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Synthesis and Reactivity of the Putative Neurotoxin Tryptamine-4,5-dione

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In dilute aqueous solution at physiological pH tryptamine-4,5-dione (**1**; ≤200 μM) slowly reacts to give 3-(2-aminoethyl)-6-[3′-(2-aminoethyl)-indol-4′,5′-dione-7-yl]-5-hydroxyindole-4,7-dione (**7**) as the major product. Attempts to isolate pure solid samples of **1** by freeze-drying a chromatographically pure solution of the dione always results in the formation of significant (15–35%) amounts of 7,7′-bi-(5-hydroxytryptamine-4-one) (**10**). Similarly, relatively high concentrations of **1** (e.g., 20 mM) in vehicles such as 0.5 M NH_4Cl, pH 7.0, or isotonic saline containing ascorbic acid (1 mg/ml) at pH 3 rapidly transform into dimer **10**, which subsequently polymerizes. Dione **1** has been claimed to be a neurotoxin which when administered into the brains of laboratory animals causes nerve terminal damage similar to that observed in human Alzheimer's disease (AD). However, the present study suggests that under the conditions employed to prepare and centrally adminster solutions of **1** it would be almost completely transformed to **10**. Procedures are given to prepare the solutions of dione **1** and to assess the purity of such solutions.

INTRODUCTION

Recently, Volicer *et al.* (*1*) employed high-performance liquid chromatography (HPLC) with multielectrode coulometric detection to analyze the cerebrospinal fluid (CSF) of patients with neurodegenerative Alzheimer's disease (AD). These investigators found that the levels of the indolic neurotransmitter 5-hydroxytryptamine (5-HT; serotonin) were significantly lower in the CSF of AD patients than in that of age-matched controls. These results are in accord with observations that the levels of 5-HT in the cerebral cortex of AD patients are also decreased compared to controls (*2*, *3*). Volicer *et al.* (*1*) also detected an unknown electroactive species in the CSF of AD patients which was not present in that of controls. This compound was speculated to be an oxidized form of 5-HT because it had chromatographic and electrochemical properties similar to those of *in vitro* electrochemically oxidized 5-HT (*1*, *4*). Such results suggest that oxidation reactions of 5-HT and, perhaps, of other endogenous indoles might occur in the central nervous system of AD patients. Work in this laboratory has recently been directed toward investigation of the hypothesis that aberrant oxidative metabolism of endogenous indoles in the Alzheimer brain might lead to toxins which play roles in the neuronal

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degeneration and biochemical abnormalities characteristic of AD (*5–7*). As the initial steps in this investigation we have recently reported the mechanisms and products of the electrochemical oxidation (*8–11*) and of various enzyme-mediated (*12*) oxidations of 5-HT. Tryptamine-4,5-dione (**1**) was first discovered in these studies as a product, under certain conditions the major product, of the electrochemical oxidation of 5-HT in acidic aqueous solution (*8*). Subsequently, **1** was found to be, generally, a rather minor product of both the electrochemical (*11*) and enzymatic (*12*) oxidations of 5-HT at physiological pH. Using electrochemically synthesized and very dilute solutions (10 μM–10 nM) of **1**, Chen *et al.* (*13*) found that this compound significantly increased basal 5-HT efflux from rat brain hippocampal and striatal fragments in a fashion similar to that evoked by the indolic neurotoxin 5,6-dihydroxytryptamine (*14*). Intracerebroventricular (i.c.v.) administration of very much higher concentrations of what was believed to be **1** to rats resulted in nerve terminal degeneration particularly in medial limbic system structures including some known to be affected in human AD (*15*, *16*). Furthermore, i.c.v. administration of **1** also resulted in the depletion of 5-HT and 5-hydroxyindole-3-acetic acid (5-HIAA) levels in prefrontal cortex, striatum, and hippocampus (*16*). More recently, it has been claimed that **1** binds to and inactivates G proteins, a process which may account in part for the neurotoxic properties of the dione (*17*).

The various results provided by Volicer and his co-workers (*13*, *15–17*) regarding the neurotoxic properties of **1** and possible mechanisms underlying such properties are intriguing and provide important support for the hypothesis that aberrant oxidative metabolites of 5-HT might play roles in the neuronal degeneration and biochemical changes which characterize AD. However, in our previous investigations it was noted that even in very dilute acidic aqueous solution (i.e., $\leq$30 μM), **1** was not stable and slowly decomposed (*8*). This instability was subsequently confirmed (*13*). At higher concentrations of **1** the rate of such reactions increased. Indeed, because of the complicating effects of such reactions, **1** was initially characterized as a quinoxaline derivative formed after successful trapping with *o*-phenylenediamine (*8*). The instability of dilute solutions of **1** (e.g., 100 μM) at physiological pH is even more pronounced than at low pH (*15*). Preliminary experiments in this laboratory subsequently revealed that the decomposition of **1** at physiological pH becomes very rapid at higher concentrations although the nature of the reactions involved and the products formed was not investigated (*11*). However, a knowledge of these reactions and particularly of the products formed is clearly of relevance. For example, if indeed **1** is formed endogenously in the Alzheimer brain, it is important to know whether this dione or its spontaneously formed decomposition product(s) is the neurodegenerative compound. Furthermore, such information is necessary before a search for aberrant oxidative metabolites of 5-HT is undertaken in Alzheimer brain tissue or CSF. It is also important to note that in assessing *in vivo* effects of **1** in rat brain, Crino *et al.* (*16*) injected 5–20 μg of the dione dissolved in 5 μl of a vehicle consisting of aqueous 0.5 M NH_4Cl (pH 7–7.4). Thus, the concentrations of **1** employed for these injections ranged from 5.25 to 21 mM. Accordingly, the question is raised regarding the actual chemical identity of the substance that was administered into the brains of these

laboratory animals and which resulted in nerve terminal damage. The work described in this communication, therefore, was designed to investigate the stability and solution chemistry of **1**, particularly at physiological pH. The major primary decomposition products deriving from **1** in both dilute and concentrated solutions are described.

EXPERIMENTAL

Chemicals

5-Hydroxytryptamine hydrochloride (5-HT · HCl) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Formic acid (88%), triethylamine (TEA), and trifluoroacetic acid (TFA) were obtained from Sigma (St. Louis, MO). HPLC grade acetonitrile (MeCN) and glacial acetic acid were obtained from Fisher Scientific (Springfield, NJ).

Electrochemistry

Cyclic voltammograms were obtained with a BAS-100A electrochemical analyzer (Bioanalytical Systems, West Lafeyette, IN). Voltammograms were obtained at a pyrolytic graphite electrode (PGE; Pfizer Minerals, Pigments and Metals Division, Easton, PA) having an approximate surface area of 3.6 mm^2. A conventional three-electrode electrochemical cell that contained a platinum gauze counter electrode and a saturated calomel reference electrode (SCE) was employed for voltammetry. All voltammograms were corrected for ir drop. Potentials were referenced to the SCE at ambient temperature (22 ± 2°C). Test solutions were thoroughly deaerated with a vigorous stream of N_2 gas for ca. 5 min before voltammograms were recorded.

Controlled potential electrolyses employed a Princeton Applied Research Corp. (Princeton, NJ) Model 174A polarographic analyzer. A three-compartment cell was used in which the working, counter, and reference electrode compartments were separated by a Nafion membrane (Type 117, DuPont Co., Wilimington, DE). The working electrode compartment had a capacity of 40 ml. The working electrode consisted of several plates of pyrolytic graphite having a total surface area of ca. 60 cm^2.

HPLC Chromatography

HPLC employed a Bio-Rad (Richmond, CA) gradient system equipped with dual Model 1330 pumps, and Apple Model IIe controller, a Rheodyne (Cotati, CA) Model 7125 loop injector (2.0- and 10.0-ml sample loops were used) and an Isco (Lincoln, NE) Model 226 or a Bio-Rad Model 1305A uv monitor (254 nm). In order to monitor the electro-oxidation of 5-HT, as well as for other analytical studies, HPLC Method I was employed. In the method a reversed phase column (Brownlee Laboratories, RP-18, 5 μm particle size, 25 × 0.7 cm) was used. This was protected by a short guard column (Brownlee, RP-18, 5 μm, OD-GU, 50–5 mm). Two mobile

phase solvents were employed. Solvent A was prepared by adding 11.0 ml of HPLC grade MeCN and 1.1 ml of TEA to 988 ml of deionized water. The pH of this solution was adjusted to 3.30 with glacial acetic acid. Solvent B was MeCN. The following gradient was employed: 0–4 min, 100% solvent A and a linear increase of flow rate from 2.0 to 2.5 ml/min; 4–15 min, linear gradient to 12% solvent B and a linear increase of flow rate to 3.0 ml/min; 15–24 min, linear gradient to 24% solvent B; 24–30 min, linear gradient to 25% solvent B. The mobile phase was then linearly returned to 100% solvent A over the course of 5 min (2.0 ml/min flow rate); the column was equilibrated for another 5 min (2.0 ml/min) before another sample was injected. The sample volume injected was typically 2.0 ml.

In order to isolate and purify reaction products HPLC Method II was employed. This method used a preparative reversed phase column (J. T. Baker, Bakerbond C_{18}, 10 μm particle size, 25 × 2.12 cm) protected by a guard column (5 × 0.9 cm) packed with the same stationary phase. Two mobile phase solvents were used. Solvent C was prepared by adding 50 ml of MeCN to 950 ml of deionized water. The pH of the solution was then adjusted to 2.1 with TFA. Solvent D was prepared by adding 400 ml of MeCN to 600 ml of deionized water. The pH of the resulting solution was then adjusted to 2.1 with TFA. The following gradient system was employed: 0–5 min, 100% solvent C, flow rate linearly increased from 4.0 to 5.0 ml/min; 5–10 min, linear gradient to 12% solvent D and a linear increase of flow rate to 5.5 ml/min; 10–22 min, linear gradient to 25% solvent D and a linear increase of flow rate to 6.0 ml/min; 22–35 min, linear gradient to 35% solvent D; 35–40 min, 35% solvent D; 40–50 min, linear gradient to 65% solvent D; 50–55 min, 65% solvent D. The mobile phase was then linearly returned to 100% solvent C over 5 min and the column was equilibrated for a further 5 min (6.0 ml/min) before another sample was injected. The injection volume was 10.0 ml.

Spectroscopy

Fast atom bombardment mass spectrometry (FAB-MS) was carried out with a VG Instruments (Manchester, UK) Model ZAB-E spectrometer. Liquid chromatography-mass spectrometry (LC-MS) utilized a Kratos MS 25/RFA spectrometer equipped with a thermospray source. The source was maintained at 325°C and the thermospray capillary tip at 195°C. The mobile phase was 0.1 M ammonium acetate in water at a flow rate of 0.9 ml/min. All LC-MS experiments were performed by an off-line method. Thus 0.2- to 2.0 ml aliquots of fractions collected from conventional HPLC separations were injected directly into the thermospray source using a Rheodyne 7125 loop injector. NMR spectra were recorded on a Varian XL-300 spectrometer. Ultraviolet–visible spectra were recorded on a Hewlett–Packard Model 8452A diode array spectrophotometer.

*Synthesis of Tryptamine-4,5-dione (**I**)*

A 33 μM solution of 5-HT · HCl (0.28 mg) in 0.01 M HCl (40 ml) was electrooxidized at pyrolytic graphite electrodes at +0.68 V. Upon completion of the oxidation (ca. 20–30 min, as judged by HPLC analysis using Method I) another 0.28 mg of 5-HT · HCl was added to the working electrode compartment of the

electrochemical cell. The electrolysis was then continued until 5-HT was again completely oxidized. This procedure was repeated until 2.5 mg (0.3 mM) 5-HT had been oxidized, which required approximately 3 h. Throughout the electrolysis the solution in the working electrode compartment was bubbled vigorously with N_2 gas and stirred with a Teflon-coated magnetic stirring bar. The counter and reference electrode compartments contained 0.01 M HCl. The conversion of 5-HT into **1** using this procedure was ≥95% (HPLC Method I).

Repetitive 10-ml injections of the bright purple solution (λ_{max} in 0.01 M HCl: 538, 352 nm) into a preparative HPLC system (Method II) were then employed to purify **1**. The chromatographic peak corresponding to **1** (retention time, t_R = 29.5 min) was collected, frozen, and promptly freeze-dried. The resulting product was obtained as a fluffy brown-purple powder. Analysis of the product obtained from many such syntheses using HPLC Method I revealed that it consisted predominantly of **1** (65–85%) and dimer **10** (35–15%).

A freshly chromatographed solution of **1** in the HPLC mobile phase (Method I; pH 3.3) exhibited a characteristic purple color (λ_{max}: 538, 354 nm). LC-MS on a sample of this solution exhibited an intense ion at *m/e* 193 (86%). This corresponds to the pseudomolecular ion (MH^+) of the reduced form of **1**, i.e., 4,5-dihydroxytryptamine. FAB-MS (3-nitrobenzyl alcohol matrix) of a solid sample of **1** (which was always contaminated with dimer **10**) exhibited an intense pseudomolecular ion (MH^+) at 191 (77%). Accurate mass measurements on MH^+ gave *m/e* 191.0819 ($C_{10}H_{11}N_2O_2$; calcd. *m/e* 191.0821). FAB-MS also exhibited a second pseudomolecular ion at *m/e* 381 (MH^+, 30%) corresponding to dimer **10**. ^1H NMR (Me_2SO-d_6) of a sample of **1** (contaminated with 15% dimer **10**) showed δ 11.96 (bs, 1H, N(1)-H), 7.79 (bs, 3H, NH_3^+), 7.35 (d, $J_{6,7}$ = 9.9 Hz, 1H, C(7)-H), 6.93 (s, 1H, C(2)-H), 5.95 (d, $J_{6,7}$ = 9.9 Hz, 1H, C(6)-H), 3.12 (t, J = 7.2 Hz, 2H, C(β)-H_2), 2.88 (t, J = 7.2 Hz, 2H, C(α)-H_2). In D_2O, δ 7.31 (d, J_6, 7 = 9.9 Hz, 1H, C(7)-H), 6.83 (s, 1H, C(2)-H), 6.00 (d, $J_{6,7}$ = 9.9 Hz, 1H, C(6)-H), 3.23 (t, J = 6.9 Hz, 2H, C(β)-H_2), 2.98 (t, J = 6.9 Hz, 2H, C(α)-H_2). Minor resonances observed in these spectra of **1** corresponded to **10**. The NMR spectra of **1** are in accord with those expected for this compound and are in close agreement with that reported for the *N*-ethylcarbamate derivative of **1** (*18*) and for tryptophol-4,5-dione (*19*).

3-(2-Aminoethyl)-6-[3'-(2-aminoethyl)-indole-4',5'-dione-7'-yl]-5-hydroxyindole-4,7-dione (*7*)

Compound **7** was initially observed as the major decomposition product of **1** in dilute (0.2 mM) aqueous solution at pH 7.2 using HPLC Method I (t_R = 17.8 min). This compound was collected following decomposition of 0.2 mM **1** in pH 7.2 phosphate buffer for 12 h using HPLC Method II. After freeze-drying **7** was obtained as a red-brown solid. At pH 7.4 **7** gave a red solution, λ_{max}, nm (log ε_{max}, M^{-1} cm^{-1}): 518(3.30), 484(3.25), 346(3.58), 300(3.77), 238(4.00). FAB-MS (3-nitrobenzyl alcohol matrix) of **7** gave a pseudomolecular ion (MH^+) at *m/e* 395(16%). Accurate mass measurements on MH^+ gave *m/e* 395.1346 ($C_{20}H_{19}N_4O_5$; calcd. *m/e* 395.1355). Thus, **7** has a molecular formula $C_{20}H_{18}N_4O_5$ and a molar mass of 394 g and hence consists of one residue each of **1** and 5-hydroxytryptamine-

4,7-dione (**4**). ^{1}H NMR (Me_2SO-d_6) δ 11.88 (bs, 1H, N(1)-H), 11.01 (bs, 1H, N(1′)-H), 7.75 (bs, ca. 6H, $2NH_3^+$), 6.80 (s, 1H, C(2)-H), 6.65 (s, 1H, C(2′)-H), 5.96 (s, 1H, C(6′)-H), 3.06 (m, 4H, C(β)-H_2 and C(β′)-H_2), 2.90 (m, 4H, C(α)-H_2 and C(α′)-H_2). Addition of D_2O caused the signals at 11.88, 11.01, and 7.75 ppm to disappear. The structure of **7** was confirmed by synthesis of this compound. A solution of **4** (0.05 mM) and **1** (0.1 mM) in pH 7.2 phosphate buffer was stirred for 2–3 h. HPLC analysis (Method I) revealed that after this time all **1** (t_R = 12 min) and **4** (t_R = 16.2 min) had disappeared and that the sole product was **7** (t_R = 17.8 min). Compound **7** was isolated using HPLC Method II. After freeze-drying the product obtained was spectrally (uv, FAB-MS, ^{1}H NMR) and electrochemically (e.g., cyclic voltammetry) identical to that formed from **1** alone in dilute solution.

7,7′-Bi-(5-hydroxytryptamine-4-one) (***10***)

Dimer **10** was most conveniently obtained as a byproduct of the synthesis of **1**. The solid samples of **1** always contained 15–35% **10**. Thus, HPLC Method II was employed to isolate **10** (t_R = 40.8 min). After freeze-drying, **10** was obtained as a deep red-brown fluffy solid. In acidic aqueous solution **10** gave a bright purple color, λ_{max}, nm (log ε_{max} M^{-1} cm^{-1}) in 0.01 M HCl, pH 2.0, = 538 (4.04), 368 (3.70), 302 (3.79), and 226 (4.07). FAB-MS (3-nitrobenzyl alcohol matrix) gave an intense pseudomolecular ion (MH^+) at *m/e* 381 (100%). Accurate mass measurements on MH^+ gave *m/e* 381.1557 ($C_{20}N_{21}N_4O_4$; calcd. *m/e* 381.1563). Thus, **10** has a molecular formula $C_{20}H_{20}N_4O_4$ and a molar mass of 380 g. ^{1}H NMR (D_2O) δ 6.88 (s, 2H, C(2)-H and C(2′)-H), 6.51 (s, 2H, C(6)-H and C(6′)-H), 3.27 (m, 4H, C(β)-H_2 and C(β′)-H_2), 3.00 (m, 4H, C(α)-H_2 and C(α′)-H_2).

RESULTS

Controlled potential electro-oxidation of very dilute solutions of 5-HT (ca. 30 μM) in 0.01 M HCl at sufficiently positive potentials (+0.68 V) leads to a very high conversion (≥95%) of the indoleamine to **1**. By repeatedly adding 5-HT to the resulting product solution, such that the concentration of the indoleamine does not exceed ca. 30 μM, followed by further electrolysis it is possible to obtain a ca. 0.3 mM solution of **1**. Based upon HPLC (Method I) and spectral analysis the latter solution was of relatively high purity (≥90%) and was stable under ambient conditions for approximately 4 h. After longer periods of time HPLC analyses revealed that dimers **7** and **10** were formed from **1**. A cyclic voltammogram of such a solution is shown in Fig. 1A. After adjusting the pH of this solution to 7.2, the cyclic voltammogram shown in Fig. 1B was obtained. Thus, at pH 2.0 and pH 7.2 **1** exhibits reduction peaks at peak potentials (E_p) of +0.085 V and −0.23 V, respectively. These peaks correspond to the 2*e*, $2H^+$ reduction of **1** to 4,5-dihydroxytryptamine (4,5-DHT) (*8*). After scan reversal, quasireversible oxidation peaks at E_p = +0.100 and −0.162 V appear in cyclic voltammograms at pH 2.0 and 7.2, respectively. These peaks correspond to oxidation of 4,5-DHT to **1**. The $E^{\circ\prime}$ values for the **1**/4,5-DHT couple at pH 2.0 and 7.2 are, therefore, 0.092 V and −0.197 V, respectively.

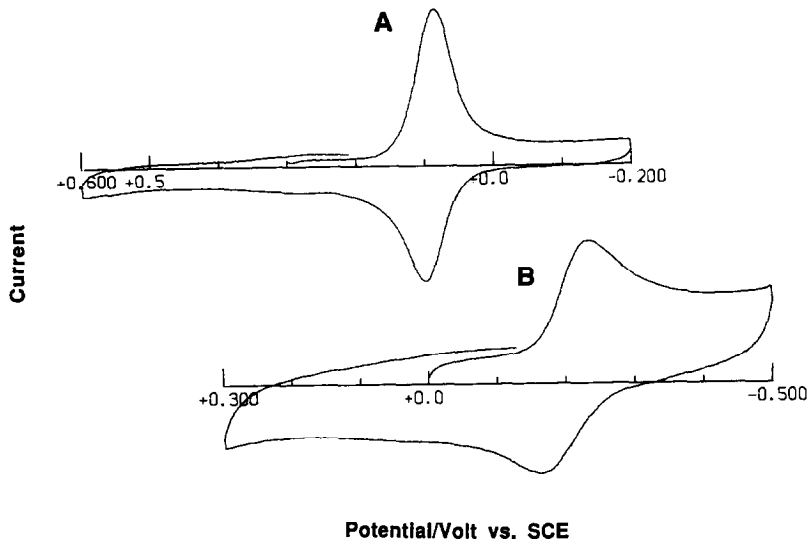


FIG. 1. Cyclic voltammograms at the PGE of 0.2 mM tryptamine-4,5-dione (**1**) in (A) 0.01 M HCl, pH 2.0, and (B) after neutralizing the solution in (A) (9.0 ml) with 4.0 M KOH to pH 7 followed by addition of 1.0 ml of pH 7.2 phosphate buffer (μ = 1.0). Sweep rate; 200 mV s^{-1}.

Using preparative HPLC Method II a solution of **1** (ca. 0.3 mM) in 0.01 M HCl can be further concentrated. By injecting 10-ml aliquots, the resulting peak of **1** contained concentrations of **1** of about 0.5–0.6 mM. The solution containing **1** obtained in this fashion consisted of about 16% MeCN in water at an apparent pH (TFA) of 2.1. The latter solution represents the highest concentration of pure **1** that could be prepared and was stable under ambient conditions for about 6 h. (After longer time periods dimers **7** and **10** and other minor unidentified products began to be detectable by HPLC analysis.) Following freeze-drying of such a solution of **1** a solid product could be isolated. However, HPLC analysis (Method I) of this solid dissolved in 0.01 M HCl at a concentration of ca. 0.1 mM revealed that it consisted primarily of a mixture of **1** and dimer **10**. Typically, such products contained 65–85% of **1** and 35–15% of **10**.

It is important to note that controlled potential electro-oxidations of 5-HT in 0.01 M HCl at concentrations >100 μM under the conditions described earlier yield other products in addition to dione **1**. For example electrolyses of 1 mM solutions of 5-HT yield a very complex mixture of products the major of which is 5,5′-dihydroxy-4,4′-bitryptamine (*10*).

HPLC analyses (Method I) of a 0.2 mM solution of **1** in pH 7.2 phosphate buffer (μ = 0.1) reveal that over the course of several hours the peak corresponding to the dione systematically decreases and several minor peaks and one major new peak appears (Fig. 2). The major product of the spontaneous but slow decomposition of **1** in pH 7.2 phosphate buffer is dimer **7**, which consists of the one residue each of **1** and 5-hydroxytryptamine-4,7-dione (**4**). A minor product of the decomposition reaction is dimer **10**, although throughout the reaction the peak correspond-

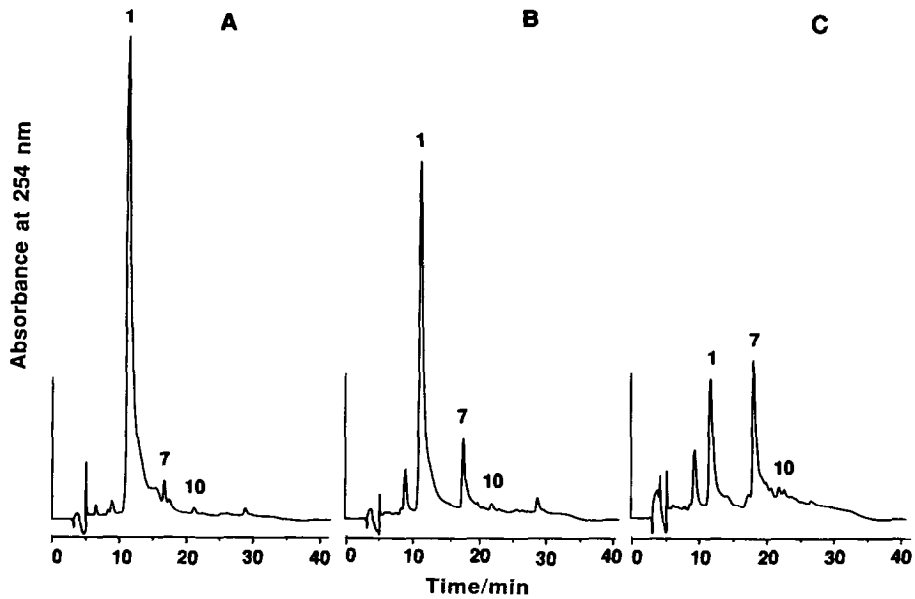


FIG. 2. HPLC chromatograms (Method I) of a solution initially 0.2 mM in **1** in pH 7.2 phosphate buffer (μ = 0.1) after (A) 1 h, (B) 8 h, and (C) 25 h. Injection volume, 2.0 ml.

ing to this dimer does not grow. It will be demonstrated subsequently that this is due to the instability of **10** at physiological pH. The other minor decomposition products of **1** remain to be determined. Cyclic voltammograms of 0.2 mM **1** in pH 7.2 phosphate buffer recorded at various time intervals are presented in Fig. 3. Thus, immediately after preparation of a solution of **1** only the characteristic quasi-reversible couple of this compound, $E^{\circ\prime}$ = −0.197 V, can be observed (Fig. 3A). However, after 21 h the peaks corresponding to the 1/4,5-DHT couple significantly decrease and two new reversible couples at $E^{\circ\prime}$ = −0.28 and −0.54 V appear (Fig. 3B). The latter couples are characteristic of dimer **7** (Fig. 3C). The uv–visible spectrum of a freshly prepared bright purple solution of **1** (0.2 mM) in pH 7.2 phosphate buffer exhibits bands at λ_{max} = 532, 350, and 234 nm (Fig. 4A). After 21 h the band at 352 nm shifts to 346 nm and new bands at 484 and 300 nm appear (Fig. 4B). These new bands correspond to those expected for dimer **7** (Fig. 4C). The latter solution was red-purple in color.

Dimer **10** was always obtained when a solution of **1** was freeze-dried (see Experimental). This dimer could be isolated from the latter mixture, using HPLC Method II, as a red-brown solid. Cyclic voltammograms and uv–visible spectra of the purple solutions of **10** at pH 2.0 and 4.5 are shown in Fig. 5. These cyclic voltammograms of **10** exhibit reversible couples at $E^{\circ\prime}$ = +0.213 V and +0.008 V (pH 2.0) and +0.01 V and −0.20 V (pH 4.5). The λ_{max} values for **10** were 538, 368, 302, and 226 nm at pH 2.0 and 542, 366, 302, and 226 nm at pH 4.5. In pH 7.2 phosphate buffer **10** initially gave a bright blue solution (λ_{max} = 590, 366, 314, and 224 nm) but during the course of a few minutes this color faded, and,

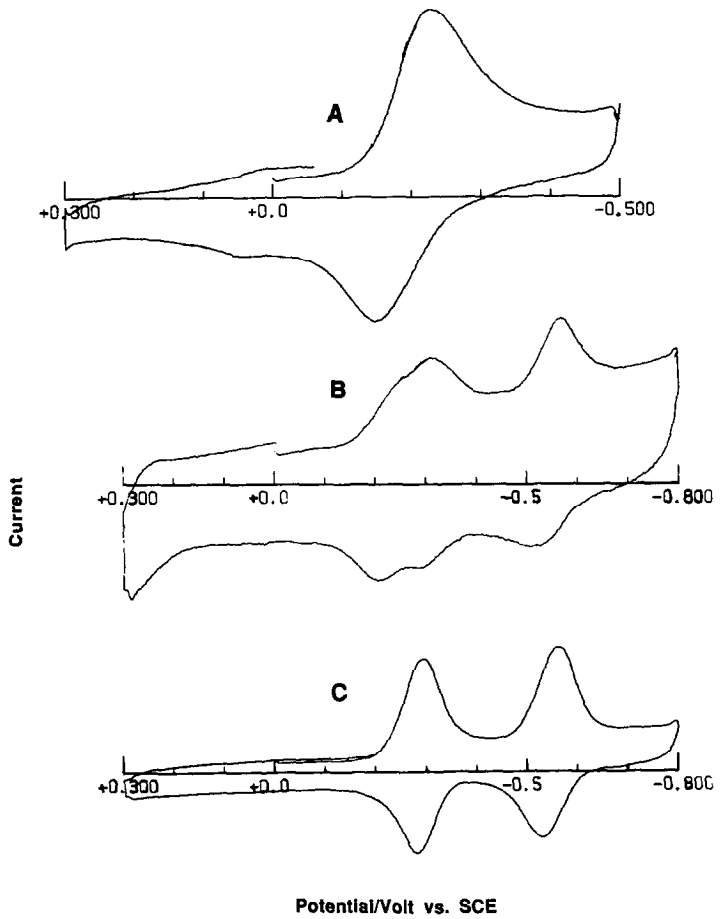
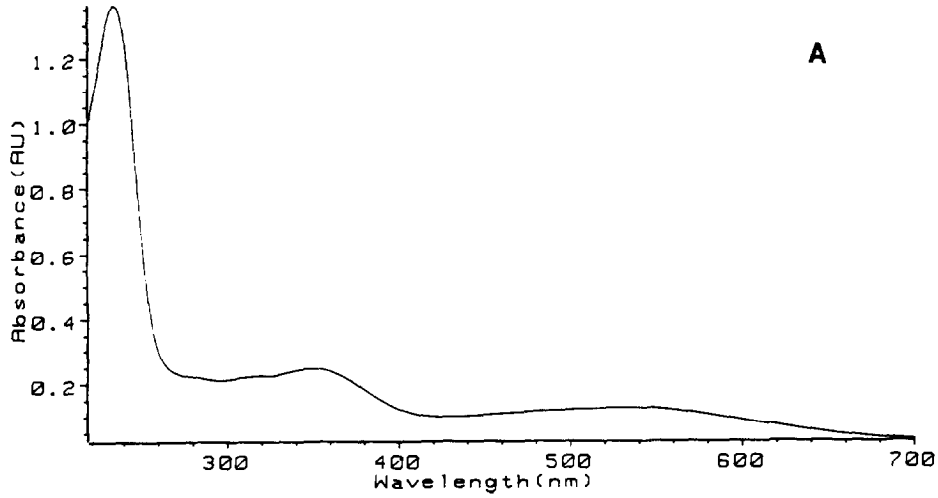


FIG. 3. Cyclic voltammograms at the PGE of (A) a freshly prepared solution of 0.2 mM **1** in pH 7.2 phosphate buffer (μ = 0.1), (B) the same solution after 21 h; and, (C) a 0.2 mM solution of dimer **7** in pH 7.2 phosphate buffer (μ = 0.1). Sweep rate, 200 mV s^{-1}.

ultimately, a gray precipitate was formed in the solution. HPLC analysis (Method I) of a solution initially 1.0 mM **10** (t_R = 19.6 min) in pH 7.2 phosphate buffer revealed that within 15 min no trace of the dimer remained and no new chromatographic peaks appeared. Taken together these observations suggest that under these conditions **10** rather rapidly polymerizes.

In their recent studies into the *in vivo* neurotoxicity of **1** Crino *et al.* (*16*) claimed to have prepared up to 21 mM solutions of the dione in 0.5 M NH_4Cl which were injected into the brains of laboratory animals. In the present studies we have been unable to prepare pure solutions of **1** at any pH value at such concentrations. However, it has been possible to prepare solid samples of **1** contaminated with 15–35% of dimer **10** following freeze-drying of dilute acidic solutions of the dione. Figure 6 shows an HPLC chromatogram recorded 1 min after preparation of a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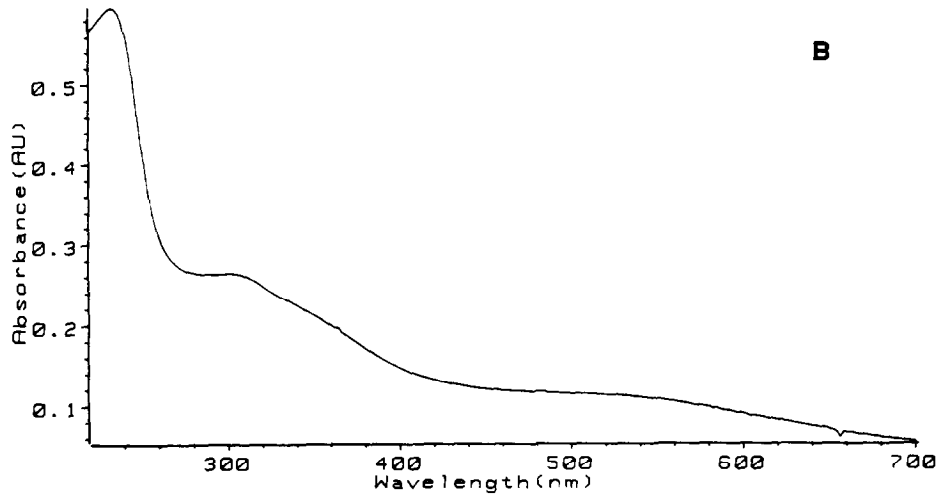
700
Wavelength(nm)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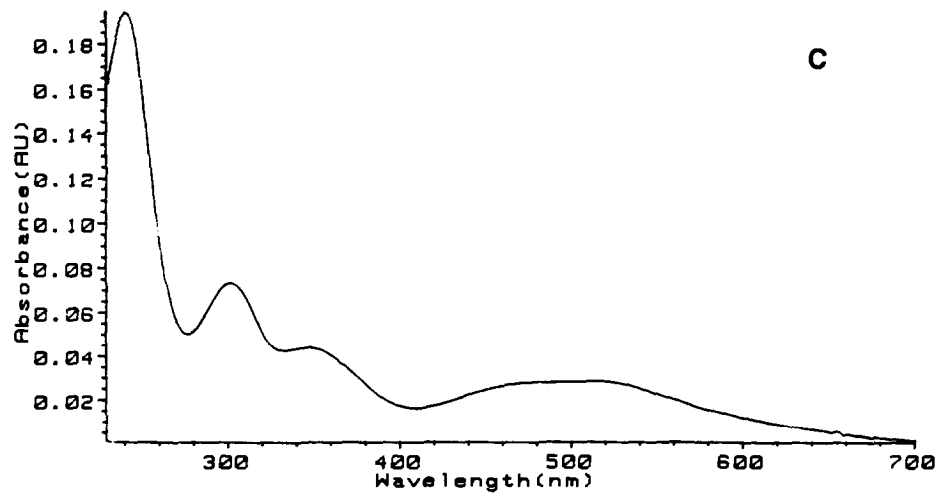
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600
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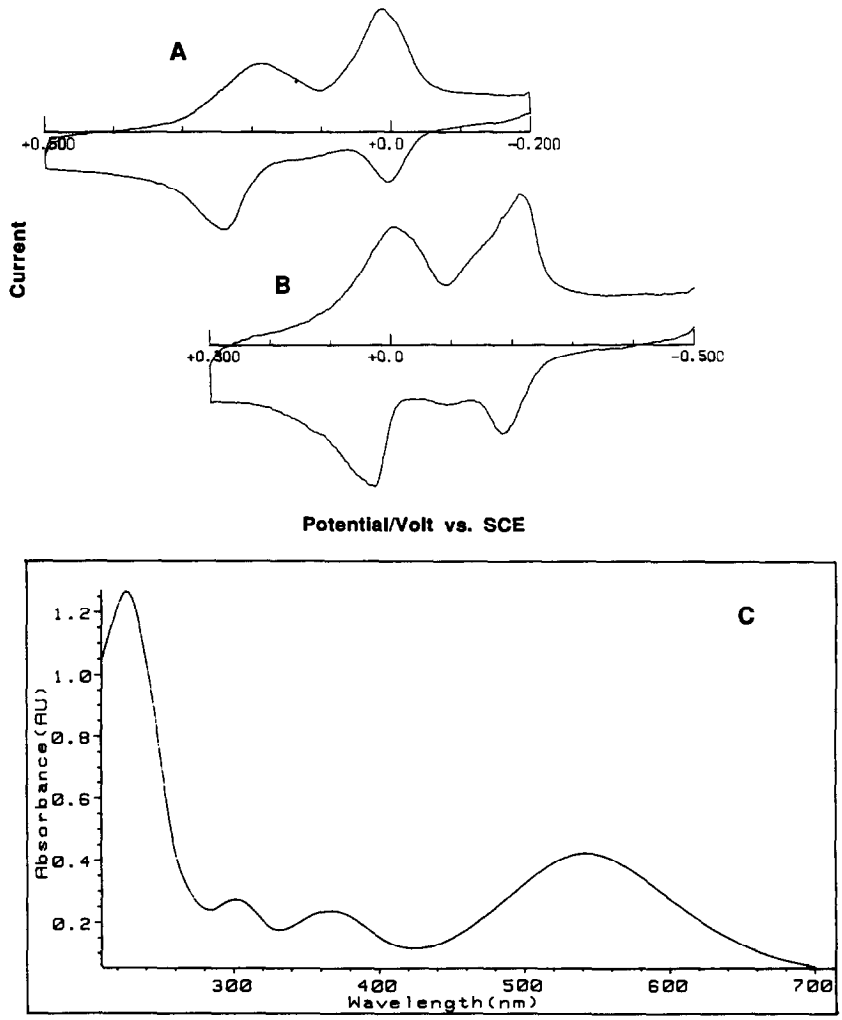


FIG. 5. Cyclic voltammograms at the PGE of 0.2 mM **10** in (A) 0.01 M HCl, pH 2.0, and (B) pH 4.5 phosphate buffer at a sweep rate of 200 mV s^{-1}. (C) Ultraviolet–visible spectra of 0.2 mM **10** at pH 4.5.

solution from such a sample containing initially 20 mM **1** (and 3.5 mM **10** contaminant) in 0.5 M NH_4Cl (pH 7.0). Clearly, even after this very short time lapse approximately 97% of **1** had disappeared. The major chromatographic peak in Fig. 6 corresponds to dimer **10**. In 0.5 M NH_4Cl solution (pH 7.0) dimer **10** slowly polymerized. For example, HPLC analysis of 20 mM **10** in the latter medium

FIG. 4. Ultraviolet–visible spectra of (A) a freshly prepared solution of 0.2 mM **1** in pH 7.2 phosphate buffer (μ = 0.1); (B) the same solution after 21 h; and (C) a 0.02 mM solution of dimer **7** in pH 7.2 phosphate buffer (ν = 0.1).

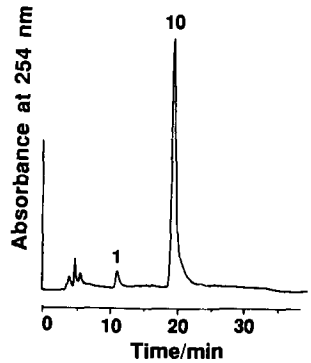
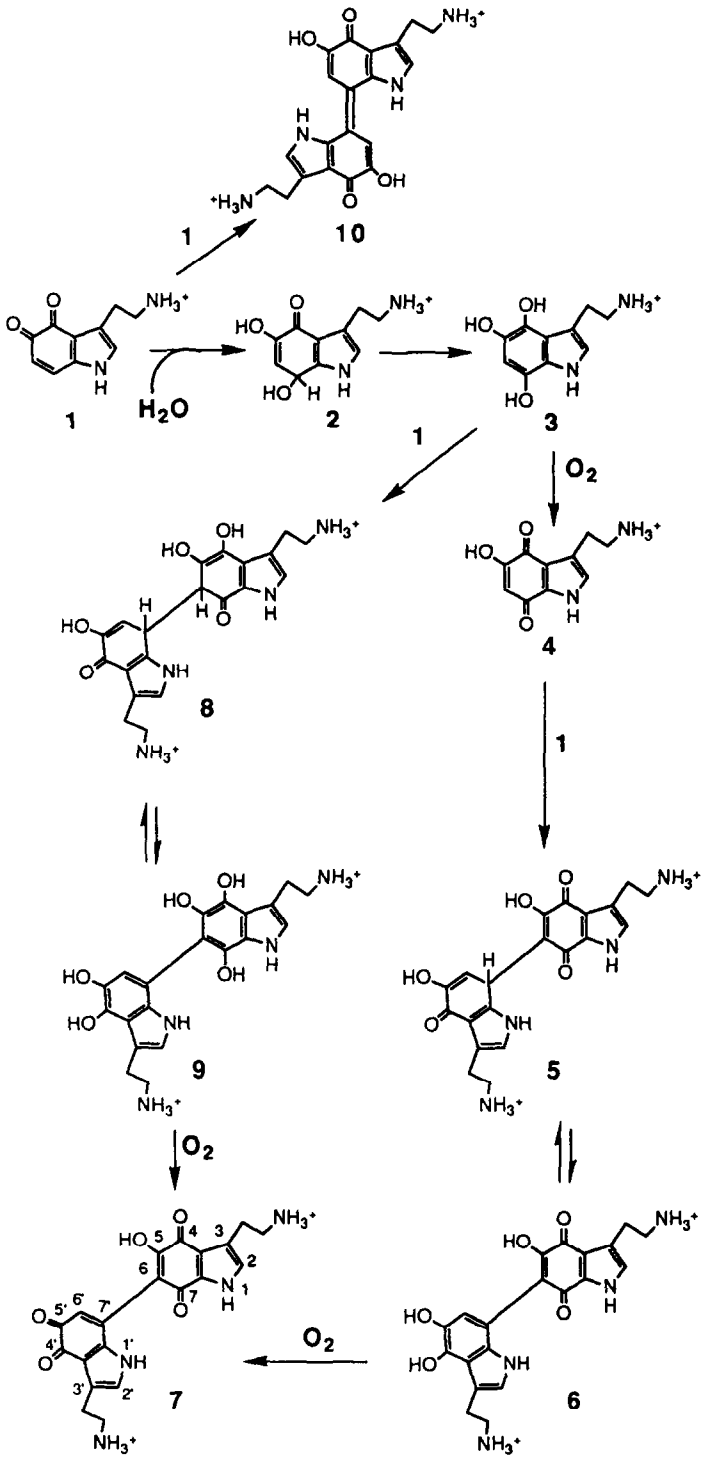


FIG. 6. HPLC chromatogram (Method I) recorded 1 min after preparation of a 20 mM solution of **1** in 0.5 M NH_4Cl (pH 7.0). This solution was prepared from a freeze-dried and chromatographically purified (HPLC Method II) solution of **1** and contained 65% **1** and 35% **10**. The actual concentration of **1** was 20 mM, that of **10** was 3.5 mM. Injection volume: 1.0 ml (12.5 μl diluted to 1.0 ml).

showed that after 40 min and 60 min approximately 50 and 74%, respectively, of the dimer had disappeared. A more typical vehicle employed to deliver drugs into the central nervous system of laboratory animals is isotonic saline (0.9% NaCl) containing 1 mg/ml of ascorbic acid. HPLC analysis of a solution initially 20 mM in **1** (contaminated with 3.5 mM **10**) in the latter vehicle (final pH 3.0) revealed that after 1, 5, and 10 min the concentration of the dione had decreased to 13.2, 5.6, and 0.5 mM, respectively. As the concentration of **1** decreased during this time period that of dimer **10** increased. However, during the subsequent 60 min nearly 75% of the **10** so formed disappeared, presumably as a result of polymerization. A 20 mM solution of **1** (contaminated with 3.5 mM **10**) in isotonic saline (final pH 3.3) in which no ascorbate was present was appreciably more stable. Thus, after 20 min the concentration of **1** decreased to 16 mM and the concentration of dimer **10** correspondingly increased.

DISCUSSION

The above results indicate that at physiological pH dilute solutions of **1** slowly react to give predominantly dimer **7**. There are two possible routes by which **7** can be formed from **1**, both of which involve initial nucleophilic attack by water on the latter dione to give 4,5,7-trihydroxytryptamine **3** (Scheme I). The latter triol is an extremely easily oxidized compound and is autoxidized to 5-hydroxytryptamine-4,7-dione (**4**) ($E^{\circ\prime}$ for the **3**/**4** couple at pH 7.4 is -0.51 V vs SCE) (*20*). Independent experiments reveal that *para*-quinone, **4**, reacts directly with **1** to give **7** (see Experimental) presumably by the reaction sequences **5**–**7** (Scheme I). Alternatively, nucleophilic addition of triol **3** to **1** would be expected to yield dimer **9** which contains one residue each of **3** and 4,5-DHT. The expected facile autoxidation of **9** then yields **7**.



SCHEME I

Following freeze-drying of a purified, dilute solution of **1** significant quantities of the 7,7-linked dimer **10** are formed. Similarly, relatively high concentrations of **1** (e.g., 20 mM) in either 0.5 M NH_4Cl (pH 7.0) or in isotonic saline containing 1 mg/ml of ascorbic acid are quite rapidly transformed into dimer **10**, which subsequently polymerizes. The precise mechanism by which **1** is transformed into **10** remains to be determined. In isotonic saline a 20 mM solution of **1** (pH 3.3) appears to be much more stable. However, it is important to note that in the present study it has not been possible to obtain solid samples of **1** in the pure state; rather, they were always contaminated with significant quantities of dimer **10**.

There are some important conclusions which can be drawn from the results reported here. For example, it seems probable that the drug injected into rat brains in an aqueous 0.5 M NH_4Cl vehicle by Crino *et al.* (*16*) that caused nerve terminal degeneration was not dione **1** but rather **10** and/or oligomeric/polymeric products derived from the latter dimer. It is of importance to note that both **1** and **10** produce intense purple/blue solutions at pH 7.2. Thus, visually, it would be very difficult to judge whether a concentrated solution (20 mM) contained predominantly **1** or **10**. Should dione **1** be formed endogeneously in the Alzheimer brain it is extremely unlikely that it could ever reach concentration levels remotely approaching 20 mM, i.e., those thought to be administered by Crino *et al.* (*16*). Rather, it is more likely that very low concentrations would be formed during very long periods of time (i.e., years or decades). Thus, it would seem that a more appropriate method to investigate the *in vivo* effects of **1** would be to infuse very dilute solutions of the dione into the brains of laboratory animals over quite long time periods. The work reported here suggests that dilute solutions of **1** in either 0.01 M HCl or in isotonic saline should be sufficiently stable for such infusion experiments. It is planned to report on the results of such *in vivo* experiments at some future date.

It is also of relevance to note that **1** is an extremely electrophilic species and, therefore, would be expected to be avidly scavenged by intraneuronal nucleophiles. Perhaps the most abundant such nucleophile is the tripeptide glutathione (GSH). Indeed, it has recently been demonstrated that at physiological pH, **1** reacts rapidly with GSH to yield 7-*S*-glutathionyl-tryptamine-4,5-dione. This glutathionyl conjugate is a very potent toxin when centrally administered to mice (*21*).

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