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INTERACTIONS OF SALSOLINOL WITH OXIDATIVE ENZYMES

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Abstract—Tyrosinase (EC 1.14.18.1)/O_2, ceruloplasmin (human type X)/O_2, and peroxidase (EC 1.11.1.7)/H_2O_2 oxidized the endogenous central nervous system alkaloid salsolinol (SAL) at physiological pH. The proximate oxidation product was an electrophilic *ortho*-quinone (**4**) which at pH 7.0 rapidly tautomerized. Four major initial products were formed from **4**: *cis*- and *trans*-1,2,3,4-tetrahydro-1-methyl-4,6,7-isoquinolinetriol (**A** and **B**, respectively), 2,3,4-trihydro-1-methyl-7-hydroxy-6-oxy-isoquinoline (**C**), and 1-methyl-6,7-isoquinoline diol (**D**). Mechanisms describing the formation of these products have been presented. *Ortho*-quinone **4**, formed in the enzyme-mediated reactions, was rapidly attacked by glutathione to yield the 5-*S*-, 8-*S*-, and 5,8-bi-*S*-glutathionyl conjugates of SAL. Preliminary experiments indicated that injection of **A**, **B** and **C** into the CNS of mice evoked profound behavioral effects. Quinone methide **C** was toxic. The potential role of the oxidation of salsolinol in the neurodegenerative and behavioral effects associated with chronic alcoholism is discussed.

The tetrahydroisoquinoline (TIQ†) alkaloid salsolinol (SAL; 1,2,3,4-tetrahydro-1-methyl-6,7-isoquinoline diol) is a normal, trace constituent of the mammalian central nervous system (CNS) [1–3]. Levels of SAL increase in certain brain regions of rats which chronically consume ethanol [4–6]. In humans, urinary levels of SAL become elevated during ethanol intoxication and decline during detoxification [3, 7]. A very simple route for the formation of SAL is by the Pictet–Spengler condensation reaction between the catecholamine neurotransmitter dopamine (DA) and acetaldehyde (Scheme I) [8, 9]. In chronic alcoholism, it has been suggested that acetaldehyde, the proximate metabolite of ethanol in the liver, can to some degree escape into the circulatory system, enter the brain, and condense with DA (and other biogenic catecholamines) to form TIQ alkaloids which might contribute to the behavioral changes, physical dependence and addictive properties of ethanol [10]. Chronic intracerebroventricular infusions of SAL to the rat evoke a profound increase in the preference of the animal for ethanol, an effect which continues long after termination of drug treatment [11, 12]. This observation provides significant support for the role of SAL in the addictive properties of ethanol.

Long-term chronic alcoholism leads to a general decline of intellectual abilities attributed to organic brain damage [13]. Neuronal damage, particularly to hippocampal regions of the brain, has been noted [14]. Indeed, the effects of chronic alcoholism have been likened to a premature aging of the brain [15]. Collins [16] has proposed that oxidations of SAL, and perhaps other TIQs which are elevated in the brains of chronic alcoholics, might lead to the formation of toxic metabolites which are responsible for neuronal damage. For example, it was suggested that *in vivo* oxidation of SAL might yield the quinone methide **C** (Scheme II). This compound bears a structural similarity to the putative *para*-quinone formed upon intraneuronal oxidation of the catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) [17, 18], and thus might be expected to possess neurotoxic properties as a result of its ability to alkylate and cross-link cellular proteins, perhaps as conceptualized in Scheme II. However, little is known about the oxidation chemistry and biochemistry of SAL in the CNS. Furthermore, if a neurotoxic oxidation product of SAL, such as **C**, is formed *in vivo*, it would necessarily be extremely difficult to detect in the free state if it did indeed react with protein nucleophiles. Thus, analyses of cerebrospinal fluid (CSF) or of CNS tissue would be unlikely to detect such species. Such analyses, therefore, would be incapable of providing direct insights into either the role of oxidation reactions of SAL in the neurodegenerative aspects of chronic alcoholism or, indeed, the identities of potentially toxic metabolites. Accordingly, as an alternative approach, we have initiated a program to investigate the fundamental oxidation chemistry and biochemistry of SAL and related endogenous central alkaloids. Initially, the oxidation chemistry of SAL in aqueous solution was investigated using

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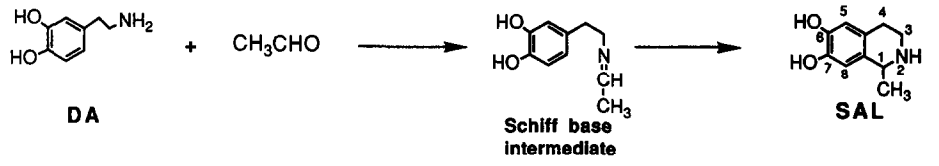
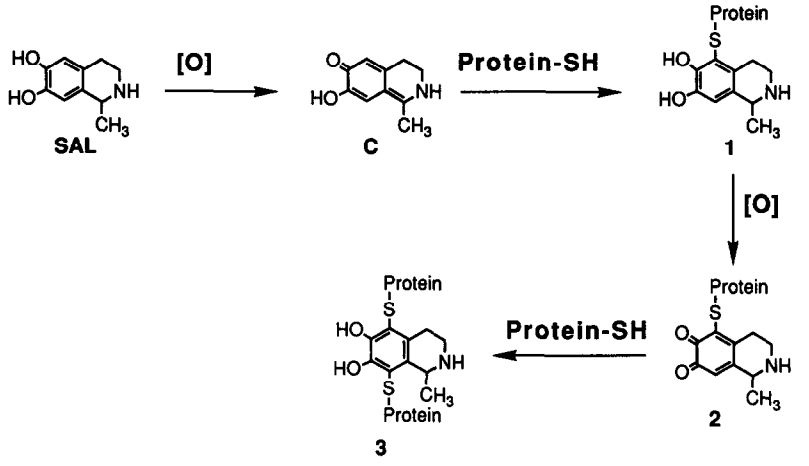
† Abbreviations: TIQ, tetrahydroisoquinoline; SAL, salsolinol; DA, dopamine; 6-OHDA, 6-hydroxydopamine; CSF, cerebrospinal fluid; TFA, trifluoroacetic acid; NOED, nuclear Overhauser effect difference; PGE, pyrolytic graphite electrode; SCE, saturated calomel reference electrode; FAB–MS, fast atom bombardment–mass spectrometry; EI–MS, electron impact–mass spectrometry; and GSH, glutathione.



Scheme I.



Scheme II.

electrochemical approaches [19]. This study revealed that SAL is a rather easily oxidized compound and, because of the very wide range of experimental conditions which can be employed in electrochemical investigations, it was possible to characterize a key intermediate and to isolate and identify the major initial oxidation products. However, in view of the implication that oxidation reactions of SAL might play functional roles in the neurodegenerative aspects of chronic alcoholism it was of considerable interest to investigate such reactions mediated by enzymes. Accordingly, in this report the oxidation reactions mediated by three enzyme systems are described. Evidence is provided to support the conclusion that the proximate oxidation product of SAL is an unstable but extremely electrophilic *ortho*-quinone which can react with intraneuronal nucleophiles such as glutathione (GSH) and sulfhydryl-containing proteins. Preliminary data are reported indicating that three oxidation products of SAL are centrally active and/or toxic when administered into the mouse CNS.

MATERIALS AND METHODS

L-Salsolinol, tyrosinase (from mushroom, EC 1.14.18.1; 3430 units/mg; mol. wt = 200,000), ceruloplasmin (human type X in 0.25 M sodium chloride plus 0.05 M sodium acetate at pH 7.0), peroxidase (type IV from horseradish, EC 1.11.1.7), alcohol dehydrogenase (from Bakers Yeast; EC 1.1.1.1), and glutathione were obtained from Sigma (St. Louis, MO) and were used without further purification. Phosphate buffers of known pH and ionic strength (μ) were prepared according to Christian and Purdy [20].

HPLC employed a Bio-Rad (Richmond, CA) computer-controlled binary gradient system equipped with two model 1300 pumps, a Gilson (Middleton, WI) Holochrome variable wavelength UV detector set at 280 nm and a Rheodyne model 7125 loop injector. HPLC separations employed two reversed-phase columns. The first was a preparative column (J. T. Baker, Bakerbond, Phillipsburg, NJ, C_{18}, 10 μm particle size, 21.2 × 250 nm). A semi-preparative column (Brownlee Laboratories, Santa Clara, CA, RP-18, 5 μm particle size, 25 × 0.7 cm) was also used. For the initial separation of the products of various enzyme-catalyzed oxidations of SAL, Gradient System I was employed with the preparative HPLC column. Two mobile phase solvents were used. Solvent A was prepared by adding 30 mL of concentrated ammonium hydroxide (NH_4OH) to 3970 mL of deionized water. The pH of this solution was adjusted to 3.0 with concentrated trifluoroacetic acid (TFA). Solvent B was prepared by adding 15 mL of NH_4OH and 1200 mL of HPLC grade methanol (MeOH) to 2785 mL of water. The resulting solution was then adjusted to pH 3.0 with TFA. The following gradient was employed: 0–20 min, linear gradient from 100% solvent A to 60% solvent A and 40% solvent B; 20–30 min, linear gradient to 100% solvent B; 30–40 min, 100% solvent B. The flow rate was constant at 8 mL/min. Gradient Systems II and III were used to desalt and purify oxidation products and both employed the semi-preparative HPLC column. For Gradient System II two mobile phase solvents were employed. Solvent

C was prepared by adding 4 mL of concentrated formic acid (HCOOH) to 4000 mL of deionized water. Solvent D was prepared by adding water to 8 mL of HCOOH and 800 mL of HPLC grade acetonitrile (MeCN) until the total volume was 4000 mL. The following gradient was employed: 0–5 min, 100% solvent C; 5–15 min, linear gradient to 100% solvent D; 15–25 min, 100% solvent D. The flow rate was constant at 3 mL/min. Solvents C and D were also used with Gradient System III but the following gradient was employed: 0–15 min, linear gradient from 100% solvent C to 100% solvent D; 15–25 min, 100% solvent D. The flow rate was 3 mL/min. Gradient System I was also used to separate the glutathione adducts of SAL. Gradient Systems II and III were used to desalt and purify these compounds.

Low and high resolution fast atom bombardment–mass spectrometry (FAB–MS) and electron impact–mass spectrometry (EI–MS) were performed on a VG Instruments (Manchester, U.K.) model ZAB-E mass spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer. Nuclear Overhauser effect difference (NOED) free induction decays were obtained using a gated decoupling program. For each measurement 100 scans with irradiation were subtracted from those with irradiation on resonance. A decoupler amplitude of up to 74 Hz was employed and a flip angle of 32° was applied. The reaction delay time was set at 4.0 sec. UV-visible spectra were recorded on a Hewlett–Packard model 8452A diode array spectrophotometer.

Cyclic voltammograms were obtained with a BAS-100A Electrochemistry System (Bioanalytical Systems, West Lafayette, IN). All voltammograms were corrected for uncompensated cell resistance. A pyrolytic graphite electrode (PGE; Pfizer Minerals, Pigments and Metals Division, Easton, PA) having an approximate surface area of 12.6 mm^2 was used for voltammetry and was resurfaced before recording each voltammogram as described previously [21]. Controlled potential electrolysis employed a Princeton Applied Research Corp. (Princeton, NJ) model 173 potentiostat. Several plates of pyrolytic graphite were used as the working electrode having a total surface area of *ca.* 200 cm^2. Conventional three electrode electrochemical cells were used for voltammetry and controlled potential electrolyses and contained platinum counter electrodes and a saturated calomel reference electrode (SCE). All potentials were referred to the SCE at ambient temperature (22 ± 2°). Solutions for voltammetry were always deaerated by bubbling N_2 for *ca.* 5 min before the voltammogram was recorded. Controlled potential electrolyses were performed on solutions which were bubbled continuously with N_2 and stirred with a Teflon-coated magnetic stirring bar.

All enzyme-mediated oxidations were performed at ambient temperature (22 ± 2°).

Formation of glutathione adducts of SAL

A solution (15–30 mL) containing SAL (0.99 mM), tyrosinase (183 units/mL) and GSH (4.7 mM) in pH 7.0 phosphate buffer ($\mu = 0.15$) was stirred vigorously, in a vessel open to the atmosphere, at ambient temperature for 4 hr. Then, aliquots of the resulting solution (2–30 mL) were separated using a preparative reversed-phase HPLC column and Gradient System I. The chromatographic peaks corresponding to glutathione conjugates **E**, **F**, and **G** were collected separately and lyophilized. The resulting dry, solid materials were then dissolved in the minimum volume of water and desalted using a semi-preparative reversed-phase column and Gradient Systems II and III. The resulting solutions were lyophilized to give pure, dry samples of **E**, **F**, and **G**.

Product identifications

In the absence of glutathione, oxidations of SAL by tyrosinase/O_2, ceruloplasmin/O_2, and peroxidase H_2O_2 gave the same products which have been reported previously in the electrochemical oxidation of the alkaloid. These products are *cis*-1,2,3,4-tetrahydro-1-methyl-4,6,7-isoquinoline triol (**A**), *trans*-1,2,3,4-tetrahydro-1-methyl-4,6,7-isoquino-isoquinoline triol (**B**), 2,3,4-trihydro-1-methyl-7-hydroxy-6-oxyisoquinoline (**C**), and 1-methyl-6,7-isoquinoline diol (**D**). Spectroscopic evidence employed to elucidate the structures of these compounds has been presented elsewhere [19].

5-S-(*Glutathionyl*)-1,2,3,4-*tetrahydro*-1-*methyl*-6,7-*isoquinoline diol* (**E**). Compound **E** was isolated as a fluffy white solid. In pH 7.0 phosphate buffer ($\mu = 0.15$) λ_{max}, nm (log ε_{max}): 300 (3.36), 260 (3.34), 230 (3.73). FAB–MS (3-nitrobenzyl alcohol matrix) gave m/e = 485.1712 (MH^+, 100% $C_{20}H_{29}N_4O_8S$; calcd. m/e = 485.1706). Thus, **E** had a molar mass of 484 g and an elemental formula $C_{20}H_{28}H_4O_8S$ and, therefore, consisted of one residue each of SAL and GSH. ^1H NMR (D_2O) δ 6.82(s,1H,C(8)-H), 4.51(q,J = 6.2 Hz, 1H, C(1) - H), 4.35–4.28(m, 1H, C(a) - H), 3.72(t, J = 6.3 Hz, 1H, C(d) - H), 3.57(s, 2H, C(f) - H_2), 3.53–3.41(m, 2H, C(3) - H_2), 3.27–3.18(m, 2H, C(e) - H_2), 3.16–3.12(m, 2H, C(4) - H_2), 2.47–2.42(m, 2H, C(c) - H_2), 2.07(dt, J = 6.6 Hz, J = 6.6 Hz, 2H, C(b) - H_2), 1.60(d,J = 6.2 Hz, 3H, C(1) -CH_3). The assignment of the various resonances was based initially on comparisons with the ^1H NMR spectra of SAL and GSH in D_2O (compound **E** was not soluble in non-exchanging solvents) and by homonuclear selective decoupling experiments. The appearance of only one resonance in the aromatic region of the spectrum indicated that the GSH residue was linked to either position 5 or 8 of the SAL residue. The actual site of linkage between the SAL and GSH residues was established using NOED experiments. Pre-irradiation of the signal at 1.6 ppm (C(1)-CH_3) resulted in a significant enhancement of the signal at 6.82 ppm. Accordingly, it was concluded that the resonance at 6.82 ppm corresponded to C(8)-H and, therefore, that the glutathionyl residue is attached to the SAL residue at the C(5)-position.

8-S-(*Glutathionyl*)-1,2,3,4-*tetrahydro*-1-*methyl*-6,7-*isoquinoline diol* (**F**). Compound **F** was isolated as a white solid. In pH 7.0 phosphate buffer (μ = 0.15), **F** exhibited a characteristic spectrum with λ_{max}, nm (log ε_{max}): 300 (3.33), 260 (3,34), 226 (3,79). FAB–MS (3-nitrobenzyl alcohol matrix) gave m/e = 485.1710 (MH^+, 100% $C_{20}H_{29}N_4O_8S$; calcd. m/e =

485.1706). Thus, **F** had a molar mass of 484 g and a molecular formula $C_{20}H_{28}N_4O_8S$ and, therefore, consisted of one residue each of SAL and GSH. ^1H NMR (D_2O) δ 6.79(s, 1H, C(5) - H), 5.03(q, J = 6.8 Hz, 1H, C(1) - H), 4.41(t, J = 5.4 Hz, 1H, C(a)-H), 3.73(t, J = 6.3 Hz, 1H, C(d) - H), 3.56–3.43(m, 4H, C(3) - H_2, C(f) - H_2), 3.16(d, J = 4.8 Hz, 1H, C(e) - H_a), 3.11(d, J = 4.5 Hz, 1H, C(e) - H_b), 3.04–2.90(m, 2H, C(4) - H_2), 2.47(t, J = 7.2 Hz, 1H, C(c) - H_a), 2.45(t, J = 6.9 Hz, 1H,(c) - H_b), 2.12–2.04(m, 2H, C(b) - H_2), 1.53(d, J = 6.8 Hz, 3H, C(1) - CH_3). In NOED experiments pre-irradiation of the signal at 1.53 ppm (C(1)-CH_3)caused no enhancement of the only aromatic resonance at 6.79 ppm confirming, therefore, that the latter resonance corresponds to C(5)-H. Accordingly, the glutathionyl residue must be attached to the C(8)-position of the SAL residue.

5,8-Di-glutathionyl-1,2,3,4-tetrahydro-1-methyl-6,7-isoquinoline diol (**G**). Compound **G** was isolated as a white solid. In pH 7.0 phosphate buffer (μ = 0.15) λ_{max}, nm (log ε_{max}): 314 (3.51), 280 (4.04), 214 (4.3). Negative ion FAB–MS (3-nitrobenzyl alcohol matrix) gave m/e = 789 (M^-, 4%) indicating that **G** consisted of one SAL residue and two residues of GSH. ^1H NMR (D_2O) δ 5.08(q, J = 6.5 Hz, 1H, C(1)-H), 4.40(t, J = 6.9 Hz, 1H, C(a) - H), 4.38(t, J = 6.3 Hz, 1H, C(a') - H), 3.82–3.02(m, 14H, C(d) - H, C(d') - H, C(f) - H_2, C(f') - H_2, C(e) - H_2, C(3) - H_2, C(4) - H_2), 2.48(t, J = 6.9 Hz, 2H, C(c) - H_2), 2.46(t, J = 6.9 Hz, 2H, C(c') - H_2), 2.11–2.09(m, 4H, C(b) - H_2,C(b') - H_2), 1.56(d, J = 6.5 Hz, 3H, C(1) - CH_3). The absence of any proton resonances in the aromatic region indicates that glutathionyl residues are attached to the SAL residue at both the C(5)- and C(8)-positions.

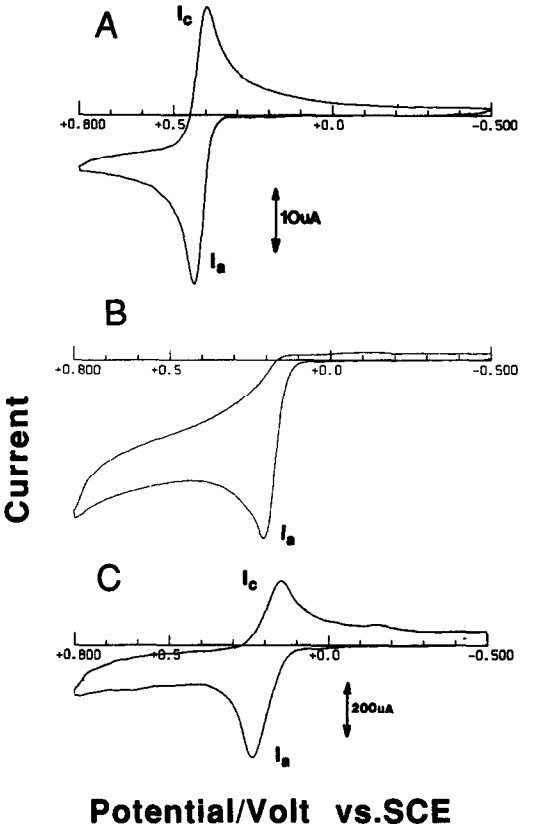


Fig. 1. Cyclic voltammograms at the pyrolytic graphite electrode of 1.39 mM salsolinol in phosphate buffers (μ = 1.0) at (A) pH 3.0, (B) and (C) pH 7.0. Sweep rates: (A) 10 mV/sec; (B) 10 mV/sec; and (C) 1000 mV/sec.

RESULTS

Electrochemical studies

Cyclic voltammograms of SAL in aqueous solutions buffered at pH 3.0 and 7.0 are presented in Fig. 1. The peak potential (E_p) for the single oxidation peak I_a of SAL at pH 3.0 was +436 mV at a sweep rate of 10 mV/sec; at pH 7.0 E_p for peak I_a was +208 mV. Thus, particularly at pH 7.0, SAL may be regarded as a quite easily oxidized compound. When the potential sweep was reversed after scanning through peak I_a at low pH, reversible reduction peak I_c appeared even at very slow sweep rates (Fig. 1A). However, at pH 7, peak I_c could only be observed at much faster sweep rates (Fig. 1C). Such cyclic volammetric behaviors indicated that the proximate electrochemical oxidation product of SAL responsible for reduction peak I_c was appreciably more stable in acidic solution than in neutral (or basic) aqueous solutions. A chromatogram obtained following an incomplete electro-oxidation of SAL at +290 mV at pH 7.0 showed that four major products were formed, **A**, **B**, **C**, and **D** (Fig. 2). When exhaustive electro-oxidations of SAL were carried out, **A–D** ultimately disappeared and were replaced by a large number (> 20) of secondary products along with a black, insoluble, polymeric material. The structures of **A–D** have been elucidated using spectroscopic methods [19] and are presented in Table 1. The identities of the numerous secondary products, however, remain to be determined.

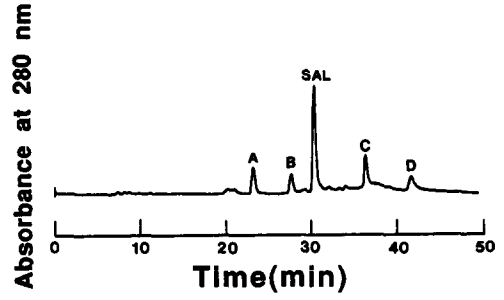
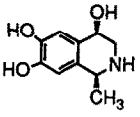
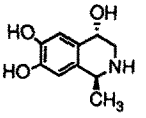
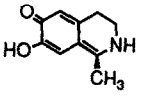
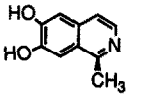


Fig. 2. HPLC chromatogram of the product solution formed by controlled potential electro-oxidation of 0.94 mM SAL in pH 7.0 phosphate buffer for 6 hr at +290 mV. Injection volume: 2.0 mL. Chromatography employed Gradient System I.

Enzyme-mediated oxidations of SAL

Oxidations of SAL mediated by tyrosinase/O_2, ceruloplasmin/O_2, and peroxidase/H_2O_2 were studied in pH 7.0 phosphate buffer (μ = 0.15) in

Table 1. Products of electrochemical oxidation of salsolinol at pH 7.0

Compound	Chemical name	Structure*
A	*cis*-1,2,3,4-Tetrahydro-1-methyl-4,6,7-isoquinoline triol	
B	*trans*-1,2,3,4-Tetrahydro-1-methyl-4,6,7-isoquinoline triol	
C	2,3,4-Trihydro-1-methyl-7-hydroxy-6-oxyisoquinoline	
D	1-Methyl-6,7-isoquinoline diol	

* All structures were ascertained using FAB–MS, exact mass measurements, ^{1}H NMR [including homonuclear selective decoupling, and 2-D correlated spectroscopy (COSY) measurements], ^{13}C NMR spectroscopy, UV-visible and i.r. spectroscopy [19].

vigorously stirred solutions at ambient temperature (23 ± 2°) which were exposed to the atmosphere. In the absence of an enzyme catalyst, auto-oxidation of SAL at pH 7.0 could not be detected for periods up to 10 hr. Similarly, SAL was not oxidized by H_2O_2 in the absence of peroxidase under the conditions used in this report. Representative chromatograms of the product solutions obtained from the enzyme-mediated oxidations of SAL are presented in Fig. 3. Clearly, all three enzyme systems catalyzed the oxidation of SAL to yield **A**, **B**, **C**, and **D**, i.e. the same products formed in the electrochemically driven reaction. Increasing the concentrations of tyrosinase, ceruloplasmin and peroxidase resulted in an increase in the rate of the oxidation reaction to **A**, **B**, **C** and **D**. However, it was observed that the HPLC peaks corresponding to compounds **A–D** systematically increased during the early stages of the enzyme-mediated reactions, reached a maximal height, and subsequently decreased and disappeared and were replaced by many peaks due to secondary products. No attempts were made to isolate and identify these secondary products. Such behavior was also noted during the electrochemical oxidation of SAL [19]. The E_p values for electrochemical oxidation of SAL, **A**, **B**, **C** and **D** in pH 7.0 phosphate buffer at the PGE at comparable concentrations (*ca.* 1 mM) and sweep rate (10 mV/sec) were +208, +193, +197, +198 and +450 mV, respectively. Thus, the initial oxidation products are oxidized at very similar potentials to SAL. Accordingly, it may be concluded that **A–D** undergo further oxidation reactions to form secondary products which remain to be identified.

When SAL (0.93 mM) was incubated with H_2O_2 (0.52 mM) and peroxidase (9.3 units/mL) in pH 7.0 phosphate buffer with N_2 bubbling vigorously through the solution (i.e. in the absence of molecular oxygen), compounds **A–D** were not formed as products. The major initial product formed eluted at a retention time of 36 min (Gradient System I) and had a characteristic UV spectrum (λ_{max} at pH 3.0: 298, 274 nm). Unfortunately, the latter compound was too unstable to permit its isolation and structure elucidation. The anaerobic peroxidase/H_2O_2 oxidation of SAL ultimately gave an extremely complex mixture of products which remain to be identified.

Enzyme-mediated oxidations of SAL in the presence of glutathione

The above results indicated that the tyrosinase/O_2, ceruloplasmin/O_2 and aerobic peroxidase/H_2O_2

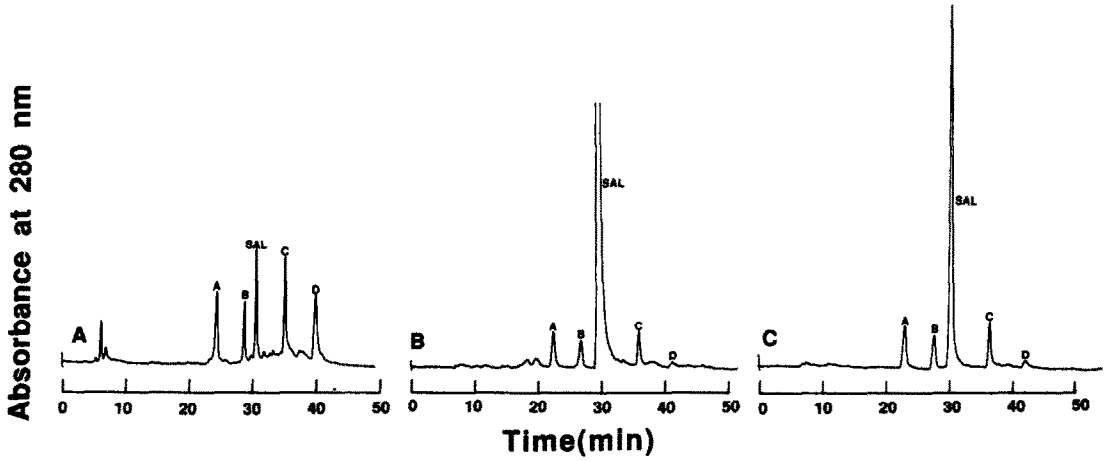


Fig. 3. HPLC chromatograms of product solutions formed by the oxidation of SAL (0.99 mM) in pH 7.0 phosphate buffer (μ = 0.15) at 23° in the presence of (A) tyrosinase (182.9 units/mL) for 4 hr; (B) type IV peroxidase (9.3 units/mL) and H_2O_2 (0.52 mM) for 6 hr; and (C) ceruloplasmin (29.8 units/mL) for 8 hr. Injection volume in each case: 2.0 mL. Chromatography employed Gradient System I.

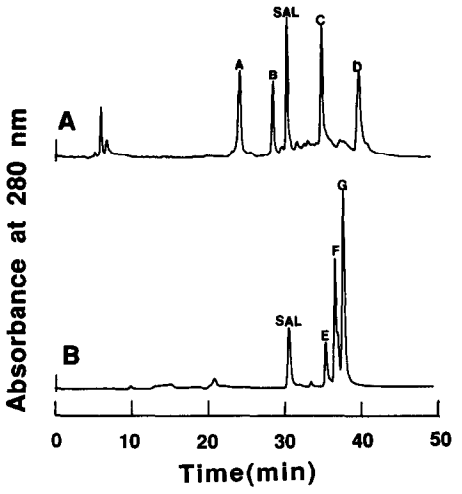


Fig. 4. HPLC chromatograms of the product solutions formed by oxidation of SAL (0.99 mM) in pH 7.0 phosphate buffer in the presence of (A) tyrosinase (182.9 units/mL) and (B) tyrosinase (182.9 units/mL) and glutathione (4.71 mM) at 23° for 4 hr. Injection volume: 2.0 mL. Chromatography employed Gradient System I.

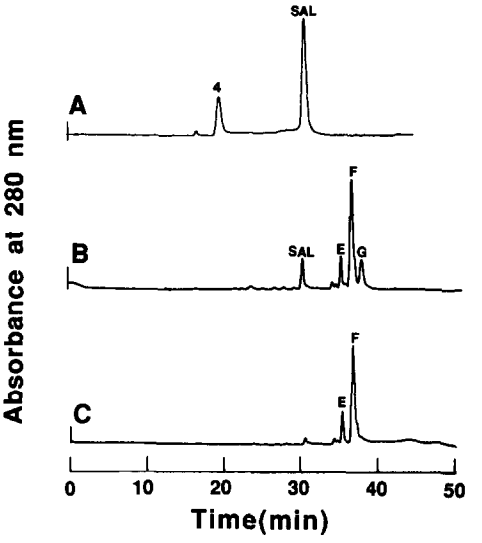


Fig. 5. HPLC chromatograms of the product solution obtained (A) following controlled potential electro-oxidation of 0.93 mM SAL in 0.1 M HCl at +620 mV for 20 min; (B) after addition of six, 10-μL aliquots (10-min intervals) of a solution of GSH (0.197 M in 0.1 M HCl) to 4 (*ca.* 0.2 mM) dissolved in the chromatographic mobile phase (pH 3.0; *ca.* 30 mL); and (C) after a single 60-μl addition of solution of GSH (0.197 M in 0.1 M HCl). Injection volume: 2.0 mL. Chromatography employed Gradient System I.

oxidations of SAL yielded the same initial products, **A–D**, as the electrochemically-driven reaction. These observations suggested that all of the oxidation reactions proceeded, in a *chemical sense*, by similar reaction pathways. Cyclic voltammograms of SAL showed that at physiological pH (Fig. 1, B and C) the proximate electrochemical oxidation product was very easily reducible (peak I_c) but also very reactive having a lifetime of approximately 1 sec [19]. This species has been unequivocally shown to be *ortho*-quinone **4** [19]. Compound **4** is an electron-deficient species and, accordingly, would be expected to undergo facile reactions with nucleophiles. To ascertain whether in fact **4** was the proximate oxidation product in the enzyme-mediated oxidations of SAL, the reactions were carried out in the presence of the cellular nucleophile GSH. GSH would be expected to trap **4** as a glutathionyl conjugate of SAL. In such studies tyrosinase/O_2 was employed most extensively (see Materials and Methods) because it was the most effective catalyst for oxidation of SAL although all of the other enzyme systems studied gave the same results. Chromatograms of the product mixture formed when SAL (0.99 mM) was incubated with tyrosinase (182.9 units/mL) in the absence and presence of GSH (4.7 mM) are presented in Fig. 4, A and B, respectively. In the presence of GSH, compounds **A–D** were not observed as products but three new products, **E**, **F** and **G**, appeared. These compounds were isolated and spectroscopically characterized as the 5-*S*-, 8-*S*-, and 5,8-bi-*S*-glutathionyl conjugates of SAL, respectively. Addition of GSH to solution of quinone methide **C** at pH 7.0 resulted in no reaction. These results, therefore, strongly suggested that *ortho*-quinone **4** was generated in the enzymatic oxidation of SAL and that this quinone avidly reacted with GSH before it could decompose to **A–D**. That this is almost certainly true was confirmed by additional electrochemical experiments. Electrochemical oxidation of SAL at low pH has been shown to yield *ortho*-quinone **4** as the major initial product [19]. While it was not possible to isolate **4** in the solid state, a pure solution of the *ortho*-quinone could be prepared using HPLC. Thus, SAL (30 mL, 0.93 mM in 0.1 M HCl) was electro-oxidized at +620 mV for 20 min. A single injection of the entire product solution into a preparative HPLC system was made using Gradient System I. The resulting chromatogram exhibited one major product peak corresponding to *ortho*-quinone **4** (Fig. 5A). In the chromatographic mobile phase (pH 3.0), **4** gave a pale yellow solution (λ_{max}: 400, 280, 220 nm). However, even under these conditions **4** decomposes at a significant rate [19]. The structure of **4** was confirmed from its cyclic voltammetric behavior, which was identical to that of SAL, its facile two-electron, $2H^+$ electro-reduction to SAL, liquid chromatography–mass spectrometry, and its characteristic UV-visible spectrum [19]. Stepwise addition (10 μL aliquots) of an aqueous solution of GSH (60 μL total; 0.197 M) to *ca.* 30 mL of **4** (estimated to be about 0.2 mM) dissolved in the chromatographic mobile phase (pH 3.0) caused decoloration of the solution. HPLC revealed that this solution contained SAL, **E**, **F** and **G** (Fig. 5B). Such results clearly demonstrated that at pH 3.0 GSH reacted avidly with **4** to give glutathionyl conjugates **E**, **F** and **G**. Unfortunately, at pH 7.0 **4** is very unstable such that experiments similar to those carried out at pH 3.0

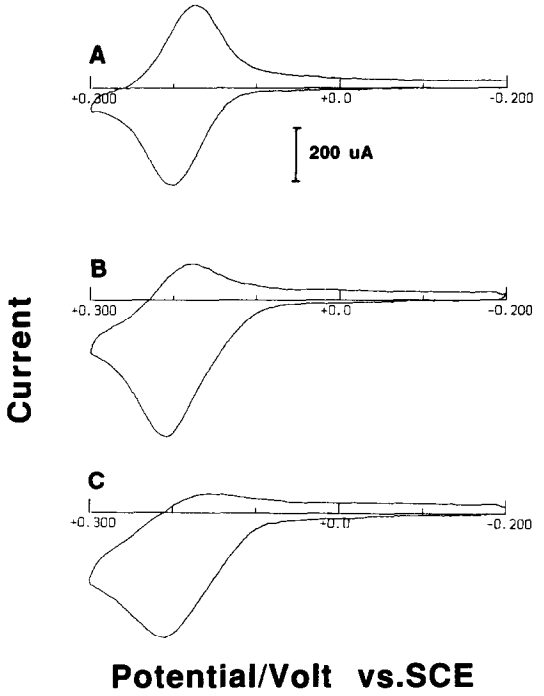


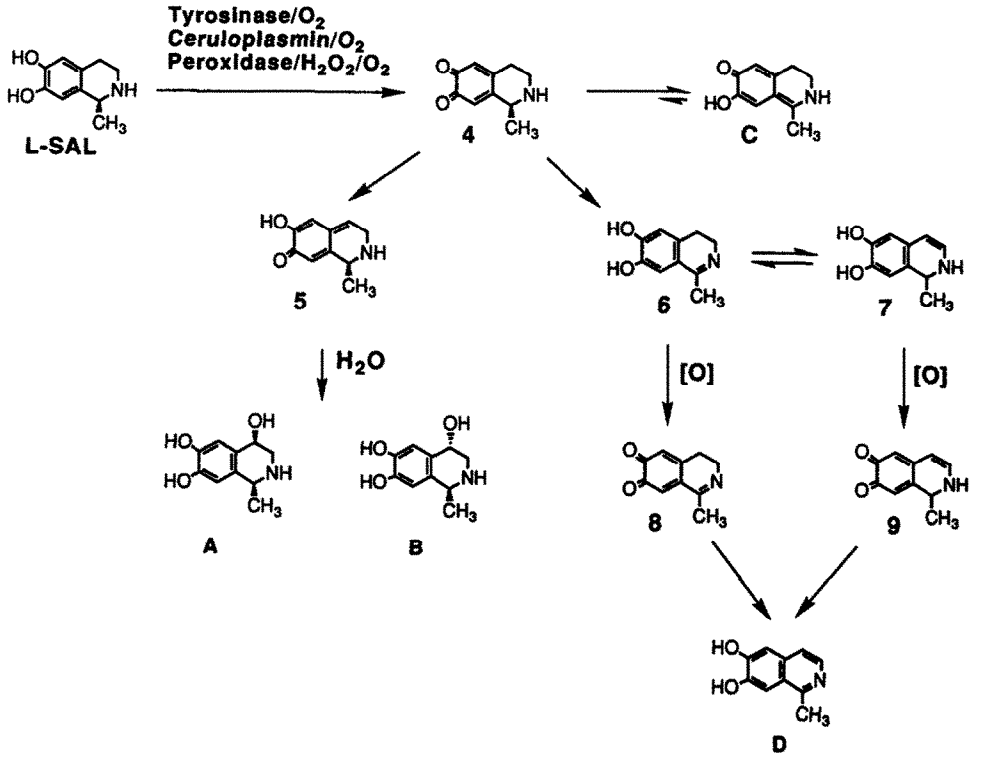
Fig. 6. Cyclic voltammograms at the pyrolytic graphite electrode of 0.42 mM SAL in pH 7.0 phosphate buffer (μ = 1.0) in the presence of (A) 0 mM, (B) 3.25 mM, and (C) 13.0 mM GSH. Sweep rate: 1 V/sec.

could not be performed. However, at sufficiently fast sweep rates cyclic voltammograms of SAL at pH 7.0 exhibited reduction peak I_c coupled to oxidation peak I_a. Peak I_c was due to the reduction of **4** formed in the initial peak I_a oxidation of SAL [19]. Addition of increasing concentrations of GSH to a solution of SAL at pH 7.0 resulted in a systematic decrease in the height of reduction peak I_c compared to oxidation peak I_a (Fig. 6). At a sufficiently high concentration of GSH, reduction peak I_c was eliminated completely. These results indicated that a very rapid reaction occurred between **4** and GSH at pH 7.0. Similar cyclic voltammetric experiments on SAL at pH 7.0 were also carried out in the presence of alcohol dehydrogenase, an enzyme known to contain numerous (36) sulfhydryl residues [22]. With increasing concentrations of alcohol dehydrogenase, reduction peak I_c also systematically decreased suggesting that **4** reacted rapidly with the sulfhydryl residues of this protein.

DISCUSSION

In the physiological pH domain, tyrosinase/O_2, ceruloplasmin/O_2 and peroxidase/H_2O_2 in the presence of molecular oxygen oxidized the endogenous CNS alkaloid SAL to four major initial products **A**, **B**, **C** and **D**. These are exactly the same initial products formed in the electrochemical oxidation of SAL [19]. Cyclic voltammetry of SAL over a wide pH range has shown that the proximate electro-oxidation product was reversibly reducible in the peak I_c process (Fig. 1). At low pH the proximate electro-oxidation product was sufficiently stable to permit it to be characterized as *ortho*-quinone **4** [19]. At pH 7.0 **4**, at about the 1 mM concentration level, has a lifetime of $\leq$ 1 sec. Both tyrosinase and ceruloplasmin are known to oxidize *ortho*-diphenols to the corresponding *ortho*-quinones in the presence of molecular oxygen [23, 24]. Peroxidase/H_2O_2 is known to generally catalyze one-electron oxidations to give radical intermediates [25, 26]. In the absence of molecular oxygen, peroxidase/H_2O_2 oxidized SAL but, unfortunately, the primary product was too unstable to be isolated and chemically characterized. Ultimately, a rather complex mixture of products was formed. However, in the presence of molecular oxygen, peroxidase/H_2O_2 oxidized SAL to **A**, **B**, **C**, and **D** as the major initial products. Thus, it can be concluded that under such a condition a peroxidase-oxidase oxidation of SAL occurred. There are many examples of such peroxidase-oxidase oxidations of organic substrates [27]. It was not the purpose of this study to elucidate the precise mechanistic aspects of the enzyme-mediated oxidations of SAL but simply to demonstrate that a representative group of oxidative enzyme systems were capable of oxidizing the alkaloid. However, the fact that the initial products of the enzymatic and electrochemical oxidations of SAL were identical suggests that all of the basic chemical reaction pathways were similar. Accordingly, by analogy with the proposed electrochemically-driven pathway [19], it may be concluded that the enzyme-mediated oxidations proceed by primary oxidation of SAL to the corresponding *ortho*-quinone **4** (Scheme III). Based upon the identities of the products derived from **4**, it must rapidly enolize. A major product derived from **4** was quinone methide **C**. This compound was quite stable at pH 7.0 no doubt owing to both resonance and intramolecular hydrogen bonding effects. Both resonance and steric effects protect the C(8a) = C(1) double bond of **C** from nucleophilic attack. At pH 7.0, for example, **C** was unaffected by GSH, a relatively strong nucleophile. There are, however, several other tautomers of *ortho*-quinone **4**. Resonance and steric effects cannot protect the C(4) = C(4a) double bond of quinone methide tautomer **5** and it was rapidly attached by water to yield the *cis*- and *trans*-trihydroxy-TIQs **A** and **B** (Scheme III). The final product of both the enzyme and electrochemical oxidation of SAL was the fully aromatized isoquinoline **D**. The exact chemical/biochemical routes leading to **D** are not known at this time. However, neither controlled potential electro-oxidation nor enzymatic oxidation of **C** gave **D** as a detected product. Accordingly, it has been concluded that tautomers **6** and/or **7** are formed which were further oxidized to **D** via putative *ortho*-quinone intermediates **8** and **9**, respectively (Scheme III).

Evidence for the intermediacy of **4** in the enzymatic oxidations of SAL has been provided by the isolation of glutathionyl conjugates **E**, **F** and **G** when the reactions were carried out in the presence of GSH. Such conjugates were formed by initial nucleophilic attack by GSH on **4** to give the 5-*S*- and 8-*S*-glutathionyl derivatives **E** and **F** as conceptualized



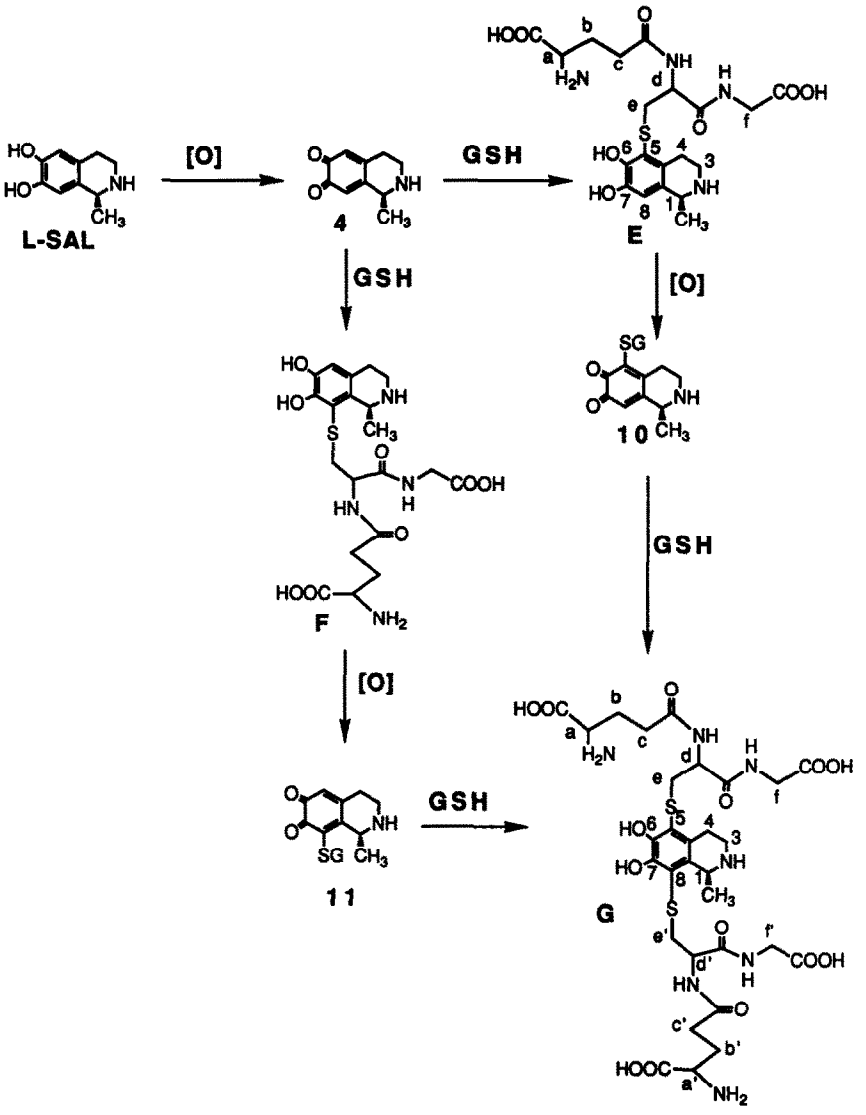
Scheme III.

in Scheme IV. Both **E** and **F** were also oxidized in the presence of tyrosinase, for example, and GSH to give the 5,8-bi-*S*-glutathionyl conjugate **G**. There are, therefore, two routes leading to the formation of **G**. These involve oxidations of both **E** and **F** to the corresponding electrophilic *ortho*-quinones **10** and **11**, respectively, which are attacked by GSH to give **G** (Scheme IV). At pH 3.0 a solution of **4**, formed by electrochemical oxidation of SAL in 0.1 M HCl and purified by HPLC, also reacted avidly with GSH to give a mixture of **E**, **F**, **G** and SAL, a reaction which occurred even in the absence of molecular oxygen. Cyclic voltammograms of SAL, **E** and **F** (2000 mV/sec) all exhibited reversible behavior. These reversible cyclic voltammograms were used to measure $E^{\circ\prime}$ values for the **4**/SAL, **E**/**10**, and **F**/**11** couples and, at pH 7.0 for example, were +192, +179 and +177 mV, respectively.* These $E^{\circ\prime}$ values indicated that **4** should be capable of chemically oxidizing **E** and **F** to *ortho*-quinones **10** and **11**, respectively. Accordingly, it can be concluded that GSH initially attacks **4** to give **E** and **F** which are then chemically oxidized by the remaining **4** to quinones **10** and **11** which are further attacked by GSH to give diglutathionyl conjugate **G** as outlined in Scheme V. A mixture of **E**, **F**, **G** and SAL was only obtained when **4** was in a molar excess over the GSH added, i.e. under the conditions described in Fig. 5B. However, when a solution of GSH (60 μL, 0.197 M) was added in one aliquot to a solution of **4** (30 mL of *ca.* 0.2 mM in the chromatographic mobile phase, pH 3.0), HPLC analysis of the product solution showed formation of only **E** and **F** (Fig. 5C). Under the latter experimental conditions, GSH was in a molar excess such that all of the **4** present in the solution was attacked to give **E** and **F**. In the absence of unreacted **4**, the latter conjugates could not be chemically oxidized to quinones **10** and **11** and, hence, neither **G** nor SAL was formed.

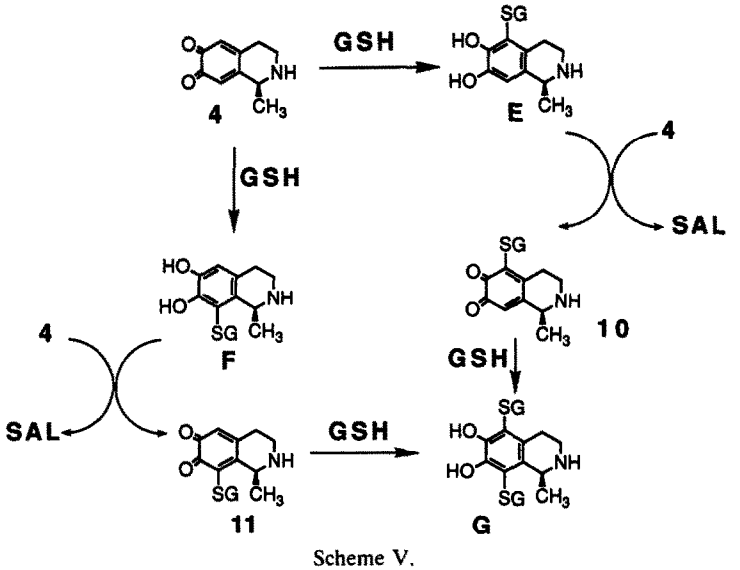
CONCLUSIONS

The results reported here indicate that the endogenous CNS alkaloid SAL is an easily oxidized compound. And, the oxidations of SAL can be mediated by several oxidative enzyme systems. The proximate oxidation product in the enzyme-mediated and electrochemically-driven oxidations of SAL was *ortho*-quinone **4** which, in the physiological pH domain, rapidly tautomerized to give quinone methide **C** and several other tautomers which react further ultimately yielding **A**, **B** and **D**. It is not yet known whether SAL can undergo oxidative transformations in the CNS although the ease of oxidation of the alkaloid certainly indicates that such reactions are feasible. The suggestion that oxidation reactions of SAL elevated in the brain in chronic

* $E^{\circ\prime}$ values were calculated from cyclic voltammograms using the expression $E^{\circ\prime} = [(E_p)_a + (E_p)_c]/2$, where $(E_p)_a$ was the peak potential for the oxidation peak and $(E_p)_c$ the peak potential for the reduction peak.



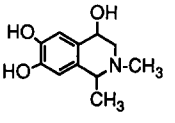
Scheme IV.



Scheme V.

alcoholism might lead to toxic metabolites responsible for the neuronal damage associated with the disease [16], therefore, appears to have some merit. Collins [16] has suggested that quinone methide **C** might be a CNS toxin as a result of its reactions with protein nucleophiles as conceptualized in Scheme II. However, **C** is a quite stable compound in aqueous solution at physiological pH and exhibited little tendency to react with a typical cellular nucleophile such as GSH. However, the proximate oxidation product of SAL, *ortho*-quinone **4**, reacted avidly with GSH (Scheme IV) and sulfhydryl-containing proteins. Formation of 5,8,bi-*S*-glutathionyl conjugate **G** clearly demonstrated that **4** has the potential to not only alkylate but also to cross-link membrane and other cellular proteins. Several well known neurotoxins, such as 5,6-dihydroxytryptamine, are believed to express their neurodegenerative effects as a result of intraneuronal oxidation to electrophilic quinones which alkylate and cross-link key cellular proteins [28–30]. Thus, intraneuronal oxidation of SAL to **4** has the potential to evoke neurodegenerative consequences. Compounds **A–D**, initial oxidation products of SAL, and glutathione conjugates **E–G** may provide useful analytical markers to assess whether oxidation reactions of SAL do indeed occur in the mammalian brain.

Several years ago a condensate of epinephrine and acetaldehyde, which contained 1,2,3,4-tetrahydro-1,2-dimethyl-4,6,7-isoquinoline triol (**12**), was



12

administered into the CNS of laboratory animals and caused profound physiological and behavioral changes and depletion of hypothalamic norepinephrine [31] and selective degeneration of adrenergic neurons [32]. These effects were similar to those evoked by the catecholaminergic neurotoxin 6-OHDA [33]. Compound **12** is structurally similar to the *cis*- and *trans*-trihydroxy-TIQs **A** and **B** formed upon oxidation of SAL. At this stage of our investigations only very small quantities of **A–D** have been isolated and, hence, it has not been possible to study in detail the effects of these compounds in the CNS. Very preliminary experiments, however, have been performed in which **A**, **B** and **C** have been administered intracranially in the vicinity of the left lateral ventricle to mice (Sprague–Dawley, male albino, Hsd:ICR strain, weighing approximately 30 g) in a vehicle (5 μL) consisting of isotonic saline (0.9% NaCl) containing 1 mg/mL ascorbic acid. Compounds **A** and **B** at doses between 80 and 200 μg evoked extreme hyperactivity and tremor. At a dose of 32 μg, quinone methide **C** also evoked extreme hyperactivity (rolling, jumping) and caused difficulty in walking, effects which persisted for *ca.* 1 hr. With a 50 μg dose animals exhibited similar hyperactivity and defecated repeatedly. With a 79-μg dose an initial sedative effect (40–50 min) was followed by extreme hyperactivity and at $\leq$ 3 hr death. The behavioral effects evoked by central administration of **A**, **B** and **C** and other potential oxidative metabolites of SAL, their toxicity and their effects on neurotransmitter/metabolite levels will be reported on more extensively in the near future. However, such preliminary observations suggest that SAL can be oxidized into several centrally active/toxic drugs. These results provide the first support for Collins' hypothesis [16] that oxidation reactions of SAL, and perhaps other TIQs elevated in the brain following chronic heavy consumption of ethanol, could lead to formation of toxic metabolites which might play a role in the neurodegenerative aspects of alcoholism.

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