5
	SOD (0.32 mg)‡	86.8 ± 1.3

Abbreviations: SOD, superoxide dismutase; DPTA, diethylenetriaminepentaacetic acid; and DMSO, dimethyl sulfoxide.

* Reactants were dissolved in 3.0 mL of air-saturated pH 7.4 phosphate buffer ($\mu = 0.2$) in the chamber of a Clark-type oxygen electrode assembly at 37°.

† Initial rates are given as the mean ($N \geq 3$) ± SD.

‡ One thousand units.

§ Added as $FeCl_3$.

‖ Added as $CuSO_4$.

agents. In an earlier report [22], it was demonstrated that Compound **3** was very rapidly oxidized to Compound **2** by molecular oxygen at physiological pH. Taken together, these observations suggested that Compound **2** should be capable of redox cycling reactions in the presence of intraneuronal reductants such as GSH, NADPH or ascorbic acid and molecular oxygen. This possibility was investigated by incubating Compound **2** with each of these reductants at pH 7.4 and monitoring the initial rate of oxygen consumption with a Clark-type oxygen electrode. In all such experiments, pH 7.4 phosphate buffer (μ = 0.1) was used. The volume of solutions contained in the oxyen electrode assembly chamber was 3.0 mL and the temperature was 37°. Incubations of Compound **2** (100 μM) with GSH, NADPH, cysteine or ascorbic acid (2.0 mM) resulted in considerable increases in the initial rates of oxygen consumption (Table 1). Compound **2** also greatly accelerated the rate of oxygen consumption by a mouse brain homogenate preparation (Table 1). Addition of catalase (50 μL of a 10 mg/mL solution in water) to a solution of Compound **2** (100 μM) and GSH (2.0 mM) contained in the reaction chamber of an oxygen electrode assembly after 5 min resulted in the liberation of 45 ± 4% of the consumed oxygen. The same amount of catalase added to a solution of Compound **2** (100 μM) and NADPH (2.0 mM) after 5 min resulted in the return of 39 ± 2% of the consumed oxygen. Catalase also resulted in the liberation of molecular oxygen when added a few minutes after the reaction between Compound **2** (100 μM) and ascorbic acid (2.0 mM) had been initiated. However, the rate of oxygen consumption in this system was so rapid (Table 1) that the rise in oxygen level evoked by catalase was only very brief. These observations, particularly with the Compound **2**/GSH and Compound **2**/NADPH systems, suggested that during the redox cycling reactions consumed oxygen was almost quantitatively converted to H_2O_2. This conclusion was confirmed by incubating catalase with the Compound **2**/GSH,

Compound 2/NADPH and Compound 2/ascorbic acid systems where it was observed that the initial rates of oxygen consumption decreased by about 50% compared to the rates measured in the absence of this enzyme (Table 2). Although Compound 2 catalyzed the rate of oxygen consumption by cysteine (Table 1), the effect was relatively modest. Furthermore, addition of catalase after the reaction had proceeded for several minutes did not result in the liberation of oxygen, i.e. either H_2O_2 was not produced as a byproduct or it was rapidly consumed in secondary reactions. HPLC analysis of the blue product solution revealed the presence of a mixture of unstable products that have not been characterized yet. In view of the low intraneuronal levels of cysteine [19, 20] and the complexity of the reactions between this amino acid and Compound 2, this system was not studied further.

Superoxide dismutase (SOD) had only a minor influence on the initial rate of oxygen consumption by the Compound 2/GSH, Compound 2/NADPH and Compound 2/ascorbate systems (Table 2), suggesting that superoxide radical anion ($O_2^{\cdot}$), even if formed, had no significant effects on redox cycling reactions. Added transition metal ions (Fe^{3+}, Cu^{2+}), the transition metal ion chelating agent DTPA [33], and the hydroxyl radical (HO·) scavengers ethanol, mannitol and dimethyl sulfoxide all had very little effect on the initial rate of oxygen consumption by the Compound 2/GSH and Compound 2/ascorbate systems (Table 2). Thus, although formation of HO· is likely as a result of well-known Fenton chemistry involving trace transition metal ions [34], which always contaminate the buffer system employed, and H_2O_2 formed as a byproduct, this radical appeared to play no significant role in the basic redox cycling processes.

Reactions between Compound 2 and glutathione

The studies described above indicated that in the presence of an excess of GSH and molecular oxygen Compound 2 redox cycled and formed H_2O_2 as the major byproduct. Incubation of Compound 2 (100 μM) and GSH (2.0 mM) in thoroughly deoxygenated pH 7.4 phosphate buffer resulted in the disappearance of its characteristic spectrum (λ_{max} = 544, 362 sh, 230 nm; Fig. 2A) and the appearance of the spectrum of Compound 3 (λ_{max} = 316, 272 nm; Fig. 2B) within a few minutes. Thus, Compound 2 was readily reduced to 3 by GSH. Figure 3 presents a series of HPLC chromatograms recorded at various times when a solution of Compound 2 (100 μM) and GSH (2.0 mM) was stirred at 37° and exposed to the atmosphere. Clearly, in addition to redox cycling processes, other chemical reactions occurred. Thus, after 15 min approximately 55% of Compound 2 had been consumed and several products were formed. After about 2 hr Compound 2 had virtually disappeared from the solution. Compound 4 was one of the initial products to be detected and increased in concentration for about 45 min after which it decreased and almost disappeared. After 2 hr the three major products were Compounds 5, 6 and 7. Methods were devised to optimize the formation of Compounds 4, 6 and 7 such that they could be isolated and structurally characterized by spectroscopic methods (see Materials and Methods). Compound 5 was not sufficiently stable to permit its isolation and structure determination. However, it was found that Compound 5 was formed from both Compound 2 and Compound 4, reactions that provided information bearing on the structure of this compound. For example, Fig. 4A shows the UV-visible spectrum of a freshly prepared solution of Compound 4 at pH 7.4. Within 1 hr the spectrum of Compound 4 was replaced by that of Compound 5 (Fig. 4C). HPLC analysis (Method 1) confirmed that Compound 4 was spontaneously transformed into Compound 5 at pH 7.4. Compound 5 was also formed from Compound 2. Thus, Fig. 5A shows a cyclic voltammogram of a freshly prepared solution of Compound 2 at pH 7.4. The quasi-reversible peaks at $E^{o\prime} = -248$ mV correspond to the Compound 2/Compound 3 couple. After 6 hr the peak currents for the latter couple had decreased markedly and a new couple appeared at $E^{o\prime} = -500$ mV (Fig. 5b). A freshly chromatographed solution of Compound 5 adjusted to pH 7.4 exhibited a reversible couple at $E^{o\prime} = -500$ mV. HPLC analyses also revealed that at pH 7.4 Compound 2 slowly reacted to give Compound 5 and at least two additional unstable products. In addition a dark, presumably polymeric, precipitate was formed. Accordingly, although Compound 5 could not be isolated in sufficient purity to permit the use of spectroscopic methods to elucidate its structure, it was concluded from the above observations that this compound was 7-*S*-glutathionyl-6-hydroxytryptamine-4,5-dione.

Cyclic voltammetry at pH 7.4 revealed that Compounds 4, 5 and 6 exhibited reversible couples at $E^{o\prime} = -510$, -500 and -570 mV vs SCE, respectively (Fig. 6). The reduction peaks in these voltammograms represent the $2e$-$2H^+$ reductions of the *ortho* quinone residues of Compounds 4, 5 and 6 to the corresponding dihydroxy forms and the oxidation peaks to the reverse reactions. Compound 7 also exhibited a reduction peak at $E_{p\,Red} = -510$ mV but, following scan reversal, quite complex electrochemistry appeared. Nevertheless, the electrochemical behaviors and redox potentials of Compounds 4, 5 and 6 suggest that these compounds would be expected to redox cycle with physiological reductants.

Preliminary biological studies

Compound 2 was the initial product formed by reactions of Compound 1 with GSH. Based upon the preceding chemical studies, it appeared that Compound 2 might have an appreciable intraneuronal lifetime before being converted into more structurally complex products. As a consequence, biological studies at this stage of the work were focussed on Compound 2.

The experimental LD_{50} for Compound 2 in the mouse (30 ± 5 g) was 21.2 ± 1.1 μg (mean ± SD). Doses of Compound 2 ranging from 10 to 30 μg (free base) administered in the vicinity of the right lateral ventricle were employed to determine this LD_{50} value.

The behavioral responses evoked by central administration of Compound 2 to mice, and described below, employed a dose of 21 μg. Behavioral effects

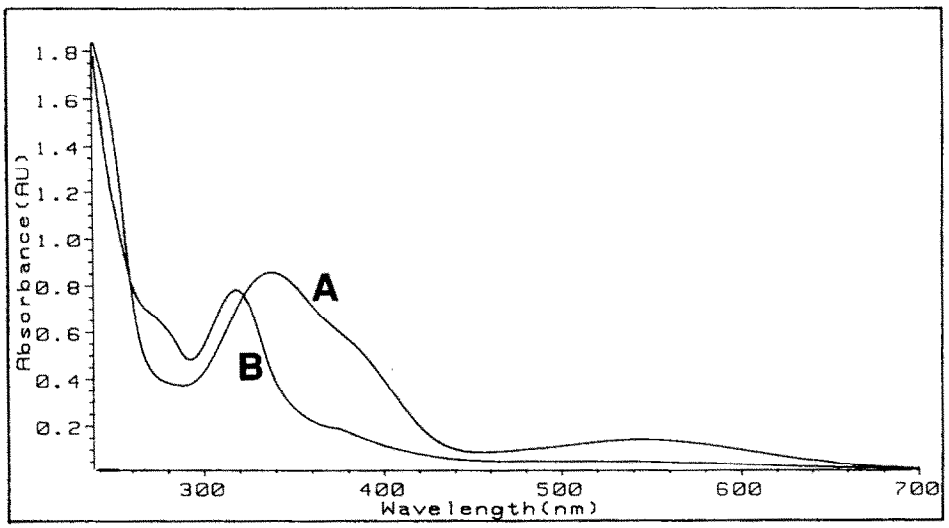


Fig. 2. UV-visible spectra of 0.1 mM 7-*S*-glutathionyl-tryptamine-4,5-dione (Compound **2**) and 2.0 mM glutathione in pH 7.4 phosphate buffer ($\mu = 0.1$) at room temperature (A) immediately upon preparation of the solution and (B) 15 min later.

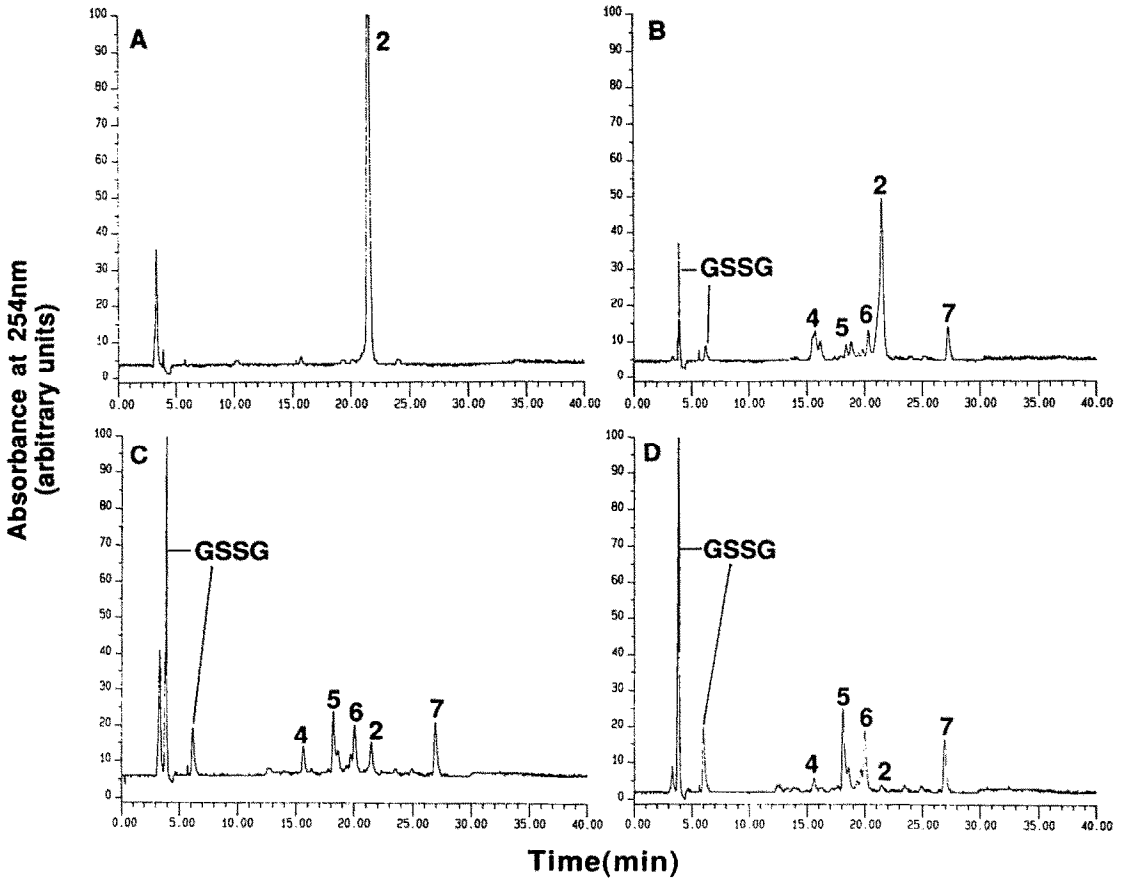


Fig. 3. HPLC chromatograms of a solution initially containing 0.1 mM Compound **2** and 2.0 mM GSH in pH 7.4 phosphate buffer ($\mu = 0.1$) at 37° exposed to the atmosphere with constant stirring (A) immediately after preparation, (B) after 15 min, (C) after 1 hr and (D) after 2 hr. HPLC Method I was employed. Injection volume: 2 mL.

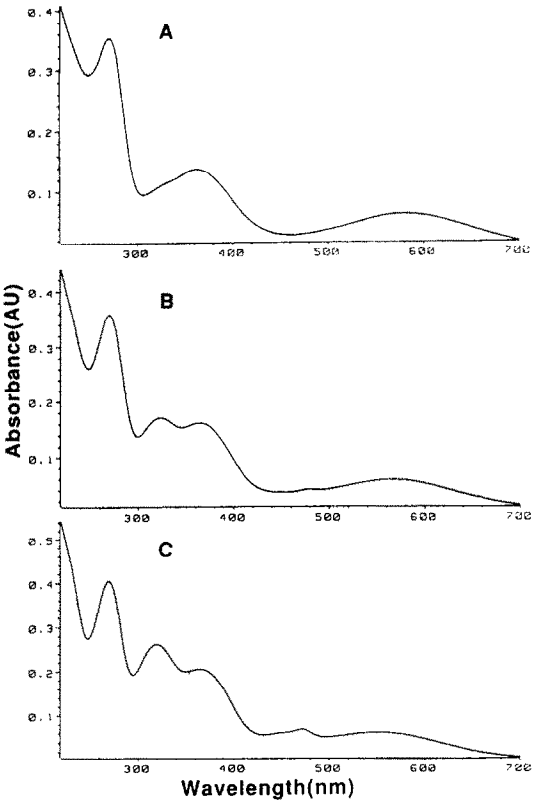


Fig. 4. UV-visible spectra of (A) a freshly prepared solution of Compound **4** in pH 7.4 phosphate buffer ($\mu = 0.1$), (B) the same solution after 10 min, and (C) after 1 hr.

occurred primarily between the time animals recovered from a light ether anesthesia (<5 min) and 30 min. At times greater than 1 hr after injection of Compound **2** no discernible behavioral effects different from controls were observed. Between *ca.* 5 and 30 min following injection of Compound **2**, the limbs of the animals were frequently extended. Animals periodically ran extremely rapidly, occasionally jumped vertically to a height of 3–4 in. and/or rapidly rolled over about the long axis of the body and tail completing 3–6 rotations in a single episode. Between these periods of hyperactivity animals lay on their side or back. Often animals would violently rub the side of their heads against the cage bars with resultant bleeding. The tails of the animals were often stiff and pointed upward. If death occurred, it did so normally during the 5–30 min period following administration of Compound **2**. Control animals treated with 5 μL of vehicle alone exhibited none of these behavioral responses and all survived.

Whole brain neurotransmitter/metabolite analyses

Levels of NE, DA, 5-HT, ACh and related metabolites in whole mouse brain were determined 30 min, 1, 2, 3, 4, 8, 1 day and 1 week following intracerebral injection of 21 μg of Compound **2** in 5 μL of vehicle. Controls were treated with 5 μL of vehicle alone. Measured whole brain levels of NE, DA, 5-HT, ACh and appropriate metabolites are shown in Table 3. The most striking neurochemical effects appeared 30 min after administration of Compound **2** and corresponded approximately to the period during which marked behavioral effects were observed. Thus, at 30 min all biogenic amine transmitter levels were reduced, whereas the levels of corresponding metabolites were elevated. These results suggested that Compound **2** evoked the release of NE, DA and 5-HT and that such release

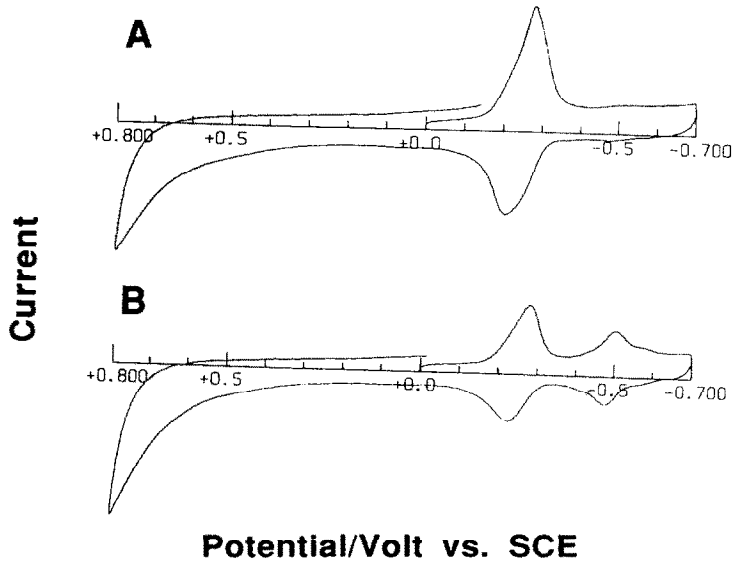


Fig. 5. Cyclic voltammograms at the PGE of (A) a freshly prepared solution of Compound **2** in pH 7.4 phosphate buffer ($\mu = 0.1$) at 37°, and (B) after stirring the solution for 6 hr. Sweep rate: 200 mV sec^{-1}.

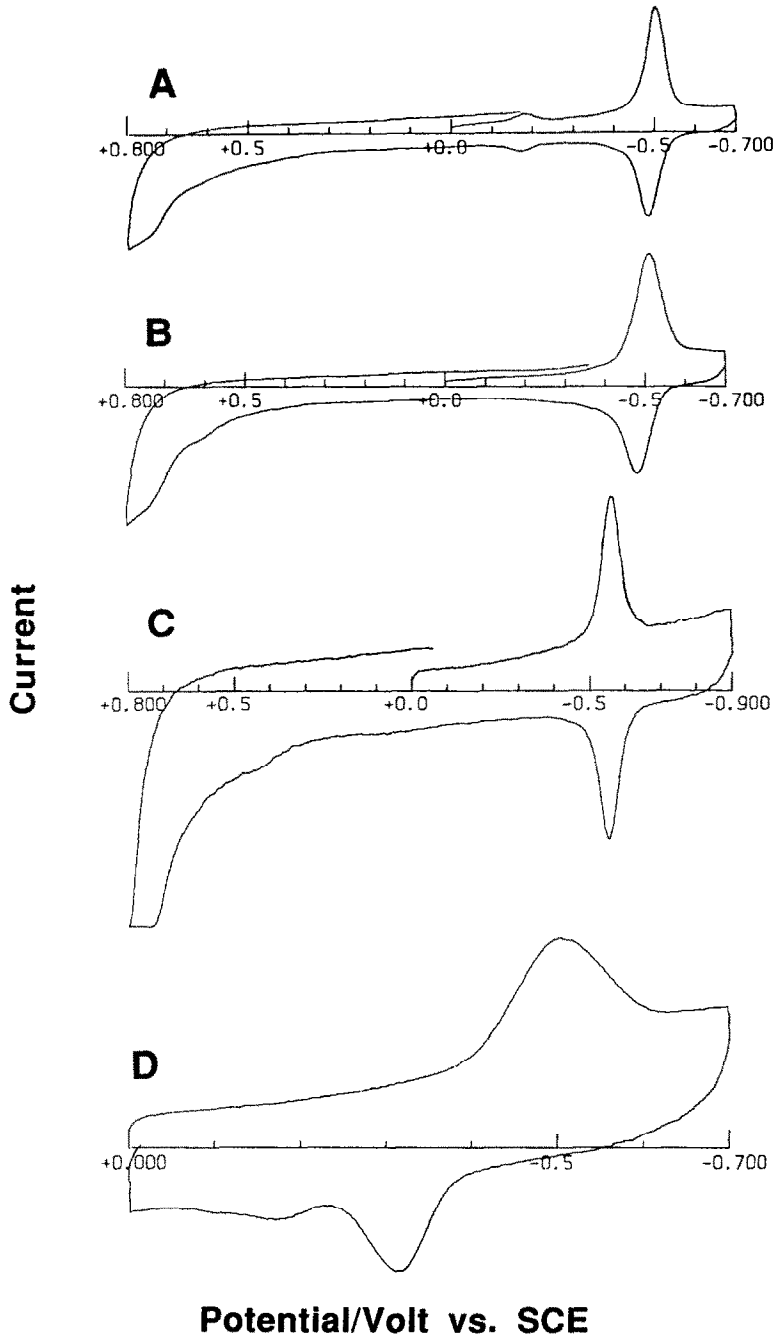


Fig. 6. Cyclic voltammograms at the PGE of (A) Compound **4**, (B) Compound **5**, (C) Compound **6**, and (D) Compound **7** in pH 7.4 phosphate buffer ($\mu = 0.1$). Sweep rate: 200 mV sec^{-1}.

was related to the observed hyperactivity. The whole brain levels of NE were reduced most profoundly after 30 min and corresponded to only 51% of control levels. One hour after drug administration of the DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) remained elevated and ACh levels were significantly above control levels. At longer times the level of many of the transmitters and metabolites exhibited considerable oscillations, although they appeared to return to control levels 1 week after treatment.

It has not yet been possible to chemically synthesize Compound **1** without significant contamination from **7,7-D** [18]. Accordingly, to determine whether Compound **1** was converted into Compound **2** *in vivo* in mouse brain, it was necessary to inject a mixture of Compound **1** (97 μg) and **7,7'-D** (145 μg) dissolved in 5 μL of isotonic saline. This was the

Table 3. Effects of central administration of 7-*S*-glutathionyl-tryptamine-4,5-dione (Compound **2**) on whole mouse brain neurotransmitter/metabolite levels

			Neurotransmitter/Metabolite levels (% of control)							
Time*	Group	N	NE	DA	DOPAC	HVA	5-HT	5-HIAA	ACh	Ch
30 min	Controls	6	100 ± 5	100 ± 3	100 ± 2	100 ± 4	100 ± 1	100 ± 2	ND¶	ND
	2	6	51 ± 6†	80 ± 11	135 ± 11‡	157 ± 14§	81 ± 7‖	115 ± 8	ND	ND
1 hr	Controls	12	100 ± 5	100 ± 5	100 ± 8	100 ± 4	100 ± 5	100 ± 6	100 ± 3	100 ± 5
	2	12	119 ± 11	101 ± 7	129 ± 9‖	149 ± 12§	98 ± 5	114 ± 6	126 ± 6§	116 ± 12
2 hr	Controls	6	100 ± 5	100 ± 2	100 ± 3	100 ± 10	100 ± 6	100 ± 11	100 ± 7	100 ± 5
	2	7	101 ± 9	87 ± 6	140 ± 19	96 ± 10	86 ± 6	78 ± 10	69 ± 10§	97 ± 5
3 hr	Controls	12	100 ± 5	100 ± 4	100 ± 6	100 ± 12	100 ± 2	100 ± 6	ND	ND
	2	11	89 ± 3‖	98 ± 7	96 ± 4	94 ± 9	92 ± 4	84 ± 6	ND	ND
4 hr	Controls	6	100 ± 9	100 ± 5	100 ± 26	100 ± 9	100 ± 3	100 ± 22	100 ± 4	100 ± 10
	2	6	122 ± 10	111 ± 6	68 ± 3	126 ± 9‖	116 ± 7‖	99 ± 8	105 ± 7	99 ± 10
8 hr	Controls	6	100 ± 17	100 ± 16	100 ± 17	100 ± 14	100 ± 12	100 ± 12	100 ± 16	100 ± 12
	2	6	82 ± 7	82 ± 4	80 ± 9	71 ± 5‖	79 ± 4	81 ± 7	80 ± 9	82 ± 6
1 day	Controls	12	100 ± 8	100 ± 4	100 ± 8	100 ± 7	100 ± 5	100 ± 10	100 ± 14	100 ± 3
	2	11	106 ± 14	128 ± 6§	102 ± 19	104 ± 5	114 ± 5‖	100 ± 13	118 ± 12	110 ± 13
1 week	Controls	12	100 ± 9	100 ± 8	100 ± 5	100 ± 7	100 ± 7	100 ± 13	100 ± 5	100 ± 9
	2	11	117 ± 5	111 ± 4	105 ± 11	96 ± 17	98 ± 5	92 ± 14	88 ± 2	138 ± 5

All animals were treated with 21 μg of 7-*S*-glutathionyl-tryptamine-4,5-dione (free base). After being lightly anesthetized with ether, the agent was delivered in 5 μL of isotonic saline solution containing 1 mg/mL ascorbic acid in the vicinity of the right lateral ventricle. Results are means ± SEM.

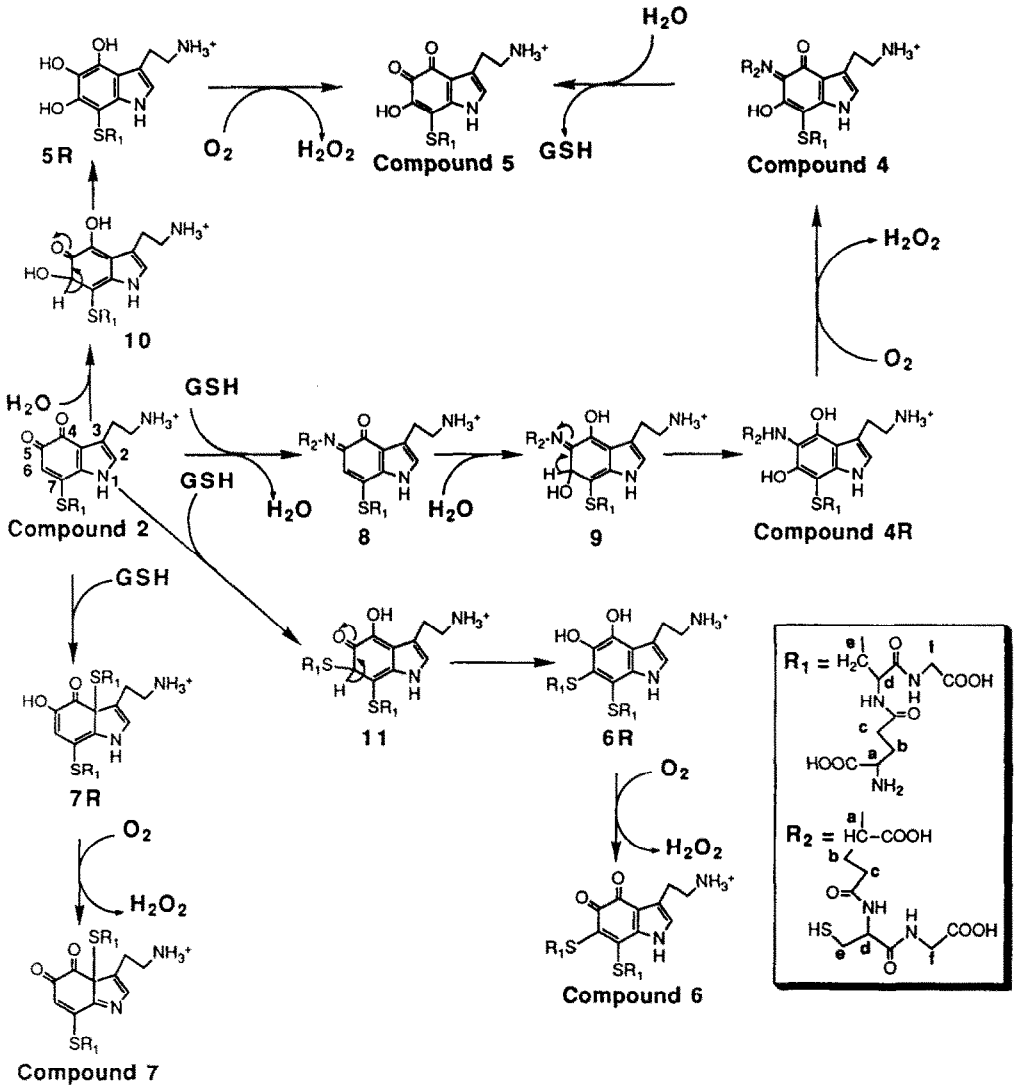
Typical concentrations (mean ± SEM) found in whole brain analyses for controls, expressed as nmol/g of wet tissue weight, were: NE, 2.33 ± 0.12; 3,4-dihydroxyphenylacetic acid (DOPAC), 0.82 ± 0.07; DA, 4.82 ± 0.19; 5-HIAA, 2.15 ± 0.26; homovanillic acid (HVA), 1.56 ± 0.06; 5-HT, 2.90 ± 0.20; ACh, 38.7 ± 1.2; and Ch, 57.2 ± 5.4.

* Time between treatment and sacrifice.

†–‖ Significantly different from control at: †$P < 0.001$, ‡$P < 0.01$, §$P < 0.005$, and ‖$P < 0.05$.

¶ ND, not determined.

Scheme I



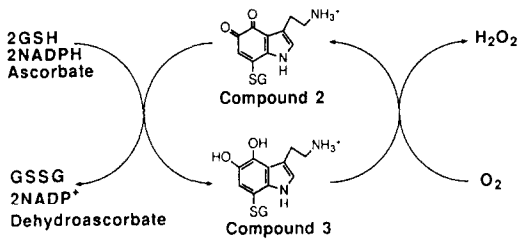
maximum dose of the mixture that mice could survive for *ca.* 15 min. Fifteen minutes following such an injection, animals were decapitated with a guillotine. Brains were rapidly removed, homogenized and filtered, and aliquots of the homogenate were analyzed by HPLC with electrochemical detection (see Materials and Methods). Chromatographic peaks corresponding to Compound **1** (t_R = 8.3 min) or Compound **2** (t_R = 24.7 min) were not observed in any of the experimental or control brain homogenate samples. Studies with standard samples of Compound **2** indicated that the detection limit for this drug was *ca.* 50 ng in 5 μL of the homogenization solution. Because of the relative insensitivity of this analytical method, it was not possible to definitively conclude that no Compound **2** was formed from Compound **1**. However, if Compound **2** was formed, it accounted for ≤10% of Compound **1** injected.

DISCUSSION

This study indicated that when Compound **2** was incubated with an excess of GSH at physiological pH two types of reaction occurred: redox cycling, leading to the formation of H_2O_2 and oxidized glutathione (GSSG) as byproducts, and further conjugation of Compound **2** by GSH. There appear to be at least three pathways by which GSH further conjugated Compound **2**. One of the major initial products of reaction between Compound **2** and GSH was Compound **4**, which also serves as a precursor of Compound **5**. A plausible reaction pathway leading to Compound **4** would be condensation between the primary amine residue of GSH to yield the Schiff base diglutathionyl conjugate (Compound **8**) which following nucleophilic attack by water yields Compound **4R** as conceptualized in Scheme

I. The latter was a very easily oxidized compound ($E^{o\prime}$ for the Compound **4**/Compound **4R** couple at pH 7.4 was −510 mV vs SCE). Thus, air oxidation of Compound **4R** yielded Compound **4**. It should be noted that the available spectroscopic evidence was unable to distinguish between a structure for Compound **4** in which the Schiff base residue was located at C(4)- or C(5) and hence the structures proposed for Compounds **4** and **4R** must be regarded as tentative. An unequivocal structure for Compound **4** could, in principle, be obtained using single cyrstal X-ray diffraction methods. Unfortunately, Compound **4** was not sufficiently stable to permit its crystallization. The Schiff base residue of Compound **4** was not very stable and could be hydrolytically cleaved to yield Compound **5** (Scheme I) [35, 36]. Compound **5** was formed relatively rapidly by hydrolysis of Compound **4** at physiological pH. An alternative but much slower pathway leading to Compound **5** involved nucleophilic attack by water on Compound **2** to give the 4,5,6-trihydroxytryptamine, Compound **5R**, which was rapidly air oxidized to Compound **5** ($E^{o\prime}$ for the Compound **5**/Compound **5R** couple at pH 7.4 was −510 mV vs SCE). Nucleophilic attack by the sulfhydryl residue of GSH at the C(6)- and C(3a)-positions of Compound **2** yielded Compounds **6R** and **7R**, respectively. The ease of air oxidation of the latter intermediates

Scheme II



led to the 6,7-di-*S*-glutathionyl and 3a,7-di-*S*-glutathionyl Compounds **6** and **7** (Scheme I).

Compound **2** exerted a significant catalytic effect on the reaction between molecular oxygen and intracellular reductants such as GSH, NADPH and ascorbic acid as a result of redox cycling processes. In these reactions Compound **2** was reduced to Compound **3** by these physiological reductants that were, correspondingly, oxidized (Scheme II). Molecular oxygen then oxidized Compound **3** back to Compound **2** with concomitant formation of H_2O_2 in almost quantitative yield. The reduction of Compound **2** to Compound **3** by GSH, NADPH or ascorbate might proceed in two one-electron steps via a radical intermediate. Similarly, oxidation of Compound **3** to Compound **2** by molecular oxygen might also proceed by two one-electron oxidations with resultant formation of $O_2^{\cdot}$. The available electrochemical and chemical evidence, however, does not permit this mechanistic nuance to be resolved. In the event that $O_2^{\cdot}$ was formed, it would very rapidly be disproportionate to H_2O_2 (Eq. 1) [37].

$$2O_2^{\cdot} + 2H^+ = H_2O_2 + O_2 \; K_{pH7} = 4 \times 10^{20} \quad (1)$$

Previous results [1–6] have indicated that not only are the levels of 5-HT reduced in the brains of AD patients but also that unknown oxidized forms of the transmitter are present in CSF [5, 6]. Based upon earlier results from this laboratory [12, 13], the *in vitro* oxidation products of 5-HT at physiological pH include tryptamine-4,5-dione (Compound **1**). In the event that Compound **1** was formed within serotonergic nerve terminals, which probably contain relatively high concentrations of GSH [19, 20], it might be expected to react at least in part to form Compound **2** [22]. The results reported here have demonstrated that centrally administered Compound **2** was toxic in laboratory mice and evoked hyperactivity for up to approximately 30 min after drug administration. During this hyperactive phase, Compound **2** caused whole brain levels of NE, 5-HT and perhaps DA to be decreased along with a significant elevation of the normal catecholamine metabolites (Table 3). The facile reactions of Compound **2** with GSH to form the diglutathionyl Compounds **4**, **4R**, **6** and **7** suggest that this conjugate might similarly react with intraneuronal protein nucleophiles.

Chromatographic analysis of homogenized mouse brains 15 min following injection of a rather large dose of Compound **1** revealed that neither Compound **1** nor Compound **2** was present at experimentally detectable levels. Because most brain GSH is probably stored within nerve terminals, axons and glia [20, 38] and hence not readily extraneuronally accessible, this result suggests that Compound **1** was rapidly and irreversibly bound to extraneuronal membrane components or other proteins. A similar result has been observed following injection of 6-hydroxydopamine (6-OHDA) into rat brain when only about 0.2% of the administered drug could be detected as its 2-*S*-glutathionyl conjugate [39]. Apparently, most 6-OHDA was covalently bound to membrane components [40].

In vitro, Compound **2** undergoes redox cycling reactions with typical intraneuronal reductants and molecular oxygen. Intraneuronal redox cycling of Compound **2** with GSH, ascorbate and NADPH would result in the rapid accumulation of H_2O_2 and, perhaps, intermediary formation of $O_2^{\cdot}$, with concomitant depletion of these physiological reductants. In the presence of trace concentrations of transition metal ions, H_2O_2 is known to decompose to yield the hydroxyl radical (HO·) by Fenton chemistry [34]. Similarly, transition metal ion catalyzed reactions between $O_2^{\cdot}$ and H_2O_2 yield HO· as a result of the Haber–Weiss reaction [41]. The hydroxyl radical is the most active of all reduced oxygen species and is responsible for cellular damage that accompanies many redox cycling reactions, particularly peroxidation of membrane lipids [42] and damage to proteins [43, 44]. Cytotoxicity deriving from redox cycling reactions of Compound **2** would depend upon intraneuronal generation of H_2O_2, $O_2^{\cdot}$, (and hence HO·) at rates which exceed the capacity of protective enzymes such as glutathione peroxidase and SOD to destroy these species. Whether the rates of oxygen consumption, and

hence H_2O_2 production, measured *in vitro* when Compound **2** was incubated with GSH, NADPH or ascorbate (Table 2) reflect *in vivo* rates that would be sufficiently rapid to overwhelm such protective mechanisms is presently unanswerable. However, Compound **2** clearly evoked rapid oxygen consumption in the presence of a brain homogenate preparation (Table 1). The redox potential of the Compound **2**/Compound **3** system at pH 7.4 is such that the reduction of Compound **2** should be catalyzed by a number of intraneuronal flavoprotein enzymes of low substrate specificity [42]. Such enzyme-mediated reductions of Compound **2** would be expected to greatly accelerate redox cycling reactions and thus the rate of production of H_2O_2 and hence HO·. Secondary consequences of redox cycling reactions of Compound **2** with physiological reductants would be depletion of these reductants and oxygen.

It is not yet known whether Compound **2** or other more complex species derived from this conjugate as a result of its reactions with GSH (i.e. Compounds **4R**, **4**, **5**, **6** and **7**) possess additional neuropharmacological properties that might contribute to the toxic and behavioral effects evoked by central administration of Compound **2** to laboratory mice. We hope to report on such properties in a future paper.

CONCLUSIONS

Previous investigators have provided evidence for the occurrence of unknown oxidized forms of 5-HT in the CSF of AD patients [5, 6]. At physiological pH, *in vitro* oxidations of 5-HT yield very complex mixtures of products that include tryptamine-4,5-dione (Compound **1**) [12, 13]. There have been claims that Compound **1** is a neurotoxin that in the rat damages some brain structures that are degenerated in human AD [16]. In the event that Compound **1** is formed as a result of aberrant oxidative transformation of 5-HT within serotonergic nerve terminals or axons, there can be little question that it would be a very short-lived species because of its facile reaction with available nucleophiles. Such nucleophiles could include both membrane and cytoplasmic proteins. Covalent binding of Compound **1** to such nucleophiles could account in part for its toxic properties. Another likely reaction of Compound **1** would be conjugation by intraneuronal GSH to yield Compound **2**. Results reported here indicate that Compound **2** is a potent behavioral toxin when directly administered into mouse brain evoking extreme levels of hyperactivity. Based on whole brain analyses, Compound **2** causes a rapid decrease of NE, 5-HT and DA with a concomitant rise in the levels of corresponding metabolites. Such neurochemical changes appeared to coincide with the behavioral hyperactivity evoked by Compound **2**. A possible explanation for these observations is that Compound **2** interacts with presynaptic receptors that control the release of the biogenic amine and, perhaps, other transmitters. Compound **2** also redox cycles in the presence of intraneuronal reductants and molecular oxygen and rapidly generates H_2O_2. Rapid intraneuronal accumulation of H_2O_2 and, perhaps $O_2^{\cdot}$, could overwhelm protective mechanisms and result in the formation of cytotoxic HO·. Compound **2** can also be further attacked by GSH to yield at least three diglutathionyl conjugates, i.e. Compounds **4**, **6** and **7**. The ability of GSH to further conjugate Compound **2** suggests that this compound might similarly alkylate key intraneuronal proteins. Both the intraneuronal generation of HO· and alkylation of protein nucleophiles could have neurodegenerative consequences.

The actual chemical identities of oxidized forms of 5-HT present in the CSF of AD remain to be determined. In the event that aberrant oxidations of 5-HT occur in the cytoplasm of certain serotonergic neurons in the Alzheimer brain and that these reactions mimic the *in vitro* oxidations of the transmitter, Compound **1** would be a likely product. Based on previous results [22] and those reported here, it is very unlikely that Compound **1** would exist in brain tissue for any significant period of time. Rather, it would be expected to react rapidly with protein nucleophiles or, in GSH-rich regions, it would readily form Compound **2**. Thus, it is not surprising that a previous search for Compound **1** as a marker of aberrant oxidation of 5-HT in the CSF of AD patients was not successful [14, 15]. Compound **2** is clearly a very toxic substance and if formed within serotonergic neurons could potentially cause cellular damage or death as a result of mechanisms that include redox cycling and alkylation of key protein nucleophiles. Release of Compound **2** might permit it to interact with other neuronal systems selectively affected in AD. In the presence of GSH, Compound **2** would react to give Compounds **4**–**7**. The toxicity, neuropharmacological properties and stability of Compounds **4**–**7** in the CNS remain to be determined. However, it is conceivable that these compounds may play some roles in the neuronal degeneration or other biochemical changes that characterize AD or serve as analytical markers for intraneuronal formation of Compound **1** in the Alzheimer brain.

Acknowledgements—This work was supported by National Institutes of Health Grant GM-32367. Additional support was provided by the Vice President for Research and Research Council at the University of Oklahoma.

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