Potential New Insights Into the Molecular Mechanisms of Methamphetamine-Induced Neurodegeneration

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INTRODUCTION

In humans, methamphetamine evokes alertness, a decreased sense of fatigue, increased ability to concentrate or perform physical tasks, euphoria, and increased initiative and confidence (McGeer et al. 1987). Probably because of its mood-elevating and antifatigue effects, methamphetamine is self-administered by humans and a number of animal species in experimental models (Schuster 1981). Methamphet-amine is recognized as a positively reinforcing drug, and it continues to be extensively abused in many countries. However, high doses or continuous use of methamphetamine can lead to a variety of undesirable side effects. These include paranoid delusions, disordered thought, aggression, and hallucinations (Seiden et al. 1988). Neurotoxicological studies have established that certain dose regimens of methamphetamine evoke the degeneration of dopaminergic and serotonergic fibers in the brains of many animal species including the monkey (Seiden et al. 1975/1976), rats, mice, and cats (Ricaurte et al. 1980; Wagner et al. 1980; Levine et al. 1980; Seiden and Ricaurte 1987). These observations suggests that methamphetamine might also evoke similar neurodegenerative effects in the human brain.

For a variety of reasons, most studies aimed at understanding the neurodegenerative effects of methamphetamine have employed the rat. Repeated low doses or a single large dose of methamphetamine to this animal results in the degeneration of serotonergic (Ricaurte et al. 1980; Seiden et al. 1988; Bakhit and Gibb 1981; Hotchkiss and Gibb 1980*a*, 1980*b*; Commins and Seiden 1986) and dopaminergic (Seiden et al. 1988; Ricaurte et al. 1982; Wagner et al. 1980; Fibiger and McGeer 1971; Buening and Gibb 1974) nerve terminals in several areas of the brain and of a subpopulation of cell bodies in the somatosensory cortex (Commins and Seiden 1986). The latter region of the rat cortex contains no serotonergic or catecholaminergic perikarya and hence these cell bodies must be associated with another,

as yet unknown, neurotransmitter system. Based on measurements of residual levels of 5-hydroxytryptamine (5-HT) and dopamine (DA) in many brain areas following methamphet-amine administration, it appears that the most profound degeneration of serotonergic terminals occurs in the frontal cortex, hippocampus, and amygdala, whereas dopaminergic terminals are most severely degenerated in the caudate nucleus, amygdala, and nucleus accumbens (Seiden et al. 1988). Such studies have also suggested that serotonergic terminal fields are more susceptible than dopaminergic terminal fields to the neurotoxic effects of methamphetamine. Furthermore, for the various brain areas examined, it appears that depletions of DA and 5-HT are proportional. For example, if DA is not depleted then 5-HT is depleted by only a small amount; when significant dopaminergic degeneration occurs, serotonergic degeneration is even more pronounced (Seiden et al. 1988). These observations could imply that the methamphetamine-induced degeneration of dopaminergic and serotonergic terminals are not entirely independent processes, but are in some way related or connected phenomena.

The fundamental molecular mechanisms that underlie the neurodegenera- tive properties of methamphetamine are unknown, although they are probably not directly caused by the drug or its normal metabolites (Gibb et al. 1994). However, several lines of evidence suggest that oxygen radical species contribute directly or indirectly to methamphetamine-induced neuronal damage (Cadet et al. 1994). For example, pretreatment of rats with antioxidants attenuates the neurodegenerative effects of methamphetamine (DeVito and Wagner 1989). It seems to be widely accepted that oxygen radical species limit their damage in biological systems to lipids, proteins, and deoxyribonucleic acid (DNA) (Halliwell 1992). However, the two neuronal systems severely damaged as a result of methamphetamine administration utilize neurotransmitters that are very easily oxidized (i.e., 5-HT and DA). Thus, it appears unlikely that these neurotransmitters would be spared from oxygen radical-mediated oxidation reactions unless there are very sophisticated mechanisms available for their protection. Support for this view can be drawn from the observation that, following the administration of a single large dose of methamphetamine to the rat, the catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) has been detected in the caudate nucleus (Seiden and Vosmer 1984) and the serotonergic neurotoxin 5,6-dihy-droxytryptamine (5,6-DHT) has been detected in the cortex and hippocampus (Commins et al. 1987).

The only known direct chemical pathway leading to 6-OHDA and 5,6-DHT is attack of the hydroxyl radical (HO·) on DA (Slivka and Cohen 1987) and 5-HT (Wrona et al. 1995), respectively. The detection of 6-OHDA (Seiden and Vosmer 1984) and 5,6-DHT (Commins et al. 1987) in rat brain following methamphetamine administration has been reported to be somewhat sporadic and the concentrations measured extremely low. Indeed, several investigators have been unable to detect 6-OHDA in rat brain following methamphetamine administration (Rollema et al. 1986; Evans and Cohen 1989; Karoum et al. 1993). Nevertheless, a hypothesis has been advanced that under conditions of massive methamphetamine-induced release of DA and 5-HT and monoamine oxidase (MAO)-A and MAO-B inhibition (Suzuki et al. 1980), these neurotransmitters are non-enzymatically oxidized in the synaptic cleft to give 6-OHDA and 5,6-DHT, respectively (Seiden et al. 1988; Seiden and Vosmer 1984; Commins et al. 1987). Subsequent carrier-mediated uptake of 6-OHDA and 5,6-DHT into dopaminergic and serotonergic terminals, respectively, have been proposed to lead to the degeneration of these neurons. Some support for this hypothesis is provided by the fact that selective DA and 5-HT uptake inhibitors protect dopaminergic and serotonergic terminals, respectively, against metham-phetamine-induced damage (Marek et al. 1990*a*; Ricaurte et al. 1983).

On the assumption that methamphetamine in some way evokes aberrant oxidation of DA to 6-OHDA and 5-HT to 5,6-DHT, it is of interest to consider whether such reactions would occur within the cytoplasm of nerve terminals rather than in the synaptic cleft. In the dopaminergic system methamphetamine participates in an exchange-diffusion process (Raitiri et al. 1979) and displaces DA from cytoplasmic storage sites, presumably resulting in elevated levels of free or unbound neurotrans-mitter prior to its massive efflux through the uptake carrier site (Marek et al. 1990*a*; Liang and Rutledge 1982). Elevated cytoplasmic concen- trations of DA appear to expose this neurotransmitter to oxidation. To illustrate, pretreatment of rats with pargyline (which increases the cytoplasmic pool of DA) followed by methamphetamine results in increased levels of 6-OHDA in the striatum compared to those observed in animals not treated with this MAO-A and MAO-B inhibitor (Marek et al. 1990*c*). Similarly reserpine, which disrupts catecholamine storage vesicles and increases cytoplasmic levels of DA, potentiates metham-phetamine-induced damage to dopaminergic terminals (Wagner et al. 1983). Indeed, treatment of guinea pigs with reserpine alone results in a marked elevation of striatal levels of 5-*S*-cysteinyldopamine, indicative of increased oxidation of cytoplasmic DA (Fornstedt and Carlsson

1989). In contrast, the catecholamine synthesis inhibitor -methyl-*p*-tyrosine (MpT), which depletes the cytoplasmic pool of DA, attenuates methamphetamine-induced damage to dopaminergic terminals (Wagner et al. 1983) and decreases 6-OHDA formation (Axt et al. 1990). Reinstating DA synthesis by administration of L-dopa reverses the protective effects of MpT against methamphetamine-induced damage to dopaminergic terminals (Schmidt et al. 1985). However, administration of L-dopa without methamphetamine does not appear to cause any neurodegenerative effects, indicating that DA is not a neurotoxic agent (Schmidt et al. 1985).

Taken together, these lines of evidence tend to support the idea that elevated cytoplasmic concentrations of DA might favor the intraneuronal oxidation of this neurotransmitter and that such reactions are linked to the degeneration of dopaminergic terminals. The influence of manipulations of the cytoplasmic pool of 5-HT on the methamphetamine-induced oxidation of 5-HT to 5,6-DHT have not been studied. However, in view of the many apparent similarities between the neurodegenerative and other biochemical effects of methamphetamine on dopaminergic and serotonergic terminals, it might also be concluded that oxidation of 5-HT to 5,6-DHT occurs intraneuronally.

The hypothesis that 6-OHDA and 5,6-DHT are responsible for mediating the methamphetamine-induced degeneration of dopaminergic and serotonergic terminals, respectively, has been challenged on the basis of the sporadic detection and exceedingly low levels of these neurotoxins as measured in the brain (Evans and Cohen 1989). However, in the event that these neurotoxins are formed only at, or in, axon terminals, it is not inconceivable that they might transiently reach lethal localized concen-trations. Furthermore, 6-OHDA and 5,6-DHT are believed to be neuro-toxic because of their facile intraneuronal autoxidation to cytotoxic products and byproducts. Thus, the highly localized sites of formation of 6-OHDA and 5,6-DHT and their chemical instability would be expected to make the detection of these neurotoxins in the brain a significant analytical challenge.

Inhibition of DA synthesis by the tyrosine hydroxylase inhibitor MpT attenuates not only 6-OHDA formation and degeneration of striatal dopaminergic terminals, but also 5,6-DHT formation and the degeneration of serotonergic terminals and cell bodies of unknown neurotransmitter content in the somatosensory cortex (Commins and

Seiden 1986; Axt et al. 1990). These observations suggest that either DA and/or an aberrant metabolite of this neurotransmitter might play a role in the processes that result in the degeneration of both dopaminergic and serotonergic terminals and certain cell bodies in the somatosensory cortex. Selective DA uptake inhibitors such as amfolenic acid (AFA) protect striatal dopaminergic terminals against methamphetamine-induced damage (Marek et al. 1990*a*) but do not block the release of DA or 6-OHDA formation (Marek et al. 1990*b*). However, AFA does not protect striatal serotonergic terminals against methamphetamine-induced damage (Marek et al. 1990*a*).

Significantly, AFA prevents the degeneration of striatal dopaminergic terminals when administered as late as 8 hours after methamphetamine (Marek et al. 1990*a*). Selective 5-HT uptake inhibitors such as fluoxetine protect serotonergic terminals against methamphetamine-induced damage but exacerbate damage to striatal dopaminergic terminals (Ricaurte et al. 1983). Taken together, these results suggest a number of possible conclusions:

1. Methamphetamine evokes an initial increase in the cytoplasmic concentrations of free or unbound DA and 5-HT prior to their massive release;

2. Increased cytoplasmic levels of DA and 5-HT in some way permit oxidation of these neurotransmitters as evidenced by the formation of 6-OHDA, 5-*S*-cysteinyldopamine, and 5,6-DHT;

3. The toxic effects of methamphetamine toward dopaminergic and serotonergic terminals require intact and functioning neurotransmitter uptake systems;

4. DA or a toxic DA metabolite might contribute to the degeneration of both dopaminergic and serotonergic nerve terminals; and,

5. A toxic metabolite of 5-HT might contribute to the degeneration of serotonergic and dopaminergic terminals and certain cell bodies in the somatosensory cortex (there is virtually no dopaminergic input into this region of the cortex) (Lindvall and Björklund 1978).

The latter two conclusions imply that toxic aberrant metabolites of DA and 5-HT are transferred between connected dopaminergic and seroto-nergic terminals and between serotonergic terminals and

connected cell bodies in the somatosensory cortex. However, while 6-OHDA and 5,6-DHT might in principal contribute to the degeneration of dopa-minergic and serotonergic terminals, respectively, it is not likely that 6-OHDA contributes to the degeneration of serotonergic terminals or that 5,6-DHT contributes to the degeneration of dopaminergic terminals or neuronal perikarya in the somatosensory cortex. This is so because 6-OHDA (Johnsson et al. 1975) and 5,6-DHT (Baumgarten and Lachenmeyer 1972) are selective catecholaminergic and serotonergic neurotoxins, respectively, except when present in the brain in very high concentrations. Indeed, it might be argued that 6-OHDA may not be responsible for the degeneration of dopaminergic terminals because this neurotoxin cannot be detected in rat brain after methamphetamine administration at times when AFA is able to block the neurodegenerative process (Marek et al. 1990*b*).

It is possible that toxic metabolites of DA and 5-HT other than, or in addition to, 6-OHDA and 5,6-DHT might contribute to the neuronal degeneration evoked by methamphetamine. Several lines of experimental evidence provide some support for this possibility. For example, the methamphetamine-induced degeneration of dopaminergic and serotonergic terminals is significantly attenuated by N-methyl-D-aspartate (NMDA) receptor antagonists (Sonsalla et al. 1991; Farfel et al. 1992). However, there is no evidence available to suggest that 6-OHDA, 5,6-DHT, or methamphetamine are NMDA receptor agonists. Some evidence has been presented that methamphetamine evokes elevated release of glutamate in the striatum of rats (Sonsalla et al. 1991; Nash and Yamamoto 1992) that might result in enhanced activation of NMDA receptors located on dopaminergic and serotonergic terminals and resultant excitotoxic damage (Choi 1987; Lafon-Cazal et al. 1993). However, there are relatively few NMDA receptors located on these terminals in the striatum, whereas gamma aminobutyric acid (GABA), cholinergic, and substance P cell bodies in this structure have high densities of these receptors (Coyle and Schwarz 1976; Sonsalla et al. 1991). Thus, if methamphetamine evokes widespread release of glutamate from glutamatergic terminals throughout the striatum, activation of NMDA receptors and excitotoxic damage would be expected to cause serious damage to GABAergic, cholinergic, and substance P cell bodies. However, these neurons suffer no long-lasting damage following methamphetamine administration (Hotchkiss et al. 1979; Sonsalla et al. 1986).

As noted by Sonsalla and colleagues (1991), if dopaminergic and serotonergic terminal damage induced by methamphetamine is due to the direct effects of glutamate on NMDA receptors, it would clearly have to be a very discrete and highly localized action. In principle, such a localized action could be caused by aberrant metabolites of DA and/or 5-HT formed at dopaminergic or serotonergic terminals, respectively, that are either potent NMDA receptor agonists or in some way potentiate the release of glutamate from connected glutamatergic terminals. A possible clue to the mechanism that might evoke the latter process may be drawn from the observation that the methamphetamine-induced damage to dopaminergic terminals is blocked by coadministration of pharmacologic agents that elevate extraneuronal levels of GABA (Hotchkiss and Gibb 1980*a*).

This finding suggests that interactions of GABA with GABA receptors can block the neurotoxic effects of methamphetamine. However, there are no known interactions between 6-OHDA, 5,6-DHT, or methamphet-amine and GABA receptors. Interestingly, $GABA_B$ receptors are located both on nerve terminals and at postsynaptic sites in many brain regions (Bowery 1989). Activation of presynaptic $GABA_B$ receptors by GABA reduces the evoked release of biogenic amine, excitatory amino acid (including glutamate), and neuropeptide neurotransmitters by blockade of calcium channels (Bowery 1989; Lev-Tov et al. 1988). Conversely, therefore, a $GABA_B$ inverse receptor agonist would be expected to potentiate the opening of the calcium channel and evoke elevated release of neurotransmitters. Following methamphetamine administration, formation of an aberrant metabolite of DA and/or 5-HT that was a potent $GABA_B$ receptor inverse agonist at dopaminergic and/or serotonergic terminals could, in principal, account for the release of glutamate from anatomically connected glutamatergic terminals leading to very localized NMDA receptor-mediated excitotoxicity and neuronal damage.

Available evidence thus suggests that methamphetamine evokes aberrant oxidations of DA to 6-OHDA and 5-HT to 5,6-DHT, probably in the cytoplasm of dopaminergic and serotonergic terminals, respectively. While these two neurotoxins might contribute to the degeneration of the nerve terminals where they are formed, it is not likely that they are involved with neurodegenerative mechanisms mediated by NMDA or GABA receptors. The influence of MpT and selective DA uptake inhibitors on the neurodegenerative effects of methamphetamine tend to support the notion that aberrant metabolites derived from this neurotrans-mitter

(other than or in addition to 6-OHDA) not only contribute to the degeneration of dopaminergic terminals, but also play a role in the degeneration of connected serotonergic terminals. Similarly, the effects of selective 5-HT uptake inhibitors suggest that aberrant metabolites of 5-HT (other than or in addition to 5,6-DHT) might contribute to the degeneration of serotonergic terminals, connected dopaminergic terminals, and certain cell bodies in the somatosensory cortex. These putative aberrant metabolites of DA and/or 5-HT might include compounds that either activate NMDA receptors and evoke excitotoxic damage or facilitate the release of glutamate, but only in the immediate vicinity of their sites of formation at dopaminergic and/or serotonergic terminals.

In the event that aberrant metabolites of DA and 5-HT do play roles in mediating the neurodegenerative effects of methamphetamine, it becomes of key interest to know the identities of the compounds and mechanisms that might be responsible for their formation. Extensive studies (Seiden et al. 1988; Seiden and Vosmer 1984; Commins et al. 1987; Marek et al. 1990*a*, 1990*b*; Axt et al. 1990) indicate that methamphetamine evokes oxidation of DA to 6-OHDA and 5-HT to 5,6-DHT. However, the autoxidation (i.e., oxidation by molecular oxygen in the absence of enzyme catalysis) of DA (Graham 1978) and the electrochemically driven oxidation (Zhang and Dryhurst 1993) of this neurotransmitter in aqueous solution at physiological pH do not give 6-OHDA as a detectable product. Similarly, the autoxidation (Wrona et al. 1992) and enzyme-mediated (Wrona and Dryhurst 1991) and electrochemical (Wrona and Dryhurst 1990) oxidations of 5-HT at physiological pH do not give 5,6-DHT. However, HO· attack on DA does give 6-OHDA, although in much lower yields than 2-OHDA and 5-OHDA (Slivka and Cohen 1987). The latter observation, therefore, might indicate that 6-OHDA and 5,6-DHT are marker molecules for the methamphetamine-induced HO· oxidation of DA and 5-HT, respectively. Indeed, there is good—albeit indirect—evidence that methamphetamine evokes formation of oxygen radicals in the brain (DeVito and Wagner 1989; Cadet et al. 1994). However, remarkably little is known about the HO·-mediated oxidations of DA and 5-HT, the products (i.e., putative aberrant oxidative metabo-lites) that are likely to be formed in vivo, and the neurobiological properties of these products. Accordingly, the authors have recently initiated studies aimed at elucidating the oxidation chemistry of 5-HT and DA, including that mediated by HO·, and assessing the neurobiological activities of the resulting putative metabolites and their possible roles in methamphetamine-evoked neurodegenerative processes and other neurodegenerative brain disorders.

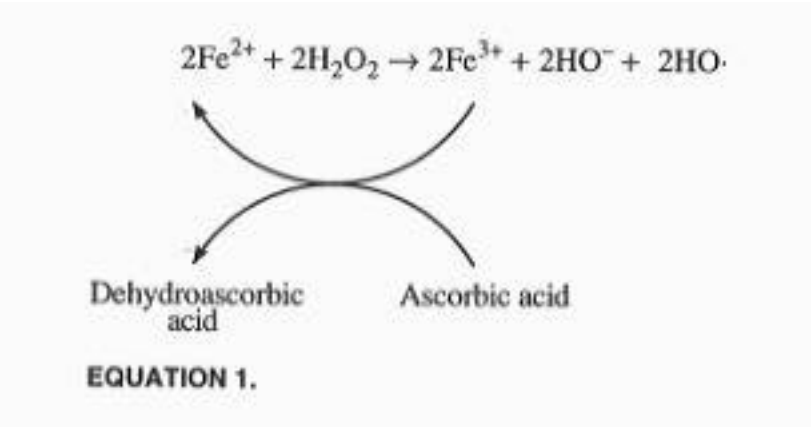
OXIDATION CHEMISTRY OF 5-HT AND DA

Location is a central question relevant to studies of the oxidation reactions of 5-HT and DA and the role of such reactions (and resulting products) in mediating methamphetamine's neurodegenerative properties. This question is important because the course of these reactions and the putative aberrant oxidative metabolites formed are likely to be strongly dependent on whether they occur in the synaptic cleft (Seiden et al. 1988; Seiden and Vosmer 1984; Commins et al. 1987; Marek et al. 1990*c*) or intraneuronally. For example, the cytoplasm of dopaminergic and serotonergic nerve terminals contain relatively high concentrations of ascorbic acid (AA) (Spector and Eells 1984), glutathione (GSH), L-cysteine (CySH) (Slivka et al. 1987), hydrogen peroxide (H_2O_2, a byproduct of many intraneuronal metabolic processes), molecular oxygen, and traces of low molecular weight Fe^{2+} species (Halliwell 1992). By contrast, extraneuronal levels of AA (Spector and Eells 1984), GSH, and CySH (Slivka et al. 1987), for example, are probably appreciably lower than cytoplasmic concentrations.

A widely used HO·-generating system consists of AA, traces of Fe^{2+}-ethylenediaminetetraacetic acid (EDTA), H_2O_2, and molecular oxygen (Udenfriend et al. 1954). In this medium, HO· is formed by decomposition of H_2O_2 by the Fenton reaction (Walling 1975) and the resulting Fe^{3+} is reduced back to Fe^{2+} by AA (equation 1). Thus, as long as AA and H_2O_2 are available, HO· is formed by this cyclic reaction. When incubated with this HO·-generating system, 5-HT is oxidized extremely rapidly (Wrona et al. 1995). The initial step in the reaction involves HO· attack on 5-HT to give the 4,5-(**1**), 2,5-(**2**) (1) and 5,6-(**3**)-dihydroxytryptamine radicals, which are then oxidized by a second molecule of HO· to give 2,5-DHT, 4,5-DHT, and 5,6-DHT in approximate yields of 60 percent, 24 percent, and 11 percent, respectively (figure 1). However, 4,5-DHT is not an isolatable product owing to its very facile oxidation by molecular oxygen to give tryptamine-4,5-dione (T-4,5-D) in a reaction that forms H_2O_2 as a byproduct (Wong and Dryhurst 1990). Furthermore, 2,5-DHT exists predominantly in solution at pH 7.4 as its 2-keto tautomer, 5-hydroxy-3-ethylamino-2-oxindole (5-HEO). The latter compound readily deprotonates to give the C(3)-centered carbanion **4** which attacks T-4,5-D to give dimer **5**. Autoxidation of the 4,5-DHT residue of **5** then yields **6**, forming H_2O_2 as a byproduct. Dimer **6** undergoes a slow intramolecular cyclization reaction to give the pyrrolo[2,3-*f*]quinoline **7**, in which the 4,5-DHT residue is autoxidized

to *o*-quinone **8**, again forming H_2O_2 as a byproduct. A relatively minor amount of T-4,5-D also dimerizes to give the 7,7.-linked dimer **9**, which is immediately autoxidized to 7,7.-D forming H_2O_2 as a byproduct. Because of its reactions with 5-HEO and its dimerization to 7,7.-D, T-4,5-D is not observed as a product of the HO·-mediated oxidation of 5-HT. 5,6-DHT is clearly formed as a result of oxidation of 5-HT by HO·, but it represents only a rather minor product of this reaction. Although 5,6-DHT can be oxidized by molecular oxygen to give *o*-quinone **10**, which subsequently polymerizes to indolic melanin, this is a relatively slow reaction in vitro (Singh and Dryhurst 1990). Furthermore, detection of this neurotoxin as a product of the HO·-mediated oxidation of 5-HT presumably reflects the reduction of *o*-quinone **10** by AA.

The rate of the reaction shown in figure 1 is dependent on the concen-tration of Fe^{2+} in the reaction medium. However, even in the absence of added Fe^{2+}, 5-HEO **6**, and 5,6-DHT are observed as products after several



EQUATION 1.

minutes, indicating that submicromolar concentrations of iron and/or other transition metal ions that always contaminate buffer constituents are sufficient to catalyze the decomposition of H_2O_2 to HO·. Similarly, in the presence of Fe^{2+} but without added H_2O_2, 5-HT is oxidized to 5-HEO, **6** (subsequently **8** forms), and 5,6-DHT. This is because autoxidation of AA to dehydroascorbic acid provides the initial source of H_2O_2 and hence HO·. When 5-HT is oxidized in the same HO·-generating system in the presence of GSH, the yields of 5,6-DHT are not significantly altered. However, yields of 5-HEO increase, those of **6** (and **8**) decrease, and a new product, 7-*S*-glutathionyltryptamine-4,5- dione (7-*S*-Glu-T-4,5-D), appears. In the presence of sufficiently high concentrations of GSH,

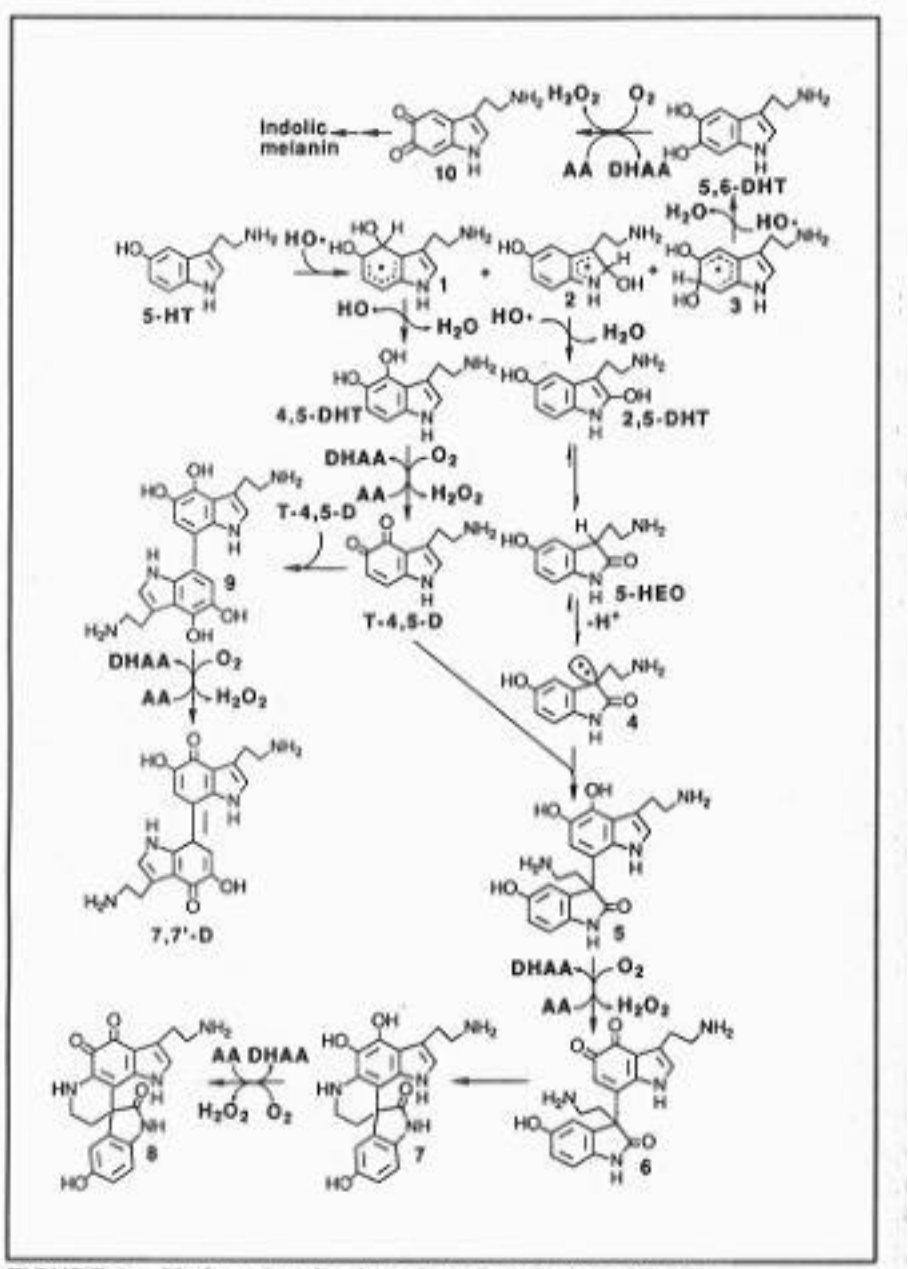


FIGURE 1. *Hydroxyl radical-mediated oxidation of 5-HT.*

formation of **6** (and **8**) is almost completely blocked and yields of 5-HEO and 7-*S*-Glu-T-4,5-D reach maximal levels. These observations are explained by the fact that GSH efficiently scavenges T-4,5-D to give

7-*S*-glutathionyl-4,5-dihydroxytryptamine (**11**, figure 2), which is then autoxidized to 7-*S*-Glu-T-4,5-D and forms H_2O_2 as a byproduct (Wong et al. 1993).

Quantitative measurements of the yields of products formed when 5-HT is oxidized with the AA/Fe^{2+}-EDTA/H_2O_2/O_2 system reveal that the initial yields of 5-HEO, 5,6-DHT, and **6** (and 7-*S*-Glu-T-4,5-D when GSH is present) are larger than expected based on the H_2O_2 (and hence HO·) concentrations employed (Wrona et al. 1995). This additional HO· probably results from autoxidations of 4,5-DHT (Wong and Dryhurst 1990), 5,6-DHT (Singh and Dryhurst 1990), **5** (and **8**) (Wrona et al. 1995), **11** (Wong et al. 1993), and redox cycling reactions of the T-4,5-D/4,5-DHT (formal potential (E%.) = -240 millivolts (mV) versus the saturated calomel reference electrode (SCE) at pH 7.4), **6**/**5** (E%. = -495 mV), **8**/**7** (E%. = -495 mV), 7-*S*-Glu-T-4,5-D/**11** (E%. = -248 mV), **10**/5,6-DHT, and 7,7./**9** (Singh et al. 1992) couples which form H_2O_2 as a byproduct.

These autoxidation-redox cycling reactions and resultant H_2O_2 and HO· formation apparently continue the oxidation of 5-HT until AA is exhausted. Although 5,6-DHT is an initial but minor product of the HO·-mediated oxidation of 5-HT, this neurotoxin almost completely disappears within 2 to 3 hours (Wrona et al. 1995) because of its autoxidation to an insoluble brown-black indolic melanin polymer (Singh and Dryhurst 1990; Singh et al. 1990). By contrast, the major reaction product, 5-HEO, is an appreciably more stable compound.

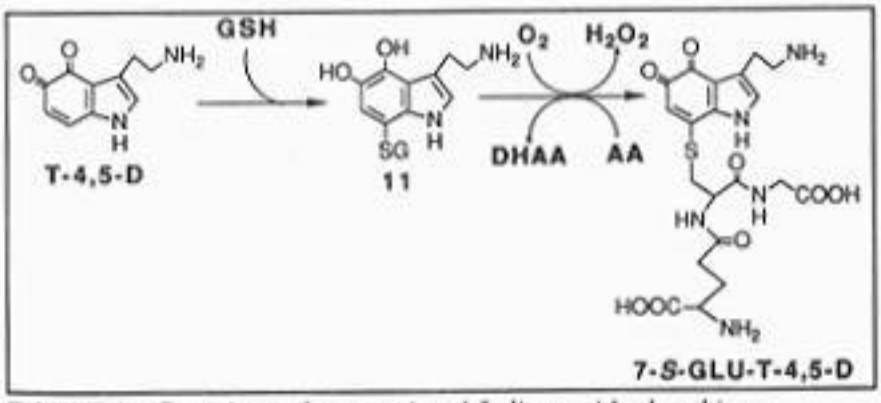


FIGURE 2. *Reactions of tryptamine-4,5-dione with glutathione.*

The oxidation of DA at physiological pH by HO·-generating systems consisting of $Fe^{2+}/H_2O_2/O_2$ or $Fe^{2+}/AA/O_2$ (H_2O_2 and hence HO· being formed as a result of AA autoxidation) has been reported to give a mixture of 2-OHDA, 5-OHDA, and 6-OHDA in relative yields of 3:2:1 (Slivka and Cohen 1987). However, the yields of all of these products were low based upon the HO· concentrations generated. The mechanism of these hydroxylation reactions is not completely clear. However, Richter and Waddell (1983) have reported that addition of HO·, generated by pulse radiolysis, to each of the available ring positions of DA is equally probable. Under the conditions employed by these investigators, which were carried out in the absence of molecular oxygen and iron salts, the intermediate radical species (**12**-**14**, figure 3) eliminate the elements of water to give semiquinones of DA. The dehydration of the 6-hydroxy- lated radical (**14**) was faster than for **12** and **13**. Slivka and Cohen (1987) have suggested that radicals **12**-**14** are oxidized by molecular oxygen or Fe^{3+} to give 2-OHDA, 5-OHDA, and 6-OHDA (figure 3). The lower yield of 6-OHDA was attributed to the more rapid water elimination reaction of **14**. In the event that methamphetamine evokes oxidation of DA by HO· in the cytoplasm of dopaminergic nerve terminals, then such reactions must occur in the presence of CySH and GSH (Slivka et al. 1987) and it is probable that these sulfhydryl compounds would influence the products formed.

Information bearing on such reactions in vivo can be drawn from reports that the 5-*S*-cysteinyl conjugates of DA and other endogenous catechols are present in human and other mammalian brains (Rosengren et al. 1985; Fornstedt et al. 1986). These conjugates are probably formed either by nucleophilic addition of CySH to the *o*-quinones formed by oxidation of these catechols or by addition of GSH to yield the 5-*S*-glutathionyl conjugates which are then hydrolyzed by peptidase enzymes (Rosengren et al. 1985).

Several lines of evidence suggest that the oxidation of DA and other catechols and formation of their 5-*S*-cysteinyl conjugates occurs in the cytoplasm of dopaminergic neurons. To illustrate, both GSH and CySH, which are clearly required to form these cysteinyl conjugates, are located in the cytoplasm of dopaminergic terminals and axons (Slivka et al. 1987). Furthermore, treatment of guinea pigs with reserpine, which disrupts DA storage vesicles and elevates cytoplasmic levels of this neurotransmitter, evokes a marked elevation of striatal levels of

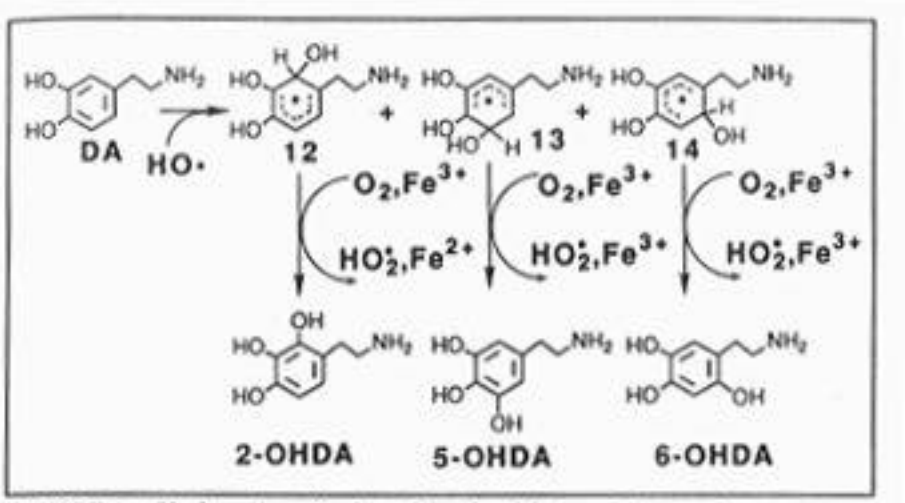


FIGURE 3. *Hydroxyl radical-mediated oxidation of dopamine.*

5-S-cysteinyldopamine (5-*S*-CyS-DA) (Fornstedt and Carlsson 1989). The mechanism of the HO·-mediated oxidation of DA in the presence of molecular oxygen and Fe^{2+}/Fe^{3+} does not appear to involve an electro- philic *o*-quinone intermediate (figure 3), the apparent precursor of 5-S-CyS-DA (Rosengren et al. 1985; Fornstedt et al. 1986, 1989). Autoxidation of DA apparently does proceed via such an intermediate, but is a relatively slow reaction at physiological pH and hence unsuitable for isolation and identification of reaction products. However, the electrochemically driven oxidation of DA is not only very facile but undoubtedly proceeds by initial formation of an *o*-quinone intermediate (Zhang and Dryhurst 1993). Accordingly, the authors have recently studied the influence of CySH on the electrochemical oxidation of DA at physiological pH (Zhang and Dryhurst 1994). This reaction results in formation of a very complex mixture of products. However, among the major products are the dihydrobenzothiazines (DHBTs) **17** to **19** and **22** (figure 4). In this reaction, DA-*o*-quinone is initially attacked by CySH to give 5-*S*-CyS-DA, which is appreciably more easily oxidized than DA to give *o*-quinone **15**. This intermediate very rapidly cyclizes to give the bicyclic *o*-quinone imine **16**. In the presence of free CySH **16** can either be reduced to DHBT **17** or nucleophilic addition of a second CySH molecule occurs to give DHBTs **18** and **19**. The latter compounds are even more easily oxidized than DA and 5-*S*-CyS-DA to *o*-quinone imines **20** and **21**, respectively, which can be either reduced by CySH or further

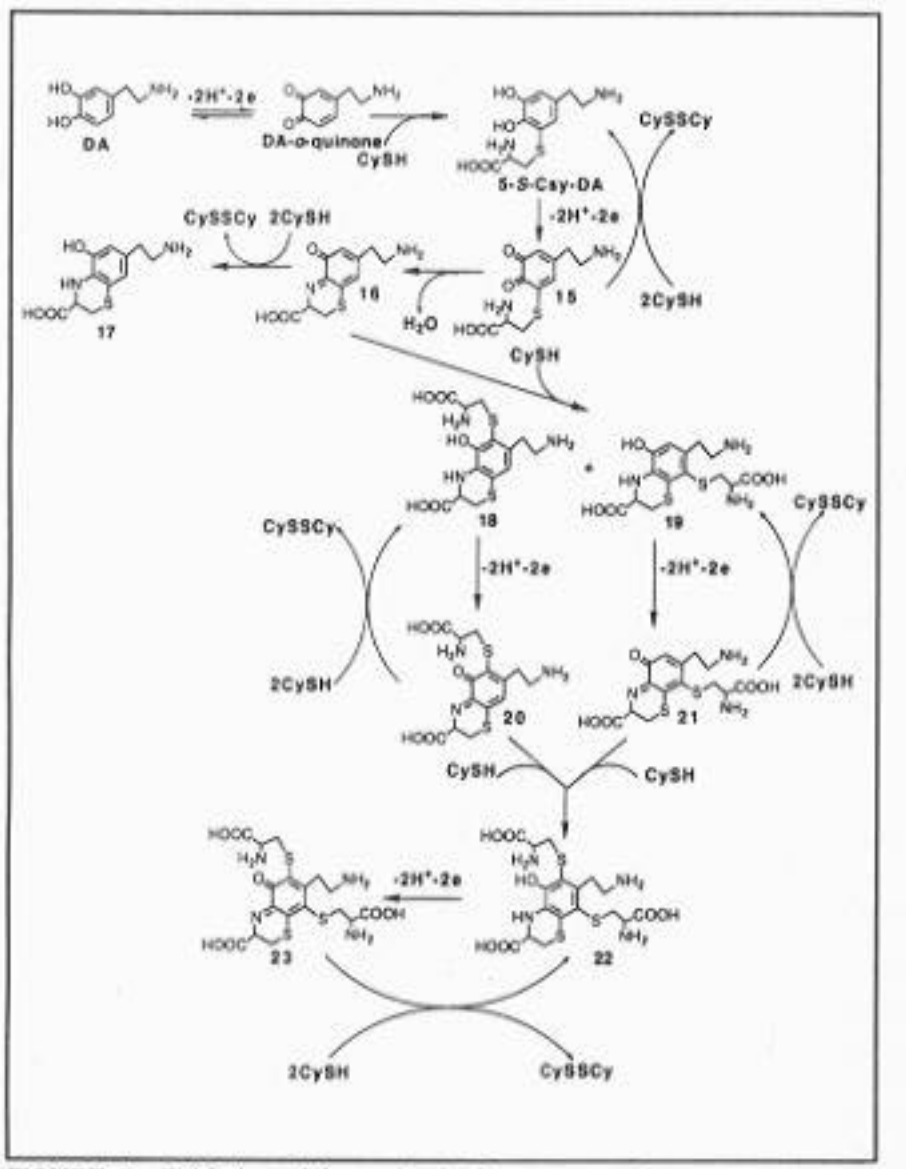


FIGURE 4. *Oxidation of dopamine in the presence of cysteine.*

attacked to give DHBT **22**. DHBTs **17** to **19** and **22** survive in the oxidation reaction simply because their oxidized forms are readily reduced by free CySH. DA-*o*-quinone is also scavenged by GSH to give a number of glutathionyl conjugates that include 5-*S*-glutathionyl- dopamine (5-*S*-Glu-DA) (Zhang and Dryhurst, in press). As suggested by Rosengren and colleagues (1985), if formed in the cytoplasm of dopaminergic terminals, 5-*S*-Glu-DA might be hydrolyzed by peptidase enzymes to 5-*S*-CyS-DA (figure 5). Under conditions when DA is oxidized, the latter conjugate must also be

oxidized because it is a significantly more easily oxidized compound (Zhang and Dryhurst 1994). In the event that this reaction is mediated by molecular oxygen, the expected byproducts would be superoxide anion radical (O_2-·) and/or H_2O_2, both precursors of HO·. The resulting products would include DHBTs **17** to **19** and **22** as conceptualized in figure 5. Again, the facile autoxidation of these DHBTs and their ability to redox cycle would be expected to potentiate additional formation of HO· precursors. The oxidation of DA in the presence of CySH in an HO·-generating system consisting of AA/Fe^{2+}/H_2O_2/O_2 also forms 5-*S*-CyS-DA in high yield; this is further oxidatively cyclized to DHBT **17** (Zhang and Dryhurst, unpublished results). Preliminary results do not indicate that 6-OHDA is a significant reaction product.

Studies in this laboratory aimed at understanding the in vitro oxygen radical-mediated oxidations of 5-HT and DA under experimental conditions that might mimic those in the cytoplasm of serotonergic and dopaminergic nerve terminals in vivo are at an early stage. Nevertheless, these investigations have established that 5,6-DHT is formed as a result of HO· attack on 5-HT. However, this neurotoxin is the most minor of the identified products of the reaction and is also a rather unstable species owing to its oxidative polymerization to indolic melanin. Furthermore, intraneuronal formation of 5,6-DHT as a result of methamphetamine administration would necessarily expose this substance to mitochondria which are known to catalyze its oxidation by molecular oxygen (Klemm et al. 1980; Singh and Dryhurst 1990). Taken together, these facts might account for the sporadic and low levels of 5,6-DHT detected in rat brain following an acute dose of methamphetamine (Commins et al. 1987).

These observations led Evans and Cohen (1989) to question the hypothesis that this neurotoxin mediates the methamphetamine-induced degeneration of serotonergic terminals. Assuming that formation of 5,6-DHT in rat brain following methamphetamine administration reflects intraneuronal oxidation of 5-HT by HO· (this is the only known chemical reaction that directly converts 5-HT and 5,6-DHT), the reaction pathways shown in figures 1 and 2 (Wrona et al. 1995) predict that 5-HEO in particular (and perhaps **6** and **8**) should be formed as major and more stable aberrant metabolites. However, intraneuronal GSH (Slivka et al. 1987) would be expected to scavenge T-4,5-D to give 7-*S*-Glu-T-4,5-D.

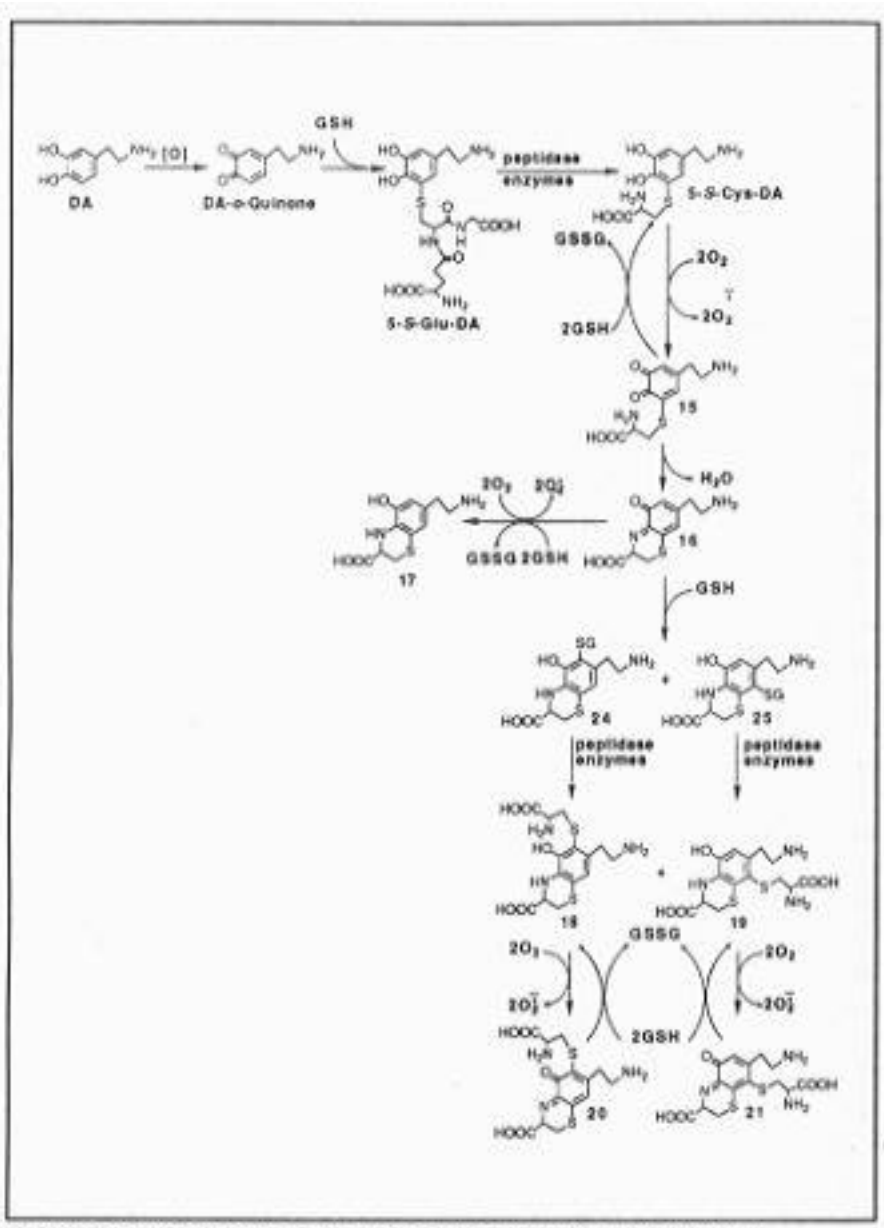


FIGURE 5. *Oxidation of dopamine in the presence of glutathione and possible in vivo effects of peptidase enzymes.*

Thus 5-HEO, **6**, **8**, or 7-*S*-Glu-T-4,5-D might represent valuable analytical marker molecules in brain tissue to provide evidence in support of the idea that methamphetamine does indeed evoke intraneuronal formation of HO·. Furthermore, the fact that the in vitro HO·-mediated oxidation of 5-HT gives additional products in higher yields than

5,6-DHT raises the possibility that one or more of these other putative aberrant oxidative metabolites might play roles in the neurodegenerative effects evoked by methamphetamine. The influence of CySH on the HO·-mediated oxidation of 5-HT remains to be studied. However, recent studies into the electrochemically driven oxidation of 5-HT in the presence of CySH suggest that many additional products are likely to be formed (Wrona et al. 1994).

In vitro studies reveal that the HO·-mediated oxidation of DA at physiological pH gives 6-OHDA (Slivka and Cohen 1987) (figure 3). Accordingly, detection of 6-OHDA in the rat striatum shortly following methamphetamine administration (Seiden and Vosmer 1984) suggests that this drug also evokes formation of HO·. Nevertheless, this conclusion must be tempered by the fact that several investigators have been unable to detect even extremely low levels of 6-OHDA in rat brain following administration of methamphetamine or other drugs that release DA into the synaptic cleft (Rollema et al. 1986; Evans and Cohen 1989; Karoum et al. 1993). However, using gas chromatography-mass spectrometry, Karoum and colleagues (1993) have observed a fluoro-propionylated derivative of an unknown compound having a retention time very similar to pentafluoropropionylated 6-OHDA in the brains of methamphetamine treated rats. In the event that methamphetamine evokes the intraneuronal oxidation of DA, it seems plausible to suggest that CySH and or GSH might intervene in the reaction as shown in figures 4 and 5 to give 5-*S*-CyS-DA and 5-*S*-Glu-DA, precursors of DHBTs such as **17** to **19** and **22**. This suggestion raises the possibility that these putative aberrant oxidative metabolites of DA, formed only from endogenous substances, might contribute to the neurodegenerative effects evoked by methamphetamine.

IN VIVO PROPERTIES OF PUTATIVE ABERRANT OXIDATIVE METABOLITES OF 5-HT AND DA

Investigations into the neurotoxicology and neuropharmacology of putative aberrant oxidative metabolites of 5-HT and DA are at a very preliminary stage. 5-HEO, the major product of the in vitro HO·-mediated oxidation of 5-HT, is not toxic (lethal) when administered into the brains of mice weighing ~ 30 grams (g) at doses as high as 100-200 micrograms (g) (Dryhurst et al., unpublished results). However, several other intermediates/products of this reaction are active in the brain. For example, T-4,5-D is lethal when injected into the brains of mice (Wong et al. 1993). Furthermore,

intracerebroventricular injections of T-4,5-D into rat brain have been claimed to evoke long-lasting decreases in 5-HT levels in the hippocampus, striatum, and cortex; reduced activity of tryptophan hydroxylase (Chen et al. 1992); and degeneration of nerve terminals (Crino et al. 1989). 7-*S*-Glu-T-4,5-D is also lethal (median lethal dose (LD_{50}) = 21 g) when administered into mouse brain, evoking extreme excitation and hyperactivity for about 30 minutes (Wong et al. 1993). During this period, 7-*S*-Glu-T-4,5-D evokes statistically significant declines of whole brain levels of norepinephrine, DA, and 5-HT and elevation of the metabolites of these neurotransmitters (Wong et al. 1993). This might suggest that 7-*S*-Glu-T-4,5-D potentiates the release and elevated turnover of the monoamines although its influence on the release of other neurotransmitters, especially glutamate, remains to be determined. 7-*S*-Glu-T-4,5-D also redox cycles in the presence of cellular antioxidants/reductants and molecular oxygen and when incubated with brain homogenates forming H_2O_2 as a byproduct (Wong et al. 1993). Dimer **6** is also lethal (LD_{50} = 19 g) when administered into mouse brain (Dryhurst et al., unpublished results). Furthermore, the E%. values of **6** and **8** (both -495 mV at pH 7.4) are such that these compounds might be expected to redox cycle under intraneuronal conditions (Yamazaki and Piette 1963) forming O_2-· and/or H_2O_2 as byproducts, that is, precursors of cytotoxic HO·.

5-*S*-CyS-DA, a putative aberrant oxidative metabolite of DA in dopaminergic terminals, is not lethal when administered into the brains of laboratory mice. DHBTs **17** (LD_{50} = 14 g), **18** (LD_{50} = 17 g), and **19** (LD_{50} = 70 g) are lethal (Zhang and Dryhurst 1994).

SUMMARY

In the event that methamphetamine evokes HO· formation within serotonergic axon terminals, the resultant oxidation of 5-HT would be expected to generate not only 5,6-DHT but also T-4,5-D, 7-*S*-Glu-T-4,5-D, **6**, **8**, and 7,7T-D (figure 1), at least three of which (T-4,5-D, 7-*S*-Glu-T- 4,5-D, and **6**) are lethal in mouse brain. Furthermore, several intermed- iates/products formed in the in vitro oxidation of 5-HT by HO· are readily autoxidized (4,5-DHT, 5,6-DHT, **5**, **7**, and **9**) or redox cycled (T-4,5-D, **6**, **8**, 7,7T-D, 7-*S*-Glu-T-4,5-D) in reactions that would be expected to yield O_2-· and/or H_2O_2 as byproducts. These byproducts, in the presence of trace levels of transition metal ion catalysts, would be readily converted into HO· (Walling 1975; Halliwell and Gutteridge 1984). Together these

putative aberrant oxidative metabolites of 5-HT and HO·-forming reactions might contribute to the degeneration of serotonergic nerve terminals. Similarly, the methamphetamine-induced intraneuronal formation of HO· in dopaminergic terminals might be expected to generate not only 6-OHDA (and 2-OHDA and 5-OHDA, figure 3) but also 5,-*S*-CyS-DA and 5-*S*-Glu-DA, precursors of DHBT **17** and other more complex dihydrobenzothiazines (figure 4).

DHBTs **17** to **19** are lethal in mouse brain, although at this time the biochemical/chemical mechanisms underlying this toxicity and specific neuronal systems affected are unknown. However, 5-*S*-CyS-DA and **17** to **19** are much more easily oxidized than DA, and the latter DHBTs appear to be capable of redox cycling reactions (Zhang and Dryhurst 1994). Thus, the HO·-mediated oxidation of DA in dopaminergic nerve terminals induced by methamphetamine might be expected to generate aberrant oxidative metabolites that (as a result of autoxidation and redox cycling reactions) potentiate formation of O_2-· and/or H_2O_2, and then HO· and neuronal damage.

A number of lines of evidence, discussed previously, suggest that aberrant metabolite(s) of DA (other than or in addition to 6-OHDA) might contribute to the methamphetamine-induced degeneration of not only dopaminergic terminals but also serotonergic terminals. Similarly, aberrant metabolite(s) of 5-HT (other than or in addition to 5,6-DHT) might be involved in the degeneration of serotonergic and dopaminergic terminals and a subpopulation of cell bodies in the somatosensory cortex. Experimental evidence indicates that some of the neurodegenerative effects evoked by methamphetamine are mediated by NMDA and GABA receptors. Thus, it will be of considerable interest to investigate the neuro- toxicity of putative aberrant oxidative metabolites of 5-HT (figures 1 and 2) and DA (figures 4 and 5) towards serotonergic, dopaminergic, and other neuronal systems and their interactions with NMDA, GABA, and other brain receptors.

A central question relates to mechanisms by which methamphetamine might evoke the intraneuronal formation of oxygen radicals that appear to play important roles in the overall neurodegenerative processes evoked by this drug (DeVito and Wagner 1989; Cadet et al. 1994). Once putative oxidative metabolites of 5-HT such as T-4,5-D, 7-*S*-Glu-T-4,5-D, 5,6-DHT, **6**, **8**, and 7,7T-D (figure 1) are formed intraneuronally, autoxidation/redox cycling reactions should, in principle, be capable of generating O_2-· and/or H_2O_2, the precursors of HO·. Similarly, intraneuronal formation of 6-OHDA, 5-*S*-CyS-DA,

and DHBTs **17** to **19** and **22** would also be expected to potentiate elevated fluxes of O_2-·, H_2O_2, and HO· as a result of the facile autoxidation/redox cycling reactions of these putative aberrant metabolites. The presence of very low concentrations of 5-*S*-CyS-DA in DA-rich regions of human and other mammalian brains suggests that autoxidation (Rosengren et al. 1985; Fornstedt et al. 1986, 1989, 1990) or perhaps some other form of DA oxidation is a normal reaction in vivo. Furthermore, available evidence suggests that it is cytoplasmic DA that is oxidized to give 5-*S*-CyS-DA (Fornstedt et al. 1989; Fornstedt and Carlsson 1989). Presumably, only free or unbound cytoplasmic DA is available for such oxidation reactions.

The ability of methamphetamine to displace DA from cytoplasmic storage sites prior to efflux through the uptake carrier site (Raitiri et al. 1979; Marek et al. 1990*a*; Liang and Rutledge 1982) would be expected to elevate the levels of this neurotransmitter available for autoxidation. Furthermore, reuptake of released DA through the uptake carrier site might continue to provide elevated cytoplasmic levels of the neurotrans-mitter for autoxidation and oxidation by HO·, generated as a result of autoxidation/redox cycling reactions of aberrant oxidative metabolites of DA. Such a sequence of events might result in the gradual increase in the levels of toxic aberrant oxidative metabolites of DA and account for the observation that administration of the uptake inhibitor AFA up to 8 hours after methamphetamine is capable of protecting dopaminergic terminals (Marek et al. 1990*b*).

There is presently no experimental information concerning the normal autoxidation of cytoplasmic 5-HT. However, 5-HT autoxidizes at about the same rate as DA at physiological pH (Creveling et al. 1975; Rotman et al. 1976). Methamphetamine displacement of 5-HT from its cytoplasmic storage sites and reuptake of the released neurotransmitter might lead to 5-HT autoxidation to aberrant metabolites; as a result of autoxidation/redox cycling reactions, these metabolites form elevated fluxes of O_2-·, H_2O_2, and HO· and thereby potentiate their own synthesis and neuronal damage.

Until recently it was believed that intraneuronal proteins known as serotonin binding proteins (SBP) were involved in the storage, protection, and/or transport of 5-HT (Tamir et al. 1976; Gershon and Tamir 1984) and catecholamines (Jimenez Del Rio et al. 1992, 1993*a*, 1993*b*; Pinxteren et al. 1993). The binding of 5-HT and DA to SBP is increased by Fe^{2+} but not Fe^{3+}. Thus, it was generally

believed that Fe^{2+} initially binds to sulfhydryl groups of SBP and that the monoamine neurotrans- mitters form reversible coordinate bonds with the trapped iron. However, more recent results (Jimenez Del Rio et al. 1993*a*) have revealed that in the presence of Fe^{2+} and molecular oxygen, SBP covalently (irreversibly) binds the oxidation products of 5-HT and DA. The latter reactions are apparently mediated by oxygen radicals formed by reaction between Fe^{2+} and molecular oxygen. These observations might imply that the physiological role for SBP is to act as a scavenger of electrophilic oxidation products of 5-HT (e.g., T-4,5-D, **10** (figure 1)) and DA (e.g., DA-*o*-quinone, figure 4), which are themselves cytotoxins or precursors of other toxic metabolites. Interestingly, reserpine inhibits the binding of 5-HT (or its oxidation products) to SBP in the presence of Fe^{2+} and molecular oxygen (Tamir et al. 1976) and hence might account, in part, for increased formation of 5-*S*-CyS-DA in the striatum of reserpinized rats (Fornstedt and Carlsson 1989). Thus, reserpine might not only evoke increased cytoplasmic levels of DA, but also impair the ability of SBP to bind electrophilic oxidation products. Similarly, elevated cytoplasmic levels of free DA and 5-HT caused by methamphetamine, followed by intraneuronal oxidation of these neurotransmitters, might result in formation of electrophilic intermediates at concentrations that exceed the scavenging capacity of SBP with resultant neurodegenerative consequences.

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