Oxidation Chemistry and Biochemistry of the Central Mammalian Alkaloid 1-Methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline

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The electrochemical oxidation of the central mammalian alkaloid 1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline (1) has been studied in neutral aqueous solution at a pyrolytic graphite electrode (PGE). Voltammograms of 1 show two closely spaced oxidation peaks, I_a and II_a. At potentials less positive than the peak potential (E_p) for peak I_a, 1 is oxidized to a radical intermediate which dimerizes to give two diastereomers of 5,5′-bi(1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline) (5 and 6). At potentials more positive than E_p for peak I_a the putative radical intermediate is further electrooxidized to a C(5)-centered carbocation which reacts with 1 in an ion–substrate reaction to give 5 and 6 or with water to give, ultimately, 1-methyl-1,2,3,4-tetrahydro-β-carboline-5,6-dione (12). Dimers 5 and 6 give two reversible oxidation peaks at the PGE, the second of which corresponds to peak II_a observed in voltammograms of 1. Because 5 and 6 are easily oxidizable compounds they are only observed as products in the initial stages of the controlled potential electrooxidation of 1. Tyrosinase/O_2, human ceruloplasmin/O_2, and peroxidase/H_2O_2 also oxidize 1 to 5, 6, and 12 as the initial products. In the presence of glutathione the electrochemically driven and enzyme-mediated oxidations of 1 result in the formation of 5-*S*-glutathionyl-1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline as a major product. Central administration of diastereomer 5 or 6 to mice evoked behavioral responses similar to those caused by the opioid analgesics. These behavioral effects, which include spatial disorientation and a characteristic ducklike walk, became most pronounced approximately 3 h after drug administration and continued for about 3 days. Neurotransmitter and related metabolite analyses of whole brain reveal that 5 and 6 cause a general increase in dopaminergic and serotonergic activity and a small but significant decrease in cholinergic activity. These transmitter/metabolite disturbances appear to parallel the time course of the observed behavioral effects. The possible roles of in vivo oxidations of 1, an alkaloid which is elevated in mammalian brain following ethanol consumption, in the addictive, behavioral, and neurodegenerative consequences of chronic alcoholism are discussed.

Several tetrahydro-β-carbolines are endogenous, albeit trace, constituents of the mammalian brain. These include 1,2,3,4-tetrahydro-β-carboline (THβC),[1,2] 1-methyl-THβC,[3] 2-methyl-THβC,[2] 6-methoxy-THβC,[2] 6-hydroxy-THβC,[3,4] and 1-methyl-6-hydroxy-THβC.[3] These so-called mammalian THβC alkaloids probably arise endogenously from the condensation of central nervous system indolamines (or their precursor amino acid L-tryptophan) with an aldehyde or α-keto acid via the Pictet–Spengler reaction.[5,6] 1-Methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline (1; 2,3,4,9-tetrahydro-1-methyl-1*H*-pyrido[3,4-*b*]indol-6-ol) can be formed as a result of the reaction between the indolic neurotransmitter 5-hydroxytryptamine (5-HT) and acetaldehyde. An intermediate Schiff base is formed in this reaction which rapidly cyclizes to give 1 (Scheme I). Recent studies have shown that 1 not only occurs in the mammalian brain[3] but also in body fluids and other tissues.[7,8] The two enantiomers of the alkaloid occur in unequal amounts in the urine of some species and are formed in vivo in a reaction sequence originating from tryptophan.[9] These observations suggest that an enzymatic process is probably involved to some extent in the formation of 1. Beck et al.[9] have proposed that 1 is formed in vivo in a reaction between pyruvate and 5-HT giving the 1-carboxylic acid derivative of 1 which is subsequently decarboxylated (Scheme I). A major metabolite of 1-methyl-THβC is 1.[10] It has also been noted that incubations of 5-HT and acetaldehyde in rat brain preparations give 1.[11] Further, in vivo experiments with the rat indicate that dietary ethanol, presumably via acetaldehyde formation, stimulates the production of 1.[12] And, urinary excretion of 1 in intoxicated human alcoholics is elevated and decreases upon detoxification.[7] Other THβCs also appear to be formed after ingestion of ethanol. For example, 1-methyl-THβC has been identified in the urine of humans after ingestion of a large dose of ethanol.[13] Such observations lend support to the hypothesis that, following ingestion of large quantities of ethanol, some of its proximate metabolite, acetaldehyde, formed in the liver, escapes into the circulatory system, enters the brain, and condenses with the biogenic catecholamines and indol-

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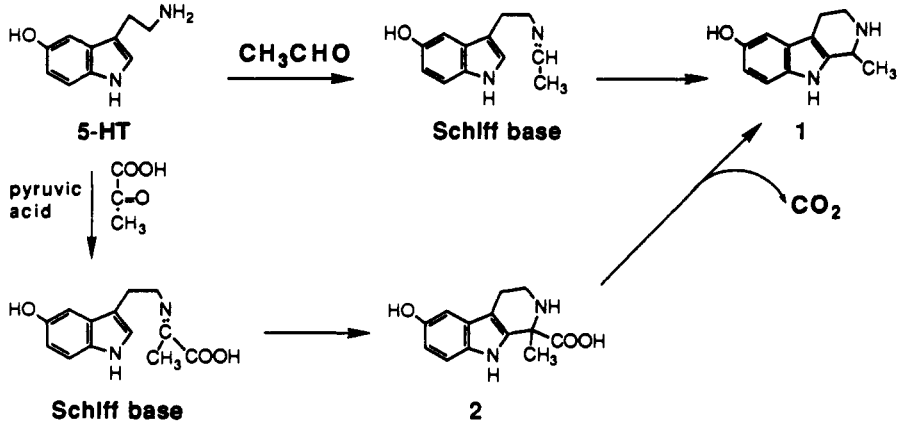
Scheme I



amines to form tetrahydroisoquinoline (TIQ) and THβC alkaloids and that these alkaloids contribute to the behavioral changes, physical dependence, and addictive properties of ethanol (for reviews see refs 14 and 15). Further support for this hypothesis is provided by the observations that chronic intracerebroventricular infusions of minute amounts of THβC (and some TIQs) into the rat results in a large increase in the animal's preference for and consumption of ethanol in free-choice situations.[16,17] This preference continues long after infusion of the alkaloids is discontinued, suggesting, perhaps, that a long-lasting alteration in the central nervous system (CNS) has occurred.

Long-term chronic alcoholism results in impaired learning ability and memory and a general decline of intellectual abilities all of which have been attributed to organic brain damage.[18] Neuronal loss, particularly in hippocampal regions of the brain, has been noted.[19] Indeed, the effects of chronic alcoholism have been likened to a premature aging of the brain.[20] While there are undoubtedly direct effects of ethanol on brain tissue,[21] it is now quite certain that the trace levels of some endogenous THβCs and TIQs are elevated as a result of chronic alcohol consumption. It is also known that many THβCs evoke a variety of biochemical and neuropharmacological responses including the release of 5-HT from synaptosomes[22] and from hypothalamus.[23] They also function as analgesics,[4,22] alter body temperature,[24] and cause a deterioration of memory.[25] Several THβCs are toxins. For example, chronic intraperitoneal administration of 6-hydroxy-THβC to rats causes a high rate of mortality.[26] Intracisternal administration of the same compound to mice causes hypothermia at low doses and catalepsy at high doses. When combined with a monoamine oxidase inhibitor 1 evokes extreme hyperactivity in the rat.[27,28] The fundamental biochemical phenomena underlying the effects of THβCs on ethanol preference and consumption and other neuropharmacological and toxic properties of these alkaloids are very incompletely understood. Recently, Collins[29] advanced a hypothesis that oxidation reactions of the condensation products of the biogenic catecholamines and acetaldehyde, i.e., TIQ alkaloids, in the CNS might lead to toxic metabolites which are re-

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sponsible for neuronal damage. While this hypothesis remains to be experimentally confirmed, there are formidable precedents for oxidation reactions playing key roles in the pathogenesis of neuronal degeneration. For example, destruction of catecholaminergic neurons by 6-hydroxydopamine and 6-hydroxy-DOPA is probably caused by the intraneuronal oxidation of these drugs to cytotoxic products or byproducts.[30,31] Aberrant oxidation reactions of 5-HT to toxic indoles have been implicated in the neuropathology of Alzheimer's Disease.[32] The serotonergic neurotoxins 5,6-[33] and 5,7-dihydroxytryptamine[34] and the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine[36] are all converted to their active forms in vivo by intraneuronal oxidation reactions. The condensation product of 5-HT and acetaldehyde, i.e., 1, would be expected to be an easily oxidized compound. Accordingly, the in vivo oxidation of this alkaloid, which is elevated following alcohol ingestion, might similarly yield metabolites which play roles in the behavioral, addictive, or neurodegenerative consequences of chronic alcoholism. The mammalian metabolism of 1 has not been studied. That oxidation reactions of THβCs do occur in vivo, however, might be implied from the observation that 1-methyl-β-carboline *and* 1-methyl-THβC are present in human urine following ethanol consumption.[13] A fully aromatic β-carboline-3-carboxylate ester, characterized in extracts of normal human urine, might be an oxidized metabolite of a THβC precursor.[14,36] A THβC is also thought to be oxidized to a reactive intermediate which can cross-link proteins in aging human lenses, suggesting that THβCs might be involved in cellular aging phenomena.[37] However, there have been no systematic studies of the oxidation chemistry of THβCs although it appears to be rather widely believed[14] that oxidations of these alkaloids should ultimately result in their aromatization to the corresponding β-carbolines. In this report it will be demonstrated that 1 is indeed a rather easily oxidized compound at physiological pH. The oxidation chemistry has been studied using electrochemical methods and a group of oxidative enzyme systems. The dihydro or fully aromatic β-carbolines are not among the initial products of these reactions. The major products which are formed evoke profound behavioral effects and disturb the dopaminergic, serotonergic, and cholinergic transmitter systems when centrally administered to mice.

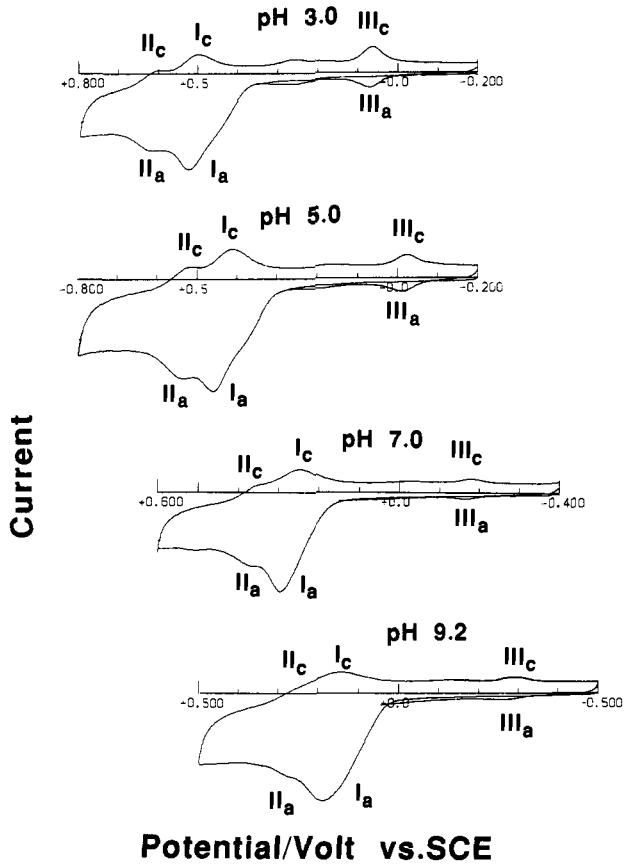

Figure 1. Cyclic voltammograms at the PGE of 0.58 mM 1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline (1) in phosphate buffers (μ = 1.0). Sweep rate: 200 mV s^{-1}.

Results

Electrochemical Oxidations. Representative cyclic voltammograms of 1 at the pyrolytic graphite electrode (PGE) are presented in Figure 1. On the first anodic sweep two overlapping oxidation peaks (I_a and II_a) appear. At pH values <7 peak I_a is distorted by a shoulder on its rising segment. After scan reversal three reduction peaks appear, I_c, II_c, and III_c. Peaks II_a/II_c and peaks I_a/I_c appear to form reversible couples. On the second anodic sweep, oxidation peak III_a appears and forms a reversible couple with reduction peak III_c. The peak potentials (E_p) for both peak I_a and peak II_a are pH-dependent according to the relationships:

peak I_a:

$$E_{p(pH\ 3.0\text{–}6.0)} = [0.665 - 0.046pH]V$$

$$E_{p(pH\ 6.0\text{–}7.4)} = [0.931 - 0.091pH]V$$

$$E_{p(pH\ 7.4\text{–}9.2)} = [0.542 - 0.039pH]V$$

peak II_a:

$$E_{p(pH\ 3.0\text{–}6.0)} = [0.792 - 0.055pH]V$$

$$E_{p(pH\ 6.0\text{–}7.4)} = [1.023 - 0.094pH]V$$

$$E_{p(pH\ 7.4\text{–}9.2)} = [0.433 - 0.017pH]V$$

These relationships are based upon voltammograms measured at the PGE at a sweep rate (ν) of 200 mV s^{-1} and using 0.6 mM 1. At pH 7.0 the E_p values for peaks I_a and

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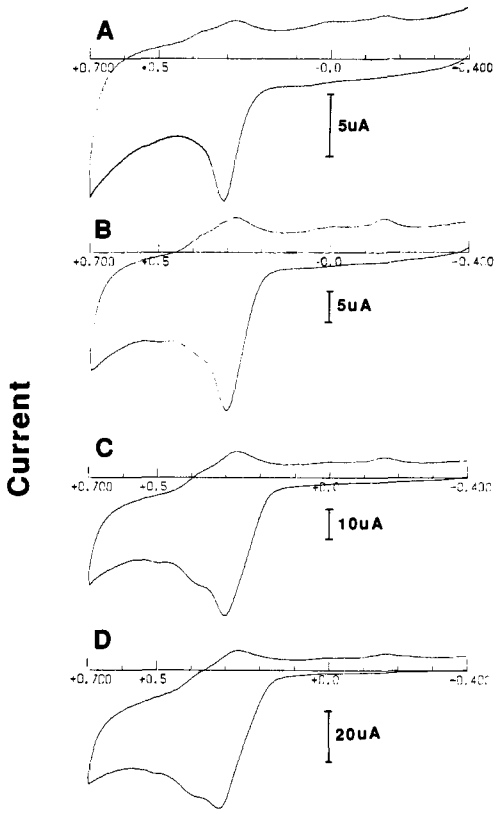

Figure 2. Cyclic voltammograms at the PGE of (A) 10 μM, (B) 100 μM, (C) 500 μM, and (D) 1000 μM 1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline (1) in pH 7.0 phosphate buffer (μ = 1.0). Sweep rate: 100 mV s^{-1}.

II_a are +0.29 V and +0.37 V, respectively.

The experimental peak current function, $i_p/AC\nu^{1/2}$ (where i_p is the peak current in microamperes; A is the electrode area, cm^2; C is the concentration of 1, mM; and ν is the voltage sweep rate, V s^{-1}) for peak I_a increased greatly with increasing ν, indicating that this peak is controlled to a significant extent by the adsorption of 1 at the surface of the PGE.[38] Because of the strong adsorption of 1 and the severe overlap between peaks I_a and II_a, it was not possible to measure voltammetric n values.

At very low concentrations of 1 (10 μM) voltammograms at pH 7.0, for example, show only oxidation peak I_a (Figure 2A). However, with increasing concentrations of 1 peak II_a appears and grows relative to peak I_a (Figure 2, parts B–D). Increasing values of ν cause peak II_a to systematically decrease and, ultimately, disappear; correspondingly, reduction peak I_c grows (Figure 3). It is interesting to note that at large values of ν (e.g., 10 V s^{-1}; Figure 3C), the E_p values for peaks I_a and I_c are virtually identical, indicative of strong adsorption of 1 and its proximate oxidation product at the PGE.[39] These various voltammetric behaviors suggest that the proximate oxidation product of 1 undergoes a second-order homogeneous chemical reaction to form the species responsible for oxidation peak II_a.

The reversible peaks III_c/III_a can only be observed in cyclic voltammograms of 1 after initially scanning through oxidation peak I_a. This couple is most prominent at low pH, although it is clearly present at pH ≥7 (Figure 1). At a representative ν of 200 mV s^{-1} and pH 5.0 the ratio of $(i_p)_{III_c}/(i_p)_{I_a}$ systematically decreased from 0.30 to 0.11 as the concentration of 1 was increased from 10 to 600 μM. Similar trends were noted at pH 3 and pH 7. Furthermore, with increasing ν not only does oxidation peak II_a systematically decrease and disappear with a corresponding decrease in reduction peak II_c but also peaks III_c/III_a decrease and ultimately disappear (see, for example, Figure 3C).

The cyclic voltammetric behaviors described above allow a number of conclusions to be drawn regarding the electrochemical oxidation of 1. First, a proximate product of the peak I_a oxidation of 1 can be reversibly reduced in the peak I_c process. Second, this proximate product can undergo follow up chemical reactions to generate both the

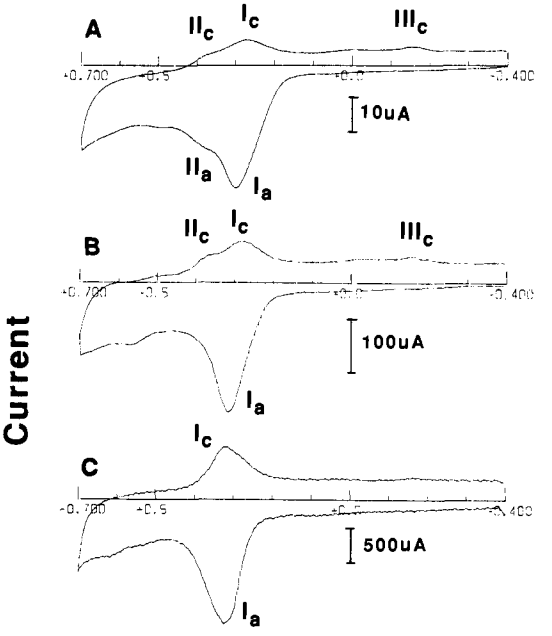

Figure 3. Cyclic voltammograms at the PGE of 0.2 mM 1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline (1) in pH 7.0 phosphate buffer (μ = 1.0) at sweep rates of (A) 100 mV s^{-1}; (B) 1000 mV s^{-1}; (C) 10240 mV s^{-1}.

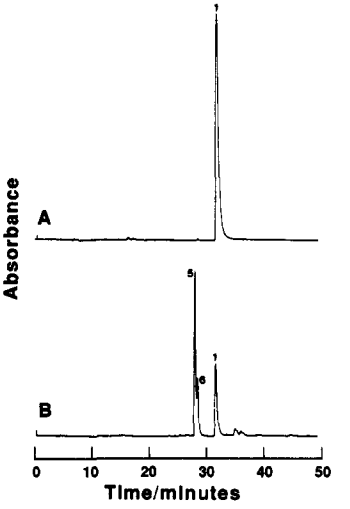

Figure 4. HPLC chromatogram of (A) 0.58 mM 1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline (1) and (B) the product mixture obtained following controlled potential electrooxidation of 1 for 30 min at +0.3 V in pH 7.0 phosphate buffer. Gradient system I was employed; injection volume: 0.2 mL.

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species responsible for oxidation peak II_a *and* the species responsible for reduction peak III_c. High concentrations of 1 and small values of ν favor the formation of the species responsible for oxidation peak II_a whereas low concentrations of 1 and small values of ν favor the formation of the compound responsible for peaks III_c/III_a.

Controlled Potential Electrooxidations and Product Isolations. HPLC analyses throughout the course of a controlled potential electrooxidation of 1 at +0.30 V (E_p for peak I_a) at pH 7.0 revealed that during the first 30 min two major products, **5** and **6**, were formed (Figure 4). However, after longer electrolysis times the chromatographic peaks of **5** and **6** decrease and ultimately disappear. Correspondingly, a large number of additional products appear, and an insoluble, black, presumably polymeric, precipitate is formed. The identities of these secondary products remain to be elucidated. This report was intended to focus on the major initial oxidation products of 1, i.e., **5** and **6**, and the species responsible for peaks III_c/III_a observed in cyclic voltammograms of 1.

Spectroscopic methods (MS and NMR) were employed to identify compounds **5** and **6** as diastereomers of 5,5′-bi(1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline), and **12** as 1-methyl-1,2,3,4-tetrahydro-β-carboline-5,6-dione (see the Experimental Section). Compound **12** is the species responsible for peaks III_c/III_a observed in cyclic voltammograms of 1. Following controlled potential electrooxidation of 1 (ca. 0.5–1 mM) at peak I_a potentials at pH 7 for ca. 30 min diastereomers **5** and **6** are clearly the major initial products observed in chromatograms of the resulting product mixture (Figure 4B). Compound **12** cannot be observed in these chromatograms because it coelutes with unreacted 1. An inspection of the cyclic voltammograms shown in Figure 1 indicates that **12**, characterized by the peaks III_c/III_a couple, is formed in higher yield at low pH than at pH 7. Accordingly, in order to prepare sufficient quantities of **12**, controlled potential electrooxidations of dilute solutions of 1 (≤0.2 mM) were performed at pH 1.5 using very positive potentials (+1.0 V). Under these conditions electrolyses of 1 were rapid (i.e., complete in ≤20 min) and formed **12** in high yield (≥80%).

Enzymatic Oxidations. The foregoing studies reveal that at physiological pH alkaloid 1 is a relatively easily oxidized compound at an electrode. However, oxidation reactions in vivo must generally occur either by direct, uncatalyzed reactions with molecular oxygen (autoxidation) or via some form of enzyme-catalyzed reaction. Experiments revealed that the autoxidation of 1 (0.5 mM) in pH 7.0 phosphate buffer (23 ± 2 °C) was insignificant over the course of at least 16 h.

Three representative enzyme systems were selected in order to assess whether enzyme-mediated oxidations of 1 were feasible. These were tyrosinase/O_2, ceruloplasmin/O_2, and peroxidase/H_2O_2. Figure 5 presents some chromatograms of the product mixtures obtained when 1 was incubated with these enzyme systems. Each of these systems oxidized 1, giving dimeric diastereomers **5** and **6** as the major primary products although many additional minor products are also formed. When the enzyme-mediated oxidations were permitted to proceed for longer periods of time, dimers **5** and **6** were further oxidized to give a large number of secondary products, the identities of which remain to be determined. All of the enzyme-mediated oxidations formed minor amounts of dione **12** (which coeluted with the chromatographic peak of unreacted 1).

Oxidation in the Presence of Glutathione. Electrochemical oxidation of 1 at pH 7.0 in the presence of glutathione (GSH) resulted in a considerable decrease in the yields of dimers **5** and **6** and the appearance of 5-*S*-glutathionyl-1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline (**14**) as a major product (Figure 6A). Oxidations of 1 by tyrosinase/O_2 (Figure 6B) and peroxidase/H_2O_2 (Figure 6D) also produced **14** as the major initial reaction product. However, ceruloplasmin/O_2 oxidation of 1 gave only minor amounts of **14** (Figure 6C).

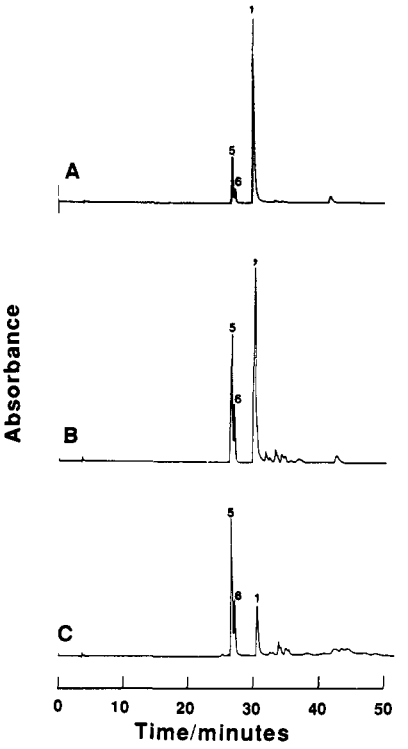


Figure 5. HPLC chromatograms of the product mixture formed when 1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline (1; 0.5 mM) was incubated (23 ± 2 °C) in pH 7.0 phosphate buffer (μ = 0.15) with (A) tyrosinase (172 units mL^{-1}) for 10 min; (B) ceruloplasmin (44.7 units mL^{-1}) for 50 min, and (C) type IV peroxidase (4 units mL^{-1}) and H_2O_2 (0.22 mM) for 2 min with N_2 bubbling through the solution. Injection volume: 0.2 mL. Gradient system I was employed.

Reaction Pathways

Strong adsorption of 1 at the PGE precluded experimental measurements of the voltammetric n value and quantitative mechanistic interpretations of voltammetric behaviors. Nevertheless, a more qualitative interpretation of the voltammetric behaviors of 1 and its initial products **5**, **6**, and **12** permits valuable insights into possible reaction pathways. Figure 7 presents cyclic voltammograms of 1, **5**, **6**, and **12** recorded at pH 7. Diastereomeric dimers **5** and **6** both give rise to two closely spaced voltammetric oxidation peaks at E_p = +0.29 and +0.37 V and, after scan reversal, two corresponding reduction peaks at E_p = +0.36 and +0.27 V. The second, more positive, oxidation peaks of **5** and **6** are the dominant peaks at the concentration (0.59 mM) employed. The E_p values for these peaks coincide with that for oxidation peak II_a observed in cyclic voltammograms of 1. Similarly, the most positive reduction peaks observed in cyclic voltammograms of **5** and **6** coincide with peak II_c observed in cyclic voltammograms of 1. The effects of ν and concentration on peak currents for the two oxidation peaks of **5** and **6** suggested that the first (less positive) peak was an adsorption prepeak. However, further studies are necessary to more completely understand the voltammetric oxidations of **5** and **6**. The reversible redox couple observed in cyclic voltammograms

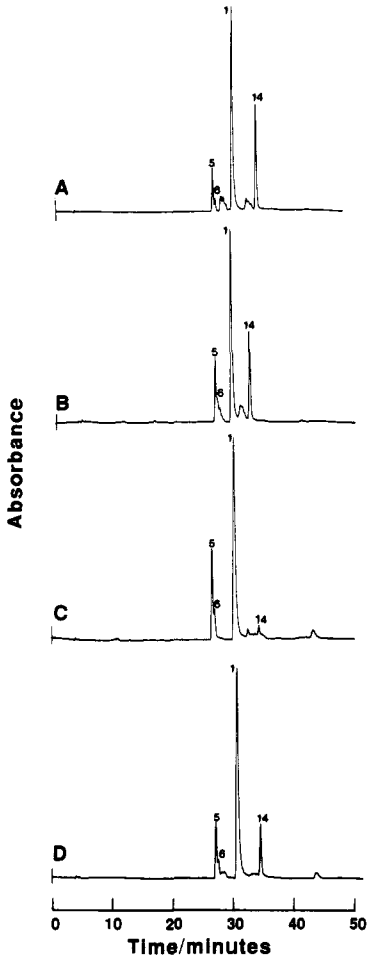


Figure 6. HPLC chromatograms of the product solution formed as a result of (A) controlled potential electrooxidation of 0.5 mM 1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline (1) in the presence of 0.76 mM glutathione at +0.30 V for 30 min; (B) 0.46 mM 1 in the presence of tyrosinase (64.6 units mL^{-1}) and GSH (0.72 mM) for 65 min; (C) 0.5 mM 1 in the presence of ceruloplasmin (44.7 units mL^{-1}) and GSH (0.72 mM) for 350 min; (D) 0.5 mM 1 in the presence of type IV peroxidase (4 units mL^{-1}), H_2O_2 (0.22 mM), and GSH (0.72 mM) for 3 min in pH 7.0 phosphate buffer at 22 ± 3 °C. Injection volume: 0.2 mL. Gradient system I was employed.

of dione **12** coincides with peaks III_c/III_a observed in cyclic voltammograms of **1**.

At sufficiently fast sweep rates cyclic voltammograms of **1** show only oxidation peak I_a on the initial anodic sweep and, after scan reversal, only reversible reduction peak I_c (Figure 3C). However, upon decreasing the sweep rate peaks II_a/II_c and III_c/III_a appear. Furthermore, at a given sweep rate, increasing concentrations of **1** result in a systematic increase in the height of peak II_a (i.e., corresponding to the oxidation of dimers **5** and **6**) relative to peak I_a and a decrease in the height of peak III_c (i.e., corresponding to the reduction of dione **12**). Peak clipping experiments reveal that in order to clearly observe peaks III_c/III_a it is necessary to reverse the initial anodic sweep at potentials equal to or more positive than E_p for peak I_a. Cyclic voltammograms of **5** and **6**, however, do not show peaks III_c/III_a. These observations indicate that at potentials more negative than E_p for peak I_a, dimers **5** and **6** are formed whereas at potentials equal to or more positive than E_p for peak I_a, **5**, **6**, and dione **12** are formed. Controlled potential electrolysis experiments at various applied potentials followed by product analyses confirmed the latter conclusions. Accordingly, it has been concluded that the initial step in the electrochemical oxidation of **1**

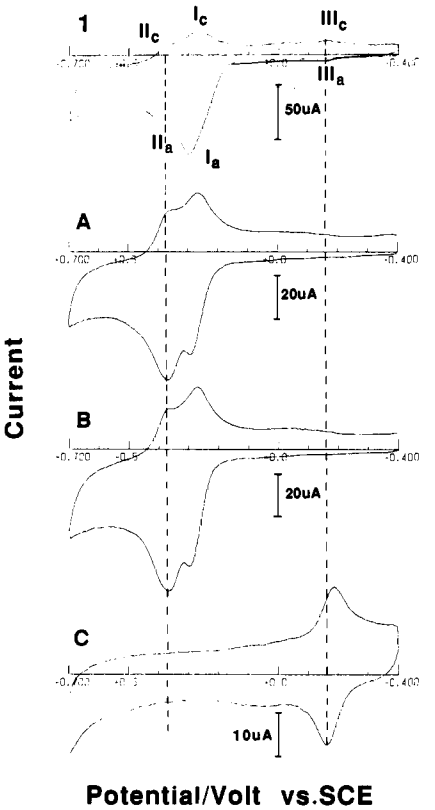
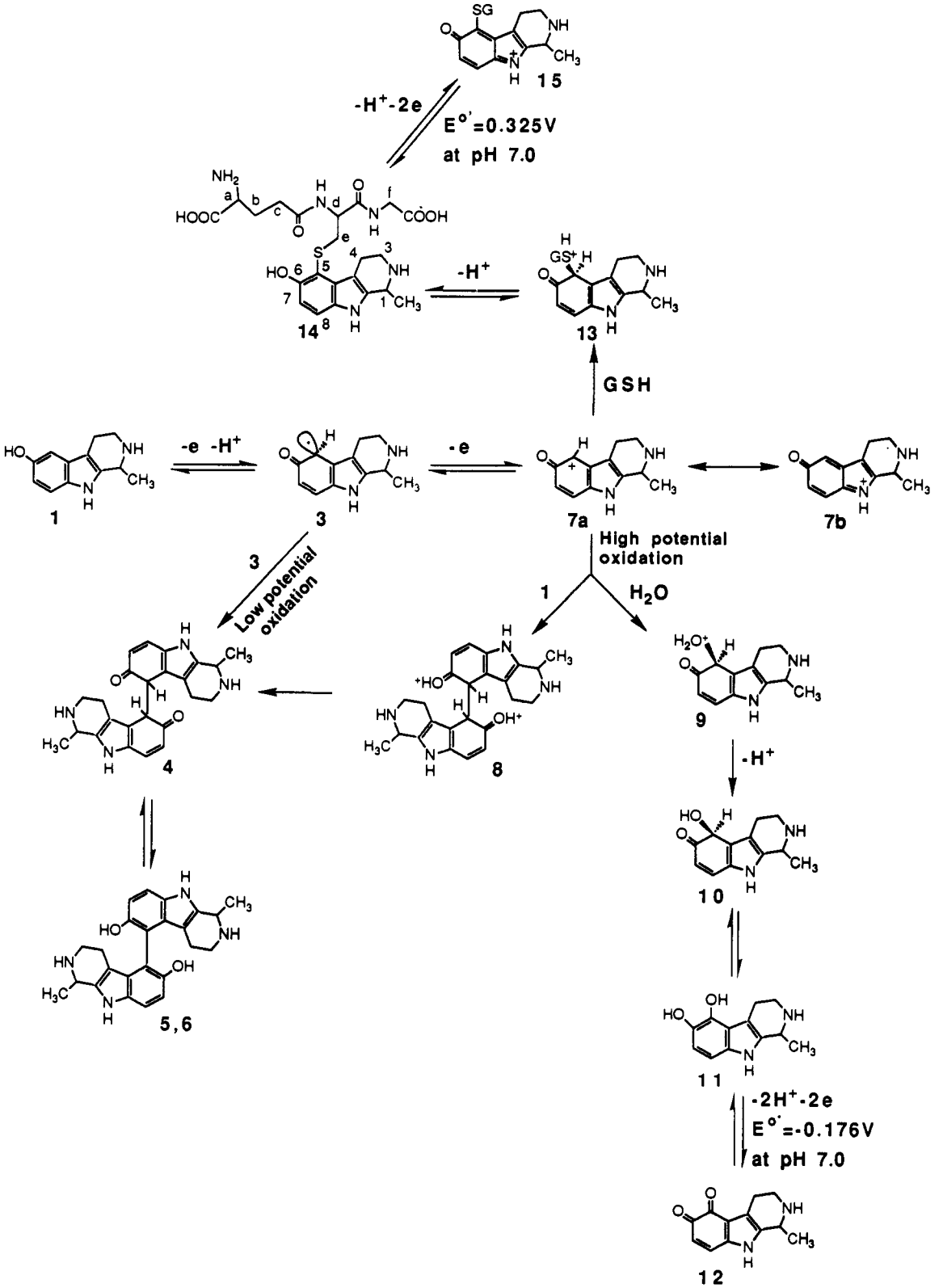


Figure 7. Cyclic voltammograms at the PGE of (1) 1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline (1; 0.58 mM), (A and B) diastereomers of 5,5′-bi(1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline) (**5** and **6**; 0.58 mM), and (C) 1-methyl-1,2,3,4-tetrahydro-β-carboline-5,6-dione (**12**) in pH 7.0 phosphate buffer (μ = 1.0). Sweep rate: 200 mV s^{-1}.

is, overall, a one-electron, one-proton abstraction reaction leading to the radical intermediate **3** which dimerizes to give diastereomers **5** and **6** as conceptualized in Scheme II. Because of the complicating effects of adsorption on the voltammetric behaviors of **1** it is not presently possible to provide a more detailed mechanism for the sequence of electron and proton transfers and the subsequent dimerization reaction using, for example, the elegant approaches of Nadjo and Savéant.[40] In order to account for the formation of dione **12** when **1** is electrooxidized at potentials equal to or more positive than E_p for peak I_a it is proposed that putative radical **3** undergoes a further one-electron oxidation to give cations **7a/7b** (Scheme II). When the initial anodic sweep in voltammograms of **1** at pH 7.0 is reversed at potentials more negative than E_p for peak I_a, there is no evidence for reduction peak I_c, whereas when the switching potential is equal to or more positive than E_p, peak I_c can be clearly observed. Accordingly, it may be concluded that peak I_c corresponds to the reversible reduction of **7a/7b**. Nucleophilic attack by **1** on carbocation **7a** leads to dimeric cation **8**, which upon deprotonation and enolization gives diastereomeric dimers **5** and **6** (Scheme II). Alternatively, nucleophilic attack by water on carbocation **7a** gives the 5,6-dihydroxy-THβC **11** by the route shown in Scheme II. The latter compound is very easily oxidized ($E^{0\prime}$ for the **11/12** couple at pH 7.0 is −0.176 V) and is therefore immediately electrooxidized

(40) Nadjo, L.; Savéant, J. M. Electrodimerization. VIII. Role of Proton Transfer Reactions in the Mechanism of Electrohydrodimerization. Formal Kinetics for Voltammetric Studies (Linear Sweep, Rotating Disc, Polarography). *J. Electroanal. Chem. Interfacial Electrochem.* **1973**, *44*, 327–366.

Scheme II



to dione 12. The decrease in the heights of reduction peaks I_c and III_c and increase in the height of oxidation peak II_a relative to that of peak I_a with increasing concentrations of 1 are in accord with the proposed reaction pathways.

At physiological pH, tyrosinase/O_2, ceruloplasmin/O_2, and peroxidase/H_2O_2 oxidize 1 primarily to 5 and 6 along with much smaller amounts of dione 12. Thus, these enzyme-mediated oxidations yield products identical to those formed in the electrochemically driven reaction. The goal of the work reported here was not to elucidate detailed mechanistic aspects of the enzyme-mediated oxidations of 1 but rather to establish that the alkaloid was susceptible to biological oxidation. However, in view of the similarities between the products formed by the electrochemical and enzymatic oxidations it seems reasonable to conclude that the *chemical* aspects of the enzyme-mediated oxidations of 1 follow the same general pathways detailed in Scheme II.

In the presence of the strong nucleophile GSH oxidations of 1 result in the formation of the glutathionyl con-

Table I. Effects of 5,5'-Bi(1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline) (5 and 6) on Whole Mouse Brain Neurochemical Levels[a]

compd	time[b]		n	NE[c]	DOPAC	DA	HVA	5-HIAA	5-HT	ACh	Ch
5 and 6	3 h										
		controls	11	100 ± 8	100 ± 11	100 ± 3	100 ± 6	100 ± 14	100 ± 6	100 ± 5	100 ± 5
		exptls	11	93 ± 6	81 ± 8	98 ± 4	90 ± 6	86 ± 10	107 ± 4	98 ± 16	83 ± 6
5 and 6	1 day										
		controls	11	100 ± 3	100 ± 6	100 ± 3	100 ± 3	100 ± 5	100 ± 2	100 ± 5	100 ± 9
		exptls	12	105 ± 4	93 ± 6	96 ± 3	107 ± 5	135 ± 5***	111 ± 4*	78 ± 8*	97 ± 113
5	1 day	controls	5	100 ± 5	100 ± 4	100 ± 3	100 ± 4	100 ± 6	100 ± 3	100 ± 8	100 ± 1
		exptls	6	102 ± 4	97 ± 7	90 ± 7	115 ± 3*	146 ± 9**	94 ± 3	87 ± 9	77 ± 6*
6	1 day										
		controls	5	100 ± 5	100 ± 4	100 ± 3	100 ± 4	100 ± 6	100 ± 3	100 ± 8	100 ± 1
		exptls	6	104 ± 6	101 ± 6	96 ± 4	132 ± 11*	163 ± 16**	107 ± 5	73 ± 14	97 ± 2
5 and 6	1 week	controls	11	100 ± 10	100 ± 9	100 ± 10	100 ± 5	100 ± 10	100 ± 9	100 ± 13	100 ± 9
		exptls	11	111 ± 8	96 ± 6	98 ± 5	100 ± 4	99 ± 5	103 ± 5	94 ± 13	107 ± 6

[a] All results expressed as percent controls ± sem. All animals treated with 40 μg of indicated compound (free base). After being lightly anesthesized with ether, the agent was delivered in 5 μL of an isotonic saline solution containing 1 mg/mL ascorbic acid in the vicinity of the left lateral ventricle. [b] Time between treatment and sacrifice. [c] Compound abbreviations used are NE, norepinephrine; DOPAC, 3,4-dihydroxyphenylacetic acid; DA, dopamine; HVA, homovanillic acid; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; ACh, acetylcholine; and Ch, choline. (*) $P < 0.05$ compared to controls. (**) $P < 0.005$ compared to controls. (***) $P < 0.001$ compared to controls.

jugate **14** at the expense of dimers **5** and **6**. Accordingly, it is proposed that GSH attacks putative carbocation **7a** to give **14** by the route conceptualized in Scheme II.

Biological Studies

The major initial electrochemical and enzymatic oxidation products of **1** are diastereomeric dimers **5** and **6**. Preliminary experiments have been carried out in order to assess the toxicity and behavioral effects evoked by **5** and **6** when centrally administered to mice and the neurochemical effects of these drugs in the CNS. In all of the biological studies reported below **5**, **6** and mixtures of **5** and **6** were administered intracranially under light ether anesthesia in the vicinity of the lateral left ventricle in 5 μL of a vehicle consisting of isotonic saline (0.9% NaCl) containing 1.0 mg/mL of ascorbic acid using the procedures described by Blank et al.[41]

LD_{50} Determination. The statistical approaches of Dixon[42,43] were employed in an attempt to measure the LD_{50} of an unseparated mixture of **5** and **6** (formed by controlled potential electrooxidations of **1**). In these studies the drugs were administered intracranially as described earlier. However, all animals survived even when the maximum dose, 200 μg, was employed.

Behavioral Effects. Behavioral effects evoked by **5**, **6**, and the unseparated mixture of **5** and **6** were very similar. At a dose of 20 μg a slight trembling of the body and limbs was observed. At doses ≥30 μg, substantial alterations in behavior were noted. Thus, after recovery from the light ether anesthesia (<15 min) animals had great difficulty walking and demonstrated a general rigidity; the tail was stiff and erect. A particularly impaired gait of the hind limbs gave the appearance that these limbs were being partially dragged during forward movement. Approximately 3 h after injection, animals exhibited spatial disorientation, walking and stumbling into objects and the side of the cage, and tremor. By this same time, all had developed a duck-like gait in the hind limbs in which the thighs were adducted to the body and the legs were extended laterally, reminiscent of the effects often evoked by the opioid analgesics.[44] The latter behavioral effects continued for approximately 3 days with recovery to normal gait and behavior for all animals being achieved by the fourth day following treatment. However, the recovery to normal behavior was usually more rapid following treatment with isomer **6** and began within 1 day and was complete within 2 days following treatment.

The minimal dose of **5** and/or **6** necessary to evoke the behavioral effects described above was 30 μg (administered intracranially in 5 μL of vehicle; $n = 6$) although, in fact, most of the detailed observations were performed following a 40-μg dose ($n = 92$). Larger doses (up to 200 μg) did not appear to appreciably intensify the observed behavioral effects or significantly increase their duration. However, neither the behavioral effects nor neurochemical effects (see the next section) evoked following intracranial administration of **5** and/or **6** were studied in any detail following doses greater than 40 μg. Intracranial injection of vehicle alone (5 μL) resulted in none of the behavioral responses described following administration of **5** and/or **6**. Indeed, animals treated with vehicle alone exhibited identical behavior to totally untreated animals once they had completely recovered from the anesthesia (ca. 30 min).

Neurochemical Effects. The effects of **5**, **6**, and the unseparated mixture of the two diastereomers on various transmitters and metabolites were assessed 3 h, 1 day, and 1 week following intracranial administration of the drugs. The concentrations of norepinephrine (NE), dopamine (DA), 5-HT, acetylcholine (ACh), and related metabolites in whole mouse brain after drug treatments are shown in Table I. No significant differences between controls and experimentals were observed in the levels of compounds measured for animals sacrificed 3 h or 1 week after drug administration. However, at 1 day following treatment there was a significant increase ($P < 0.005$) in the level of the 5-HT metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) in the treated animals of all three groups compared to controls. Similarly, there was a significant increase ($P < 0.05$) in the DA metabolite homovanillic acid (HVA) level of animals treated with diastereomers **5** and **6** and a corresponding, but not significant, increase in the HVA level for the animals treated with a mixture of **5** and **6**. There was also a significant decrease ($P < 0.05$) in the ACh level of animals treated with a mixture of **5** and **6**, a significant decrease ($P < 0.05$) in the choline (Ch) level

(41) Blank, C. L.; Murrill, E.; Adams, R. N. Central Nervous System Effects of 6-Aminodopamine and 6-Hydroxydopamine. *Brain Res.* **1972**, *45*, 635–637.
(42) Dixon, W. J. The Up-and-Down Method for Small Samples. *J. Am. Stat. Assoc.* **1965**, *60*, 967–978.
(43) Dixon, W. J.; Mood, A. M. A Method for Obtaining and Analyzing Sensitivity Data. *J. Am. Stat. Assoc.* **1948**, *43*, 109–126.
(44) Irwin, S. Comprehensive Observational Assessment: Ia. A Systematic, Quantitative Procedure for Assessing the Behavioral and Physiologic State of the Mouse. *Psychopharmacologia* **1968**, *13*, 222–257.

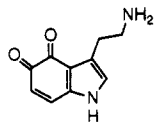
evoked by 5, and a small but statistically significant rise in the 5-HT level in animals treated with the mixture of 5 and 6. Such observations suggest a general increase in dopaminergic and serotonergic activity and a slight decrease in cholinergic activity associated with the behavioral effects evoked by the drugs during the first few days following treatment.

Conclusions

The rationale for the work described above was to explore the possibility that oxidative transformations of 1 in the brain might lead to toxic and/or neuropharmacologically active metabolites which play a role in the neurodegenerative, addictive, and behavioral effects evoked as a result of chronic consumption of ethanol. It has been established from electrochemical experiments that at physiological pH 1 is an easily oxidized compound, that key radical (3) and, more importantly, carbocation (7a) intermediates are formed. These putative intermediates react to give diastereomeric dimers 5 and 6. However, carbocation 7a also reacts with nucleophiles such as water to give, ultimately, dione 12 or glutathione to give conjugate 14. Several different representative oxidative enzyme systems mediate the same chemical transformations of 1. In the initial stages of the electrochemically driven and enzyme-mediated oxidations of 1 (in the absence of GSH) dimers 5 and 6 are quite clearly the overwhelmingly predominant products. However, because 5 and 6 are both oxidizable, more prolonged electrooxidations or enzymatic oxidations result in the formation of very much more complex mixtures of products. Perhaps surprisingly, neither the dihydro nor the fully aromatic β-carbolines are among the identified oxidation products of 1. In the presence of GSH, conjugate 14 becomes the major initial oxidation product of 1 at the expense of dimers 5 and 6 (except for the ceruloplasmin/O_2 oxidation reaction). Glutathionyl conjugate 14 is also a relatively easily oxidized compound (E_p = +0.324 V in pH 7.0 phosphate buffer; ν = 100 mV s^{-1}), although attempts to investigate the secondary oxidation reactions of this compound have not yet been carried out. The role, if any, of oxidative transformations of 1 in the etiology of chronic alcoholism remains to be established. Nevertheless, the results reported here indicate that biologically mediated oxidations of the alkaloid are certainly feasible reactions. The major oxidation products of 1, diastereomeric dimers 5 and 6, are not lethal when centrally administered to mice at doses as high as 200 μg. However, 5, 6, and a mixture of the diastereomers evoke profound and very long-lasting behavioral responses. Many of these behavioral effects are similar to those evoked by opioid analgesics.[44] These observations appear to be the first which demonstrate that a simple oxidative transformation of the endogenous central alkaloid, 1, which is significantly elevated in the brain following chronic ethanol ingestion, leads to drugs which could potentially play a functional role in the behavioral aberrations characteristic of chronic alcoholics. Neurotransmitter and metabolite analyses of whole mouse brain following treatments with 5 and 6 reveal alterations in the dopaminergic, serotonergic, and cholinergic pathways for time periods which approximately parallel the observed behavioral aberrations. It is important to note that the studies reported here describe effects evoked by acute central administration of relatively large concentrations of drugs 5 and 6. Should oxidative transformations of 1 and related THβCs occur in the brains of alcoholic individuals, it is probable that the effects of 5 and 6 are evoked by much lower concentration levels which are formed over much longer periods of time, i.e., years and decades. Thus, further studies of long-term administration of 5 and 6 at much lower concentration levels are clearly called for. In addition, efforts to detect and characterize oxidation products of 1 in the CNS of both normal and alcoholic patients are required to provide additional support for functional roles for 5 and 6 are necessary. We hope to report on such studies in the future.

A key intermediate in the electrochemical and enzymatic oxidations of 1 is carbocation 7a, an electrophilic species which reacts avidly with GSH to form, initially, glutathionyl conjugate 14. A number of pharmacologically useful neurotoxins such as 5,6- and 5,7-dihydroxytryptamine[33,34] and 6-hydroxydopamine[30,31] are widely believed to express their neurodegenerative effects as a consequence of intraneuronal oxidation to highly electrophilic intermediates which alkylate cellular proteins which consequently compromises their biological function. Thus, in the event that 1 does undergo in vivo oxidations, 7a has the potential to similarly inflict neuronal damage.

Under most of the experimental conditions investigated in this work, dione 12 was a relatively minor oxidation product of 1. A structurally similar compound, tryptamine-4,5-dione (16), is formed as a result of the electro-



16

chemical[45,46] and enzymatic[47] oxidations of the neurotransmitter 5-HT and has been claimed to be a serotonergic neurotoxin.[48] The 7-*S*-glutathionyl conjugate of 16 is a very powerful toxin when centrally administered to the mouse.[49] Thus, by analogy with these compounds it is not unlikely that 12 might possess toxic properties. Unfortunately, 12 is not a very stable compound with the result that it has not yet been possible to screen pure samples of this drug in animal experiments.

Overall, the results reported in this study provide support for a potential role for oxidative transformations of 1 into metabolites which might play roles in the behavioral and neuropathological consequences of chronic alcoholism.

Experimental Section

5-Hydroxytryptamine hydrochloride (5-HT·HCl), acetaldehyde, acetylcholine chloride, choline chloride, acetylthiocholine chloride, norepinephrine hydrochloride, 3,4-dihydroxyphenylacetic acid, dopamine hydrochloride, 5-hydroxyindole-3-acetic acid, serotonin creatinine sulfate monohydrate, homovanillic acid, disodium ethylenediaminetetraacetic acid dihydrate, guaiacol, glutathione, tyrosinase (mushroom, EC 1.14.18.1), ceruloplasmin (human type X in 0.25 M sodium chloride plus 0.05 M sodium acetate, pH 7.0), and peroxidase (type IV from horseradish, EC 1.11.1.7) were obtained from Sigma (St. Louis, MO). Glacial acetic acid, sodium acetate trihydrate, sodium perchlorate, sodium octylsulfate, di-

(45) Wrona, M. Z.; Dryhurst, G. Oxidation Chemistry of 5-Hydroxytryptamine. Part II. Mechanisms and Products Formed at Millimolar Concentrations in Acidic Aqueous Solution. *J. Electroanal. Chem. Interfacial Electrochem.* **1990**, *278*, 249–267.

(46) Wrona, M. Z.; Dryhurst, G. Electrochemical Oxidation of 5-Hydroxytryptamine in Aqueous Solution at Physiological pH. *Bioorg. Chem.* **1990**, *18*, 291–317.

(47) Wrona, M. Z.; Dryhurst, G. Interactions of 5-Hydroxytryptamine with Oxidative Enzymes. *Biochem. Pharmacol.* **1991**, *41*, 1145–1162.

(48) Crino, P. B.; Vogt, B. A.; Chen, J.-C.; Volicer, L. Partially Oxidized Serotonin: Neurotoxic Properties and Sites of Damage. *Brain Res.* **1989**, *504*, 247–257.

(49) Wong, K.-S.; Goyal, R. N.; Wrona, M. Z.; Blank, C. L.; Dryhurst, G. In preparation.

ethylamine, citric acid monohydrate, sodium hydroxide, and phosphoric acid (85%) were obtained from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade acetonitrile (MeCN) and methanol (MeOH), concentrated ammonium hydroxide (NH_4OH), trifluoroacetic acid (TFA), and sodium chloride were obtained from Fisher Scientific Company (Fair Lawn, NJ). All chemicals were of the highest possible purity available and were used without subsequent purification.

Cyclic voltammograms were obtained at a pyrolytic graphite electrode (PGE, Pfizer Minerals, Pigments and Metals Division, Easton, PA) having an approximate surface area of 12.6 mm^2. The PGE was resurfaced before recording each voltammogram as described previously.[50] A BAS Model 100A electrochemical analyzer (Bioanalytical Systems, West Lafayette, IN) was used to obtain cyclic voltammograms, which were all corrected for iR drop. Controlled potential electrolyses employed a PARC Model 173 potentiostat (PARC, Princeton, NJ) and several plates of pyrolytic graphite as the working electrode having a total surface area of ca. 150 cm^2. The latter electrodes were suspended into 30 mL of the appropriate support electrolyte solution. Conventional three-compartment electrochemical cells were used for voltammetry and controlled potential electrolysis and contained a platinum counter electrode and a saturated calomel reference electrode (SCE). All potentials are referred to the SCE at ambient temperature (22 ± 2 °C).

High-performance liquid chromatography (HPLC) used to study the oxidation chemistry and biochemistry of 1 for both analytical purposes and to preparatively isolate and purify oxidation products employed a Bio-Rad (Richmond, CA) binary gradient system equipped with dual Model 1300 pumps and a Gilson (Middletown, WI) Holochrome UV detector (typically set at 280 nm). A semipreparative reversed-phase column (25 × 1 cm, C_{18}; Spherisorb, S5 ODS 2, Queensferry, Clwyd, UK) and a preparative reversed-phase column (J. T. Baker, Phillipsburg, NJ; Bakerbond C_{18}, 25 × 2.1 cm; 10-μm particle size) were employed. Three binary gradient mobile phase systems were used which will be referred to as gradient systems I, II, and III.

For gradient systems I and II the following mobile phase solvents were employed: solvent A was prepared by adding 15 mL of NH_4OH and 1200 mL of HPLC-grade MeOH to 3785 mL of deionized water; the pH of the resulting solution was adjusted to 3.0 by careful addition of concentrated TFA. Solvent B was prepared by adding 30 mL of NH_4OH to 3970 mL of water and adjusting the pH to 3.0 with TFA. For gradient system I the following conditions were employed: semipreparative HPLC column; flow rate 3 mL min^{-1}; 0–30 min, linear gradient from 100% solvent B to 100% solvent A; 30–45 min, 100% solvent A. The mobile phase was then returned to 100% solvent B over 5 min and maintained at 100% solvent B for 5 min before the next sample was introduced.

For gradient system II the preparative HPLC column was employed at a constant flow rate of 7 mL min^{-1}; 0–20 min linear gradient from 100% solvent B to 100% solvent A; 20–40 min, 100% solvent A. The mobile phase was then returned to 100% solvent B over 5 min and maintained at 100% solvent B for 5 min before the next sample was introduced.

For gradient system III the following mobile phase solvents were used: solvent C was prepared by adding 500 mL of HPLC-grade MeCN to 500 mL of water; the pH was then adjusted to 2.0 with TFA. Solvent D was prepared by adding TFA to water until the pH was 2.0. For gradient system III the following conditions were employed: semipreparative HPLC column; flow rate 3 mL min^{-1}; 0–20 min, linear gradient from 100% solvent D to 100% solvent C; 20–40 min, 100% solvent C. The mobile phase was then linearly returned to 100% solvent D and maintained for 5 min with 100% solvent D before the next sample was introduced.

Low- and high-resolution fast atom bombardment mass spectrometry (FAB-MS) was carried out with a VG Instruments Model ZAB-E spectrometer. Liquid chromatography–mass spectrometry (LC-MS) was performed on a Kratos MS 25/RFA spectrometer equipped with a thermospray source. The solvent was 0.1 M ammonium acetate in water adjusted to pH 4.7–5.0 with acetic acid. A flow rate of 0.9 mL min^{-1} was employed. The thermospray capillary was maintained at 152 °C and the source at 250 °C. Aliquots (1–2 mL) of product solutions collected by HPLC separation and hence dissolved in the chromatographic mobile phase (pH 3.0) were injected directly into the LC–MS system using a conventional loop injector (Rheodyne Model 7125). ^1H and ^{13}C NMR spectra were recorded on a Varian Model XL-300 spectrometer. Assignments of proton resonances in NMR spectra were based upon comparison of the spectra of products with that of authentic 1 and by selective homonuclear decoupling experiments. UV–visible spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer.

Outbred adult male mice of the Hsd:ICR albino strain (Harlan Sprague–Dawley, Madison, WI) weighing 25–35 g were used in all animal experiments. The animals were housed 10 per cage, allowed access to Purina Rat Chow and water ad libitum, and maintained on a 12-h light/dark cycle with lights on at 7:00 a.m. No animals were used in experiments until at least 7 days after receipt from the supplier. All experimentals were treated with drugs by intracranial injection in the vicinity of the left lateral ventricle[42] under light ether anesthesia. Drugs were delivered in 5 μL of a vehicle consisting of isotonic saline (0.9% NaCl in water) containing 1.0 mg/mL of ascorbic acid. Control animals were treated with vehicle alone.

In order to assess neurotransmitter/metabolite effects experimental animals were treated with 5 μL of vehicle containing 40 μg of the test compound. After an appropriate time lapse (3 h, 1 day, 1 week) animals were sacrificed by exposure to 250 ms of 7.0 kW of microwave radiation (NJE-2603-10kW Microwave Irradiator, New Japan Radio Corp., Tokyo, Japan) concentrated on the head. This method of sacrifice ensures rapid inactivation of CNS enzymes which is essential for accurate determinations of the neurotransmitters and metabolites of concern.[51] 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 0.50 M acetic acid, 0.50 M sodium acetate, 0.40 M $NaClO_4$, 4.70 nmol/mL guaiacol, and 40.0 nmol/L acetylthiocholine. The latter two compounds were used as internal standards in the liquid chromatographic analysis described below. Homogenization was accomplished with a Kontes ground-glass Duall 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 (50000*g*) at 4 °C for 1 h. The supernate was filtered through a 0.45μ 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 °C in the polypropylene tube until analysis by liquid chromatography with electrochemical detection.

Liquid Chromatography with Electrochemical Detection. Two separate liquid chromatography systems were assembled in-house from commercially available equipment. The first system was devoted to the determination of acetylcholine and choline; the second to the determination of NE, DA, 5-HT, and related metabolites. The first system consisted of a Bio-Rad Model 1330 pump, a Model 7010 Rheodyne injection valve (Alltech Associates, Deerfield, IL) with a fixed 20-μL loop, a BAS polymeric guard column, a BAS 3.2 × 100 mm polymeric analytical column, a 4.0-cm BAS postcolumn reactor containing immobilized acetylcholinesterase and choline oxidase, a thin-layer platinum electrode

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cell with the electrode set at +0.50 V vs Ag/AgCl, and a BAS Model LC-4B potentiostat. The second system[55] employed a Milton Roy MiniPump Model NSI-33R, a Model 7010 Rheodyne valve with a fixed 5-μL injection loop, a BAS Phase-II reversed phase column (ODS, 3 μm, 3.2 × 100 mm), a thin-layer glassy electrode cell with the electrode set at +0.80 V vs Ag/AgCl, and a Model S-101 potentiostat (Great Plains Laboratories, Norman, OK). The output signals from both systems were monitored on Houston Instruments Omniscribe recorders.

The mobile phase for the acetylcholine/choline system was prepared by dissolving 18.6 mg of $Na_2EDTA{\cdot}2H_2O$ and 3.40 mL of concentrated (85%) H_3PO_4 in ca. 900 mL of deionized water. The pH was adjusted to 8.50 with NH_4OH and the resulting solution was diluted to 1.00 L with deionized water. The mobile phase was filtered through a 0.45-μM membrane (HAWP-047-000, Waters Associates, Milford, MA). Following filtration, 5.0 mL of 1% Kathon CG (BAS) was added to this mobile phase to retard bacterial growth. This LC system was operated with a mobile-phase flow rate of 1.5 mL/min and a pressure of ca. 1500 psi.

The mobile phase for the second system was prepared by adding 1.70 mL of diethylamine, 37.22 mg of $Na_2EDTA{\cdot}2H_2O$, 42.0 g of citric acid monohydrate, and 118.44 mg of sodium octyl sulfate to 1500 mL of deionized water. The pH was adjusted to 2.3 using dilute NaOH. The resulting solution was filtered through a 0.45-μm filter. Prefiltered HPLC grade MeCN (150 mL) and deionized water (350 mL) were added to the filtrate. The resulting solution was deaerated by bubbling with He for 30 min. This mobile phase a typically employed at a flow rate of 1.8 mL/min and a pressure of ca. 2500 psi.

The concentrations of the neurochemicals of concern were determined using the expression:

$$\text{concentration (nmol/g)} = \frac{R_{\text{sample}}}{R_{\text{external standard}}} \times \frac{\text{nmol in external standard}}{\text{weight of sample, g}}$$

where R_{sample} = the ratio of the peak height of the compound of concern to that of the internal standard in a single tissue sample; $R_{\text{external standard}}$ = average ratio of the peak height of the compound of concern to that of the internal standard in all standard samples chromatographed; nmol chemical in external standard = the number of nanomoles of the compound of concern contained in a single external standard sample; weight sample, g = weight of the tissue sample in grams.

Typical concentrations found in the whole-brain analyses for controls (nmol/g, wet tissue weight) were as follows: NE, 1.96 ± 0.09; DOPAC, 0.71 ± 0.08; DA, 5.63 ± 0.19; 5-HIAA, 0.88 ± 0.06; HVA, 1.13 ± 0.03; 5-HT, 2.89 ± 0.20; ACh, 40.5 ± 4.6; and Ch, 62.8 ± 9.2. The final results of all determinations are reported as the average plus or minus the standard error of the mean, with both quantities being expressed as percent of controls. Student's t test was used to examine statistical significance with $P < 0.05$ being taken to indicate a significant difference. The software employed for statistical evaluations was obtained from Great Plains Laboratories (Norman, OK).

Synthesis of 1-Methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline (1). There have been a few reports of attempts to chemically synthesize 1 by the Pictet–Spengler[5,6] condensation between 5-HT and acetaldehyde. These have been either unsuccessful[52] or involved extremely complex separation and purification procedures.[53] More indirect methods to synthesize 1 are complex.[52] The following is a simple, direct method for the synthesis of 1 by the Pictet–Spengler reaction. Acetaldehyde (0.44 mL) and 5-HT·HCl (200 mg) were dissolved in 24 mL of pH 4.2 phosphate buffer (ionic strength 0.5[54]). The reaction vessel was covered with Parafilm (Greenwich, CT) and maintained at 37 °C in a thermostated water bath for ca. 12 h. The reaction mixture was extracted three times with 30 mL of diethyl ether to remove unreacted acetaldehyde. The aqueous solution was then adjusted to pH 10.8 by careful addition of concentrated NH_4OH solution under a nitrogen atmosphere. The resulting solution was stored at −10 °C for approximately 30 min and a white precipitate formed. This precipitate was filtered and washed with water to remove unreacted 5-HT and was dried under vacuum. The yield of 1 so obtained was 120 mg (ca. 63%); mp 225–230 °C dec. HPLC analysis using gradient systems I and II confirmed that the product was formed in high purity. The UV spectrum of 1 in pH 7.0 phosphate buffer (μ = 1.0) showed λ_{max}, nm (log ϵ_{max}): 297 (sh, 3.72), 274 (3.90), 222 (4.30). FAB-MS (3-nitrobenzyl alcohol matrix) gave a pseudomolecular ion (MH^+) at m/e = 203.1168 (100; $C_{12}H_{15}N_2O$, calcd m/e = 203.1184). ^1H NMR (Me_2SO-d_6): δ 10.32 (s, 1 H, N(9)-H), 8.49 (s, 1 H, OH), 7.04 (d, $J_{7,8}$ = 8.4 Hz, 1 H, C(8)-H), 6.65 (s, 1 H, C(5)-H), 6.51 (d, $J_{7,8}$ = 8.4 Hz, 1 H, C(7)-H), 3.95 (q, J = 6.3 Hz, 1 H, C(1)-H), 3.34 (s, 1 H, N(2)-H), 3.11 (m, 1 H), 2.71 (m, 1 H), 2.47 (m, 2 H), 1.32 (d, J = 6.3 Hz, 3 H, CH_3). ^{13}C NMR (Me_2SO-d_6): δ 150.09 (C(6)), 138.91, 130.04, 127.81, 110.90, 110.06, 105.96, 101.82, 47.89, 42.23, 22.48, 20.51. These spectral data are in complete accord with those expected for 1 (molar mass 202 g, $C_{12}H_{14}N_2O$).

Isolation and Purification of Oxidation Products of 1. In a typical preparative electrolysis 30 mL of a solution of 1 (0.58 mM in pH 7.0 phosphate buffer, μ = 0.15) was electrooxidized at +0.30 V for 20–30 min. The electrolyzed solution was stirred vigorously with a magnetic stirring bar, and nitrogen gas was bubbled through the solution. The resulting product solution was separated using the preparative reversed-phase HPLC column and gradient system II. A single injection of the total product solution (30 mL) onto the column was made. One major product peak eluted at a retention time (t_R) of 27 min and was collected. Under the conditions employed for the controlled potential electrolysis not all 1 was oxidized. The peak corresponding to 1 eluted at t_R = 29 min. The solution collected under the product peak was freeze-dried, and the resulting solid was dissolved in the minimum volume of water (ca. 1–2 mL). This was then separated into 5 and 6 by injecting the solution (1-mL aliquots) onto the semipreparative reversed-phase HPLC column using gradient system I. Compound 5 eluted at t_R = 27.5 min; compound 6 at t_R = 28 min. The solutions containing 5 and 6 were collected individually and were freeze-dried. The resulting solids were individually dissolved in water and were desalted and purified using gradient system III and the semipreparative reversed-phase HPLC column. The solutions containing 5 and 6 were then freeze-dried. These procedures were repeated several times until sufficient quantities of 5 and 6 were obtained.

5,5′-Bi(1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline) (5). Compound 5 was isolated as a very pale yellow powder. In pH 7.0 phosphate buffer (μ = 1.0) λ_{max}, nm (log ϵ_{max}): 310 (3.76), 278 (3.83). FAB-MS (3-nitrobenzyl alcohol matrix) gave m/e = 402 (M^+, 5.6), 403 (MH^+, 7.5), 517 ($MH^+ + CF_3COOH$, 1.4). Accurate mass measurements of M^+ gave m/e = 402.2056 ($C_{24}H_{26}N_4O_2$, calcd m/e = 402.2056). Hence, 5 is a dimer of 1 having molar mass of 402 g and a molecular formula $C_{24}H_{26}N_4O_2$. ^1H NMR (Me_2SO-d_6): δ 10.75 (s, 2 H, N(9)-H and N(9′)-H), 8.11 (s, 2 H, N(2)-H and N(2′)-H), 7.16 (d, $J_{7,8}$ and $J_{7',8'}$ = 8.4 Hz, 2 H, C(7)-H and C(7′)-H), 6.78 (d, $J_{7,8}$ and $J_{7',8'}$ = 8.4 Hz, 2 H, C(8)-H and C(8′)-H), 4.56 (m, 2 H, C(1)-H and C(1′)-H), 3.21–1.61 (m, 8 H, C(3)-H_2 and C(3′)-H_2; C(4)-H_2 and C(4′)-H_2), 1.56 (d, J = 6.6 Hz, 6 H, 2 CH_3).

5,5′-Bi(1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline) (6). Compound 6 was isolated as a pale yellow powder. In pH 7.0 phosphate buffer (μ = 1.0) λ_{max}, nm (log ϵ_{max}): 310 (sh, 3.77), 276 (3.83). FAB-MS (3-nitrobenzyl alcohol matrix) gave m/e = 402 (M^+, 46), 403 (MH^+, 69), 517 ($MH^+ + CF_3COOH$, 25). Accurate mass measurements on M^+ gave m/e = 402.2101 ($C_{24}H_{26}N_4O_2$, calcd m/e = 402.2056). Thus, 6 is a dimer of 1. ^1H NMR (Me_2SO-d_6): δ 10.67 (s, 2 H, N(9)-H) and N(9′)-H), 8.04 (s, 2 H, N(2)-H and N(2′)-H), 7.13 (d, $J_{7,8}$ and $J_{7',8'}$ = 8.5 Hz, 2 H, C(7)-H and C(7′)-H), 6.75 (d, $J_{7,8}$ and $J_{7',8'}$ = 8.5 Hz, 2 H, C(8)-H and C(8′)-H), 4.50 (m, 2 H, C(1)-H and C(1′)-H), 3.20–1.78 (m, 8 H, C(3)-H_2, C(3′)-H_2, C(4)-H_2, C(4′)-H_2), 1.51 (d, J = 6.0 Hz, 6 H, 2 CH_3).

1-Methyl-1,2,3,4-tetrahydro-β-carboline-5,6-dione (12). In order to prepare 12 in relatively high yields a solution of 1 (0.2 mM in 15 mL of pH 1.5 phosphate buffer, μ = 0.1) was electrooxidized at +1.0 V for 20 min. The resulting purple solution was then purified by HPLC using the semipreparative reversed-phase

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column. A binary gradient mobile phase was used. Solvent E was prepared by adding 1200 mL of MeOH and 15 mL NH_4OH to water to make a total volume of 4000 mL. The resulting solution was adjusted to pH 3.0 with TFA. Solvent F was prepared by adding 30 mL of NH_4OH to 4000 mL of water, and then the pH was adjusted to 3.0 with TFA. The gradient profile was as follows: 0–30 min linear gradient from 100% solvent F to 100% solvent E; 30–45 min, 100% solvent E. The flow rate throughout was maintained at 3.0 mL min^{-1}. The chromatographic peak corresponding to 12 eluted at t_R = 29.0 min and was collected. In the HPLC mobile phase (pH 3.0), the solution containing 12 was bright purple; λ_{max} = 540, 350, 238 nm. Cyclic voltammetry of 12 in the same medium showed a reversible redox couple with $E^{\circ\prime}$ = +0.080 V. LC-MS of 12 dissolved in the chromatographic mobile phase showed intense ions at m/e = 217 (MH^+, 90), 219 ($MH_2{\cdot}H^+$, 100). The collected purple solution was freeze-dried, and then the resulting solid was dissolved in a minimum volume of solvent H and desalted by an HPLC method. A reversed-phase HPLC column was used (Brownlee Laboratories, Santa Clara, CA; RP-18 5 mm, 25 × 0.7 cm) with a binary gradient mobile phase system. Solvent G was prepared by diluting 800 mL of MeCN and 8 mL of concentrated formic acid (HCOOH) to 4000 mL with water. Solvent H was prepared by adding 4 mL of HCOOH to 4000 mL of water. The gradient profile was as follows: 0–15 min, linear gradient from 100% solvent H to 100% solvent G; 15–25 min, 100% solvent G. The flow rate was 3 mL min^{-1}. Typically 1-mL aliquots of the solution of 12 were injected. Ammonium trifluoroacetate eluted at t_R = 3 min; 12 eluted at t_R = 16 min. The eluant containing 12 (and formic acid) was lyophilized to give a fluffy purple solid. EI-MS (70 eV, 138 °C) gave m/e = 218 (MH_2^+, 56). In order to obtain a ^1H NMR spectrum only very dilute (ca. 100 μM) solutions of 12 could be employed. At such concentrations 12 appeared to be stable for several hours. However, ^1H NMR spectra of more concentrated solutions showed no aromatic proton resonances, suggesting that extensive polymerization occurred. ^1H NMR (D_2O): δ 7.29 (d, $J_{7,8}$ = 10.2 Hz, 1 H, C(7)-H), 6.01 (d, $J_{7,8}$ = 10.2 Hz, 1 H, C(8)-H), 3.65–3.61 (m, 1 H, C(4)-H), 3.40–3.34 (m, 1 H, C(4)-H), 3.02–2.98 (m, 2 H, C(3)-H_2), 1.60 (d, J = 6.9 Hz, 3 H, CH_3). The expected quartet for C(1)-H was masked by the large solvent peak (HOD).

Enzymatic Oxidations of 1. All enzymatic oxidations of 1 were performed in pH 7.0 phosphate buffers (μ = 0.15). Exact experimental conditions are presented elsewhere in this paper. However, oxidations by tyrosinase and ceruloplasmin were always carried out in reaction vessels which were exposed to the atmosphere. Peroxidase/H_2O_2 oxidations were carried out with N_2 gas bubbling vigorously through the reaction solution. Chromatographic and purification procedures employed to separate the products of enzyme-mediated oxidations were identical to those employed to obtain electrochemical oxidation products. That the products of the electrochemical and enzymatic oxidations of 1 were identical was always confirmed by HPLC retention times, UV–visible spectra, cyclic voltammetric behaviors, and mass and ^1H NMR spectra.

5-*S*-Glutathionyl-1-methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline (14). In order to prepare and isolate 14 several procedures were employed. In electrochemical experiments GSH (0.76 mM) and 1 (0.5 mM) dissolved in pH 7.0 phosphate buffer (30 mL) were oxidized at pyrolytic graphite electrodes at +0.3 V for 30 min. Nitrogen gas was continuously bubbled through the solution which was also vigorously stirred with a Teflon-coated magnetic stirring bar. The entire 30-mL reaction solution was then injected onto a preparative HPLC column using gradient system I. Chromatographic peak 14 (Figure 6A) was collected, and the resulting solution was freeze-dried. The dry solid residue was dissolved in the minimum volume of cold water and desalted/purified using gradient system III and a semipreparative reversed-phase column. After freeze-drying 14 was obtained as a white solid.

For enzyme-mediated reactions GSH (0.72 mM), 1 (0.46 mM), and tyrosinase (164.6 units mL^{-1}) or ceruloplasmin (44.7 units mL^{-1}) in pH 7.0 phosphate buffer were stirred in air at ambient temperature for 65 min (tyrosinase) or 350 min (ceruloplasmin). Alternatively, peroxidase (type IV; 4 units mL^{-1}), H_2O_2 (0.22 mM), GSH (0.72 mM), and 1 (0.5 mM) were incubated in pH 7.0 phosphate buffer at ambient temperature with N_2 bubbling through the solution for 3 min. The resulting product solutions were separated and compound 14 purified in the same way described previously.

In pH 7.0 phosphate buffer 14 exhibited a characteristic UV spectrum, λ_{max}, nm (log ϵ_{max}): 296 (3.79), 235 (sh, 4.04). FAB-MS (3-nitrobenzyl alcohol matrix) gave an intense pseudomolecular ion at m/e = 508.1866 (MH^+, 33; $C_{22}H_{30}N_5O_7S$, calcd m/e = 508.1866). Thus, 14 consists of one residue each of 1 and GSH and has a molecular formula $C_{22}H_{29}N_5O_7S$. ^1H NMR (D_2O): δ 7.35 (d, $J_{7,8}$ = 8.7 Hz, 1 H, C(8)-H), 6.87 (d, $J_{7,8}$ = 8.77 Hz, 1 H, C(7)-H), 4.48 (t, J = 6.0 Hz, 1 H, C(a)-H), 4.41 (t, J = 6.0 Hz, 1 H, C(d)-H), 3.80–3.69 (m, 4 H, C(f)-H_2 and C(e)-H_2), 3.43–3.37 (m, 2 H, C(c)-H_2), 3.24 (d, J = 6.0 Hz, 2 H, C(b)-H_2), 2.33–1.80 (m, 4 H, C(3)-H_2 and C(4)-H_2), 1.68 (d, J = 6.6 Hz, 3 H, C(1)-CH_3). The above assignments were based upon comparisons with the ^1H NMR spectra of 1 and GSH and by use of homonuclear selective decoupling experiments and 2D correlated spectroscopy (COSY) experiments. The latter experiments revealed that the resonance at 1.68 ppm (C(1)-CH_3) was coupled with a resonance at 4.78 ppm, which was masked by a large solvent (HOD) peak. The resonance at 4.78 is a quartet corresponding to C(1)-H.

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Registry No. 1, 3000-36-0; 5, 137743-76-1; 6, 137743-77-2; 12, 137743-78-3; 14, 137743-79-4; GSH, 70-18-8; NE, 51-41-2; DOPAC, 102-32-9; DA, 51-61-6; HVA, 306-08-1; 5-HIAA, 54-16-0; 5-HT, 50-67-9; ACh, 51-84-3; Ch, 62-49-7; acetaldehyde, 75-07-0; serotonin hydrochloride, 153-98-0; peroxidase, 9003-99-0; tyrosinase, 9002-10-2; ceruloplasmin, 9031-37-2.