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Tryptamine-4,5-dione: Properties and Reaction with Glutathione

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At physiological pH tryptamine-4,5-dione (**1**), which might be an aberrant metabolite of 5-hydroxytryptamine involved in the etiology of Alzheimer's disease, reacts rapidly with glutathione to give 7-*S*-(glutathionyl)-tryptamine-4,5-dione (**5**). Procedures have been devised to isolate **5** and to elucidate its molecular structure using FAB-MS and ^1H and ^{13}C NMR spectroscopy. The redox characteristics of **1** and **5** have been elucidated using electrochemical methods.

INTRODUCTION

More than 30 years ago it was first speculated that a defect in the metabolism of the indoleamine neurotransmitter 5-hydroxytryptamine (5-HT; serotonin) might in some way be related to various neurodegenerative, neuropsychiatric, and behavioral disorders (*1, 2*). Subsequently, such hypothetical faulty metabolic pathways have been implicated in the etiology of mental illnesses such as schizophrenia and depression (*3–6*). A recurring suggestion has been that 5-HT might be oxidatively converted to more highly hydroxylated indoleamines which are toxic in the central nervous system resulting in neuronal damage (*7, 8*). The subsequent discovery that 5,6- and 5,7-dihydroxytryptamine (5,6- and 5,7-DHT) (*9–11*) are powerful serotonergic neurotoxins provided some credence to this suggestion. Furthermore, it has long been known that 5-HT and indeed other endogenous 5-hydroxyindoles are oxidized in biological milieu, e.g., human serum and ceruloplasmin (*12–18*), although the mechanisms and products of such oxidations remain to be elucidated. Several lines of evidence, however, suggest that oxidation products of 5-HT have biological activity. For example, an unidentified product of autoxidation of 5-HT is a potent inhibitor of acetylcholinesterase (*19*). Recently, 5,6-DHT has been reported to be formed endogenously in rat brain after a single dose of methylamphetamine or *p*-chloroamphetamine (*20, 21*). Thus, the neurotoxic effects of these amphetamines may be mediated by altering the normal oxidative metabolism of 5-HT with resultant formation of a dihydroxyindoleamine neurotoxin.

Serotonergic abnormalities have been reported extensively in dementia of the Alzheimer type (*22–24*). Analysis of the cerebrospinal fluid (CSF) of Alzheimer patients by high-performance liquid chromatography with electrochemical detection (HPLC-EC) has detected an unknown electroactive species which is not

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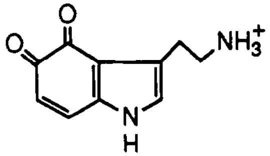
present in CSF of normal subjects (*25*). This compound has been speculated to be an oxidation product of 5-HT because it has similar chromatographic and electrochemical properties to *in vitro* electrochemically oxidized 5-HT (*26*). The electrochemical oxidation chemistry of 5-HT has recently been studied extensively (*27–30*). Under certain experimental conditions (i.e., low 5-HT concentration and high electrode potential) a major electrooxidation product is tryptamine-4,5-dione



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(**1**). This compound is also formed as a major product of oxidations of 5-HT in reactions mediated by various oxidative enzyme systems (ceruloplasmin/O_2, tyrosinase/O_2, peroxidase/H_2O_2) (*31*). Subsequent studies (*32*) have demonstrated that **1** has the ability to increase basal 5-HT release from rat hippocampal and striatal serotonergic terminals and, hence, appears to mimic the indolic neurotoxin 5,6-DHT (*33*). Compound **1** has also been found to bind sulfhydryl groups (*32*). It has been suggested that the interaction of **1** with glutathione (GSH) or proteins containing sulfhydryl groups might play a role in the mechanism by which **1** evokes 5-HT release (*32*).

In this report we describe the redox, chromatographic, and spectral properties of **1**. More importantly, the product formed by reaction of glutathione with **1** has been isolated and its structure elucidated. The redox characteristics of this adduct have been studied. These might provide some clues to the neurodegenerative properties of **1**.

EXPERIMENTAL

Chemicals

Chemicals and their sources were as follows: 5-HT (hydrochloride salt), GSH, *S*-methyl GSH, and trifluoroacetic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Formic acid (88%), ammonium hydroxide, and HPLC grade acetonitrile were obtained from Fisher Scientific (Springfield, NJ).

Apparatus

Cyclic voltammetry was carried out with a BAS-100A instrument (Bioanalytical Systems, West Lafayette, IN). All voltammograms were corrected for iR drop. A pyrolytic graphite electrode (PGE, Pfizer Minerals, Pigments and Metals Division, Easton, PA) having an approximate surface area of 3.2 mm^2 was employed for voltammetry. Procedures for preparing and resurfacing of the PGE have been described elsewhere (*34*). Controlled potential electrolyses employed a Brinkmann Instruments (Westbury, NY) Model LT73 potentiostat. A three compartment cell was used 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 (area ca. 66 cm^2). The counter electrode was platinum gauze and the reference electrode was a commercial (Fisher Scientific) saturated calomel electrode (SCE). All potentials presented in this report are referenced to the SCE at ambient temperature (22 ± 2°C). HPLC employed a Gilson (Middleton, WI) System 42 gradient system equipped with a Gilson Model 112 uv detector (254 nm) and a Rheodyne Model 7125 loop injector.

^1H and ^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer (300 MHz). Mass spectra were obtained with a VG Instruments (Manchester, UK) ZAB-E spectrometer. Ultraviolet–visible spectra were recorded on a Hewlett–Packard Model 8452A diode array spectrophotometer.

Electrochemical Synthesis of Tryptamine-4,5-dione (**1**)

A solution (35 ml) of 5-HT · HCl (ca. 0.1 mM) in 0.01 M HCl was electrolyzed at pyrolytic graphite electrodes at 0.74 V. The solution in the working electrode compartment was stirred with a Teflon-coated magnetic stirring bar and nitrogen was bubbled vigorously through the solution. The counter and reference electrode compartments contained 0.01 M HCl. Under such conditions complete electrooxidation of 5-HT required about 90 min. The yield of **1** was ≥95% as determined by HPLC analysis.

HPLC Separations

Analytical HPLC studies utilized a reversed-phase column (Brownlee Laboratories, Santa Clara, CA, RP-18, 5 μm, 25 × 0.46 cm) and a short guard column (Brownlee, RP-18, 5 μm, ODGU, 4.0 × 0.5 cm). A binary gradient mobile phase program was employed. Solvent A was prepared by adding 6.0 ml of 88% formic acid to 1 liter of 5% methanol in water. The pH of the resulting solution was adjusted to 7.0 ± 0.1 with concentrated ammonium hydroxide. Solvent B was prepared by adding 6.0 ml of 88% formic acid to 1 liter of 40% acetonitrile in water. The pH of this solution was adjusted to 7.0 ± 0.1 with concentrated ammonium hydroxide. The gradient profile employed was as follows: 0–5 min, 100% solvent A; 5–35 min, a linear gradient from 0 to 35% solvent B. A flow rate of 2 ml min^{-1} was employed.

For preparative HPLC a reversed-phase column (J. T. Baker, Phillipsburg, NJ, RP-18, 10 μm, 25 × 2.1 cm) and a short guard column (5 × 1 cm) packed with the same material were employed. A binary gradient mobile phase system was employed. Solvent C was 5% methanol in water adjusted to pH 2.5 ± 0.1 with concentrated trifluoroacetic acid. Solvent D was 40% acetonitrile in water adjusted to pH 2.5 ± 0.1 with concentrated trifluoroacetic acid. The gradient profile was as follows: 0–5 min, 100% solvent C; 5–10 min, linear gradient to 15% D; 10–12 min, 15% D; 12–22 min, linear gradient to 35% D; 22–25 min, 35% D; 25–35 min, linear gradient to 60% D. The flow rate was 9 ml min^{-1}.

Formation and Isolation of 7-S-(Glutathionyl)-tryptamine-4,5-dione (**5**)

The solution containing ca. 0.1 mM **1**, prepared by electrooxidation of 5-HT, was adjusted from pH 2.0 ± 0.1 to 7.0 ± 0.1 by careful addition of concentrated ammonium hydroxide. To the resulting solution was added ca. 3.5 ml of a freshly prepared solution of GSH (1 mM) in water; i.e., the molar ratio of **1** to GSH was 1 : 1. The solution of **1** at pH 7 was bright purple. Addition of glutathione caused a decrease in the intensity of the purple color. HPLC analysis revealed that only one major product (**5**) was formed having a retention time (t_R) of 19 min. This product was isolated by repetitively injecting 10-ml aliquots of the reaction solution onto a preparative HPLC column. Compound **5** eluted at t_R = 33.6 min (t_R for **1** was 28.0 min). The resulting bright purple solution of **5** was then freeze-dried to give a deep brown solid. HPLC analysis of the purple solution (pH 2.5) obtained following preparative chromatography and of the latter solid dissolved in water showed them to be identical to the initially formed product of the reaction of **1** and GSH.

RESULTS

Structure of **5**

The uv–visible spectrum of **5** at pH 7.4 showed, λ_{max}, nm (log ε_{max}, M^{-1} cm^{-1}): 544 (3.05), 370sh (3.70), 342 (3.82), 244 (4.03). Fast atom bombardment mass spectra (3-nitrobenzyl alcohol matrix) gave a cluster of three intense ions at m/e = 498 (82%), 497 (100%), and 496 (52%). Compound **5** is a very easily reducible compound (see Discussion) and under FAB–MS conditions not only the pseudomolecular ion (MH^+) at m/e = 496 is expected but also reduced species corresponding to MH_2^+ (m/e = 497) and MH_3^+ (m/e = 498) (*34–37*). Accurate mass measurements on MH^+ gave m/e = 496.1537 ($C_{20}H_{25}N_5O_8S$; calcd m/e = 496.1502).

The ^1H NMR spectrum of **5** in D_2O gave: δ 6.80 (s, 1H, C(2)—H), 5.88 (s, 1H, C(6)—H), 3.85 (s, 2H, C(f)—H), 3.71 (t, J = 6.9Hz, 1H, C(a)—H), 3.55 (m, 1H, C(d)—H), 3.35 (m, 2H, C(e)—H), 3.16 (t, J = 6.9Hz, 2H, C(β)—H), 2.89 (t, J = 6.9Hz, 2H, C(α)—H), 2.38 (t, J = 7.5Hz, 2H, C(c)—H), 2.17 (dt, J = 6.9Hz, J = 7.5Hz, 2H, C(b)—H). Assignments of the various resonances in the spectrum of **5** were based upon homonuclear decoupling experiments and comparisons with the ^1H NMR spectra (in D_2O) of GSH, **1** (*32*), and tryptophol-4,5-dione, a structurally related compound which can be isolated in the pure, solid form (*35*). Compund **5** is insoluble in, for example, deuterated dimethyl sulfoxide, dimethylformamide, chloroform, and carbon tetrachloride and hence it was not possible to obtain ^1H NMR results in a nonexchanging solvent. Fully coupled ^{13}C NMR (D_2O) δ, ppm: 184.03 (s, C=O), 179.64 (s, C=O), 179.34 (s, C=O), 178.70 (s, C=O), 178.12 (s, C=O), 175.76 (s, C=O), 156.03 (s, C—7), 139.82 (s, C—7′), 128.63 (d, C—2), 122.91 (s, C—4′), 119.08 (d, C—6), 112.23 (s, C—3), 58.27 (d, C—a), 55.98 (d, C—d), 46.30 (t, C—f), 43.47 (t, C—e), 36.75 (t, C—β), 35.92 (t, C—α), 30.57 (t, C—c), 28.06 (t, C—b). FAB-MS data indicate that **5** has a molar mass of 495 g and an elemental composition $C_{20}H_{25}N_5O_8S$ in complete accord with the ^{13}C NMR results. Thus, **5** must consist of one residue each of **1** and GSH. The ^{1}H NMR spectrum of **1** exhibits two doublets at 7.08 ppm (C(7)—H) and 5.77 ppm (C(6)—H). The upfield location of the latter resonance is a result of the adjacent carbonyl group at C(5). A similar upfield shift has been observed for the α proton of many similar *o*-diketones such as tryptophol-4,5-dione (*35*) and 1,2-naphthoquinone (*38*). The ^{1}H NMR spectrum of **5** shows only a singlet at 5.88 ppm indicating that the proton originally at C(7) has been replaced. Accordingly, the GSH residue must be attached to C(7) of the **1** residue. ^{13}C NMR data also indicate that following reaction of GSH with **1** the resulting product, **5**, contains two carbonyl groups associated with the indolic residue; i.e., the tryptamine-4,5-dione residue remains. *S*-Methylglutathione did not react with **1** under the conditions described previously. This indicates that it is the sulfhydryl residue of the GSH which attacks **1**. The ^{1}H NMR spectrum of **5** shows that the C(e)—H_2 resonance (3.35 ppm) is shifted downfield compared to the same residue (2.92 ppm) in GSH hence providing additional support for the proposed structure of **5**.

Spectral and Electrochemical Properties of **1** *and* **5**

Because of its tendency to dimerize and form more complex oligomers when concentrated it is not possible to isolate **1** in the pure, solid state (*28*). Using preparative HPLC techniques it is possible, however, to obtain an approximately 0.1 mM solution of **1**. A cyclic voltammogram (CV) of such a solution adjusted to pH 7.4 with a 0.5 M solution of 3-[*N*-morpholino]propanesulfonic acid (Mops) is shown in Fig. 1. This CV shows that **1** exhibits a voltammetric reduction peak at a peak potential (E_p) of -0.27 V. After scan reversal a quasi-reversible peak appears at $E_p = -0.210$ V.

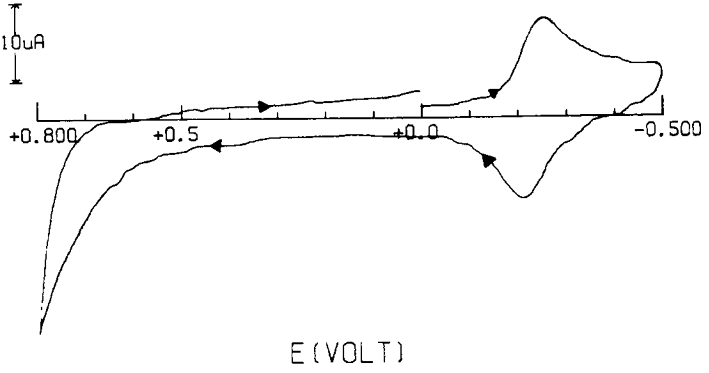


FIG. 1. Cyclic voltammogram at the PGE of ca. 0.1 mM tryptamine-4,5-dione (**1**) in pH 7.4 Mops buffer containing 22% acetonitrile. Sweep rate: 200 mV s^{-1}.

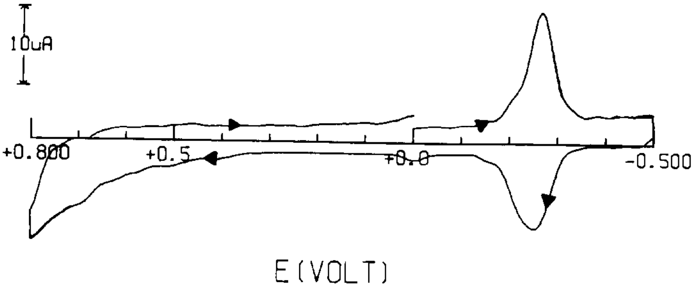


FIG. 2. Cyclic voltammogram at the PGE of 0.1 mM 7-*S*-(glutathionyl)-tryptamine-4,5-dione (**5**) in pH 7.40 phosphate buffer (μ = 0.1). Sweep rate: 200 mV s^{-1}.

Controlled potential electroreduction of **1** dissolved in the preparative HPLC mobile phase adjusted to pH 7.4 with 0.5 M Mops buffer at -0.4 V with N_2 bubbling vigorously through the solution caused its characteristic spectrum (λ_{max} = 542, 352, and 236 nm) to disappear. Coulometric measurements showed that 2 ± 0.2 electrons per molecule of **1** reduced were transferred. The resulting solution was colorless (λ_{max} = 300 and 218 nm). The CV of this solution was qualitatively identical to that shown in Fig. 1. These results indicate that electrochemical reduction of **1** forms 4,5-dihydroxytryptamine (4,5-DHT). If the solution containing the latter compound was exposed to air it rapidly became purple; HPLC and spectral analysis confirmed that **1** was formed. However, only about 70% of the initial **1** was regenerated following these reduction and oxidation steps. It was noted that in a pH 7.4 phosphate buffer (μ = 0.1) **1** was considerably less stable than in a Mops buffer at the same pH. Furthermore, electrochemical reduction of **1** in phosphate buffer did not yield 4,5-DHT but rather the solution turned blue and a considerable amount of black precipitate was formed. The chemistry associated with these observations in phosphate buffer remains to be elucidated.

A CV of **5** at pH 7.4 (Fig. 2) shows a reduction peak at $E_p = -0.272$ V and, following scan reversal, a quasi-reversible oxidation peak at $E_p = -0.226$ V. Controlled potential electroreduction of **5** caused its uv–visible spectrum (λ_{max} at pH 7.4: 544, 370sh, 342, 244 nm; Fig. 3) to disappear and, following transfer of 1.7 ± 0.3 electrons per molecule, the resultant spectrum (λ_{max}: 318, 272 nm; Fig. 3) was that of 7-*S*-(glutathionyl)-4,5-dihydroxytryptamine (**4**). A CV of the solution containing **4** was qualitatively identical to that of **5**. Compound **4** could be oxidized back to **5** in about 90% yield either electrochemically (at a potential ≥ -0.10 V) or by exposure of the solution to air. Unlike **1**, **5** was quite stable in pH 7.4 phosphate buffer and could be smoothly electroreduced to **4**.

DISCUSSION

The structure of 7-*S*-(glutathionyl)-tryptamine-4,5-dione (**5**) has been established using FAB–MS and ^{1}H and ^{13}C NMR spectroscopy. A probable reaction pathway by which the nucleophile GSH attacks electrohilic dione **1** to give **5** is presented in Scheme I.

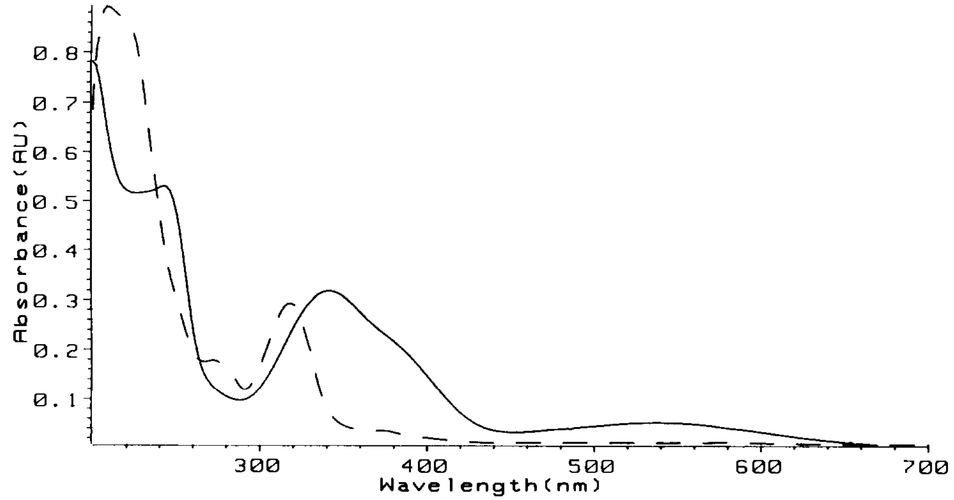
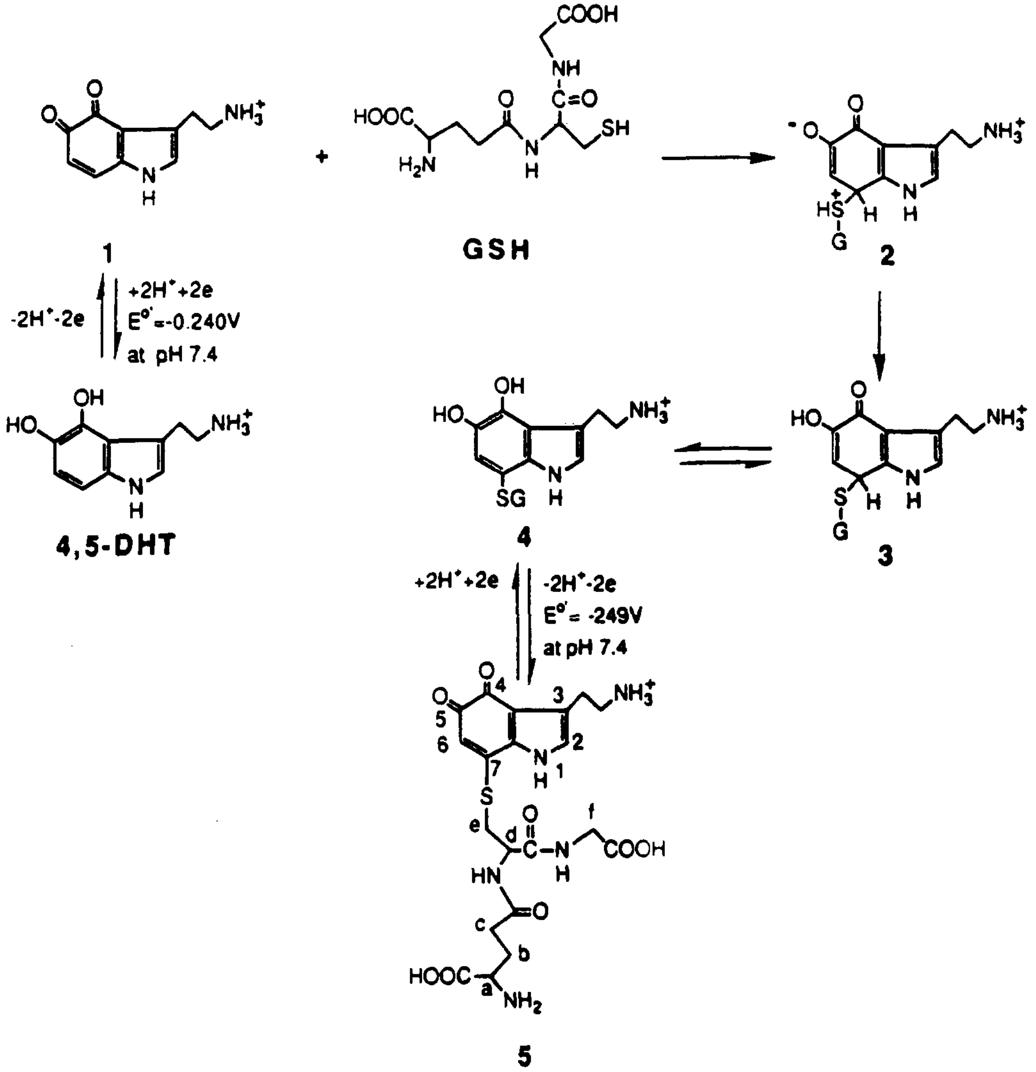


FIG. 3. Ultraviolet–visible spectrum of (—) 0.1 mM 7-*S*-(glutathionyl)-trypamine-4,5-dione in pH 7.4 phosphate buffer ($\mu = 0.1$) and (---) after controlled potential electroreduction at -0.40 V.

A recent report suggests that CSF of Alzheimer patients contains a species that is an oxidized form of 5-HT (*25*), which, based upon its electrochemical and chromatographic properties, has been suggested to be dione **1** (*26, 32*). The observation that **1** produces a significant efflux of 5-HT from serotonergic neurons *in vitro* similar to that caused by the indolic neurotoxin 5,6-DHT indicates that **1** might also possess neurodegenerative properties (*33*). This suggests, therefore, that **1** might play an important functional role in the etiology of Alzheimer's disease. However, GSH is known to be widely distributed throughout the brain in relatively high concentrations, estimated to be about 2 mM (*39*). The work reported here indicates that at physiological pH **1** is rapidly and irreversibly attacked by GSH to give **5**. Thus, under pathological conditions where 5-HT might be converted to its aberrant metabolite **1** in the brain it would be expected that very rapid conjugation with GSH should occur to give **5**. Indeed, there is some evidence that a conjugate such as **5** is formed after incubation of **1** with a brain homogenate (*32*). This in turn suggests that **5** is the actual species which causes the neurodegenerative effects attributed to **1**. The present work provides a convenient method to prepare and isolate pure samples of **5**. The neurodengenerative properties of **5**, however, remain to be determined.

The electrochemical properties of **1** and **5** (compare Fig. 1 and 2) are virtually identical. Accordingly, it would be very difficult to distinguish between these two compounds based upon their electrochemical behaviors.

HPLC-EC analyses of Alzheimer CSF using a highly ion-paired mobile phase has shown that an aberrant 5-HT metabolite coelutes with 5-HT (*26*). It has been suggested that this can lead to considerable errors in measurements of the endogenous neurotransmitter concentration. For such an interference to occur both 5-HT and its aberrant metabolite should both give responses at the potential



SCHEME I

employed on the electrochemical detector. A CV of 5-HT at pH 7.4 (Fig. 4) shows an oxidation peak at $E_p = 0.292$ V. After scan reversal a reduction peak appears at -0.24 V and, on the second anodic sweep, a quasi-reversible oxidation peak appears at -0.20 V. The latter peaks correspond to the **1**/4,5-DHT couple and cannot be observed unless the primary oxidation peak of 5-HT is first scanned. By contrast, **1** (Fig. 1) and **5** (Fig. 2) exhibit no oxidation peaks at potentials as positive as 0.60 V. Accordingly, at pH 7.4, 5-HT can be detected at potentials ≥ 0.200 V as a result of its electrochemical oxidation whereas **1** and **5** can be detected at potentials ≤ -0.200 V as a result of their electrochemical reduction. In other

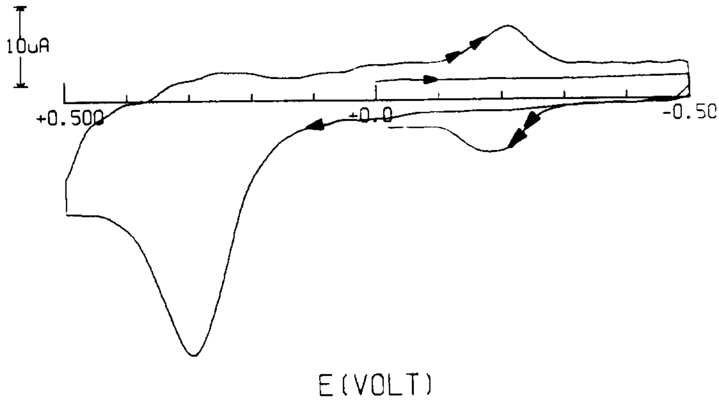


FIG. 4. Cyclic voltammogram at the PGE of 0.1 mM 5-HT in pH 7.4 phosphate buffer (μ = 0.1). Sweep rate: 200 mV s^{-1}.

words, at pH 7.4, there is no single potential between −0.50 and 0.60 V at which 5-HT and **1** (or **5**) can both give an electrochemical response. Hence, over the latter potential range, it is not possible for **1** or **5** to interfere with analyses for 5-HT. At lower pH values CVs of 5-HT, **1**, and **5** are qualitatively similar to those observed at pH 7.4. However, at pH 2.0 for example, E_p for the primary oxidation

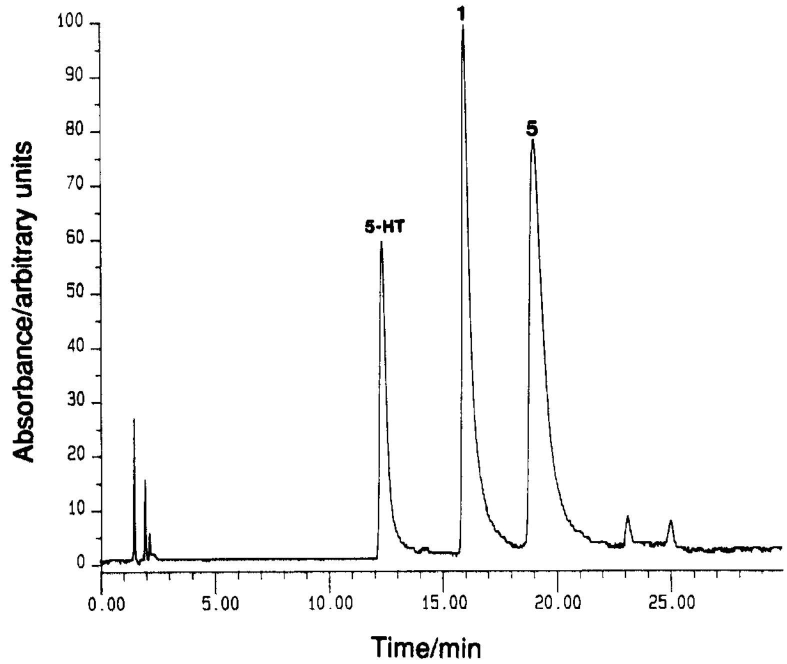
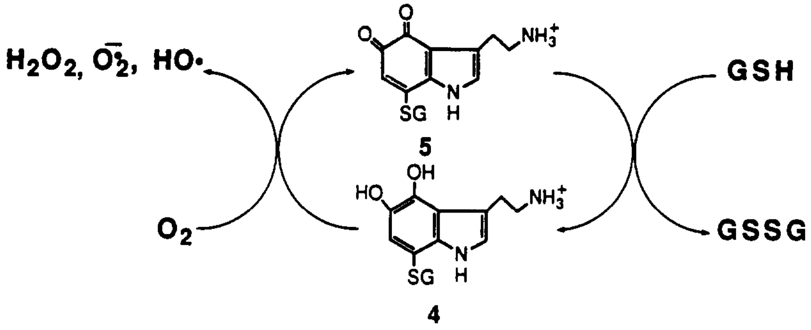


FIG. 5. High-performance liquid chromatogram of a mixture of 5-hydroxytryptamine (5-HT), tryptamine-4,5-dione (**1**), and 7-*S*-(glutathionyl)-tryptamine-4,5-dione (**5**). Conditions for analytical HPLC are given under Materials and Methods.



Scheme II

peak of 5-HT (0.1 mM, 200 mV s^{-1}) is 0.545 V. Under the same conditions E_p values for reduction of **1** and **5** are 0.087 and -0.030 V, respectively. Thus, at low pH it is very unlikely that **1** and/or **5** could interfere with HPLC-EC analyses for 5-HT. The chromatogram shown in Fig. 5 indicates that 5-HT, **1**, and **5** are easily separated from each other using a simple gradient HPLC method.

CONCLUSIONS

Abnormalities in the oxidative metabolism of the indolic neurotransmitter 5-HT have been postulated to be involved in the etiology of Alzheimer's disease (*25*). A major *in vitro* electrochemical oxidation product of 5-HT is **1** (*27–30*). This same compound has recently been shown to possess neurotoxic properties (*33*) and possibly to be formed as an aberrant metaboltic of 5-HT in Alzheimer patients (*25, 26*). The present study indicates that in brain **1** should be very rapidly attacked by endogenous GSH to yield **5**. Methods have been developed to isolate **5** and to fully elucidate its chemical structure using spectroscopic methods. The redox chemistry of **5** is virtually identical to that of **1**. The ease of reduction of **5** to **4** and the ease of oxidation of **4** back to **5** suggest that redox cycling might play an important role in the neurodegenerative activity of **5** (*40*). Indeed, preliminary experiments have revealed that **5** can be reduced to **4** upon addition of excess GSH. In the presence of air **5** is rapidly regenerated. This suggests that a purely chemical redox cycling process can occur as conceptualized in Scheme II such that GSH is converted to GSSG. Correspondingly, molecular oxygen is reduced perhaps generating cytotoxic species such as H_2O_2, $O_2^{\overline{\cdot}}$, HO ·. We hope to report further on such redox cycling processes in the near future.

ACKNOWLEDGMENTS

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