Articles

Effects of L-Cysteine on the Oxidation Chemistry of Dopamine: New Reaction Pathways of Potential Relevance to Idiopathic Parkinson's Disease

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Oxidation of the catecholaminergic neurotransmitter dopamine (1) at physiological pH normally results in formation of black, insoluble melanin polymer. In this study, it is demonstrated that L-cysteine (CySH) can divert the melanin pathway by scavenging the proximate o-quinone oxidation product of 1 to give 5-*S*-cysteinyldopamine (8). This cysteinyl conjugate is further oxidized in the presence of free CySH to give 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (11) and its 6-*S*-cysteinyl (12), 8-*S*-cysteinyl (14), and 6,8-di-*S*-cysteinyl (16) conjugates in addition to many other unidentified compounds. 5-*S*-Cysteinyldopamine (8) and dihydrobenzothiazines 11, 12, 14, and 16 are all more easily oxidized than 1. With increasing molar excesses of CySH, the formation of melanin is decreased and, ultimately, completely blocked. Preliminary experiments have revealed that when injected into the brains of laboratory mice, dihydrobenzothiazine 11 and its cysteinyl conjugates 12 and 14 are lethal and evoke profound behavioral responses including hyperactivity and tremor. On the basis of these results and other recent observations, a new hypothesis has been advanced which might help explain the selective degeneration of nigrostriatal dopaminergic neurons which occurs in idiopathic Parkinson's Disease (PD). This hypothesis proposes that in response to some form of chronic brain insult, the activity of γ-glutamyltranspeptidase is upregulated leading to an increased rate of translocation of glutathione (GSH) into the cytoplasm of dopaminergic cell bodies in the substantia nigra (SN) pars compacta. The results of this *in vitro* study predict that such an elevated translocation of GSH into heavily pigmented dopaminergic neurons would cause a diversion of the neuromelanin pathway with consequent depigmentation of these cells and formation of 8, all of which occur in the Parkinsonian SN. The further very facile oxidation of 8 which must occur under intraneuronal conditions where 1 is autoxidized, *i.e.*, in neuromelanin-pigmented cells, would lead to dihydrobenzothiazine 11 and its cysteinyl conjugates which could be the endotoxins responsible for the selective degeneration of dopaminergic SN neurons in PD. The ease of autoxidation of 8 is suggested to account for the low levels of this conjugate found in the degenerating and Parkinsonian SN. The latter reaction and the intraneuronal autoxidation/redox cycling of 11 and its cysteinyl conjugates would be expected to result in greatly increased rates of intraneuronal formation of $O_2^{\bullet-}$ which could (a) catalyze the oxidation of 1 by molecular oxygen, hence accounting for the significantly elevated 8/1 ratio observed in the Parkinsonian SN, and (b) lead to increased production of H_2O_2 and $HO^{\bullet}$, resulting in the extensive peroxidative damage which occurs in the SN in PD.

Parkinson's Disease (PD) is a degenerative neurological disorder characterized by hypokinesia, rigidity, and tremor. These symptoms are related to a severe deficiency of the neurotransmitter dopamine (1) in the striatum. The biochemical basis of this deficiency is a selective and progressive degeneration of nigrostriatal dopaminergic neurons[1] resulting from pathological processes which occur in their cell bodies located in the substantia nigra (SN) pars compacta.[2] Current information indicates that these pathological processes include oxidative stress[3-5] and a severe mitochondrial complex I (NADH CoQ_1 reductase) deficiency.[6,7] The fundamental mechanisms underlying these pathological processes are unknown. However, at autopsy, the SN of PD patients have been shown to contain increased levels of iron[8,9] and decreased levels of ferritin[10] compared to controls. In view of the ability of free or low-molecular-weight iron species to catalyze formation of the cytotoxic hydroxyl radical ($HO^{\bullet}$) from hydrogen peroxide (H_2O_2) and superoxide anion radical ($O_2^{\bullet-}$),[11] which are formed as a result of normal neuronal metabolism and autoxidation of 1, it has been suggested that nigral iron might be an endotoxin which contributes to the lipid peroxidation and the membrane damage which occurs in the Parkinsonian SN.[12-14] Dopaminergic cell bodies in the SN pars compacta are pigmented with black neuromelanin, a polymer formed in the cytoplasm of these neurons as a consequence of the autoxidation of 1 and perhaps other endogenous catechols.[15,16] Neuromelanin strongly binds Fe^{3+}, and in the presence of Fe^{3+}, synthetic 1 melanin can catalyze a Fenton-type reaction which generates $HO^{\bullet}$ and initiates lipid peroxidation.[17] Accordingly, it has been suggested that the degeneration of heavily pigmented dopaminergic neurons in the Parkinsonian SN might be caused by a similar reaction involving a neuromelanin–Fe^{3+} complex.[18] However, the SN of patients with incidental Lewy body disease, a presymptomatic stage of PD,[19] contain normal levels of iron and ferritin.[20] This suggests that increased iron levels may not be the initiator

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of PD but rather represent a secondary response to some form of primary cell damage.[21]

Normal metabolic processes in dopaminergic neurons which involve the participation of molecular oxygen such as electron transport, oxidative deamination of 1 by monoamine oxidase (MAO), and autoxidation of the neurotransmitter result in the intraneuronal formation of H_2O_2 and $O_2^{\bullet-}$. These neurons are therefore potentially susceptible to damage by $HO^{\bullet}$ which could be formed from the latter reduced oxygen species by Fenton or Haber–Weiss reactions.[11] Protection against radical species is believed to be provided by endogenous antioxidants such as glutathione (GSH), ascorbate, α-tocopherol, and ubiquinols.[22] Furthermore, GSH in conjunction with the GSH peroxidase/GSSG reductase system is responsible for elimination of intraneuronal H_2O_2 and organic peroxides. Thus, an impairment of these defense mechanisms as a consequence of inadequate levels of GSH resulting from decreased activities of the enzymes responsible for its biosynthesis or decreased activities of GSH peroxidase or GSSG reductase might be expected to lead to progressive and irreversible neuronal damage. The SN of rodents, primates, and humans contain lower levels of GSH than all other brain regions.[23,24] Furthermore, the levels of GSH in the SN of patients who died with idiopathic PD are greatly reduced compared to those of age-matched controls.[20,25] Thus, it has been suggested that low levels of nigral GSH and consequent oxidative stress might contribute to the degeneration of dopaminergic SN neurons in idiopathic PD.[23,26–28] However, several factors tend to argue against this suggestion. For example, reduced brain levels of GSH as a result of a GSH synthetase deficiency do not cause SN cell loss or Parkinsonism.[29,30] Similarly, injection of mice with diethyl maleate causes a profound depletion of brainstem (of which SN is part) GSH but no alteration in striatal levels of 1, an effect which would be expected if dopaminergic SN cell bodies had been damaged or destroyed.[31] In the Parkinsonian brain, only the SN is depleted of GSH and this depletion is not accompanied by a corresponding increase in GSSG.[20,25,32] Furthermore, the levels of GSH in the SN of patients with incidental Lewy body disease are decreased to the same extent as those in patients with advanced PD without any corresponding increase in GSSG.[20] Thus, decreased GSH levels in the SN appear to represent a very early component in the pathological processes which underlie PD. The decrease in nigral GSH in PD is not related to decreased activity of γ-glutamylcysteine synthetase, the rate-limiting enzyme for biosynthesis of the tripeptide, or to alterations in GSH peroxidase, GSSG reductase, or GSH tranferase activities.[32–34] Thus, in the Parkinsonian SN, the biochemical systems for GSH biosynthesis and for conversion of GSSG to GSH are not compromised, suggesting, perhaps, that the tripeptide is being siphoned off by reaction with some exogenous or endogenous species.

An interesting feature of dopaminergic SN pars compacta neurons is their pigmentation by neuromelanin. Chemical degradation of human SN neuromelanin yields products which suggest that this polymer is composed of indolic residues and residues derived from 5-*S*-cysteinyldopamine.[35] The indolic residues in neuromelanin result from a pathway where 1 is initially autoxidized to an *o*-quinone intermediate which undergoes an intramolecular cyclization to an indoline followed by further reactions leading to 5,6-dihydroxyindole which is oxidatively polymerized.[15,16,36,37] Residues derived from 5-*S*-cysteinyldopamine result from the facile nucleophilic addition of CySH or GSH to the o-quinone intermediate. In the latter case, 5-*S*-glutathionyldopamine is apparently hydrolyzed by peptidase enzymes to the corresponding cysteinyl conjugate. However, *in vitro* experiments reveal that oxidations of 1 by tyrosinase give a black, insoluble polymer only when the concentrations of CySH are low relative to that of the neurotransmitter.[35] When the concentration of CySH is greater than that of 1, an insoluble, black melanin polymer is not formed. On the basis of these observations, Carstam *et al.*[35] have concluded that when indolic residues are incorporated into neuromelanin, CySH and GSH must be virtually absent in the dopaminergic cell body. Indeed, these investigators have suggested that neuromelanin formation might indicate the exhaustion of CySH and GSH in SN cell bodies. Histochemical studies also reveal that there is little or no GSH in neuronal somata in any region of the brain and that the tripeptide is largely localized in glia, axons, and nerve terminals.[38,39] It is also of relevance to note that in the mouse SN, the levels of ubiquinols Q_9 and Q_{10} are lower than in any other region of the brain and that the quinol/quinone ratio is heavily skewed in favor of the oxidized forms of these compounds.[24,40] Similarly, the concentrations of α-tocopherol in the SN are low compared to in other brain structures.[40] Taken together, these observations suggest that SN neurons probably have a rather weak endogenous antioxidant defense system. This suggestion is supported by the observation that the SN has by far the highest levels of malondialdehyde, an index of peroxidative damage, of any brain region.[41] Furthermore, intraneuronal autoxidation of 1 to neuromelanin can be expected to occur at a significant rate only in the absence of an effective antioxidant system and when the cytoplasmic levels of CySH or GSH are very low.[35] Indeed, the normal loss of dopaminergic SN neurons with age[42] could well be a consequence of their suboptimal antioxidant defense system which permits autoxidation of 1 and other endogenous catechols to cytotoxic products (*e.g.*, *o*-quinones)[43–45] and byproducts ($O_2^{\bullet-}$, H_2O_2, $HO^{\bullet}$).[44] However, the regional pattern of age-dependent dropout of nigrostriatal dopaminergic neurons is different to that observed in idiopathic PD[2] and occurs at a much lower rate.

Recent reports have indicated that a significant decrease in the neuromelanin content of surviving pigmented cells occurs in the SN of PD patients.[46,47] Furthermore, it has been reported that the more heavily neuromelanin-pigmented dopaminergic SN neurons appear to be the most vulnerable to degeneration in PD.[47,48] This observation cannot exclude the possibility that factors other than neuromelanin content might be involved in the differential susceptibility of dopaminergic neurons in PD. However, one interpretation of this observation might be that intraneuronal conditions which favor neuromelanin formation, *i.e.*, a weak antioxidant system which permits high levels of autoxidation of cytoplasmic 1, predispose these cells to degeneration in PD. The depigmentation of SN neurons further suggests that the fundamental mechanism(s) underlying PD might partially or completely block the neuromelanin pathway.

Recently, very low levels of the 5-*S*-cysteinyl conjugates of 1, L-3,4-dihydroxyphenylalanine (L-DOPA) and 3,4-dihydroxyphenylacetic acid (DOPAC), have been detected

in human and various other mammalian brains.[49,50] These conjugates are probably formed either by nucleophilic addition of CySH to o-quinones formed by autoxidation of these catechols or by addition of GSH to give the 5-*S*-glutathionyl conjugates which are then hydrolyzed by peptidase enzymes.[49,50] Subsequently, it has been reported that the 5-*S*-cysteinyldopamine/1 ratios in the striatum and SN are appreciably higher in human patients with more extensively depigmented and degenerated SN cells.[51] These observations were interpreted to reflect an increase in the rate of autoxidation of 1 with the degree of depigmentation and degeneration of the SN. The higher 5-*S*-cysteinyldopamine/1 ratio measured in the SN compared to that observed in the dopaminergic terminal regions in the striatum, where 1 is largely sheltered in vesicles, suggests that only the unsheltered cytoplasmic pool of the neurotransmitter is available for autoxidation.[51] Furthermore, the increase in the 5-*S*-cysteinyldopamine/DOPAC ratio measured in severely dopamine-depleted and degenerated SN tissue was interpreted to reflect a shift in intraneuronal metabolism of 1 from the MAO-mediated oxidative deamination pathway leading to DOPAC and homovanillic acid (HVA) in favor of an autoxidation pathway.[51] Significantly, the 5-*S*-cysteinyldopamine/1 ratio in the SN of a patient with PD was an order of magnitude greater than in patients without depigmentation and degeneration. This result was suggested to be indicative of a greatly increased rate of autoxidation of 1 in the Parkinsonian SN.[51]

Taken together, available lines of evidence suggest that pigmented dopaminergic SN neurons normally contain little GSH or CySH and suboptimal levels of several antioxidants with the result that 1 and other cytoplasmic catechols are autoxidized to neuromelanin. A correlation has been clearly established between the depigmentation and degeneration of these neurons and an increase in the 5-*S*-cysteinyldopamine/1 ratio, an increase which becomes particularly pronounced in the surviving dopaminergic SN cell bodies in the Parkinsonian brain.[49-51] While this increase might reflect an elevated rate of autoxidation of cytoplasmic 1 in degenerating SN neurons, chemical and/or biochemical reasons for such an effect are not obvious. Furthermore, an increased rate of autoxidation of 1 would be expected to result in more rapid formation of neuromelanin and increased pigmentation of degenerating SN neurons even in the presence of low relative concentrations of CySH or GSH.[35] However, in PD, dopaminergic SN neurons become depigmented as the disease progresses,[46,47] and the more heavily neuromelanin-pigmented cells appear to be particularly vulnerable to degeneration.[47,48] A plausible interpretation of these apparently conflicting observations might be that a key step in the pathoetiology of PD is an increased translocation of GSH, biosynthesized in glial cells,[52] into the cytoplasm of dopaminergic cell bodies. Such an elevated influx of GSH might account for an increase in the 5-*S*-cysteinyldopamine/1 ratio by scavenging the *o*-quinone proximate autoxidation product of 1 and, hence, diversion of the neuromelanin pathway leading to depigmentation of SN neurons. Support for this suggestion is provided by the recent observation that γ-glutamyltranspeptidase activity is significantly elevated in the Parkinsonian SN.[32] An important function of this enzyme is translocation of extracellular GSH into cells *via* the γ-glutamyl cycle.[53] A consequence of elevated translocation of GSH into SN cell bodies might be the formation of endotoxins which contribute to the degeneration of nigrostriatal dopaminergic neurons in PD.

The goals of the work described in this communication, therefore, were to provide experimental evidence in support of the above hypothesis. Because cysteinyl conjugates of 1 and other cytoplasmic catechols have been unequivocally detected in human brain tissue and implicated in the pathology of PD, initial experiments have focused on the influence of this sulfhydryl compound on the oxidation chemistry of 1. In view of the fact that the autoxidation of 1 is relatively slow in aqueous solution at physiological pH, the electrochemically driven oxidation of 1 in the presence of CySH has been studied. Experiments were designed to determine whether CySH could indeed divert the oxidation of 1 to melanin polymer, to determine the conditions necessary to effect this diversion, and to elucidate the broad outlines of the chemistry involved. It was judged to be of key importance to isolate and structurally characterize major initial reaction products and to determine whether one or more of the products of oxidation of 1 in the presence of CySH were toxic and/or neuropharmacologically active when administered into the brains of laboratory animals.

Results

Voltammetric Studies. A cyclic voltammogram of 1 (1.0 mM) dissolved in pH 7.4 phosphate buffer (μ = 1.0) at a pyrolytic graphite electrode (PGE) using a sweep rate (ν) of 100 mV s^{-1} is presented in Figure 1A. Under these conditions, 1 exhibits a single voltammetric oxidation peak (I_a) having a peak potential (E_p) of 134 ± 4 mV. Following scan reversal, reduction peak I_c appears and forms a reversible couple with oxidation peak I_a (ΔE_p between peak I_a and peak I_c was 33 ± 4 mV). At the value of ν employed, the peak current (i_p) for peak I_c was clearly less than that for peak I_a. However, at more negative potentials, reduction peak II_c appears (E_p = −311 ± 1 mV) and, on the second anodic sweep, oxidation peak II_a (E_p = −290 ± 7 mV). Peaks II_c and II_a also represent a reversible couple at the PGE (E_p between peaks II_c and II_a = 21 ± 8 mV). On the basis of previous reports,[36,37,53] the peak I_a electrooxidation of 1 is a 2e, $2H^+$ reaction to give *o*-quinone 2 (Scheme 1). Peak I_c corresponds to the reduction of 2 back to 1. Deprotonation of 2 yields 3 which undergoes a facile intramolecular cyclization reaction to give 5,6-dihydroxyindoline (4) which is immediately further chemically oxidized by 2 at the electrode surface to give the orange dopaminochrome 5 and 1 (Scheme 1). Aminochrome 5 is responsible for peak II_c observed in cyclic voltammograms of 1 (Figure 1A) in which it undergoes a 2e, $2H^+$ reduction to 4. Peak II_a, observed on the second anodic sweep, corresponds to the reversible 2e, $2H^+$ oxidation of 4, formed in the peak II_c process, to give 5. The electrochemical behaviors of the 1/2 and 4/5 couples are both reversible at the PGE. Earlier investigators[36,37] used a carbon paste electrode and found that the ΔE_p values between peaks I_a and I_c and between peaks II_c and II_a were considerably greater than would be expected for electrochemically reversible processes. Nevertheless, with these differences, the cyclic voltammetric behavior of 1 at the PGE is in agreement with that reported previously.

The presence of CySH caused some significant changes in cyclic voltammograms of 1 (1.0 mM) at pH 7.4 (Figure 1B–D). Thus, with increasing concentrations of CySH (0.5–5 mM), the reduction peak I_c and the peak II_c/peak

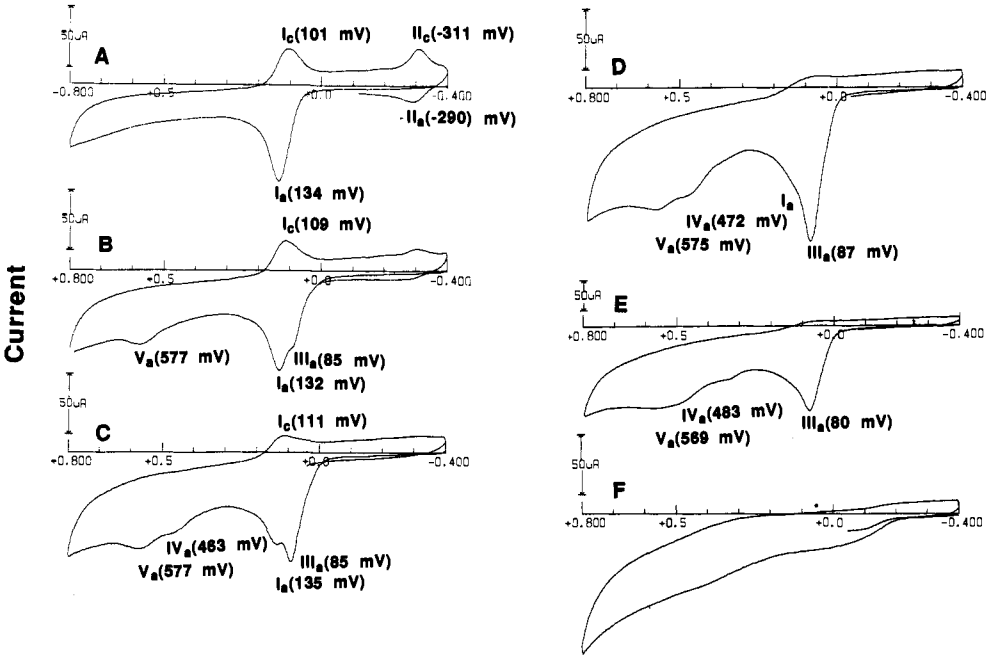
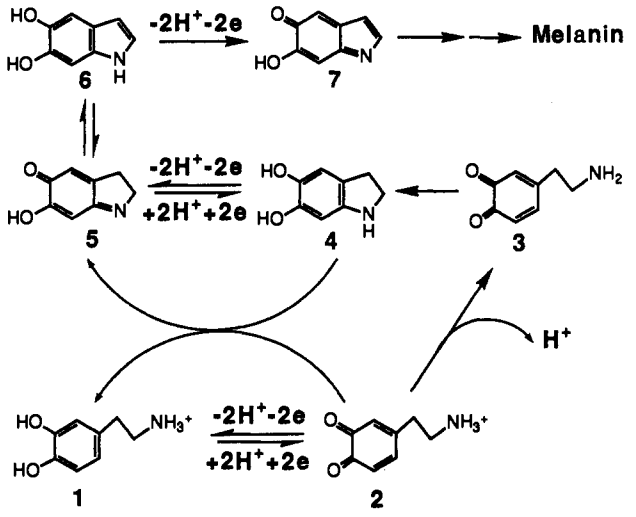


Figure 1. Cyclic voltammograms at the PGE of (A) 1.0 mM dopamine (1), (B) 1.0 mM 1 and 0.5 mM L-cysteine (CySH), (C) 1.0 mM 1 and 1.0 mM CySH, (D) 1.0 mM 1 and 2.0 mM CySH, (E) 1.0 mM 1 and 5.0 mM CySH, and (F) 5.0 mM CySH in pH 7.4 phosphate buffer (μ = 1.0). Sweep rate: 100 mV s^{-1}.

Scheme 1



II_a couple decrease and ultimately disappear (at CySH concentrations > 1 mM, Figure 1D,E). Correspondingly, at a CySH concentration ≥ 0.5 mM, oxidation peak III_a appears at more negative potentials (E_p = 85 ± 4 mV; ν = 100 mV s^{-1}) than peak Ia (E_p = 134 ± 1 mV). In the presence of ≥1 mM CySH, i_p for oxidation peak III_a exceeds that for peak I_a. Furthermore, oxidation peaks IV_a (E_p = 473 ± 10 mV; ν = 100 mV s^{-1}) and V_a (E_p = 574 ± 5 mV; ν = 100 mV s^{-1}) appear at more positive potentials than peak I_a (Figure 1C–E). In the presence of a 5-fold molar excess of CySH, oxidation peak I_a virtually disappears. A cyclic voltammogram of CySH (5 mM) at pH 7.4 (Figure 1F) at the PGE showed only a broad, rather ill-defined oxidation peak at E_p = *ca.* −100 mV. This peak corresponds to the oxidation of CySH to cystine (CySSCy). These cyclic voltammograms indicate that a rapid reaction occurs between *o*-quinone 2 and CySH. This reaction not only scavenges 2, as shown by the decrease and disappearance of peak I_c, but also blocks formation of 4 and 5, as shown by the elimination of the peak II_c/peak II_a couple. The reaction between 2 and CySH also must yield the products responsible for oxidation peaks III_a, IV_a, and V_a.

A linear sweep voltammogram of 1 (1.0 mM; ν = 5 mV s^{-1}) at pH 7.4 shows only oxidation peak I_a at E_p = 133 ± 5 mV (Figure 2A). In the presence of ≥0.5 mM CySH, oxidation peak III_a (E_p = 62 ± 10 mV) becomes clearly evident (Figure 2B–F). However, under such slow sweep-rate conditions, peaks IV_a and V_a are very poorly defined. Oxidation peaks III_a and I_a were best resolved when 1 and CySH were present at approximately equimolar concentrations (Figure 2C). However, an increase in ν resulted in an anodic shift of peak III_a (ΔE_p = 31 ± 3 mV per decade increase in ν) (Figure 3), whereas E_p for peaks I_a remained constant (E_p = 136 ± 4 mV). As a consequence, peaks III_a and I_a merged at $\nu \geq$ 500 mV s^{-1} (Figure 3D–G). Furthermore, at $\nu >$ 50 mV s^{-1}, reduction peak I_c appeared and increased in height with increasing sweep rates (Figure 3B–G). Such behaviors indicated that the reaction between 2 and CySH could be outrun to some extent with increasingly fast sweep rates.

Controlled Potential Electrooxidation and HPLC Analyses. At ν = 5 mV s^{-1}, E_p for oxidation peak I_a of 1 (1.0 mM) at pH 7.4 was 133 ± 5 mV. E_p for peak III_a observed in a voltammogram of 1 (1.0 mM) and CySH (0.5–5.0 mM) under the same conditions was 62 ± 10 mV. Accordingly, controlled potential electrooxidations of 1 in the presence of CySH were carried out at 80 mV, *i.e.*, 18 mV more positive than E_p for peak III_a but 53 mV negative of E_p for peak I_a. An HPLC chromatogram of the product solution obtained following a controlled potential electrooxidation of 1 (1.0 mM) in pH 7.4 phosphate buffer at 80 mV for 30 min revealed that under these conditions very little of the neurotransmitter was oxidized (Figure 4A). The major peak in this chromatogram having a retention time (t_R) of 13.5 min corresponded to unreacted 1. The peak at t_R = 15.7 min was due to orange dopaminochrome 5. This was characterized by its UV–visible

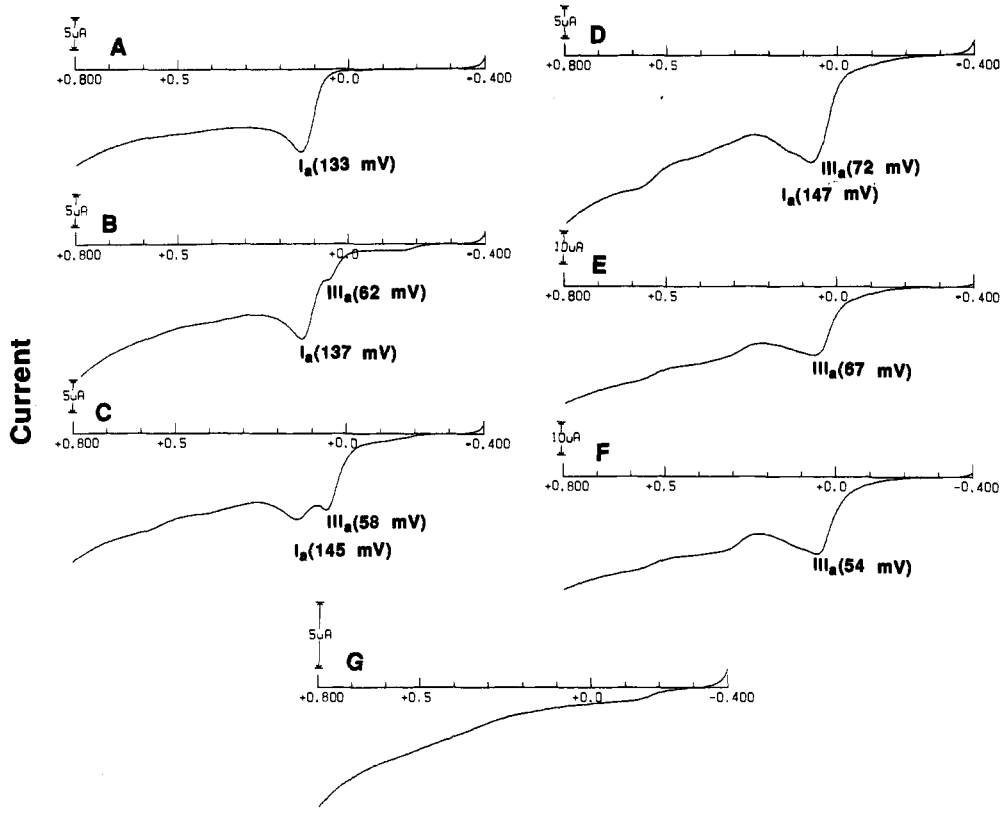


Figure 2. Linear sweep voltammograms at the PGE of (A) 1.0 mM dopamine (1), (B) 1.0 mM 1 and 0.5 mM L-cysteine (CySH), (C) 1.0 mM 1 and 1.0 mM CySH, (D) 1.0 mM 1 and 2.0 mM CySH, (E) 1.0 mM 1 and 3.5 mM CySH, (F) 1.0 mM 1 and 5.0 mM CySH, and (G) 5.0 mM CySH in pH 7.4 phosphate buffer (μ = 1.0). Sweep rate: 5 mV s^{-1}.

spectrum (λ_{max} = 300 and 476 nm in the chromatographic mobile phase, pH 3.0), thermospray mass spectrometry (m/e = 150, MH^+, 100), and cyclic voltammetry (reversible couple at $E^{\circ\prime}$ = −319 mV at pH 3.0). The species responsible for the peak at t_R = 18.7 min was identified as 5,6-dihydroxyindole (6) on the basis of its UV spectrum (λ_{max} = 299 and 276 nm in the chromatographic mobile phase, pH 3.0), thermospray mass spectrometry (m/e = 150, MH^+, 100), and cyclic voltammetry.[54] More prolonged oxidations of 1 resulted in the appearance of black, insoluble melanin resulting from further oxidative polymerization of 6 *via* quinone imine 7 (Scheme 1). These results indicate that the electrochemical oxidation[36,37,54] and the autoxidation[15,16] of 1 to melanin polymer proceed by chemically similar reaction pathways. A chromatogram of the solution obtained following a controlled potential electrooxidation of 1 (1.0 mM) in the presence of CySH (5.0 mM) at 80 mV for 30 min (Figure 4B) revealed that in excess of 60 peaks were present. An inspection of this chromatogram reveals that the peak of 1 was significantly smaller than when the oxidation was carried out in the absence of CySH (Figure 4A). Using preparative HPLC, five of the major products formed as a result of oxidation of 1 in the presence of CySH were isolated, purified, and structurally characterized (see the Experimental Section). These were 5-*S*-cysteinyldopamine (8), 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (11), 6-[(2-amino-2-carboxyethyl)thio]-7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (12), 8-[(2-amino-2-carboxyethyl)thio]-7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (14), and 6,8-di[(2-amino-2-carboxyethyl)thio]-7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (16). Structures of these compounds are shown in Figure 4B. HPLC analyses indicated that most of the many other chromatographic peaks observed on Figure 4B resulted from secondary oxidations of 11, 12, 14, and 16. The identities of these secondary products, however, remain to be identified. Oxidations of 1 (1.0 mM) in the presence of lower concentrations of CySH (0.5 and 1.0 mM) at 80 mV for 30 min resulted in significantly lower yields of 8, 12, 14, and 16, formation of relatively higher yields of 11 and secondary products, and less extensive oxidation of 1.

HPLC analysis of the product solution obtained after oxidation of 1 (1.0 mM) at 80 mV for 30 min at pH 7.4 indicated that the concentration of the neurotransmitter had decreased to 0.91 mM, *i.e.*, only 9% of the original 1 present had been oxidized. Under identical experimental conditions but in the presence of 0.5, 1.0, and 5.0 mM CySH, the concentration of 1 decreased to 0.83, 0.74, and 0.47 mM, respectively. Thus, in the presence of increasing CySH concentrations, the rate of oxidation of 1 was systematically increased.

During the controlled potential electrooxidation of 1 (1.0 mM; 30 min) at 80 mV in pH 7.4 phosphate buffer, the initially colorless solution became orange owing to the formation of aminochrome 5. Furthermore, as the oxidation progressed, a dark brown/black turbidity appeared in the solution owing to the formation of a very fine precipitate of melanin polymer. However, when the oxidation was carried out in the presence of increasing concentrations of CySH, it was noted that the orange color of 5 initially decreased in intensity (0.5 mM CySH) and was replaced by a very pale yellow color (≥1.0 mM CySH). Furthermore, with increasing concentrations of CySH, the

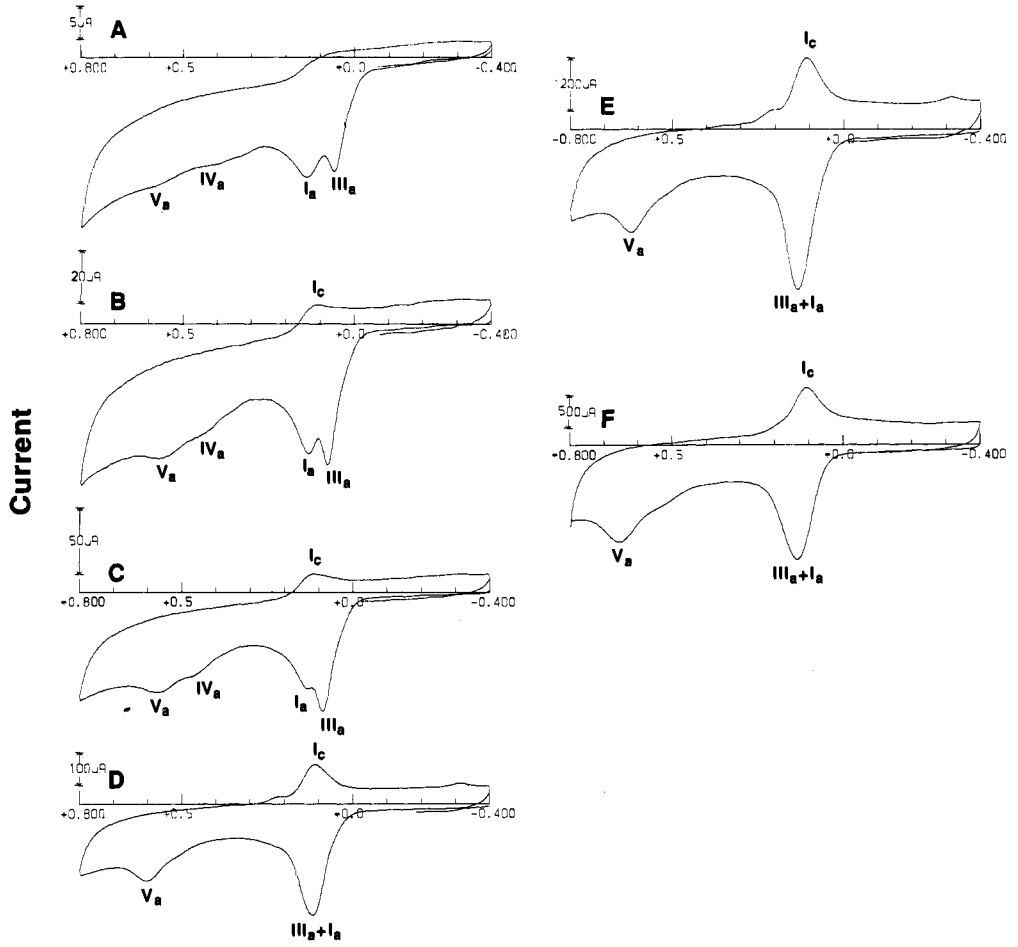


Figure 3. Cyclic voltammograms at the PGE of 1.0 mM dopamine (1) and 1.0 mM L-cysteine (CySH) in pH 7.4 phosphate buffer (μ = 1.0) at sweep rates of (A) 10 mV s^{-1}, (B) 50 mV s^{-1}, (C) 100 mV s^{-1}, (D) 500 mV s^{-1}, (E) 1 V s^{-1}, (F) 5 V s^{-1}, and (G) 10 V s^{-1}.

turbidity due to melanin decreased and, *via* >1 mM CySH, disappeared completely. The presence of the suspension of black melanin in reaction solutions caused an elevation of the background absorbance over the entire UV–visible spectral region. By monitoring the apparent absorbance of product solutions formed by controlled potential electrooxidations of 1 (1.0 mM; 30 min; 80 mV) in the absence and presence of CySH (0.1–5.0 mM) at 650 nm (none of the identified or unidentified products absorbed at this wavelength), it was found that the amount of melanin formed decreased exponentially with increasing concentrations of CySH. Each reaction product solution was also filtered through a 0.45-μm filter. A heavy black precipitate was retained on the filter membrane after filtration of the solution formed after electrooxidation of 1. The amount of black melanin retained on the filter decreased dramatically when the product solutions of oxidation of 1 in the presence of 0.5 and 1.0 mM CySH were filtered. No black precipitate was retained on the filter from the product solution obtained following oxidation of 1 in the presence of 5.0 mM CySH. With increasing excesses of CySH, however, it was observed that a small amount of a yellow precipitate was retained on the filter.

Cyclic Voltammetry of 8, 11, 12, 14, and 16. Slow sweep (ν = 10 mV s^{-1}) cyclic voltammograms of 8, 11, 12, 14, and 16, and 1 in the presence of equimolar CySH are presented in Figure 5. Between 0 and 200 mV, each product exhibited two voltammetric oxidation peaks. E_p values for the first oxidation peak for 8, 11, 12, 14, and 16 were 69 (peak 8 I_a), 53 (peak 11 I_a), 41 (peak 12 I_a), 47 (peak 14 I_a), and 67 (peak 16 I_a) mV, respectively. Under the same experimental conditions, E_p for peak I_a of 1 was 134 mV and that for peak III_a observed with 1.0 mM 1 in the presence of CySH (0.5–5.0 mM) was 60 mV. E_p values for the second oxidation peaks of 8, 11, 12, 14, and 16 were 149 (peak 8 II_a), 140 (peak 11 II_a), 108 (peak 12 II_a), 111 (peak 14 II_a), and 116 (peak 16 II_a) mV, respectively. These voltammetric results indicate that 8, 11, 12, 14, and 16 are appreciably more easy to oxidize than 1. Furthermore, E_p for peak 8 I_a of 5-*S*-cysteinyldopamine (8) corresponds closely to that of peak III_a observed in voltammograms of 1 in the presence of CySH (Figure 5G).

Cyclic voltammograms of 8, 11, 12, 14, and 16 (*ca.* 1.0 mM, pH 7.4) at a faster sweep rate (ν = 100 mV s^{-1}) are presented in Figure 6. 5-*S*-Cysteinyldopamine (8) exhibits three oxidation peaks at E_p values of 94 (peak 8 I_a), 146 (peak 8 II_a), and 565 (peak 8 III_a) mV (Figure 6A). After scan reversal, reduction peak 8 II_c (E_p = 131 mV) appears to form a reversible couple with oxidation peak 8 II_a (E_p = 146 mV). The structurally simplest dihydrobenzothiazine 11 exhibits oxidation peaks at E_p values of 82 (peak 11 I_a), 148 (peak 11 II_a), and 589 (peak 11 III_a) mV and, after scan reversal, reduction peak 11 II_c (E_p = 116 mV) which forms a reversible couple with peak 11 II_a (Figure 6B). 6-*S*-Cysteinyldihydrobenzothiazine 12 also exhibited three oxidation peaks at E_p values of 58 (peak 12 I_a), 113 (peak 12 II_a), and 507 (peak 12 III_a) mV. After scan reversal, only indistinct reduction peak 12 II_c ($E_p \approx$

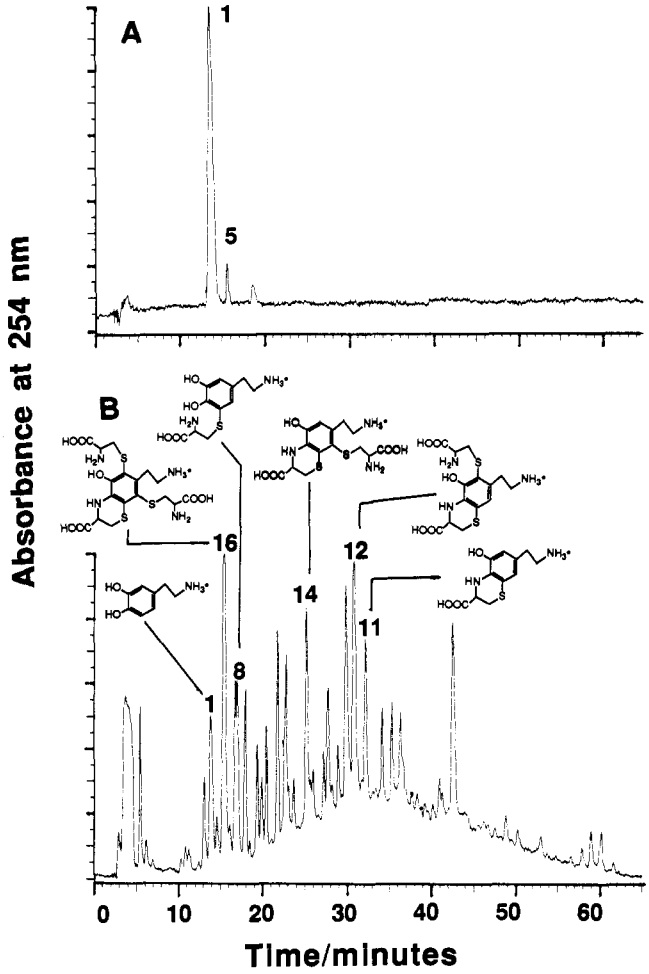


Figure 4. HPLC chromatograms of the product solutions obtained following controlled potential electrooxidation of (A) 1.0 mM dopamine (1) and (B) 1.0 mM 1 and 5.0 mM L-cysteine at 80 mV for 30 min in pH 7.4 phosphate buffer. Chromatography employed gradient system V. Injection volume: 4.0 mL.

100 mV) appeared, which apparently forms a reversible couple with oxidation peak 12 II_a (Figure 6C). 8-*S*-Cysteinyldihydrobenzothiazine 14 exhibited oxidation peaks 14 I_a, 14 II_a, and 14 III_a at E_p values of 74, 134, and 564 mV, respectively (Figure 6D). Only a very indistinct reduction peak (14 II_c) appeared after scan reversal. Di-*S*-cysteinyldihydrobenzothiazine 16 exhibited an oxidation peak at E_p = 94 mV which appeared to consist of the two overlapping peaks 16 I_a and 16 II_a (Figure 6E). At more positive potentials, oxidation peaks 16 III_a (E_p = 550 mV) and 16 IV_a (E_p = 480 mV) appeared. No significant reduction peaks were observed following scan reversal.

Reaction Pathways. Cyclic voltammograms of 1 in the presence of CySH at pH 7.4 (Figure 1B–E) support the conclusion that *o*-quinone 2 reacts with this sulfhydryl compound in a reaction that diverts the formation of 4, 5, and other intermediates believed to be formed in the melanin pathway. This conclusion is supported by the fact that oxidations of 1 in the presence of increasing concentrations of CySH cause the suppression and ultimately the elimination of black melanin polymer formation. An important experimental observation emerging from these studies is the appearance of oxidation peak III_a at more negative potentials than peak I_a in voltammograms of 1 in the presence of CySH. On the basis of a comparison of the E_p values for oxidation peaks 8 I_a, 11 I_a, 12 I_a, 14 I_a, and 16 I_a, it appears very probable that peak III_a corresponds primarily to the oxidation of 5-*S*-cysteinyldopamine (8). This is formed as a result of the nucleophilic addition of CySH to *o*-quinone 2, the proximate oxidation product of 1 (Scheme 2). In order to account for the various products formed, it appears probable that 8 is then oxidized (2e, $2H^+$) to the 5-*S*-cysteinyl-*o*-quinone 9. Cyclic voltammograms of 8 exhibit a reduction peak reversibly coupled to oxidation peak 8 I_a only at $\nu = ca.\ 10\ V\ s^{-1}$, indicating that 9 is a very reactive intermediate. Accordingly, it may be concluded that 9 rapidly cyclizes to give the bicyclic quinone imine 10 (Scheme 2). The fact that only the reduced form of the compound has been detected as a major product of electrooxidation of 1 in the presence of CySH (Figure 4B) indicates that it is partially reduced by the latter amino acid yielding CySSCy as a byproduct. However, electrophilic quinone imine 10 can also be attacked by the sulfhydryl residue of CySH yielding the 6-*S*- and 8-*S*-cysteinyldihydrobenzothiazines 12 and 14 which have been isolated as reaction products. Both 12 and 14 are more easily oxidized than 8 and 11, presumably to give the bicyclic *o*-quinones 13 and 15, respectively (Scheme 2). In principle, 13 should be capable of intramolecular cyclization to yield a tricyclic compound, although such a compound or species derived from it has not yet been detected as a product. Major reactions of 13 and 15 result from nucleophilic addition of CySH to give the 6,8-di-*S*-cysteinyldihydrobenzothiazine 16. The fact that 8, 11, 12, 14, and 16 appear as major initial products of oxidation of 1 in the presence of excess CySH even though they are all readily electrooxidized at 80 mV implies that a significant fraction of these compounds are maintained in their reduced form by CySH. It might be argued that probable precursors of 12, 14, and 16 could be the 2,5- and 5,6-di- and 2,5,6-tri-*S*-cysteinyl conjugates of 1 formed by nucleophilic addition of CySH to *o*-quinone 9 and related cysteinyl congeners. Indeed, Ito and Proto[55] have reported that the *o*-quinone formed upon oxidation of DOPA reacts with CySH to give the 5-, 2-, 6-, and 2,5-di-*S*-cysteinyl conjugates of DOPA in the ratio 74:14:1:5. However, in the present study, the only isolated cysteinyl conjugate of 1 was 8 and no evidence for formation of other conjugates in any significant yield was obtained. Accordingly, it appears that 8 represents, at least, the principal precursor of the cysteinyldihydrobenzothiazines 12, 14, and 16. Voltammograms of the type shown in Figure 2B,C clearly establish that in the presence of CySH the oxidation of 1 is facilitated as a result of the appearance of peak III_a at more negative potentials than peak I_a. This peak is almost certainly due primarily to the oxidation of 8. At the potential corresponding to peak III_a (E_p = 60 mV), only about 10–15% of 1 at the electrode surface is oxidized to 2 (see Figure 2A). However, assuming that the reaction of CySH with 2 is reasonably fast, the surface concentration of the latter *o*-quinone must be rapidly decreased. Under such conditions, Nernstian considerations require that additional 1 must be oxidized in order to reestablish the required surface concentration of 2. The rate of oxidation of 1 at peak III_a potentials, therefore, must be dependent on the rate of the reaction between CySH and 2 and also on the concentration of CySH. Thus, the i_p for peak III_a is dependent on the latter reactions and the subsequent 2e electrooxidation of 8 to cysteinyl-*o*-quinone 9. However, 9 rapidly cyclizes to give bicyclic *o*-quinone imine 10 which, based on the pathways conceptualized in Scheme 2, reacts

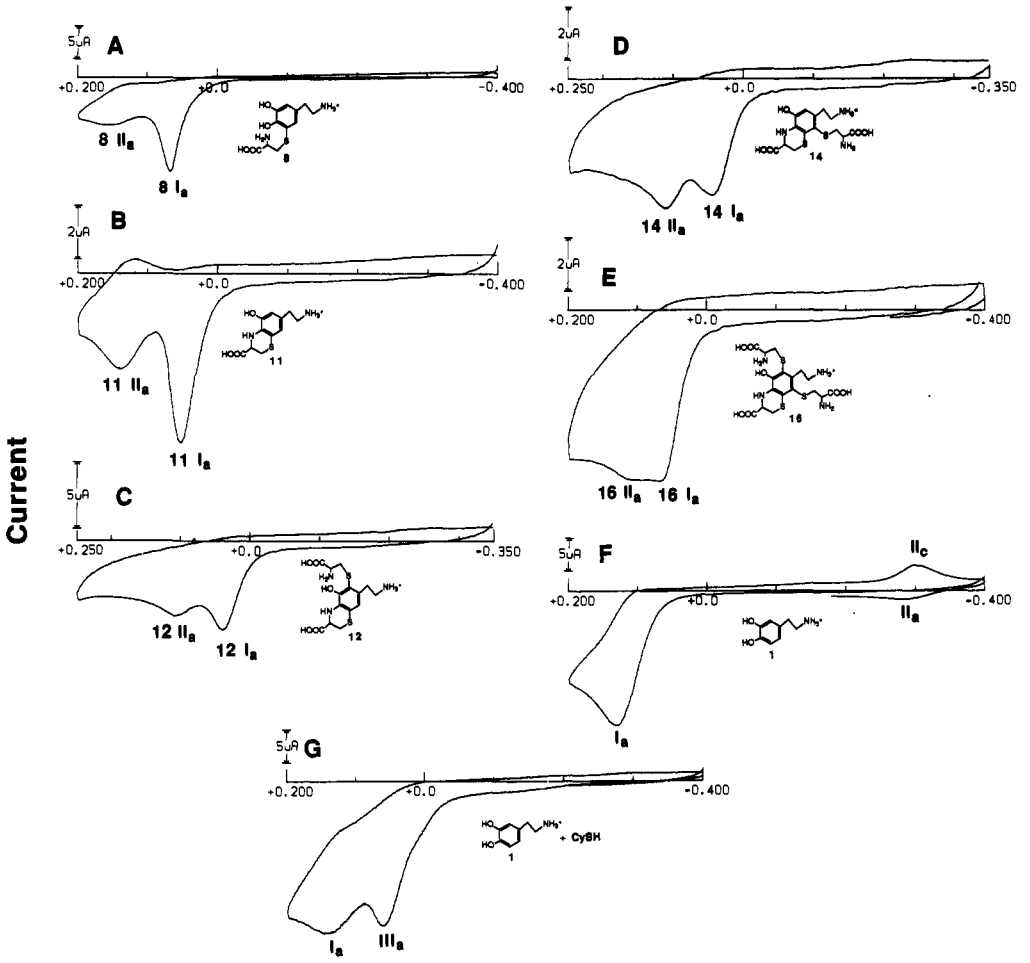


Figure 5. Cyclic voltammograms at the PGE of *ca.* 1.0 mM solutions of (A) 8, (B) 11, (C) 12, (D) 14, (E) 16, (F) dopamine (1) and (G) 1 and L-cysteine (1.0 mM) in pH 7.4 phosphate buffer. Sweep rate: 10 mV s^{-1}. Structures are shown in the figure.

to give dihydrobenzothiazine 11 and, subsequently, its cysteinyl conjugates 12, 14, and 16. Compounds 11, 12, 14, and 16 are all more easily oxidized than 8. Thus, with increasing concentrations of CySH, i_p for peak III$_a$ grows. Furthermore, in the presence of large molar excesses of CySH, the formation of 12, 14, and perhaps 16 becomes sufficiently extensive so that the voltammetric oxidation peak shifts to even more negative potentials (Figure 2), reflecting the ease of oxidation of these compounds. On the basis of the observation that 8, 11, 12, 14, and 16 are formed as major products during the initial stages of the oxidation of 1 in the presence of excess CySH (Figure 4B), it must be concluded that CySH reduces putative intermediates 9, 10, 13, 15, and 17 (Scheme 2) at the electrode surface. Hence, i_p for peak III$_a$ probably also reflects the catalytic regeneration of 8, 11, 12, 14, and 16.

Preliminary Biological Studies. The complexity of the product mixtures obtained upon oxidation of dilute solutions of 1 (1 mM) in the presence of CySH (*e.g.*, see Figure 4B) precluded the isolation of large quantities of dihydrobenzothiazines 11, 12, 14, and 16. Accordingly, it has not yet been possible to perform extensive investigations into the biological activities of these compounds. However, as an initial screen for *in vivo* activity, a 100-μg dose (free base) of each compound dissolved in 5 μL of isotonic saline was injected into the brains of laboratory mice (weighing 30 ± 5 g) in the vicinity of the right lateral ventricle. 5-*S*-Cysteinyldopamine (8) (n = 4) and the 6,8-di-*S*-cysteinyldihydrobenzothiazine 16 (n = 6) were not lethal at this dose and evoked no unusual behavioral responses. However, 100-μg doses of 11 (n = 4), 12 (n = 4), and 14 (n = 4) caused death of all experimental animals within 10–20 min. Approximate LD$_{50}$ values determined for 11, 12, and 14 in the mouse were 14, 17, and 70 μg, respectively. Behavioral responses evoked by intracerebral injection of 11 (6–100 μg) included convulsions and prolonged periods when the animals were unable to walk and lay on their sides with limbs making a continuous running motion. Animals' heads were arched backward with a concave back, and the tail was vertical and stiff. Periodically, animals exhibited episodes of violent, repetitive jumping. These behavioral responses were more pronounced the larger the dose of 11. Animals treated with 12 (12.5–100 μg) were generally unable to stand and lay on their sides with their heads arched backward. Periodically, animals jumped repeatedly and their limbs moved erratically and wildly. At times, animals rapidly rolled over along the head–tail axis, each episode involving several revolutions. These animals also exhibited episodes of repetitive head twitching and up-and-down movements of the forelimbs. A 100-μg dose of 14 evoked convulsions, episodes of circling contralateral to the site of injection, arched back, and stiff tail. At times, episodes of repetitive rolling along the head–tail axis and violent jumping occurred. With a 50-μg dose, animals remained motionless for about 15 min and then moderate tremor developed. After 1 h, such animals appeared to behave normally.

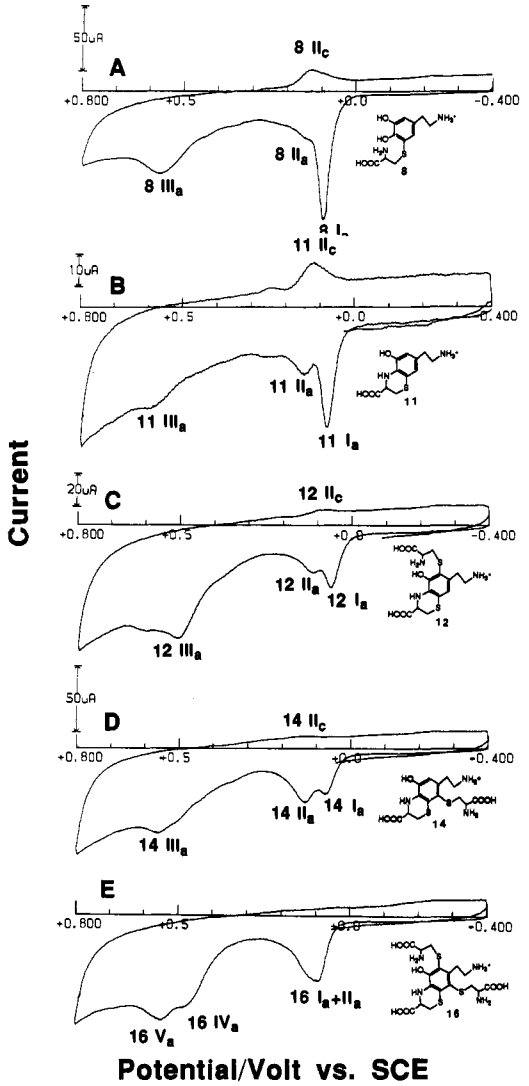


Figure 6. Cyclic voltammograms at the PGE of *ca.* 1.0 mM solutions of (A) 8, (B) 11, (C) 12, (D) 14, and (E) 16 in pH 7.4 phosphate buffer. Sweep rate: 100 mV s^{-1}. Structures are shown in the figure.

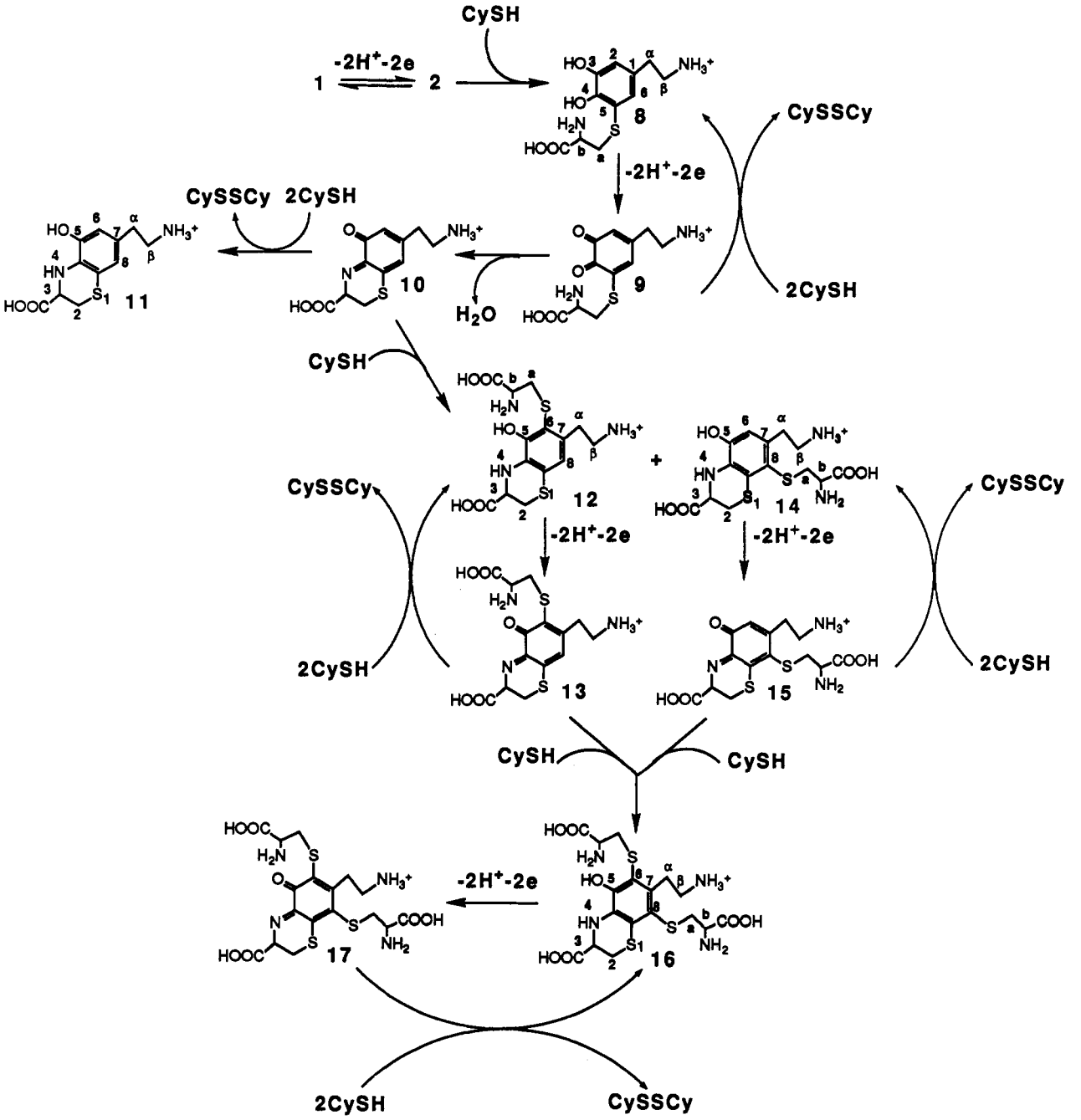
Control animals ($n > 30$) injected with 5 μL of vehicle exhibited none of the above behavioral responses, and all survived.

Discussion

Several important conclusions can be drawn from the results of this investigation. Thus, in the presence of increasing concentration of CySH, the oxidation pathway leading from 1 to black melanin polymer (Scheme 1) is first diverted and then completely blocked. Similar observations have been reported for the tyrosinase-mediated oxidation of 1 in the presence of CySH.[35] These effects result from the fact that the proximate oxidation product of 1, *o*-quinone 2, can be efficiently scavenged by CySH to give, primarily, 8. Secondly, cysteinyl conjugate 8 is appreciably more easily oxidized than 1. In the presence of free CySH, this reaction leads to dihydrobenzothiazine 11 and its cysteinyl conjugates 12, 14, and 16. Under electrochemical conditions, the facile formation of 8 and its ease of oxidation result in an increase in the rate of oxidation of 1. This increase becomes even more pronounced with increasing concentrations of CySH as a consequence of formation of 11, 12, 14, and 16, all of which are more easily oxidized than 8. Finally, *in vivo* experiments have revealed that 11 and its cysteinyl conjugates 12 and 14 are lethal and evoke profound behavioral responses when centrally administered to the mouse. It is important to emphasize, however, that at this stage of our investigations, the latter observations cannot be interpreted to mean that these compounds cause permanent neurotoxic damage to nigrostriatal or other neuronal systems. It is hoped to be able to report on the neurotoxic properties of 11 and related dihydrobenzothiazines in the near future.

Evidence has been obtained that the most heavily neuromelanin-pigmented dopaminergic SN neurons are particularly vulnerable to degeneration in PD.[47,48] Factors other than or in addition to neuromelanin content might be involved in the differential vulnerability of these neurons including their exact anatomical location in the SN.[47] However, one interpretation of this observation is that dopaminergic cell bodies equipped with particularly weak antioxidant defense systems, which would permit high rates of autoxidation of cytoplasmic 1 to neuromelanin, represent the primary targets for degeneration in PD. On the basis of the results reported in this study, an elevated influx of CySH, or more probably GSH (see later discussion), into the cytoplasm of heavily pigmented dopaminergic cell bodies should result in: (a) diversion of the neuromelanin pathway leading to progressive depigmentation, (b) endogenous formation of 8, and (c) further autoxidation of 8, a reaction which *must* occur under conditions where 1 is autoxidized, to form aberrant, toxic dihydrobenzothiazine metabolites such as 11, 12, and 14. However, the concentrations of free CySH in the brain are probably very low.[56,57] Furthermore, there do not appear to be any obvious mechanisms or any experimental data which would account for either an increase in the biosynthesis of CySH or an elevated influx of this sulfhydryl compound into the cytoplasm of SN neurons in the Parkinsonian brain. By contrast, the concentrations of GSH in glia, axons, and nerve terminals are much higher, perhaps as high as 6 mM in glia in which it is largely synthesized.[52] Furthermore, the activity of γ-glutamyltranspeptidase is significantly elevated in the Parkinsonian SN.[32] Accordingly, an alternative route, which would also lead to a diversion of the neuromelanin pathway and depigmentation of SN neurons in PD, could result from an increased rate of translocation of GSH, biosynthesized in nigral glia, into dopaminergic cell bodies. In heavily neuromelanin-pigmented SN cell bodies, in which high rates of autoxidation must occur, this increased influx of GSH would be expected to scavenge *o*-quinone 2 at least as effectively as CySH to give 5-*S*-glutathionyldopamine (18, Scheme 3). Indeed, controlled potential electrooxidation of 1 at physiological pH in the presence of GSH yields 18 as a major product.[58] Furthermore, in the presence of increasing concentrations of GSH (0.5–6 mM), the oxidation of 1 (1.0 mM) becomes increasingly rapid and the formation of melanin is decreased (0.5 mM GSH) and ultimately blocked (≥1 mM GSH).[58] Intraneuronal peptidase enzymes would be expected to hydrolyze the glutathionyl residue of 18 to give 8.[49–51] The facile autoxidation of 8 would then give *o*-quinone 9 which in turn would rapidly cyclize to *o*-quinone imine 10 (Scheme 3). Partial reduction of 10 by GSH and/or other endogenous antioxidants would then yield the lethal dihydrobenzothiazine 11. Alternatively, 10 could be attacked by GSH to give the 6-*S*- and 8-*S*-glutathionyldihydroben-

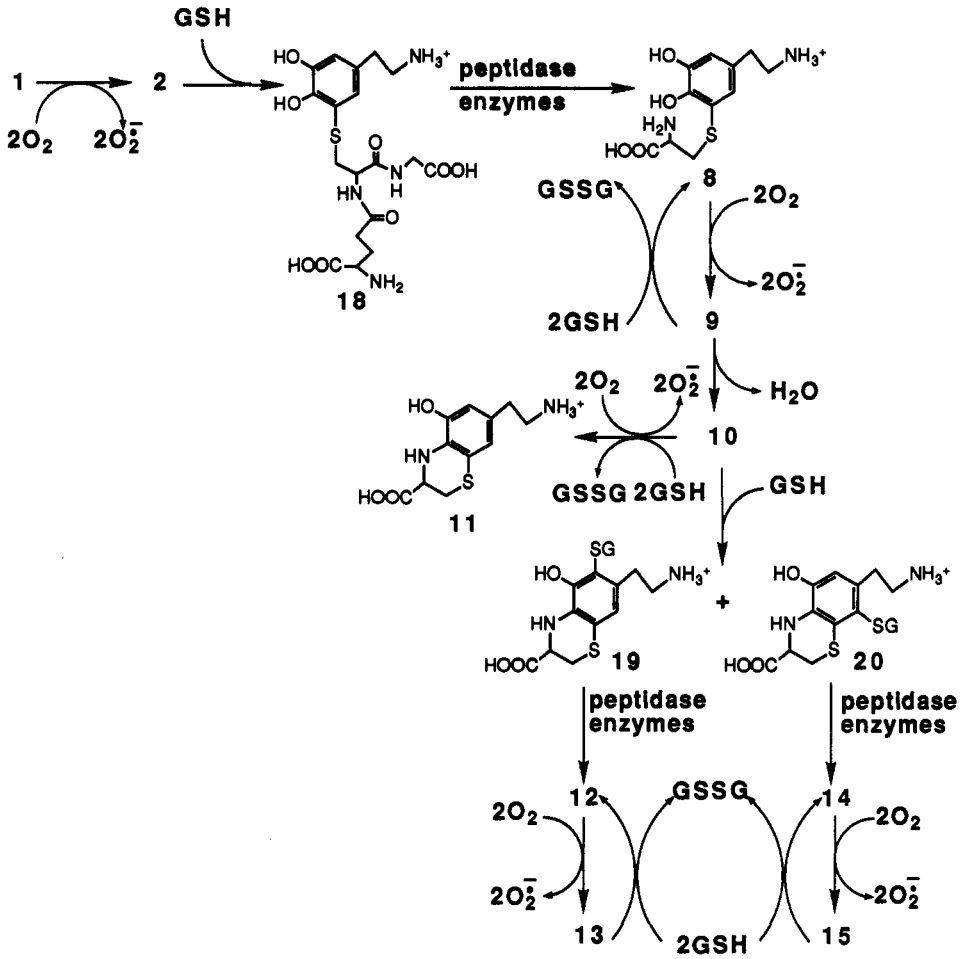
Scheme 2



zothiazines **19** and **20**. Peptidase enzymes could then convert **19** and **20** to **12** and **14**, respectively. Both **12** and **14** are more easily oxidized than **8** and **11** to give putative quinone imines **13** and **15**, respectively. However, GSH and other endogenous antioxidants would be expected to partially maintain these compounds in their reduced forms, *i.e.*, toxins **12** and **14**, respectively. Alternatively, nucleophilic addition of GSH to **13** and **15** might be expected to yield even more complex conjugates. Thus, on the basis of the largely hypothetical reactions shown in Scheme 3, an increased influx of GSH into the cytoplasm of heavily pigmented dopaminergic neurons should not only divert the neuromelanin pathway but also lead to the intraneuronal formation of toxic dihydrobenzothiazine metabolites. These reactions might also provide an explanation for the observation that the SN in PD, but not other brain regions, are depleted of GSH without a corresponding increase in GSSG,[20,25,32] a decrease which is not caused by altered activities of γ-glutamylcysteine synthetase, GSH peroxidase, GSSG reductase, or GSH transferase.[32-34] This is so because GSH would be irreversibly consumed as a result of its nucleophilic addition to cytoplasmic electrophiles such as *o*-quinone **2** or *o*-quinone imine **10** (Scheme 3).

Fornstedt *et al.*[51] have reported representative levels of **1** (pmol/g of tissue) in human pigmented, depigmented, and Parkinsonian SN tissue to be 2560 ± 456, 939 ± 361, and 231, respectively. The systematic decline in these levels of **1** reflects increasing degeneration of dopaminergic SN neurons in these three patient groups. In the same patient populations, the ratio of **8**/**1** increased from 0.0254 ± 0.006 to 0.066 ± 0.03 to 0.174, respectively. These investigators concluded that this increase in the **8**/**1** ratio with increasing degeneration and depigmentation of SN neurons reflects an increase in the rate of autoxidation of **1**, although possible chemical or biochemical mechanisms

Scheme 3



which might be responsible for this effect were not provided. An increase in the rate of autoxidation of 1 in the cytoplasm of SN neurons containing low levels of CySH or GSH would be expected to result in an increased rate of neuromelanin formation and/or increased cytoplasmic levels of 8. However, in the degenerating and Parkinsonian SN, these cells become progressively depigmented. Furthermore, while an increase in the 8/1 ratio paralleled the depigmentation and degeneration of SN neurons, the absolute concentrations of 8 in the pigmented (*i.e.*, normal), depigmented, and Parkinsonian SN were virtually identical.[51] These apparently conflicting expectations and experimental observations can be rationalized on the basis of the results reported in this investigation. Thus, depigmentation of SN cells can be explained as a result of a greatly increased influx of GSH into the cytoplasm of these neurons (Scheme 3). Furthermore, under conditions where 1 is autoxidized, cysteinyl conjugate 8, formed as a result of the GSH-mediated metabolic diversion, must be even more rapidly autoxidized. Thus, the levels of 8 measured experimentally[51] in the degenerating and Parkinsonian SN probably represent only a fraction of the conjugate that is actually formed. A significant amount of 8 must be oxidatively converted into 11, 12, 14, 16, and perhaps many other compounds which remain to be identified. The observation that the 8/1 ratio significantly increases in the degenerating and Parkinsonian SN despite the facile loss of 8 as a result of its autoxidation must imply that the levels of 1 greatly decrease. This, in agreement with earlier suggestions, could result from accelerated rates of autoxidation of 1. While increasing concentrations of CySH (and GSH) clearly evoke an elevated rate of electrochemical oxidation of 1, this effect is peculiar to the electrode reaction. Thus, the depletion of o-quinone 2, formed at the electrode surface by oxidation of 1, by reaction with CySH (or GSH) causes the rate of the electrochemical oxidation of the neurotransmitter to be accelerated. However, the rate of the *chemical* oxidation of 1 by molecular oxygen cannot be similarly increased as a result of the reaction between 2 and CySH (or GSH). Thus, the increased rate of intraneuronal autoxidation of 1 which causes the elevated 8/1 ratio in degenerating and Parkinsonian SN must be the consequence of other chemical mechanisms. Some potential insights into such mechanisms can be drawn from the oxidation reactions proposed in Scheme 3, all of which are driven by molecular oxygen. The fact that the oxidation potentials for 8, 11, 12, and 14 are significantly lower than for 1 supports the conclusion that these compounds should be easily autoxidized. By analogy with the autoxidation chemistry of 1,[15,16] the primary byproduct of autoxidation of 8, 11, 12, and 14 must almost surely be $O_2^{\cdot-}$. Furthermore, the fact that the reduced forms of the latter compounds are prominent products in the initial stages of the electrochemical oxidation of 1 in the presence of excess CySH indicates that their oxidized forms are readily reducible by the latter sulfhydryl compound. Under intraneuronal conditions, endogenous antioxidants (GSH, ascorbate) would be expected to similarly be capable of partially maintaining the oxidized forms of 8, 11, 12, and 14 in their reduced state. Thus, 8 and particularly dihydrobenzothiazine 11 and its various cysteinyl conjugates must be

capable of redox-cycling reactions in the presence of molecular oxygen and intraneuronal antioxidants. Accordingly, one probable consequence of the GSH-mediated metabolic shift which is proposed to occur in the cytoplasm of heavily pigmented dopaminergic SN neurons in PD would be a greatly increased flux of $O_2^{\bullet-}$. This reduced oxygen species is known to catalyze the oxidation of compounds such as catechols[59] and 5,7-dihydroxytryptamine[60] by molecular oxygen. This is so because $O_2^{\bullet-}$ is an extraordinarily powerful Brønsted base and abstracts a proton from such substrates to give substrate anion which is then readily oxidized by molecular oxygen.[59] Thus, one explanation of increased rates of autoxidation of 1 in the cytoplasm of degenerating SN neurons in PD might be traced to the catalytic effect evoked by elevated levels of $O_2^{\bullet-}$ produced as a byproduct of the autoxidation and redox-cycling reactions of 8 and 11 and its cysteinyl conjugates.

The SN of PD patients contain elevated levels of malondialdehyde[3] and a 10-fold increase in the levels of lipid hydroperoxides[4] compared to those of age-matched controls. These observations are compatible with the occurrence of active, toxic processes involving oxygen free radicals in the SN up to the terminal stages of the disease.[20] The autoxidation and redox-cycling reactions hypothesized in Scheme 3 would, as noted previously, be expected to produce increased fluxes of $O_2^{\bullet-}$ and, hence, H_2O_2, $^1O_2$, and, as a result of Haber–Weiss and/or transition-metal-catalyzed Fenton chemistry,[11] cytotoxic $HO^{\bullet}$. Thus, formation of metabolites such as 18, 8, and the dihydrobenzothiazines 11, 12, 14, and 16 as a consequence of an elevated influx of GSH and their facile autoxidation, coupled with the fact that 11, 12, 14, and 16 can obviously redox cycle, might account for the peroxidative damage which contributes to the degeneration of SN neurons in PD. Elevated intraneuronal levels of $O_2^{\bullet-}$ might also induce increased mitochondrial superoxide dismutase (SOD) activity which is known to occur in the Parkinsonian SN.[61] Similarly, increased $O_2^{\bullet-}$ levels and SOD activity together would act to increase intraneuronal formation of H_2O_2 which, in concert with $O_2^{\bullet-}$-stimulated release of iron from ferritin,[62] would further elevate the endogenous production of $HO^{\bullet}$[8,9,17] with concommitant damage to lipids and other cellular constituents.

A second factor which appears to contribute to the death of dopaminergic SN neurons in PD is a severe mitochondrial complex I deficiency.[6,7,63] Whether dihydrobenzothiazines such as 11, 12, 14, and 16, which might be formed intraneuronally as a consequence of the hypothesized GSH-mediated metabolic shift in these cells, inhibit mitochondrial complex I respiration remains to be determined.

Many recent studies have focused on the possibility that environmental toxins could be initiators of the degenerative processes which occur in PD. In part, this attention derives from the discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a street drug contaminant, selectively lesions nigrostriatal dopaminergic neurons and evokes Parkinsonisn in humans and primates.[64] However, using antibodies prepared to MPTP and MPP^+, Ikeda *et al.*[65] were unable to find evidence for these or related pyridinium cations in postmortem brain tissue from aged PD patients. Furthermore, the damage caused by MPTP in humans and primates is somewhat different to that observed in idiopathic PD.[66] There is also no evidence that MPTP or a similar substance is found in widely distributed man-made products or in the environment.[64] However, a number of epidemiological studies suggest a link between chronic exposure to high levels of agricultural chemicals (pesticides, herbicides, fungicides)[67,68] and industrial chemicals.[69] The possible significance of this link is strengthened by the observation that an extraordinarily high percentage of PD patients have very low activities of cysteine dioxygenase[70] and thiolmethyltransferase.[71] These are enzymes which play important roles in the detoxification and elimination of environmental toxins and xenobiotics. The genetically inherited failure of such detoxification mechanisms might permit environmental/occupational chemical toxins to enter the central nervous system.[70] Perhaps, therefore, chronic exposure of individuals genetically equipped with such compromised protective mechanisms to high levels of certain environmental or occupational chemicals might be linked to the upregulation of γ-glutamyltranspeptidase which occurs in the Parkinsonian SN. Such a combination of factors might result in elevated rates of translocation of GSH into the cytoplasm of heavily pigmented dopaminergic SN neurons, causing a diversion of the neuromelanin pathway, depigmentation, and formation of dihydrobenzothiazine endotoxins which are ultimately responsible for the selective degeneration of nigrostriatal dopaminergic neurons and PD. This sequence of events would also account for the decreased levels of GSH in the SN of patients in the early presymptomatic stage of the disease, a decrease which is not accompanied by a corresponding increase in GSSG.[20]

Experimental Section

Dopamine hydrochloride (1·HCl) and L-cysteine (CySH) were obtained from Sigma (St. Louis, MO) and used without additional purification.

Voltammograms were obtained at a pyrolytic graphite electrode (PGE; Pfizer Minerals, Pigments and Metals Division, Easton, PA) having an approximate surface area of 4 mm^2. The PGE was constructed and resurfaced prior to recording each voltammogram, as described previously.[72] A conventional three-electrode voltammetric cell containing a platinum wire counter electrode and a saturated calomel reference electrode was used. Linear sweep and cyclic voltammograms were obtained with a BAS-100A (Bioanalytical Systems, West Lafeyette, IN) electrochemistry system. All voltammograms were corrected for iR drop. Controlled potential electrooxidations employed a Princeton Applied Research Corporation (Princeton, NJ) Model 173 potentiostat. The working electrode consisted of several plates of pyrolytic graphite having a total surface area of *ca.* 400 cm^2. A three-compartment cell was used in which the counter, working, and reference electrode compartments were separated with a Nafion membrane (Type 117, DuPont Co., Wilmington, DE). The working electrode compartment had a capacity of 80 mL. The counter electrode was several plates of pyrolytic graphite suspended into a solution of the supporting electrolyte. The solution in the working electrode compartment was bubbled with a vigorous stream of N_2 and stirred with a Teflon-coated magnetic stirring bar. ^1H NMR spectra were recorded on a Varian XL-300 spectrometer. Low- and high-resolution fast atom bombardment mass spectrometry (FAB-MS) was carried out with a VG Instruments (Manchester, U.K.) ZAB-E spectrometer. Thermospray mass spectrometry utilized a Kratos (Manchester, U.K.) MS25/RFA spectrometer equipped with a thermospray source. The mobile phase was 0.1 M ammonium acetate in deionized water at a flow rate of 1.0 mL min^{-1}. Typically, 2-mL aliquots of fractions collected from conventional HPLC separations were injected directly into the thermospray source using a Rheodyne (Cotati, CA) 7125 loop injector. UV–visible spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer.

Preparative HPLC employed a Gilson (Middleton, WI) binary gradient system equipped with Model 305 and 306 pumps (25-mL pump heads) and a Gilson holochrome UV detector set at 254 nm. Some samples (≤10 mL) were introduced onto the columns by means of a Rheodyne Model 7125 injector equipped with a 10-mL sample loop. Larger sample volumes (10–80 mL) were pumped onto the column through one of the HPLC pumps. Analytical HPLC employed a Gilson System 42 binary gradient chromatograph equipped with Model 302 pumps and 10-mL pump heads, a Rheodyne 7125 injector (2.0-mL loop), and a Gilson holochrome detector set at 254 nm. Four reversed-phase HPLC columns were used, a Spherisorb S_{10}, ODS-2 preparative column (25 × 2 cm; Phase-Sep, Queensferry, Clwyd, U.K.); a Regis ODS preparative column (10 μm, 25 × 2.1 cm; Morton Grove, IN); a Spherisorb semipreparative column (C_{18}, S5, ODS-2, 25 × 1.0 cm); and a semipreparative Brownlee column (RP-18, 5 μm, 25 × 0.7 cm; Brownlee Laboratories, Santa Clara, CA). Four binary gradient mobile-phase systems were used for product isolation and purification; these will be referred to as gradient systems I–IV. For gradient system I, solvents A and B were used as the mobile phases. Solvent A was prepared by adding 30 mL of concentrated ammonium hydroxide solution (NH_4OH) to 4 L of deionized water. The pH of this solution was adjusted to 3.0 by addition of concentrated trifluoroacetic acid (TFA). Solvent B was prepared by adding 30 mL of NH_4OH to 1 L of HPLC grade acetonitrile (MeCN), 1 L of HPLC grade methanol (MeOH), and 2 L of deionized water. The pH of the resulting solution was adjusted to 3.0 with TFA. The following mobile-phase gradient was employed, 0–2 min, 100% solvent A; 2–4 min, linear gradient to 50% solvent B; 40–50 min, linear gradient to 100% solvent B; 50–60 min, 100% solvent B. For gradient system I, the Spherisorb preparative HPLC column was employed. A constant flow rate of 7 mL min^{-1} was used.

For gradient systems II and III, solvents C and D were used for the mobile phase. Solvent C was prepared by adding TFA to deionized water until the pH was 3.0. Solvent D was prepared by adding TFA to 2 L of MeCN and 2 L of deionized water until the pH was 3.0. For gradient system II, the following mobile-phase gradient was used, 0–2 min, 100% solvent C; 2–20 min, linear gradient to 10% solvent D; 20–25 min, linear gradient to 100% solvent D; 25–30 min, 100% solvent D. For gradient system III; 0–2 min, 100% solvent C; 2–20 min, linear gradient to 40% solvent D; 20–25 min, linear gradient to 100% solvent D; 25–30 min, 100% solvent D. For gradient systems II and III, the Regis preparative HPLC column was used; the flow rate was 7 mL min^{-1}. Gradient system IV employed solvent A and solvent E. Solvent E was prepared by adding 15 mL of NH_4OH to 2 L of MeOH and 2 L of deionized water. The pH of the resulting solution was adjusted to 3.0 by addition of TFA. The following mobile-phase gradient was employed, 0–2 min, 100% solvent A; 2–30 min, linear gradient to 20% solvent E; 30–35 min, linear gradient to 100% solvent E; 35–45 min, 100% solvent E. For gradient system IV, the Spherisorb semipreparative HPLC column was employed; the flow rate was 2 mL min^{-1}.

In order to monitor the course of the oxidation of 1 in the presence of CySH, the Brownlee semipreparative HPLC column was employed. The binary gradient used, gradient system V, employed solvents A and B. The following mobile-phase gradient was used, 0–2 min, 100% solvent A; 2–15 min, linear gradient to 15% solvent B; 15–70 min, linear gradient to 100% solvent B; 70–75 min, 100% solvent B. The flow rate was constant at 2.3 mL min^{-1}.

Animals. *In vivo* experiments employed outbred adult male mice of the Hsd: Icr albino strain (Harlan Sprague–Dawley, Madison, WI) weighing 30 ± 5 g. The animals were housed 10/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. Mice were not used in experiments until at least 7 days after receipt from the supplier. Experimental animals were treated with test drugs dissolved in 5 μL of isotonic saline (0.9% NaCl in deionized water). Animals were first anesthetized with ether for 45–55 s. Injections were performed freehand; the site of puncture being approximately 3.5 mm anterior to the interaural line and 1 mm right lateral of the midline perpendicular to the scalp to a depth of 3 mm. The exact procedure used has been described elsewhere.[73] Control animals were treated in an identical fashion, except only 5 μL of vehicle was injected. All animal procedures were approved by the Institutional Animal Use and Care Committee at the University of Oklahoma.

Isolation, Purification, and Structural Characterization of Reaction Products. In order to prepare sufficient quantities of products formed upon electrochemical oxidation of 1 in the presence of CySH, 17 mg of 1·HCl and 60 mg of CySH were dissolved in 80 mL of pH 7.4 phosphate buffer (μ = 0.15). Controlled potential electrooxidation of this solution was then carried out at 80 mV at pyrolytic graphite electrodes for 1 h. During the course of the oxidation, the initially colorless solution turned pale yellow. After termination of the reaction, the entire solution was pumped onto the reversed-phase column and the components were separated using gradient system I. The solutions eluted under the chromatographic peaks corresponding to compounds **8**, **11**, **12**, **14**, and **16** were collected individually in separate flasks cooled in a bath of dry ice and acetone (*ca.* −80 °C). Following several repetitive experiments, the combined solutions containing **8**, **14**, and **16** were lyophilized. *Via* preparative gradient system I, **16** was contaminated with unreacted **1**. Further purification of **16** employed gradient system IV. The solution containing **16**, which eluted at t_R = 33 min, was collected at −80 °C and lyophilized. The solid, dry residues containing **8** and **16** were individually dissolved in <10 mL of deionized water adjusted to pH 3.0 with TFA and desalted using gradient system II. Pure, solid samples of **8** and **16** were then obtained following lyophilization of the resulting solutions. The residue containing **14** was dissolved in the minimum volume of deionized water adjusted to pH 3.0 with TFA and desalted and purified using gradient system III. The desalted eluent containing **14** was then lyophilized.

The eluents containing **11** or **12** (2–40 mL) were desalted and purified using gradient system III. The desalted eluents were then lyophilized.

Dihydrobenzothiazines **11**, **12**, **14**, and **16** were all rather unstable compounds and rapidly became brown and then black in the presence of atmospheric oxygen. Because of the complexity of the products formed when **1** was oxidized in the presence of CySH (see Figure 4B), it was possible only to isolate very small quantities (1–2 mg) of each compound. For the latter reasons, it was not possible to obtain sufficient quantities of each product in a pure stable form to permit combustion analysis to determine elemental compositions. However, ^{1}H NMR (1D and 2D) spectra, low- and high-resolution FAB-MS, and HPLC were employed to ascertain purities and to elucidate chemical structures. ^{1}H NMR spectra and FAB-MS for **8**, **11**, **12**, **14**, and **16** are provided as supplementary material. The complete chemical name for each product is given below. For simplicity, however, the atom-numbering systems shown in Scheme 2 are employed for assigning ^{1}H NMR proton resonances.

5-*S*-Cysteinyldopamine (8). Compound **8** was isolated as a white solid. In pH 7.0 phosphate buffer, **8** exhibited UV bands at λ_{max}, nm (log ϵ_{max}, M^{-1} cm^{-1}), 294 (3.22), 256 (3.37), and 214 (4.04). FAB-MS (3-nitrobenzyl alcohol matrix) gave *m/e* = 273.0889 (MH^+, 100, $C_{11}H_{17}N_2O_4S$; calcd *m/e* = 273.0909). ^{1}H NMR (D_2O): δ 6.97 (d, $J_{2,6}$ = 2.1 Hz, 1H, C(2)-H), 6.83 (d, $J_{2,6}$ = 2.1 Hz, 1H, C(6)-H), 3.89 (dd, *J* = 6.8 Hz, *J* = 4.8 Hz, 1H, C(b)-H), 3.40 (dd, *J* = 14.9 Hz, *J* = 4.8 Hz, 1H, C(a)-H), 3.33 (dd, *J* = 14.9 Hz, *J* = 6.8 Hz, 1H, C(a)-H), 3.21 (t, *J* = 7.0 Hz, 2H, C(β)-H_2), 2.84 (t, *J* = 7.0 Hz, 2H, C(α)-H_2).

7-(2-Aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic Acid (11). Compound **11** was isolated as a very pale yellow solid. In pH 7.0 phosphate buffer, **11** exhibited UV bands at λ_{max}, nm (log ϵ_{max}, M^{-1} cm^{-1}), 307 (3.28) and 234 (4.06). FAB-MS (dithiothreitol/dithioerythritol matrix) gave *m/e* = 255.0818 (MH^+, 100, $C_{11}H_{15}N_2O_3S$; calcd *m/e* = 255.0803). ^{1}H NMR (Me_2SO-d_6): δ 9.57 (s, 1H, N(4)-H), 7.58 (b s, 3H, NH_3^+), 6.36 (d, $J_{6,8}$ = 1.8 Hz, 1H, C(8)-H), 6.30 (d, $J_{6,8}$ = 1.8 Hz, 1H, C(6)-H), 5.23 (b s, 1H, O-H), 4.19 (m, 1H, C(3)-H), 3.16 (dd, $J_{2,2'}$ = 12.6 Hz, $J_{2,3}$ = 3.0 Hz, 1H, C(2)-H), 2.97 (dd, $J_{2,2'}$ = 12.6 Hz, $J_{2,3}$ = 6.9 Hz, 1H, C(2)-H), 2.91 (t, *J* = 7.7 Hz, 2H, C(β)-H_2), 2.59 (t, *J* = 7.7 Hz, 2H, C(α)-H_2). Addition of D_2O caused the resonances at δ 9.57, 7.58, and 5.23 ppm to disappear. ^{1}H NMR (D_2O): δ 6.57 (s, 2H, C(2)-H and C(6)-H), 4.48 (t, $J_{2,3}$ = 4.05 Hz, 1H, C(3)-H), 3.28 (d, $J_{2,3}$ = 4.05 Hz, 2H, C(2)-H_2), 3.17 (t, *J* = 7.13 Hz, 2H, C(β)-H_2), 2.78 (t, *J* = 7.13 Hz, 2H, C(α)-H_2).

Two-dimensional (2D) correlated spectroscopy (COSY) experiments agreed with the proton couplings assigned.

6-[(2-Amino-2-carboxyethyl)thio]-7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic Acid (12). Compound **12** was isolated as a very pale yellow solid. In pH 7.0 phosphate buffer, the UV spectrum of **12** showed λ_{max}, nm (log ϵ_{max}, M^{-1} cm^{-1}), 316 (3.26), 280 (sh, 3.61), and 248 (4.09). FAB-MS (thioglycerol/glycerol matrix) gave m/e = 374.0813 (MH^+, 100, $C_{14}H_{20}N_3O_5S_2$; calcd m/e = 374.0844). ^1H NMR (D_2O): δ 6.80 (s, 1H, C(8)-H), 4.55 (t, J = 4.2 Hz, 1H, C(3)-H), 3.79 (t, J = 5.7 Hz, , C(b)-H), 3.28 (dd, J = 13.1 Hz, J = 4.2 Hz, 2H, C(2)-H_2), 3.24–3.13 (m, 4H, C(a)-H_2 and C(β)-H_2), 2.96–2.79 (m, 2H, C(α)-H_2). 2D COSY experiments confirmed the couplings between the resonances at δ 4.55 and 3.28, 3.79 and 3.20, 3.17 and 2.87.

8-[(2-Amino-2-carboxyethyl)thio]-7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic Acid (14). Compound **14** was isolated as a very pale yellow solid. In pH 7.0 phosphate buffer, the UV spectrum showed bands at λ_{max}, nm (log ϵ_{max}, M^{-1} cm^{-1}), 318 (3.10), 280 (sh, 3.48), and 248 (3.92). FAB-MS (3-nitrobenzyl alcohol matrix) gave m/e = 374.0872 (MH^+, 18, $C_{14}H_{20}N_3O_5S_2$; calcd m/e 374.0844). ^1H NMR (D_2O): δ 6.67 (s, 1H, C(6)-H), 4.47 (t, J = 3.75 Hz, 1H, C(3)-H), 3.80 (t, J = 5.85 Hz, 1H, C(b)-H), 3.25 (m, 2H, C(2)-H_2), 3.19–3.03 (m, 6H, C(a)-H_2, C(α)-H_2 and C(β)-H_2). 2D COSY experiments confirmed the couplings between the resonances at 4.47 and 3.25, 3.80 and 3.13.

6,8-Bis[(2-amino-2-carboxyethyl)thio]-7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic Acid (16). Compound **16** was isolated as a very pale pink solid. At pH 7.0, the UV spectrum of **16** exhibited bands at λ_{max}, nm (log ϵ_{max}), 330 (sh, 3.50), 300 (sh, 3.63), and 262 (4.26). FAB-MS (thioglycerol/glycerol matrix) gave m/e = 493.0836 (MH^+, 61, $C_{17}H_{25}N_4O_7S_3$; calcd m/e = 493.0835). ^1H NMR (D_2O): δ 4.34 (t, J = 4.2 Hz, 1H, C(3)-H), 3.85 (t, J = 5.25 Hz, 1H, C(b)-H), 3.73 (t, J = 6.63 Hz, 1H, C(b')-H), 3.33 (dd, J = 15.0 Hz, J = 6.63 Hz, 2H, C(a')-H_2), 3.24 (dd, J = 15 Hz, J = 5.3 Hz, 2H, C(a)-H_2), 3.22–3.16 (m, 6H, C(2)-H_2, C(β)-H_2, and C(α)-H_2). 2D COSY experiments confirmed the expected couplings between the resonances at δ 4.34 and 3.20, 3.85 and 3.24, 3.73 and 3.33.

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Supplementary Material Available: ^1H NMR, delayed COSY ^1H NMR spectra, and FAB-MS of compounds **8**, **11**, **12**, **14**, and **16** (18 pages). Ordering information is given on any current masthead page.

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