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INTERACTIONS OF 5-HYDROXYTRYPTAMINE WITH OXIDATIVE ENZYMES

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Abstract. Peroxidase (EC 1.11.1.7)/H_2O_2, ceruloplasmin (human type X)/O_2, and tyrosinase (EC 1.14.18.1)/O_2 all oxidized the indolic neurotransmitter 5-hydroxytryptamine (5-HT) in the physiological pH domain. Peroxidase/H_2O_2 oxidized 5-HT at pH values down to about 2.5. All oxidation reactions generated complex mixtures of products which included at least one known neurotoxin, tryptamine-4,5-dione. In general, the enzymatic oxidation pathways paralleled the *in vitro* electrochemical oxidation of 5-HT which has permitted suggestions to be made concerning the probable mechanisms of the enzyme-mediated reactions.

Many lines of evidence suggest involvement of the serotonergic system in dementia of the Alzheimer type (DAT). For example, several investigators have found that 5-hydroxytryptamine (5-HT; serotonin) levels are lower in many areas of postmortem DAT brains than in those of controls [1–3]. Analyses of biopsy samples from patients with Alzheimer's disease have also indicated that concentrations of 5-HT and its major metabolite, 5-hydroxyindoleacetic acid (5-HIAA), are reduced significantly below control values [4]. 5-HT uptake and K^+-stimulated release of endogenous 5-HT are also reduced below control values. Decreased concentrations of 5-HIAA in the cerebrospinal fluid (CSF) of Alzheimer patients compared to controls have also been reported by some workers [5, 6] although others have found no significant differences [7, 8]. Recently, Volicer *et al.* [9] used multielectrode high performance liquid chromatography with coulometric detection (HPLC-CD) to analyze CSF and found that 5-HT concentrations were significantly lower in Alzheimer patients than in controls. These workers also concluded that 5-HT, 5-HIAA and 5-hydroxytryptophan levels were overestimated markedly when HPLC with a conventional amperometric detector was used because of the presence of coeluting compounds. One of these interfering compounds was postulated to be a partially oxidized form of 5-HT since it had similar chromatographic and electrochemical properties to an *in vitro* electrochemically oxidized 5-HT [9]. A major product of electrochemical oxidation of 5-HT at low pH is tryptamine-4,5-dione (**9**) (see Table 1) [10]. *In vitro* experiments with rat brain fragments have shown that **9** increases basal 5-HT efflux [11] in a fashion similar to that evoked by the indolic neurotoxin 5,6-dihydroxytryptamine (5,6-DHT) [12]. This has led to the suggestion [11] that an alteration of the oxidative metabolism of 5-HT may form toxic indole derivatives which could have profound effects on neuronal activity. In fact, since the time when 5-HT was initially discovered in brain, many suggestions have appeared implicating a defect in its normal metabolism with mental illnesses [13–21]. Significantly, formation of 5,6-DHT has been noted in rat brain following injection of methylamphetamine or *p*-chloroamphetamine [22, 23], leading to the suggestion that the neurodegenerative properties of these amphetamines may be mediated by 5,6-DHT presumably formed by oxidation of 5-HT.

The cholinergic system also is affected profoundly in the pathogenesis of Alzheimer's disease [24]. Furthermore, there is evidence that the serotonergic and cholinergic systems interact. For example, *in vitro* 5-HT inhibits acetylcholinesterase (AChE) [25], and it has been proposed that AChE is present in the cell bodies of serotonergic neurons [26]. In addition, an unknown oxidation product(s) of 5-HT (formed at pH 2 and 8 in the presence of molecular oxygen) is a much more effective inhibitor of AChE that 5-HT [27]. Thus, there is clearly some evidence that under certain pathological conditions or drug regimens 5-HT can be oxidatively converted to toxic indoleamines. Furthermore, even molecular oxygen can lead to formation of a compound(s) from 5-HT which can interfere with the cholinergic system.

Many enzyme systems are also known to catalyze the oxidation of 5-HT. These include ceruloplasmin [28–34] and horseradish and mammalian peroxidase-H_2O_2 [35]. The products and mechanisms of these enzyme-mediated oxidations are unknown. However, one product formed in the ceruloplasmin-mediated oxidation of 5-HT has been speculated to be a dimer [34]. The yellow color formed in the peroxidase-mediated oxidation has been speculated to be a dimer linked through the hydroxyl groups of the 5-HT residues [35]. It has also been reported that 5-HT and other endogenous 5-hydroxyindoles are oxidized by mitochondrial monoamine oxidase [36–38], lysosomal peroxidase [39] and microsomes [40] to form, ultimately, melanin-like pigments. There are hypotheses that such neuromelanins are important in aging processes and diseases of the Alzheimer type and in Down's Syndrome [37, 41].

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The widely used neurotoxic dihydroxyindoleamines such as 5,6-DHT and 5,7-DHT [42, 43] are thought to express their neurodegenerative effects as a result of auto-oxidation in the central nervous system (CNS) [44]. Such auto-oxidation reactions are probably quite rapid and are thought to form very reactive quinoid intermediates. These putative electrophilic quinoid intermediates would undoubtedly react avidly with endogenous nucleophiles such as the sulfhydryl residues of peptides and proteins [44]. It thus seems unlikely that either the dihydroxytryptamines or their initial auto-oxidation products could exist for significant periods of time in the free state in the CNS. Hence, analyses of CSF or of CNS tissue are unlikely to detect such species. Therefore, these analyses are probably incapable of providing direct insights into either the role of oxidation reactions of 5-HT in neurodegenerative disease or the identity of potentially neurotoxic oxidation products. As an alternative approach to this problem, we have begun studies of the fundamental oxidation chemistry of 5-HT. These have initially employed electrochemical approaches [45–49] because of the values of such methods to probe oxidation reaction pathways [50]. Such studies have revealed that 5-HT is electrochemically oxidized to very complex mixtures of products. Mechanistic conclusions have also been developed to explain the electro-oxidation chemistry of 5-HT in acidic solution [48] and at physiological pH [49]. Because there is a clear implication that oxidation reactions of 5-HT may play functional roles in the etiology of Alzheimer's disease and related disorders, it is of interest to investigate the oxidation reactions of the neurotransmitter mediated by enzymes. Prior reports have demonstrated that ceruloplasmin and peroxidase catalyze the oxidation of 5-HT to unknown products via unknown reaction pathways. In this report, the oxidations of 5-HT by the latter two enzymes and a third, tyrosinase, are described.

MATERIALS AND METHODS

Materials. Peroxidase (type VIII from horseradish, EC 1.11.1.7; 100 units/mg, mol wt = 40,000), tyrosinase (from mushroom, EC 1.14.18.1; 2260 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; 3400 units/mL), and 5-hydroxytryptamine (creatinine sulfate salt) were obtained from Sigma (St. Louis, MO).

Stock solutions of substrate (10 mM 5-HT) were prepared in phosphate buffer, pH 6.0 or 7.2 (μ = 0.2). Solutions of H_2O_2 (10 mM) in water or phosphate buffer, pH 7.2 (μ = 0.2), were prepared by dilution of a 30% (w/v) solution of H_2O_2. These solutions were standardized by titration with 0.1 N potassium permanganate. Stock solutions of peroxidase (10 μM, 40 units/mL) and tyrosinase (20 μM, 9000 units/mL) were prepared in phosphate buffer, pH 7.2 (μ = 0.02). Ceruloplasmin was employed directly from a commercial solution. All stock solutions were stored at −10°. Before reaction mixtures were prepared, enzyme solutions were incubated at 37° for 30 min. Reaction mixtures were prepared by combining the appropriate ingredients and diluting with buffer to the desired final concentrations and pH. Reaction mixtures were incubated at 37° for selected periods of time. Control experiments were always carried out to ascertain whether substrates were oxidized directly by air and/or H_2O_2. During the time period of the enzyme-mediated reactions, none of the substrates were oxidized to a detectable extent. In the absence of H_2O_2, peroxidase did not cause any oxidation of 5-HT.

Phosphate buffers of known pH and ionic strength (μ) were prepared according to Christian and Purdy [51].

Methods. HPLC employed a gradient system consisting of dual Gilson model 302 pumps (Gilson Medical Electronics, Middleton, WI), a Rheodyne model 7125 loop injector, an IBM PS/2 model 30 System Controller and a Gilson Holochrome UV detector (260 nm). A reversed phase column (Brownlee Laboratories, RP-18, 5 μm particle size, 250 × 7 mm) was employed. A short guard column (Brownlee, RP-18, 5 μm, OD-GU, 50 × 5 mm) was always used and, when analyzing enzyme-containing solutions, this was replaced regularly.

To separate the products formed as a result of electrochemical and peroxidase-mediated oxidations at low pH (2–3), two mobile phases were employed. Solvent A was prepared by adding 70 mL of HPLC grade acetonitrile (MeCN, Fisher Scientific) and 7 mL of concentrated ammonium hydroxide to 923 mL of water. The pH of this solution was then adjusted to 3.14 by addition of concentrated formic acid (HCOOH). Solvent B was prepared by adding 400 mL of MeCN and 7 mL of ammonium hydroxide to 593 mL of water. This solution was then adjusted to pH 3.5 by addition of HCOOH. The following gradient was employed: 0–30 min 100% solvent A at a flow rate of 0.7 mL/min; 30–55 min, 100% solvent A at a flow rate of 2.5 mL/min; 55–60 min 100% solvent B at a flow rate of 3 mL/min. This procedure will be described as Method I.

The product mixtures formed by electrochemically-driven or enzyme-mediated oxidations of 5-HT at around pH 7 employed two different mobile solvents for HPLC analysis. Solvent C was prepared by adding 6 mL of concentrated ammonium hydroxide to 994 mL of water; the pH was then adjusted to 4.75 with concentrated HCOOH. Solvent D was prepared by adding 7 mL of concentrated ammonium hydroxide and 400 mL of MeCN to 593 mL of water; the pH was then adjusted to 4.55 with concentrated formic acid. The gradient profile employed was as follows: 0–4 min, linear gradient from 0 to 10% solvent D; 4–11 min, linear gradient to 13% solvent D; 11–18 min, linear gradient to 15% D; 18–19 min, linear gradient to 18% D; 19–20 min, linear gradient to 30% D; 20–32 min, linear gradient to 65% D; 32–35 min, linear gradient to 100% D; 35–38 min, 100% D. The flow rates employed were as follows: 0–11 min, 2.5 mL/min; 11–18 min, linear increase from 2.5 to 3.0 mL/min; 13–38 min, 3.0 mL/min. In all cases, the volume of solution injected was 2.0 mL. This procedure will be described as Method II.

UV-visible spectra were recorded on either a Hitachi 100-80 spectrophotometer or a Hewlett–Packard 8452A diode array spectrophotometer. Equipment and procedures employed for cyclic

voltammetry and other electrochemical experiments have been described elsewhere [48, 49]. However, all voltammograms were recorded at a pyrolytic graphite electrode (PGE, Pfizer Minerals, Pigments and Metals Division, Easton, PA) having an approximate surface area of *ca.* 5 mm^2. All potentials were referred to the saturated calomel reference electrode (SCE) at ambient temperature (22 ± 2°). These potentials may be converted to volt versus the normal hydrogen electrode (NHE) by adding 0.24 V.

Methods employed to preparatively separate, purify and isolate electrochemically or enzymatically generated oxidation products of 5-HT and spectroscopic and chemical methods employed for structure elucidation have been described elsewhere [45–49]. That a product formed in an enzyme-mediated oxidation was the same as that formed and characterized in previous electrochemical studies was confirmed by HPLC retention times, UV-visible spectra, cyclic voltammetry, mass, and ^{1}H-NMR spectra.

RESULTS

Electrochemical oxidations of 5-HT. Voltammograms of 5-HT at pH 2.75 and pH 7.2 are presented in Figs. 1A and 2A respectively. The peak potential (E_p) for the first oxidation peak was 0.50 V at pH 2.75 and 0.21 V at pH 7.2. These E_p values indicated that 5-HT is a relatively easily oxidized compound particularly at physiological pH. Controlled potential electro-oxidation of 5-HT at pH 2.75 caused the initially colorless solution to become pink, whereas at pH 7.2 the solution became bright yellow. Chromatograms of the product mixtures obtained following electro-oxidation of 5-HT at pH 2.75 and pH 7.2 are shown in Figs. 1B and 2B respectively. These chromatograms indicate that complex product mixtures were formed and that the products formed were strongly influenced by the pH of the reaction solution. The structures of all of the major products and many of the minor products of electro-oxidation of 5-HT are presented in Table 1. Each compound is identified by its chromatographic peak shown in Figs. 1B and 2B. It is worth noting that as the potential employed to electro-oxidize 5-HT at pH 2.75 was made more positive, the yield of tryptamine-4,5-dione (**9**) increased whereas the yields of dimers **1**, **7**, and **8** decreased. At pH 7.2 the yields of **4**, **9**, **13**, **14** and **20** increased, whereas the yields of dimers **1** and **18** decreased when electro-oxidations were carried out at more positive potentials.

Peroxidase-mediated oxidation of 5-HT. Per-

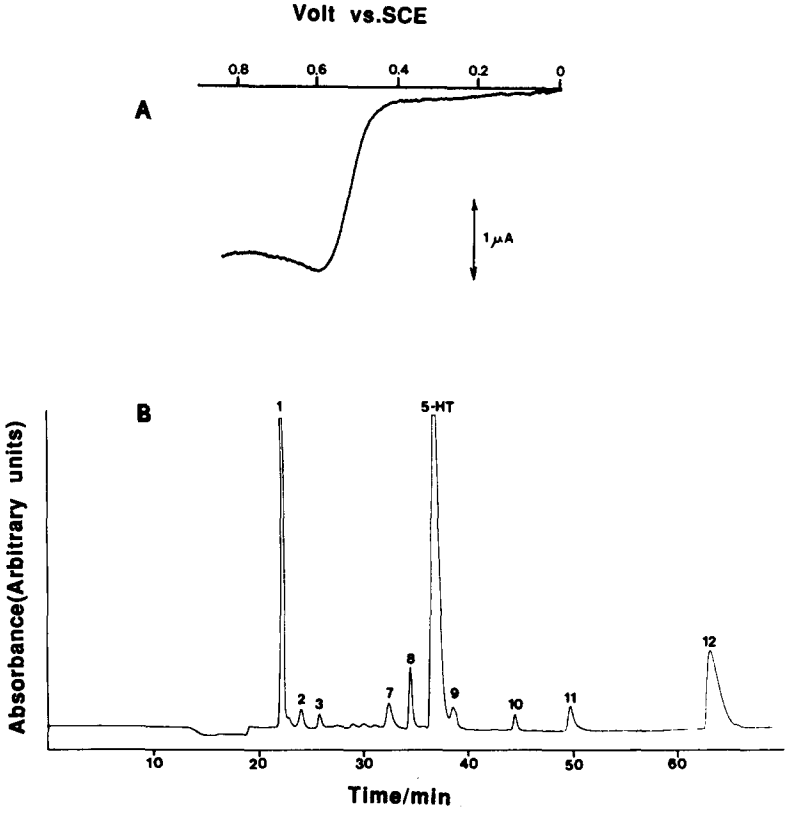


Fig. 1. (A) Linear sweep voltammogram at the pyrolytic graphite electrode (PGE) of 0.33 mM 5-HT in dilute hydrochloric acid solution, pH 2.75. Sweep rate: 5 mV/sec. (B) HPLC chromatogram of the product mixture formed upon controlled potential electro-oxidation of 0.3 mM 5-HT at 0.500 V at PGEs in dilute hydrochloric acid, pH 2.75, for 95 min. Chromatographic conditions are given in Materials and Methods.

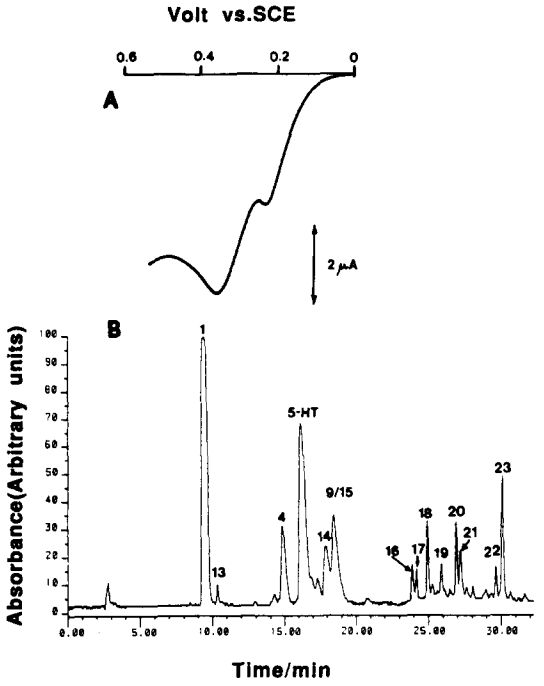


Fig. 2. (A) Linear sweep voltammogram at the PGE of 1.0 mM 5-HT in pH 7.2 phosphate buffer ($\mu = 0.2$). Sweep rate: 5 mV/sec. (B) HPLC chromatogram of the product mixture obtained following controlled potential electro-oxidation of 1 mM 5-HT at 0.210 V in pH 7.2 phosphate buffer ($\mu = 0.2$). Chromatographic conditions are given in Materials and Methods.

oxidase-H_2O_2 oxidized 5-HT over a wide pH range (2–≥7.5). In view of the oxidative conversions of 5-HT in dilute hydrochloric acid solution noted by previous workers [9, 27], it was of interest to study the peroxidase-mediated reaction in the same medium. A chromatogram of the product solution formed in dilute HCl (pH 2.75) shows that the products formed in the peroxidase-catalyzed oxidation of 5-HT (Fig. 3) were almost identical to those formed in the electrochemical reaction (Fig. 1B). One minor difference was that oxygen-bridged dimer **18** and trimer **4** were formed in the enzymatic oxidation whereas these compounds were not detected as products of the electro-oxidation at low pH. The product responsible for HPLC peak 5 was formed in higher yield in the peroxidase oxidation than in the electrochemical reaction. This compound has not been fully identified. However, the spectrum of **5** (λ_{max} at pH 3.3: 301, 274, 212 nm) is typical of a simple dimer of 5-HT [47]. Liquid chromatography–mass spectrometry of **5** showed a pseudomolecular ion (MH^+) at $m/e = 351$, confirming that this compound is a dimer.

An increase in the concentration of peroxidase and H_2O_2 caused a significant increase in the yield of dione **9** and a decrease in the yields of dimers **1**, **7** and **8**. This parallels the effect of increasingly positive potentials on the electrochemically-driven oxidation of 5-HT. Peroxidase/H_2O_2 and electrochemical oxidations of 5-HT at *ca.* pH 3 in phosphate buffers gave product profiles similar to those shown in Figs. 1B and 2B except that 4-chloro-5-hydroxytryptamine (**11**) was not formed.

At pH 7.2 the peroxidase-mediated oxidation of 5-HT was very rapid. The chromatogram shown in Fig. 4A was obtained after incubation of 5-HT (0.93 mM) with H_2O_2 (0.45 mM), i.e. $[H_2O_2]/[5\text{-HT}] = 0.48$, and peroxidase (0.04 μM) for 90 sec at 37°. The solution containing 5-HT and H_2O_2 immediately became bright yellow upon addition of per-

Table 1. Products of electrochemical oxidation of 5-hydroxytryptamine

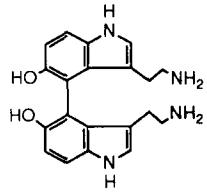
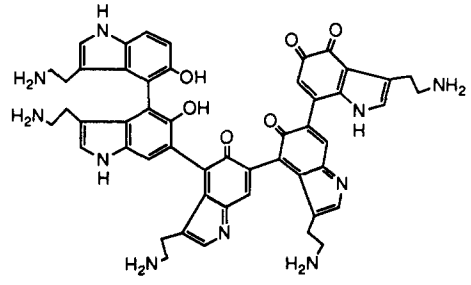
Compound No.*	Chemical name	Structure	
1	5,5′-Dihydroxy-4,4′-bitryptamine		†
2	[6,4′:6′,7″-Ter-5*H*-indole]-4″,5,5′,5″(1″*H*)-tetrone,3,3′,3″-tris(2-aminoethyl)-4-[3,3′-bis(2-aminoethyl)-5,5′-dihydroxy[4,4′-bi-1*H*-indol]6-yl]-indole		‡§

Table 1. Continued

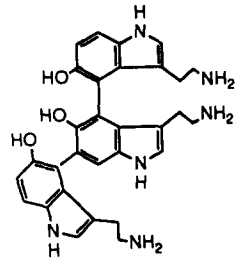
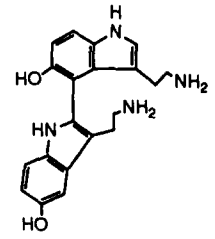
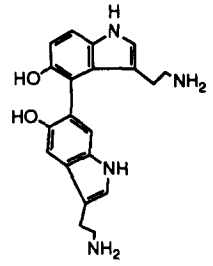
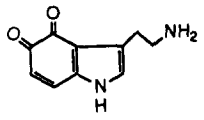
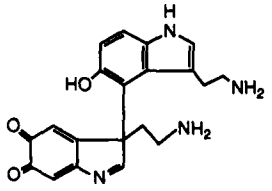
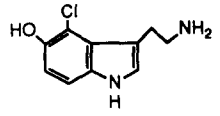
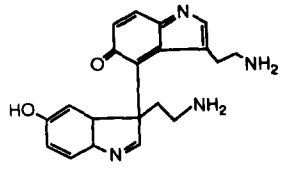
Compound No.*	Chemical name	Structure	
4	4,4′:6′,4″-Ter-5-hydroxytryptamine		‖
7	5,5′-Dihydroxy-2,4′-bitryptamine		¶
8	5,5′-Dihydroxy-4,6′-bitryptamine		¶
9	Tryptamine-4,5-dione		†
10	3-(2-Aminoethyl)-3-[3′-(2-aminoethyl)-5′-hydroxyindol-4′-yl]indolenine-5,6-dione		§
11	4-Chloro-5-hydroxytryptamine		†
12	3-(2-Aminoethyl)-3-[3′-(2-aminoethyl)-5′-oxoindol-4′-yl]-5-hydroxyindolenine		¶

Table 1. Continued

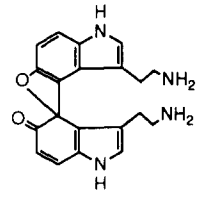
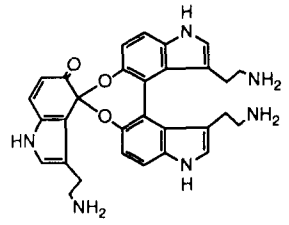
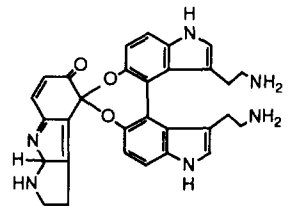
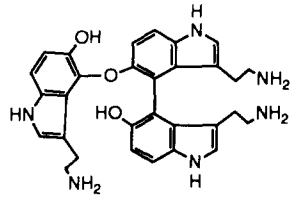
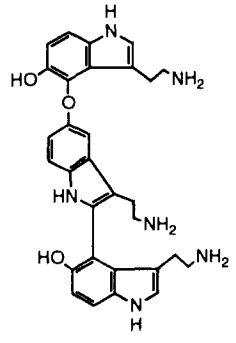
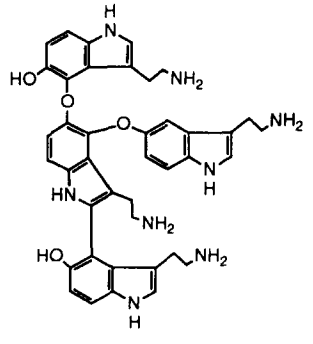
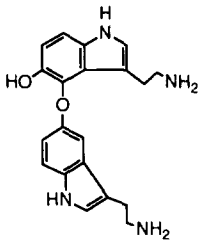
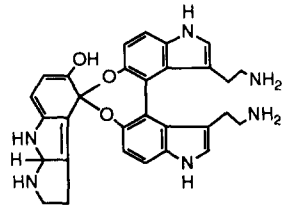
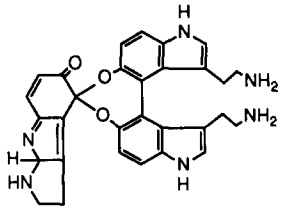
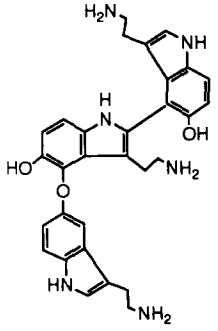
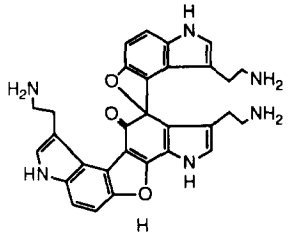
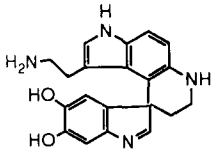
Compound No.*	Chemical name	Structure	
13	Spiro[4-*H*-indol-4,1′(5′*H*)-oxeto[3,2-*e*]indol]-5-one,3,7′-bis(2-aminoethyl)-1,5-dihydroindole		‖
14	Spiro[[1,3]dioxepino[5,4*e*:6,7-*e*′]-diindole-4,4′-[4*H*]indole]-5′(1′*H*)-one,3′,10,11-tris(2-aminoethyl)-8,13-dihydroindole		‖
15	Spiro[[1,3]dioxepino[5,4-*e*:6,7-*e*′]-diindole-4,4′(5′*H*)-pyrrolo[2,3-*b*]indol]-5′-one,10,11-bis(2-aminoethyl)-1′,2′,3′,8,8′a,13-hexahydroindole		‖**
16	[4,4′-Bi-1*H*-indole]-5-ol-3-3′-bis(2-aminoethyl)-5′-[[3-(2-aminoethyl)-5-hydroxy-1*H*-indol-4-yl]oxy]-indole		‖
17A	[4,2′-Bi-1*H*-indol]-5-ol,3,3′-bis(2-aminoethyl)-5′-[[3-(2-aminoethyl)-5-hydroxy-1*H*-indol-4-yl]oxy]-indole		
17B	[2,4′-Bi-1H-indol]-5′-ol,3,3′-bis(2-aminoethyl)-5-[[3-(2-aminoethyl)-5-hydroxy-1*H*-indol-4-yl]oxy]-4-[[3-(2-aminoethyl)-1*H*-indol-5-yl]-oxy]-indole		

Table 1. Continued

Compound No.*	Chemical name	Structure	
18	5-[[3-(2-Aminoethyl)-1*H*-indol-4-yl]-oxy]-[3-(2-aminoethyl)]-1*H*-indole		‖
19	Spiro[[1,3]dioxepino[5,4-*e*:6,7-*e*']diindole-4,4'(5'*H*)-pyrrolo[2,3-*b*]indol]-5-ol,10,11-bis(2-aminoethyl),1',2',3',8,8',8a',13-heptahydroindole		‖
20	Spiro[[1,3]dioxepino[5,4-*e*:6,7-*e*']diindole-4,4'(5'*H*)-pyrrolo[2,3-*b*]indol]-5'-one,10,11-bis(2-aminoethyl)-1',2',3',8,8a',13-hexahydroindole		‖**
21	[4,2'-Bi-1*H*-indol]-5,5'-diol,3,3'-bis(2-aminoethyl)-4'-[[3-(2-aminoethyl)-1*H*-indol-5-oxy]-yl]-indole		
22	Spiro[furo[3,2-*e*:4,5-g']diindole-4(5*H*),1'(5'*H*)-oxeto[3,2-*e*]indol]-5-one,3,6,7'-tris(2-aminoethyl)-1,8-dihydroindole		‡‖
23	Spiro[3*H*-indole-3,9'-(9*H*)pyrrolo[3,2-*f*]quinolin]-5,6-diol,1'-(2-aminoethyl)-7',8-dihydroindole		‡‖

* Compound number refers to HPLC peaks shown in Figs. 1B and 2B.
† Structure elucidation described in Ref. 45.
‡ Tentative structural assignment.
§ Structure elucidation described in Ref. 48.
‖ Structure elucidation described in Ref. 49.
¶ Structure elucidation described in Ref. 47.
** Compounds **15** and **20** are diastereomers.

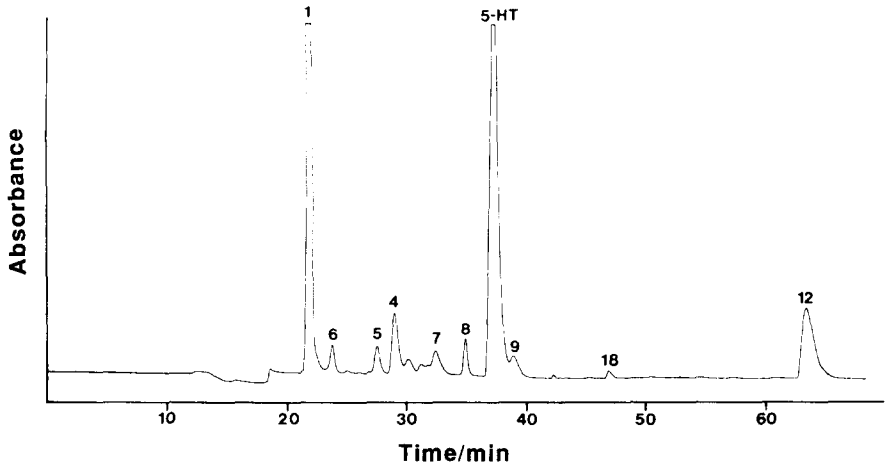


Fig. 3. HPLC chromatogram of the product mixture formed following incubation of 0.33 mM 5-HT with 0.5 mM H_2O_2 and 0.08 μM type VIII peroxidase in dilute hydrochloric acid, pH 2.75, at 37° for 4 hr. Chromatographic conditions are given in Materials and Methods.

oxidase. The product profile formed in the peroxidase/H_2O_2 oxidation of 5-HT at pH 7.2 (Fig. 4A) was very similar to that observed following electrochemical oxidation (Fig. 2B). Because of electrode filming by one or more products, the electrochemical oxidation of 5-HT was quite slow at pH 7.2. The appearance of the spiro trimer **15**, for example, as a major product in the electrochemical reaction but only a minor product of the peroxidase reaction was related to this effect (see later discus-

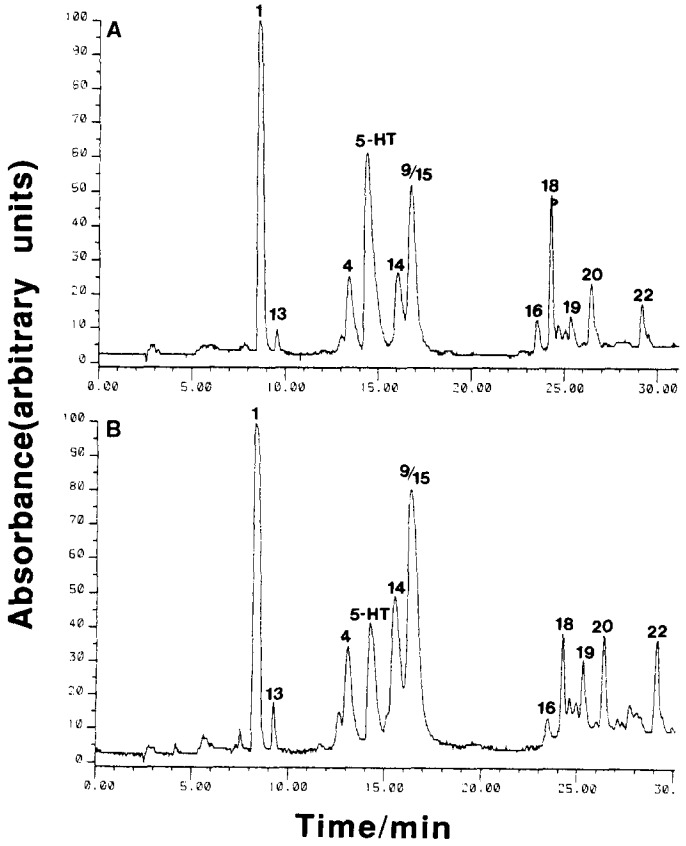


Fig. 4. HPLC chromatograms of product mixtures obtained following incubation of (A) 0.93 mM 5-HT, 0.45 mM H_2O_2 and 0.04 μM type VIII peroxidase and (B) 1.0 mM 5-HT, 1.0 mM H_2O_2 and 0.04 μM peroxidase in pH 7.2 phosphate buffer (μ = 0.2) at 37° for 90 sec. Chromatographic conditions are given in Materials and Methods.

sion). When the $[H_2O_2]/[5\text{-HT}]$ ratio was increased to ≥ 1 in the peroxidase-catalyzed reaction, the initially yellow solution rapidly changed to golden brown and at >3 min a black precipitate appeared in the solution. HPLC analysis of the soluble products (Fig. 4B) showed that compared to the reaction carried out with $[H_2O_2]/[5\text{-HT}] = 0.48$ (Fig. 4A) the product profile was the same but the yields of some products increased, notably trimer **4**, spiro dimer **13**, spiro dioxepin **14**, and dione **9**, whereas the yield of the oxygen-bridged dimer **18** decreased. The same effect was observed when electrochemical oxidations were carried out at potentials more positive than E_p for the first voltammetric oxidation peak of 5-HT.

When the $[H_2O_2]/[5\text{-HT}]$ ratio was ≤ 0.2, each mol of 5-HT oxidized consumed close to 0.5 mol of H_2O_2 and the 4,4′-linked dimer **1** was the major product along with only traces of other products. As the $[H_2O_2]/[5\text{-HT}]$ ratio increased to >0.2, more than 0.5 mol H_2O_2 was consumed per mol of 5-HT oxidized and compounds in addition to **1** appeared as major products, particularly **4**, **9** and **14**. These observations reflect the fact that increasing the $[H_2O_2]/[5\text{-HT}]$ ratio results in further oxidation of primary reaction products.

Ceruloplasmin-mediated oxidation of 5-HT. Preliminary experiments showed that the rate of 5-HT oxidation in the presence of human ceruloplasmin is maximal at pH 6. Similar reaction rates were observed in phosphate and acetate buffers. During the course of the ceruloplasmin-mediated oxidation of 5-HT, a yellow color developed in the solution. Chromatograms recorded after incubation of 5-HT in the presence of different concentrations of ceruloplasmin in pH 6.0 phosphate buffer are shown in Fig. 5. Clearly, the initial major oxidation product was dimer **1** along with smaller yields of **4**, **9**, **14**, **15**, **18**, **20** and **23** (Fig. 5A). With higher ceruloplasmin concentrations the reaction proceeded more rapidly and the relative yields of **9**, **14**, **15**, and **23** increased and **13** and **19** first appeared as products (Fig. 5B). Two additional major products responsible for HPLC peaks **24** and **25** also appeared. These products were also formed in the peroxidase/H_2O_2 oxidation when high 5-HT concentrations (>1.0 mM) and $[H_2O_2]/[5\text{-HT}] \geq 1.2$ were employed. The identity of **24** remains to be determined although it is known that it is formed as a result of further oxidation (electrochemical, peroxidase, ceruloplasmin) of dimer **1**. Compound **25** was not observed as a product of electrochemcial oxidation of 5-HT and, therefore, its structure also remains to be elucidated. It should be noted that **9** and **15** could not be properly separated using HPLC Method I (i.e. as in Fig. 5). However, these compounds could be readily separated using HPLC Method II. The latter method, however, employs mobile phases at pH 3.14 and, therefore, could not be used to separate the complete

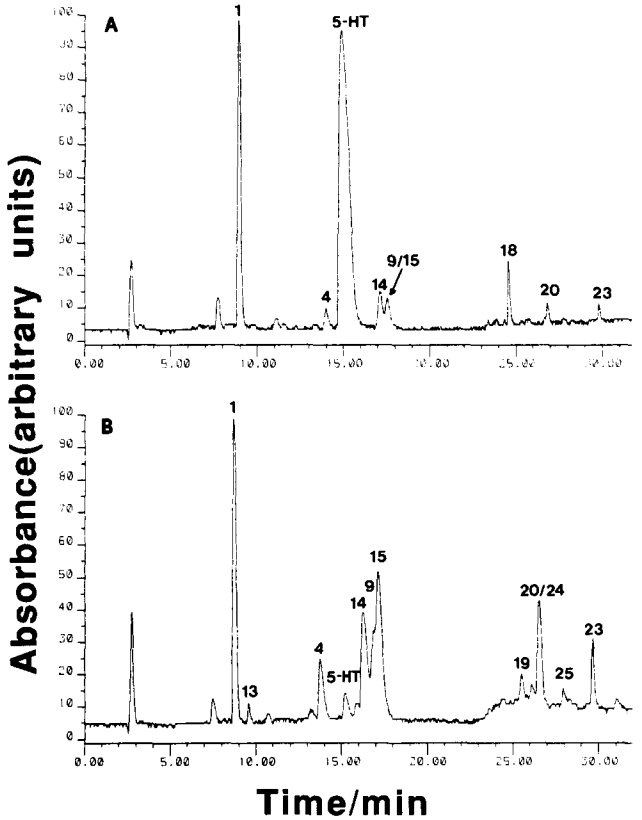


Fig. 5. HPLC chromatograms of product mixtures obtained following incubation of 1 mM 5-HT in pH 6.0 phosphate buffer ($\mu = 0.2$) at 37° for 45 min in the presence of (A) 25 μL (95 units) and (B) 50 μL (190 units) of ceruloplasmin. Chromatographic conditions are described in Materials and Methods.

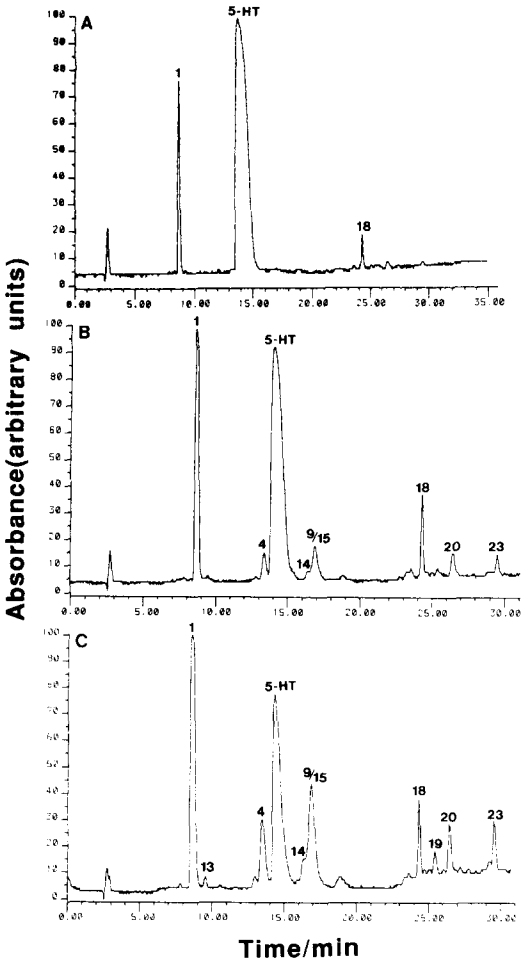


Fig. 6. HPLC chromatograms of product mixtures obtained following incubation of 1 mM 5-HT in pH 6.28 phosphate buffer at 37°: (A) 0.47 μM tyrosinase for 45 min; (B) 0.47 μM tyrosinase for 225 min; (C) 1.18 μM tyrosinase for 185 min. Chromatographic conditions are given in Materials and Methods.

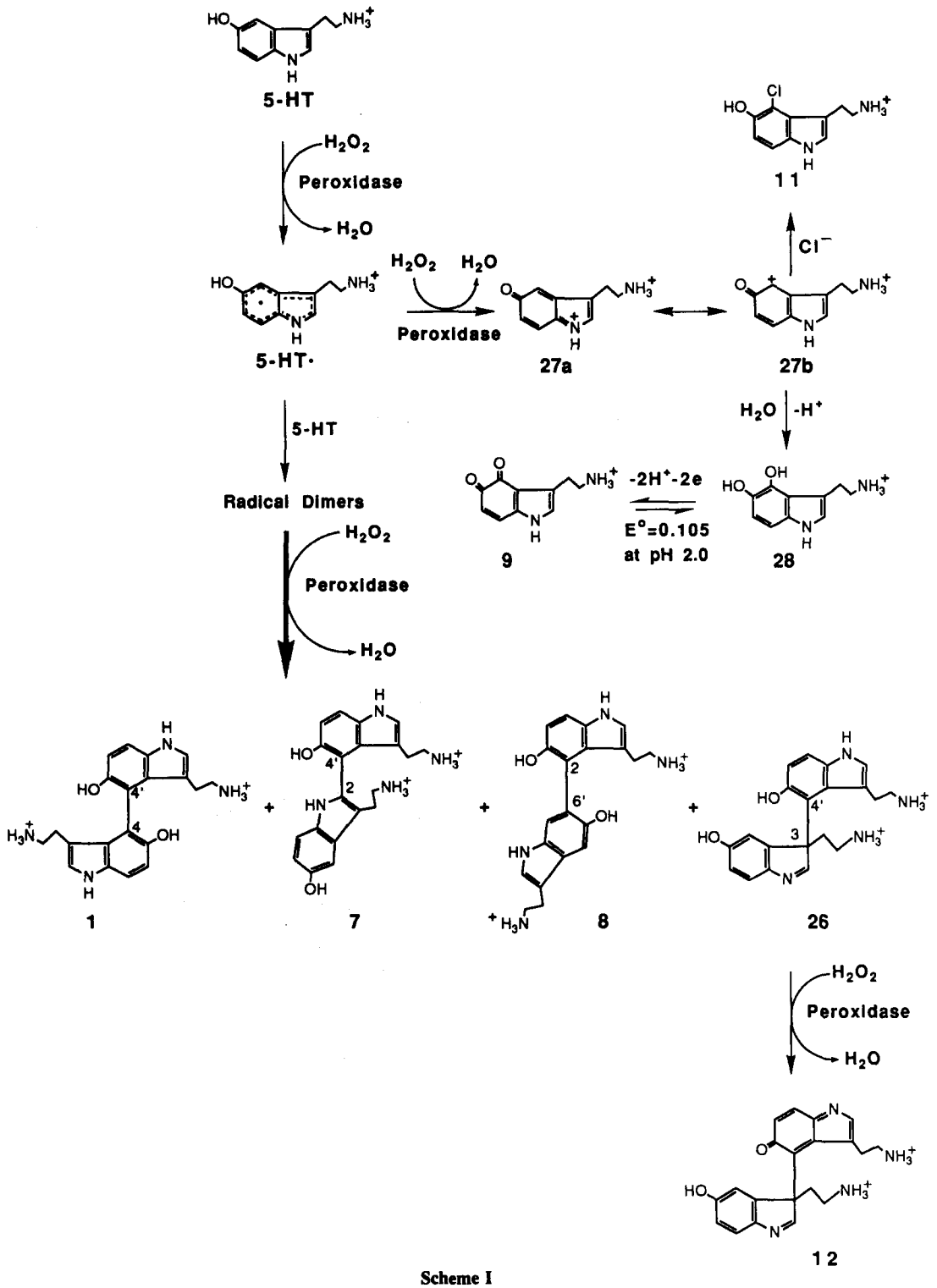
product mixtures formed at pH 6 to 7.5 because many products (e.g. **13**, **14**, **19**, **20**, and **23**) were unstable at such a low pH.

Tyrosinase-mediated oxidation of 5-HT. The rate of the tyrosinase-catalyzed oxidation of 5-HT was maximal between pH 6 and 7.5. Panels A, B, and C of Fig. 6 show a series of chromatograms of product mixtures obtained following incubation of 5-HT with various concentrations of tyrosinase at pH 6.28. The first products to appear were dimers **1** and **18** (Fig. 6A). After longer incubation times trimer **4**, spiro trimers **14**, **15** and **20**, trimer **23** and traces of dione **9** appeared (Fig. 6B). An increase in the concentration of tyrosinase accelerated the oxidation reaction and also resulted in the appearance of spiro dimer **13** and spiro trimer **19** (Fig. 6C).

DISCUSSION

Of the enzymes studied only the peroxidase/H_2O_2 system resulted in oxidation of 5-HT at low pH. The major product of the peroxidase-catalyzed and electrochemical oxidations of 5-HT was dimer **1** but other dimers (**7**, **8**) and oxidized dimers (**10**, **12**) were formed. In view of the similarity between the product profiles formed by electrochemical and peroxidase/H_2O_2 oxidations of 5-HT, it seems reasonable to conclude that at low pH the chemical aspects of the two oxidation reactions are very similar. Dimerization reactions can proceed either by an initial one-electron oxidation to a radical intermediate or by an initial two-electron oxidation to an ionic intermediate. The intermediate radical can yield dimeric species by two pathways, radical–radical coupling or radical–substrate coupling. In the case of the two-electron ionic intermediate, only an ion–substrate coupling reacton can lead to dimer. An analysis of the characteristics of the first voltammetric oxidation peak of 5-HT at low pH suggests that a radical–substrate reaction occurs leading to dimers [48]. Peroxidase enzymes are also known to generally catalyze one-electron oxidations to give radical intermediates [52, 53]. It is notable that all of the dimers formed in the peroxidase and electrochemical oxidation contained at least one 5-HT residue linked at the C(4)-position. No dimers or indeed higher oligomers were formed which contain 5-HT residues linked together at other positions (e.g. 6→6′, 2→6′, 3→3′, 2→2′, etc.). These observations argue against the intermediary of a radical in which the unpaired electron is significantly delocalized over the indolic ring system. Rather they suggest that the unpaired electron is predominantly localized at C(4). By analogy with the probable electrode reaction [48], it seems reasonable to conclude that the peroxidase/H_2O_2 oxidation of 5-HT also proceeds through an initial one-electron abstraction to yield the C(4)-centered radical 5-HT· which is then attacked to give four radical dimer intermediates which are further oxidized to stable dimers **1**, **7**, **8** and **26** (Scheme I).

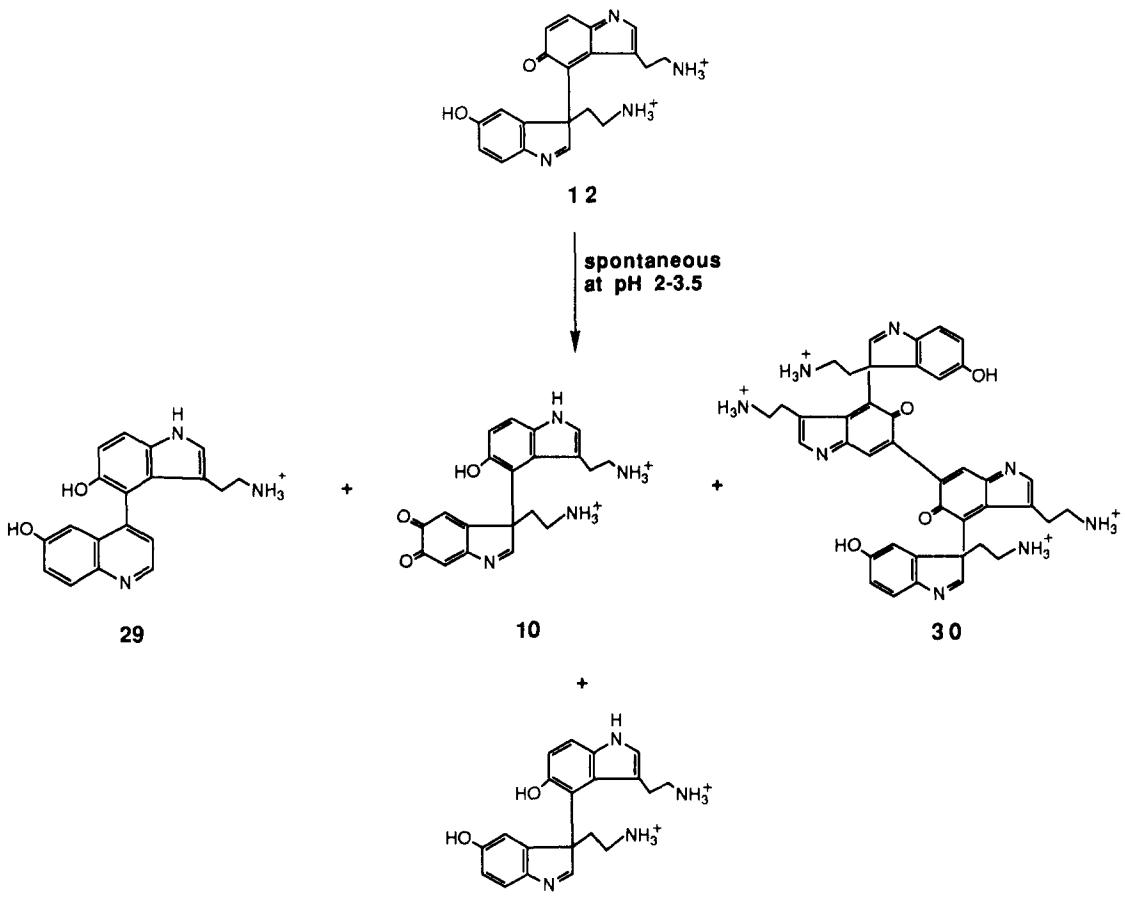
When the [H_2O_2]/[5-HT] concentration ratio is ≥0.3, the peroxidase-catalyzed oxidation of 5-HT gave dione **9** and, when Cl^- was present, 4-chloro-5-hydroxytryptamine (**11**) as significant products. An increase in the [H_2O_2]/[5-HT] ratio resulted in an increase in the yield of **9** and a corresponding decrease in the yields of dimeric products. Similarly, electrochemical oxidation of 5-HT gave dione **9** as a significant product, and its yield increased as the electrolysis potential was made more positive. These results suggest that under more oxidative conditions the primary radical intermediate, i.e. 5-HT·, is further oxidized to an intermediate which is susceptible to nucleophilic attack by water or Cl^-. This leads to the proposition that 5-HT· can be further oxidized to quinone iminium **27a** (Scheme I). Nucleophilic attack by water on resonance cation **27b** then yields 4,5-dihydroxytryptamine (**28**) which is very easily oxidized ($E^{0\prime} = 0.105$ V at pH 2) by molecular oxygen or peroxidase/H_2O_2 to dione **9**. In principle, putative radical intermediate 5-HT· could disproportionate to **27a**/**27b** and 5-HT. Several lines of evidence, however, argue against such a disproportionation. For example, assuming **1** is indeed formed by a radical mechanism of the type conceptualized in Scheme I, then if such a disproportionation reaction occurs dione **9** should always be a major oxidation product. However, in



Scheme I

peroxidase-catalyzed reactions when $[H_2O_2]/[5\text{-HT}]$ was ≤ 0.2, dimer **1** was the dominant product with only traces of other products, including **9**. Similarly, electrochemical oxidations at very low applied potentials gave the same result [48]. Further evidence favoring oxidation of 5-HT· to **27a/27b** under more strongly oxidative conditions was provided by the observation that in the presence of Cl^- **11** was a product which would be formed by direct attack of Cl^- on carbocation **27b** (Scheme I).

Indolenine-indole dimer **26** is more easily oxidized than 5-HT [48] and hence was oxidized in the presence of peroxidase/H_2O_2 to give **12**. The latter compound was formed in quite high yield in both the



Scheme II

peroxidase/H_2O_2 and electrochemical oxidation of 5-HT at low pH. Over the course of several hours at pH 2 to 3.5, **12** spontaneously decomposes to several secondary products [48]. The fully characterized products of this reaction are 4-[4′-(6′-hydroxyquinolyl)]-5-hydroxytryptamine (**29**), 3-(2-aminoethyl)-3-[3′- (2-aminoethyl)- 5′-hydroxyindol-4′-yl]- 5-hydroxyindoline (**31**), **10** and a partially characterized tetramer (**30**) (Scheme II).

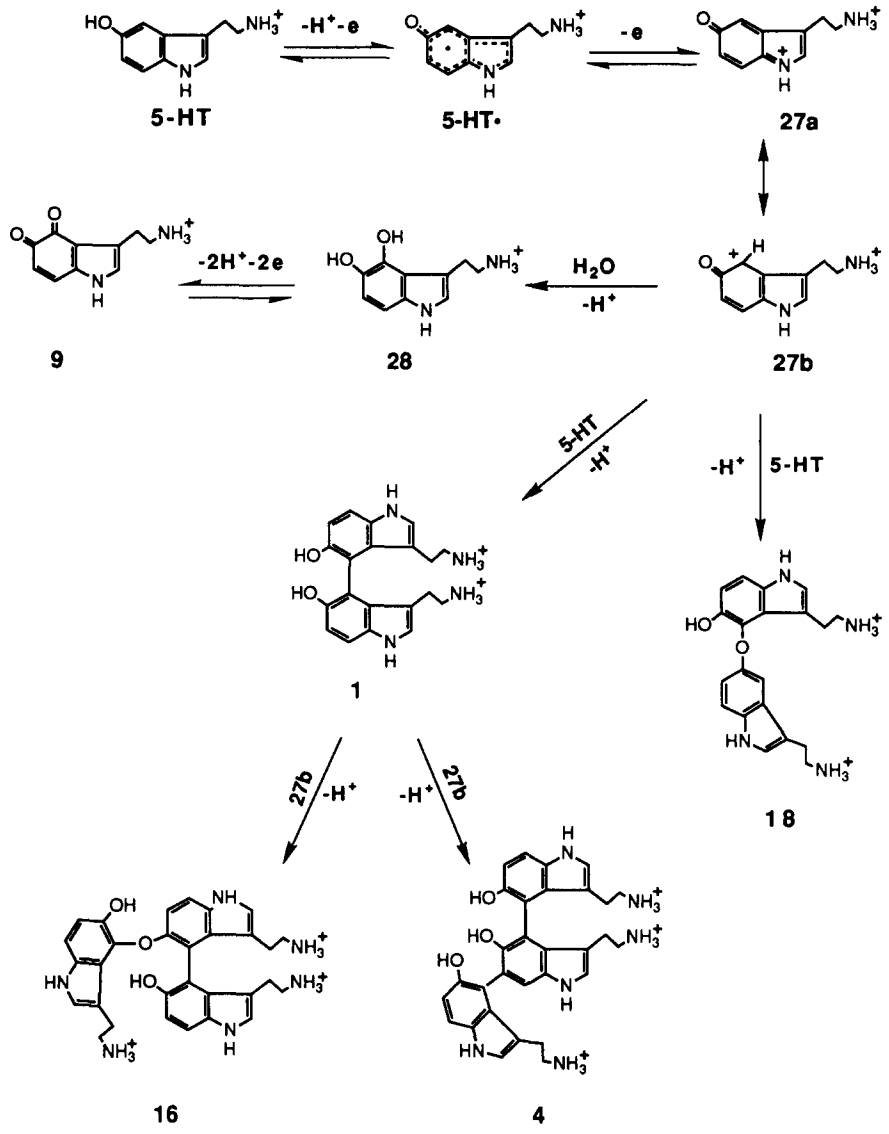
At physiological pH, peroxidase/H_2O_2, ceruloplasmin/O_2 and tyrosinase/O_2 oxidized 5-HT to complex mixtures of products. Most of these products were also formed as a result of the electrochemical oxidation of 5-HT. A summary of the product profiles formed in each of the oxidative systems studied is presented in Table 2. At pH 7.2 many of the oxidation products common to all of the enzymatic oxidations and the electrochemical

Table 2. Comparison of the product profiles formed upon oxidation of 5-hydroxytryptamine at pH 7.2* by peroxidase/H_2O_2, ceruloplasmin/O_2, tyrosinase/O_2 and electrochemical oxidation

Oxidizing system	Product profile†														
Peroxidase/H_2O_2	**1**	**13**	**4**	**14**	**9**	**15**	**16**	‡	‡	**18**	**19**	**20**	‡	**22**	‡
Ceruloplasmin/O_2	**1**	**13**	**4**	**14**	**9**	**15**	‡	‡	‡	**18**	**19**	**20**	‡	‡	**23**
Tyrosinase/O_2	**1**	**13**	**4**	**14**	**9**§	**15**	‡	‡	‡	**18**	**19**	**20**	‡	‡	**23**
Electrochemical	**1**	**13**	**4**	**14**	**9**	**15**	**16**	**17A**	**17B**	**18**	**19**	**20**	**21**	**22**	**23**

* Phosphate buffer, $\mu = 0.2$.
† Structures of products are shown in Table 1.
‡ Not detected.
§ Trace amount of dione **9** was produced.

oxidation contain one or more ether linkages, i.e. **13**, **14**, **15**, **18**, **19**, and **20**. With the exception of a trace of **18** formed in the peroxidase/H_2O_2 oxidation of 5-HT at pH 2.75, these compounds were not formed at low pH. The state of ionization of 5-HT at pH 2.75 and 7.2 remained virtually unchanged: $pK_{a_1} = 10.0$ and $pK_{a_1} = 11.1$, where pK_{a_1} refers to dissociation of the exocyclic NH_3^+ group and pK_{a_2} to dissociation of the phenolic-OH [54]. Thus, it appears that a fundamental mechanistic difference exists between the oxidations of 5-HT at low pH and at physiological pH. Cyclic voltammetry has revealed that an unstable intermediate is formed upon electrochemical oxidation of 5-HT at pH 7.2 [49]. This intermediate forms a quasi-reversible couple with 5-HT and is an electrophilic species which can be attacked by water to ultimately yield dione **9** or by 5-HT to yield, for example, the C–C linked dimer **1** and the oxygen-bridged dimer **18**. It is unlikely that 5-HT· would be susceptible to attack by water. Furthermore, it is unlikely that radical species could be detected by conventional cyclic voltammetry [55]. Hence, it has been concluded that at pH 7.2 5-HT is electro-oxidized in an overall two-electron reaction to cationic intermediate **27a**/**27b** [49] although such a reaction no doubt proceeds via transient radical intermediates such as 5-HT· (Scheme III). Nucleophilic attack by 5-HT on putative carbocation



Scheme III

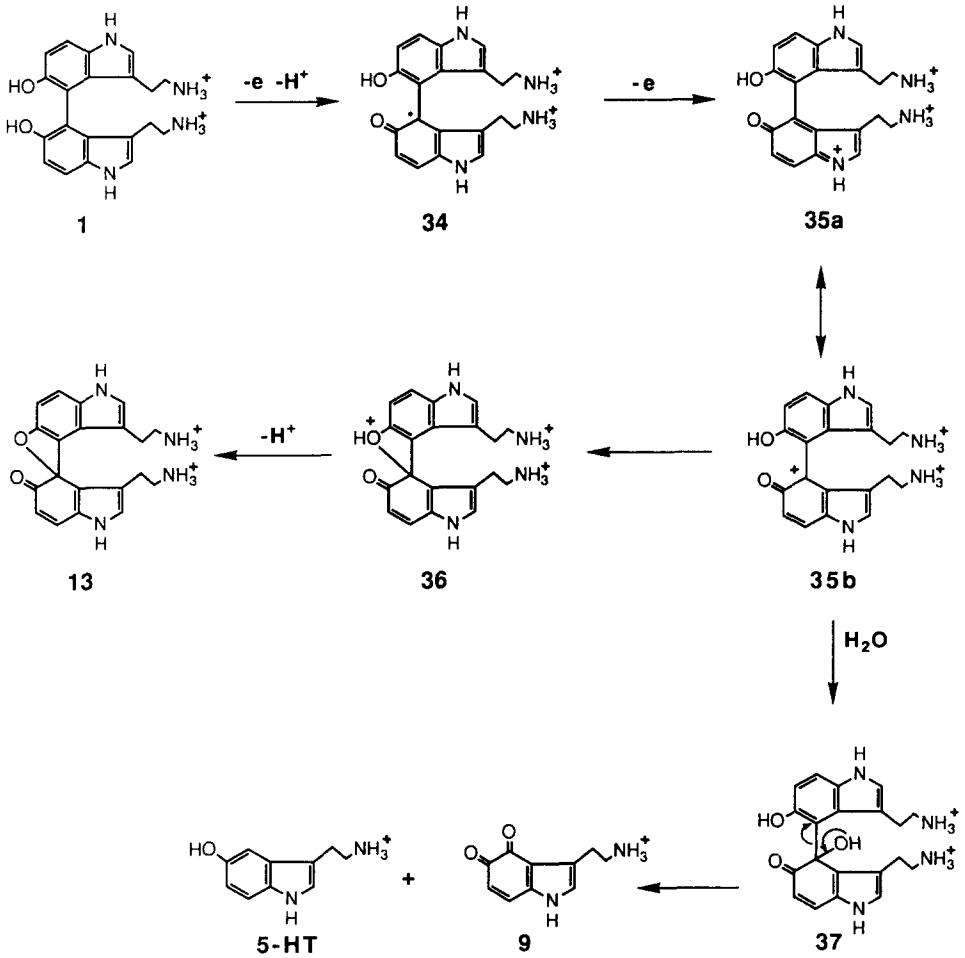
27b yielded dimers **1** and **18** or attack by water yielded **9** (Scheme III). Similarly, reactions between **27b** and **1** yielded trimers **4** and **16**.

The voltammetric oxidation peak of dimer **1** occurred at slightly more positive potentials than that of 5-HT at pH 7.2. Thus, at the potential employed to electro-oxidize 5-HT, dimer **1** was also oxidized. Similarly, peroxidase, ceruloplasmin and tyrosinase also oxidized **1**. Major products of all of these oxidations were spiro dimer **13** and dione **9**. Mechanistic information about these reactions remains to be determined. Nevertheless, by analogy with the reaction proposed for oxidation of 5-HT, it is possible that **1** is oxidized to cation **35a/35b**, perhaps via a primary radical intermediate (e.g. **34**) (Scheme IV). Intramolecular cyclization of **35b** yielded **13** or attack by water produced dione **9**.

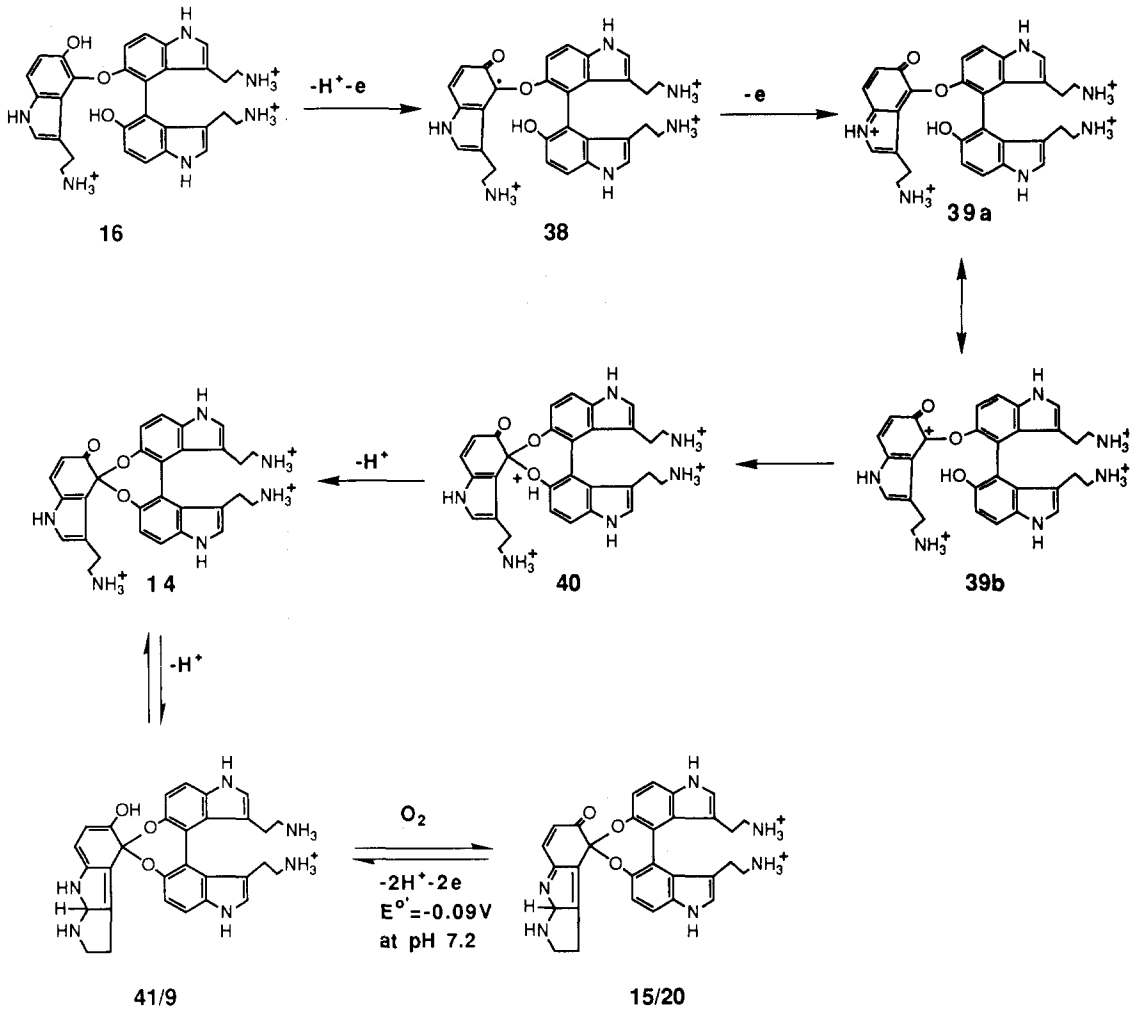
Trimer **16** exhibits a voltammetric oxidation peak at $E_p = 0.12$ V at pH 7.2 [49]. Thus, **16** was more easily oxidized than 5-HT. The major product of this oxidation was spiro dioxepin **14**. Peroxidase/H_2O_2, ceruloplasmin/O_2, and tyrosinase/O_2 also oxidized **16** largely to **14**. It is proposed that this reaction proceeds by oxidation of **16** to cations **39a/39b** probably via a transient radical such as **38** (Scheme V).

Intramolecular cyclization of **39b** then gave **14** as conceptualized in Scheme V. Trimer **16** did not appear as a significant product in the ceruloplasmin and tyrosinase oxidations of 5-HT because the rate of its oxidative cyclization to **14** was fast compared to the rate of oxidation of 5-HT. The aminoethyl side chain of the indolone residue of spiro trimer **14** slowly cyclized at pH 7.2 to yield diastereomers **15** and **20** by the route shown in Scheme V [49]. These diastereomers were products of all the enzymatic oxidations and of electrochemical oxidation of 5-HT at physiological pH.

Dimer **18** was also easily oxidized (E_p for **18** at pH 7.2 was 0.26 V; for 5HT $E_p = 0.21$ V). Enzymatic oxidation (peroxidase/H_2O_2, ceruloplasmin/O_2, tyrosinase/O_2) or electrochemical oxidation of **18** produced dione **9** as a major product by the reaction sequence shown in Scheme VI. The observed increase in the yield of dione **9** with increasing concentration of H_2O_2 in the peroxidase-catalyzed



Scheme IV



Scheme V

oxidation of 5-HT or with increasing concentrations of ceruloplasmin or tyrosinase certainly derived in large part from this reaction.

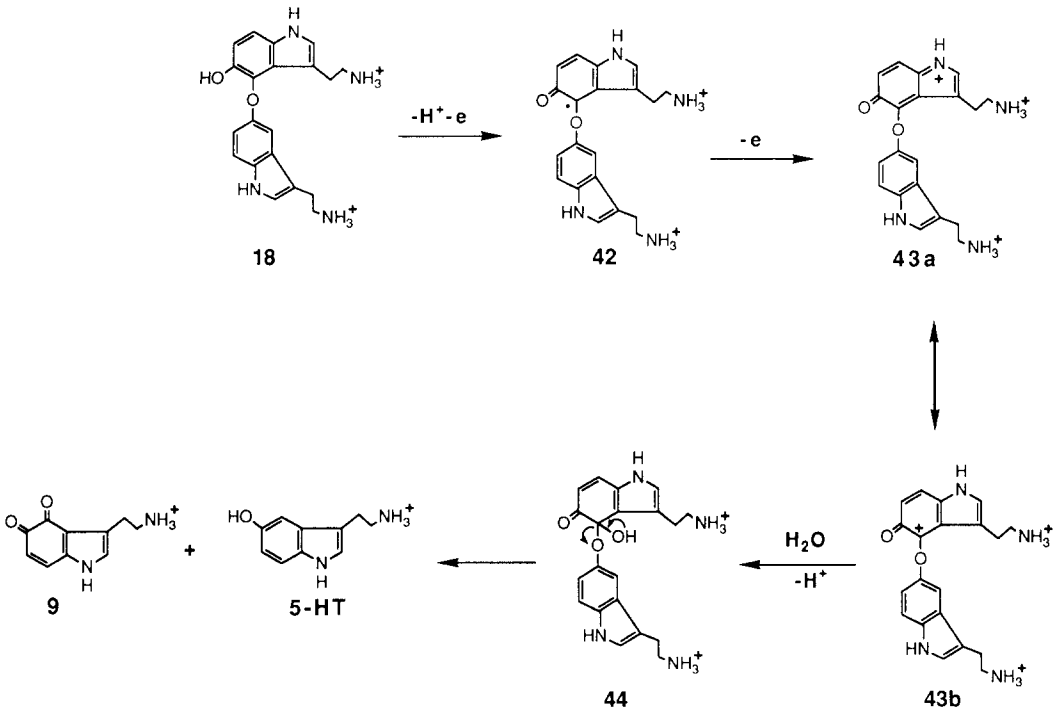
Trimers **17A** and **21** and tetramer **17B** were not detected as products of any enzymatic oxidation of 5-HT. Formation of these compounds in the electrochemical oxidation has been proposed to be due to secondary reactions between putative carbocation **27b** and dimer **18** (to give **17A** and **21**) or between **27b** and **17A** (to give **17B**) [49]. The precursors of **17A** and **21** certainly were formed in the enzymatic reactions. Thus, it must be concluded that their reactions to form these compounds are controlled by a specific electrode surface effect.

Reaction pathways leading to **22** and **23** observed in the electrochemical and some enzymatic oxidations of 5-HT remain to be elucidated.

CONCLUSIONS

Electrochemical investigations revealed that the neurotransmitter 5-HT is a rather easily oxidized compound, particularly at physiological pH. Reports that 5-HT can be oxidized in the latter pH range by peroxidase/H_2O_2 [35] and by ceruloplasmin/O_2 [28–32] have been confirmed. In addition, tyrosinase also catalyzed the oxidation of the neurotransmitter. All of the major products and many of the minor products of these enzyme-mediated oxidations were also formed upon electrochemical oxidation of 5-HT.

At low pH peroxidase was the only enzyme studied which oxidized 5-HT. By analogy with a previously elucidated electrochemical reaction mechanism it is proposed that a radical is the key intermediate in the peroxidase-mediated oxidation of 5-HT. At physiological pH values, however, many products were observed in all the enzymatic and electrochemical reactions. Most of these were not formed at low pH in either the peroxidase or electrochemical reactions. The nature of these products suggests that in the physiological pH domain the primary radical intermediate is further oxidized to a key carbocation (**27b**). This intermediate then reacts with 5-HT to yield the primary dimeric products **1** and **18** or with water to give **9**. However, none of the work reported here or in earlier electroanalytical investigations [49]

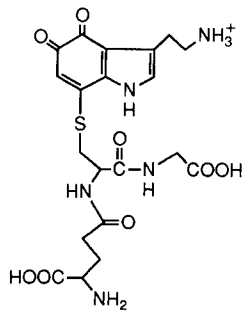


Scheme VI

can totally discount the possibility that the dimeric products are derived, at least in part, from reactions involving radical intermediates.

The results reported here indicate that 5-HT can be oxidized in the presence of many enzyme systems. Whether such oxidation can occur *in vivo*, for example, under appropriate pathological conditions remains to be determined. However, should such oxidations occur, it is clear that complex mixtures of structurally interesting products would be formed. Virtually nothing is known about the effects of these compounds in the CNS. However, tryptamine-4,5-dione (**9**) has been shown recently in both *in vitro* and *in vivo* studies to be neurotoxic [11, 56]. Furthermore, HPLC-CD analyses of CSF of Alzheimer patients have suggested the presence of **9** [57]. However, **9** is an electron deficient compound and would be expected to react avidly with nucleophiles. Indeed, it has been shown recently that **9** reacts rapidly with glutathione at physiological pH to form 7-*S*-glutathionyl-tryptamine-4,5-dione (**45**) [58]. Since glutathione is widely distributed throughout the brain at intracellular concentrations of about 2 mM [59], it appears very probable that **9**, if formed by oxidation of endogenous 5-HT, would react rapidly to form its glutathione conjugate. Indeed, the initial claim that **9** is present in the CSF of Alzheimer patients but not in that of controls [57] has not been confirmed in more recent studies [60]. Conjugate **45** has not yet been sought in CSF or CNS tissue of Alzheimer patients. Preliminary studies in this laboratory, however, indicated that **45** injected into mouse brain was extremely toxic (data not reported). In addition, *in vitro* studies have shown that **9** causes an efflux of 5-HT from rat brain fragments [11] in a manner similar to that observed with the widely used serotonergic neurotoxin 5,6-DHT [12]. It is also likely that dione **9** reacts with larger sulfhydryl-containing moieties in CNS. Interference of **9** with binding of 5-HT to 5-HT binding protein or reaction with GTP-binding proteins has been suggested [11].

Endogenous formation of **9** by oxidation of 5-HT as well as many other potentially neurotoxic oxidation products may play an important role in neurodegenerative processes particularly in brain regions innervated by 5-HT and simultaneously containing relatively low concentrations of anti-oxidants such as glutathione or ubiquinone Q10. The latter conditions occur in substantia nigra [61]. It is interesting to note that substantia nigra shows the greatest degree of neuronal degeneration with age [61].



45

An unknown oxidation product of 5-HT, formed both in dilute HCl and pH 8 phosphate buffer, is a more effective AChE inhibitor than 5-HT [27]. Insufficient information is available about the physical and chemical properties of this inhibitor for it to be identified as one of the oxidation products of 5-HT described in this report. However, several products of the electrochemical and peroxidase/H_2O_2 oxidation of 5-HT are formed at pH 2–3 and pH 7.2 (e.g. **1**, **4**, **9**, **18**). Nevertheless, further investigations to determine connections between the oxidative chemistry and biochemistry of 5-HT and, for example, the loss of a specific form of AChE in the brains of Alzheimer patients [62] are clearly appropriate.

The ceruloplasmin/O_2 oxidation of 5-HT at physiological pH is quite facile and generates reactive intermediates and many structurally interesting products including neurotoxin **9**. Ceruloplasmin has been speculated, under normal physiological conditions, to play a role in controlling endogenous levels of biogenic amines including 5-HT [31]. Thus, it is interesting to speculate further that under certain pathological conditions ceruloplasmin may play an alternative role and convert the neurotransmitter into toxic products.

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