phenomena (e.g., intermediates or transition states) may be involved. An interesting question arises as to the structure of the zwitterion since there are two phenolic OH sites for proton dissociation and the possibility of internal hydrogen bonding.[2h] According to Sinistri and Villa[2h] there is little difference in the $pK_a$ between respective *O*-monomethyl analogues, which suggests that ionization probably occurs about equally from the two sites.

### References and Notes


(1) (a) P. Pratesi, E. Grana, L. Villa, A. La Manna, and L. Villa, *Farmaco, Ed. Sci.*, **18**, 920 (1963); (b) P. Pratesi and E. Grana, *Adv. Drug Res.*, **2**, 127 (1965); (c) P. Pratesi, E. Grana, and L. Villa, *Proc. Int. Pharmacol. Meet., 3rd, 1966*, **7**, 283 (1968); (d) P. Pratesi, E. Grana, and L. Villa, *Farmaco, Ed. Sci.*, **26**, 379 (1971); (e) B. Belleau, *Pharmacol. Rev.*, **18**, 131 (1966); (f) *Ann. N.Y. Acad. Sci.*, **139**, 580 (1967); (g) B. M. Bloom and I. M. Goldman, *Adv. Drug Res.*, **3**, 121 (1966); (h) E. J. Ariens, *Ann. N.Y. Acad. Sci.*, **139**, 606 (1967); (i) A. A. Larsen, *Nature (London)*, **224**, 25 (1969).

(2) (a) E. B. Leffler, H. M. Spencer, and A. Burger, *J. Am. Chem. Soc.*, **73**, 2611 (1951); (b) A. Albert, *Pharmacol. Rev.*, **4**, 136 (1952); (c) G. P. Lewis, *Br. J. Pharmacol. Chemother.*, **9**, 488 (1954); (d) M. M. Tuckerman, J. R. Mayer, and F. C. Nachod, *J. Am. Chem. Soc.*, **81**, 92 (1959); (e) H. Shapiro, *J. Theor. Biol.*, **1**, 289 (1961); (f) S. Riegelman, L. A. Strait, and E. Z. Fishcher, *J. Pharm. Sci.*, **51**, 129 (1962); (g) C. Sinistri and L. Villa, *Farmaco, Ed. Sci.*, **17**, 949 (1962); (h) *ibid.*, **17**, 967 (1962); (i) R. F. Jameson and W. F. S. Neillie, *J. Chem. Soc.*, 2391 (1965); (j) T. Kappe and M. D. Armstrong, *J. Med. Chem.*, **8**, 368 (1965); (k) R. B. Martin, *J. Phys. Chem.*, **75**, 2657 (1971); (l) K. S. Rajan, J. M. Davis, R. W. Colburn, and F. H. Jarke, *J. Neurochem.*, **19**, 1099 (1972); (m) P. J. Antikainen and U. Witikainen, *Acta Chem. Scand.*, **27**, 2075 (1973).

(3) For reviews with leading references, see G. C. K. Roberts in "Molecular and Quantum Pharmacology", E. D. Bergman and B. Pullman, Ed., Reidel Publishing Co., Dordrecht, Holland, 1974, p 77; J. P. Green, C. L. Johnson, and S. Kang, *Annu. Rev. Pharmacol.*, **14**, 319 (1974); P. S. Portoghese, *ibid.*, **10**, 51 (1970).

(4) R. P. Ahlquist, *Am. J. Physiol.*, **153**, 586 (1948).

(5) R. T. Brittain, D. Jack, and A. C. Ritchie, *Adv. Drug Res.*, **5**, 197 (1970).

(6) R. B. Barlow, "Introduction to Chemical Pharmacology", 2nd ed, Methuen, London, 1964, p 318.

(7) J. I. Brauman and L. K. Blair, *J. Am. Chem. Soc.*, **90**, 6561 (1968).


# Isolation and Identification of an in Vivo Reaction Product of 6-Hydroxydopamine


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The product of oxidized 6-OHDA and GSH reacted in vitro has been identified by a variety of chemical and physical methods to be 2,4,5-trihydroxy-6-*S*-(glutathionyl)phenethylamine. Its chemical properties show it easily undergoes a variety of oxidative condensations and polymerization. Its oxidized form, the *p*-quinone, can be identified in small quantities in rat brain and mouse brain 1–3 h after 6-OHDA injection. This is believed to be the first report of a chemically identified species resulting from the in vivo interaction of 6-OHDA with CNS tissue.


The molecular mechanism whereby 6-hydroxydopamine (6-OHDA) exerts its powerful neurotoxicity toward catecholamine neurons is still in question. However, from the beginning it has been suggested that nucleophilic interaction (covalent bonding) of the 6-OHDA quinone with neuronal constituents might be crucial in this action.[1-4] This view has received recent support from several sources.[5,6] Our studies have shown that oxidized 6-OHDA (the *p*-quinone, 6-Q) reacts extremely rapidly with thiols. Using glutathione (GSH) as an example of a thiol known to be present in high concentration in the CNS milieu,[7,8] we characterized the rate and nature of this reaction, but the identification of the product remained somewhat uncertain.[9] We now have a complete chemical identification of the product from the in vitro reaction of oxidized 6-OHDA and GSH at pH 7.4. Further, we have been able to identify this same compound as resulting from the in vivo interaction when 6-OHDA is injected stereotaxically into rat brain.

### Results and Discussion

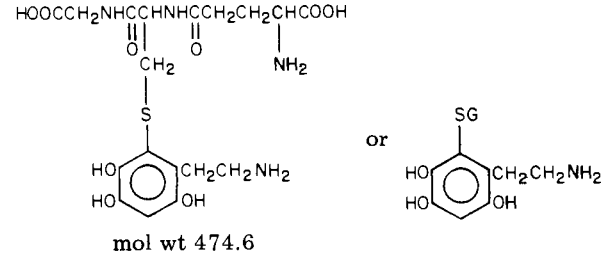
**A. Characterization of the in Vitro Reaction Product of 6-OHDA and GSH.** The reaction product (see Experimental Section) was hydroscopic and melted in air with decomposition at 195–198 °C. It gave a single peak by liquid chromatography with the same retention time as the new peak obtained when GSH is added to air-oxidized 6-OHDA solution. The UV spectrum in 0.1 M perchloric acid had a $\lambda_{max}$ at 306 nm with $\epsilon = 3.2 \times 10^3$ $M^{-1}$ $cm^{-1}$. The NMR spectrum in $D_2O$ showed one aromatic proton at 6.6 ppm which we were able previously to show was the 5-position proton;[9] thus the GSH substitution occurs on the 2 position.

The mass spectrum of 6-OHDA showed *m/e* fragments at 152, 151, 150, 149, and 139. The mass spectrum of the product gave major peaks at *m/e* of 184, 183, 182, 181, and 171, each of which are 6-OHDA fragments with an attached sulfur (mass 32), consistent with the expected fragmentation pattern.

In deaerated citric–phosphate buffer (pH 7.4) the product can be oxidized very easily at the dropping mercury electrode with an $E^{0'} = -0.20$ V vs. SCE. This shows that the product is the reduced (hydroquinone) form.

The elemental analysis provided the following data. Anal. ($C_{18}H_{26}N_4O_9S$) C, H, N, S.

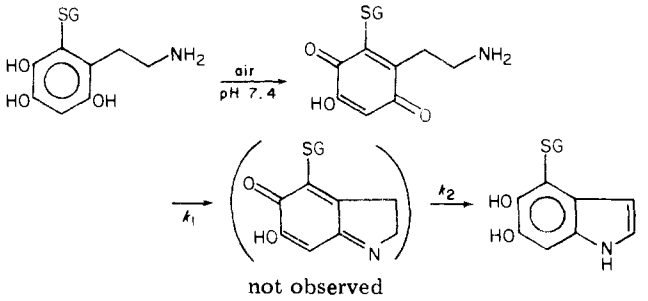
All of the above results confirm that 1 mol of oxidized 6-OHDA reacts with 1 mol of GSH by nucleophilic addition to form the RS-substituted 6-OHDA having the structure




mol wt 474.6

The correct chemical name of the product is 2-(2-*S*-glutathionyl-3,4,6-trihydroxyphenyl)ethylamine. However, since 6-hydroxydopamine nomenclature is so firmly entrenched in the literature, it is more practical herein to call it 2-*S*-(glutathionyl)-6-OHDA, with the abbreviation 2-SG-6-OHDA.

The compound 2-SG-6-OHDA, like 6-OHDA, is moderately stable in acid solution. In pH 7.4 buffer it rapidly air oxidizes and more slowly undergoes a 1,2 intracyclization in a manner similar to 6-OHDA as




not observed

The intermediate indoline is rapidly converted to the final indole. The cyclization rate was measured via electrochemical techniques similar to those used for 6-OHDA[10] and in pH 7.4 buffer, at 37 °C, $K_{obsd} = k_1 = (1.10 \pm 0.04) \times 10^{-3}$ s$^{-1}$. The corresponding $t_{1/2}$ is ca. 12 min, compared to 39 min for 6-OHDA under the same conditions. While the 2-SG-6-OHDA does undergo oxidative cyclization, the electrochemistry shows no tendency for the oxidized noncyclized form to react with another mole of GSH. However, the indole form undoubtedly undergoes oxidative polymerization as do other indoles of this nature.

**B. In Vivo Reaction Product of 6-OHDA and GSH.** To identify the in vivo reaction product, 6-OHDA was stereotaxically injected into rat or mouse brain (see Experimental Section). After a suitable time interval, the animal was sacrificed and the brain tissue examined for possible products. The identification is difficult because in a relatively short time the original 2-SG-6-OHDA is further oxidized to the quinone (2-SG-6Q) and the latter undergoes cyclization and rearrangement to the indole as discussed above. In addition, oxidative polymerization akin to melanin-style reactions presumably occurs. Fortunately, the 2-SG-6Q can be readily detected via high-performance liquid chromatography using electrochemical detection. A 5–10-μl sample of the hypothalamus homogenate was injected on the same LC column previously described.[9] In this case, the eluent was a mixture 0.05 M in hydrochloric acid and 0.06 M in ammonium chloride. The electrochemical detector was set at an applied potential of −0.10 V vs. SCE to detect reduction current for 2-SG-6Q.

Figure 1 is typical of the LC analyses of rat hypothalamus (HT) following 6-OHDA injection. The dotted line shows a clear elution peak with a retention time of 14 min for 6-OHDA-treated animals. The controls, whose chromatogram is illustrated by the solid line in Figure 1, show no such peak. This 14-min peak is identical with that of 2-SG-6Q prepared by air oxidation of authentic 2-SG-6-OHDA and eluted under the same conditions. The product 2-SG-6Q was found chromatographically in every rat HT injected but in no controls. Furthermore, ten intracranial injections of 6-OHDA in mouse brains (region unspecified and analyzed as "whole brain") gave similar results. (More concentrated samples of 2-SG-6Q gave an identical 14-min peak using standard UV detection, showing the result is no artifact of electrochemical detection.)

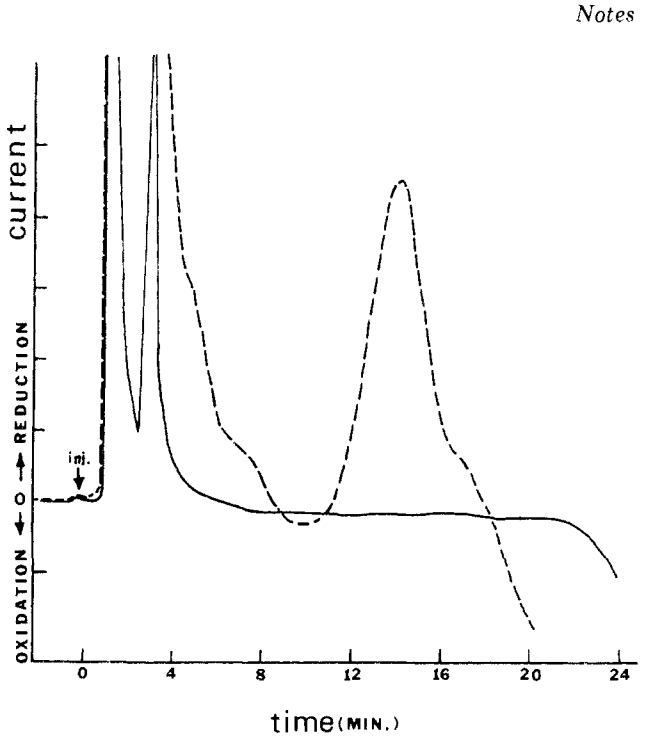



Figure 1. LC determination of the reaction product of oxidized 6-OHDA with GSH: injection point, inj; rat hypothalamus sample after 6-OHDA injection (– – –); rat hypothalamus of control animals (—).

From calibration data the total amount of 2-SG-6Q found per rat HT 1 h after injection was 0.003 μmol. Thus, from a 200-μg injection of 6-OHDA (ca. 1.2 μmol), only about 0.2% of the injected 6-OHDA can be found as 2-SG-6Q. This result is not unexpected since a considerable amount of the injected material is known to be unresolved as oxidized materials in the "solvent front" and some in the indole and polymerized indole forms of 6-OHDA and 2-SG-6Q (see Figure 1). Finally, from Saner and Thoenen's study, it is known that a considerable amount of the original 6-OHDA is covalently bound to membrane components.[1] Neither of the latter two forms would appear in the soluble chromatographic portion studied herein.

At first glance, one might consider stereotaxically injected 6-OHDA would cause a serious (albeit localized) depletion of GSH. Portions of the HT readied for the LC were assayed for GSH (and GSSG) as mentioned in the Experimental Section. Control animals showed a mean GSH content of 1.78 μmol/g (range 1.68–1.89 μmol/g of wet tissue). One hour after a 200-μg injection of 6-OHDA, this decreased to 1.50 μmol/g and after 3 h was 1.22 μmol/g. (In all cases the GSSG content was less than 10% of the total GSH.) Thus the GSH decrease is not particularly striking. Some reflection indicates that this is quite reasonable. All cells, neuronal plus glial, contain GSH and even in the catecholamine-rich HT the uptake-specific 6-OHDA depletion of GSH probably represents only a small portion of the total GSH content of the region. Also, as suggested by a reviewer, much of the GSH may be in bound form and relatively inaccessible to interaction with GSH.

## Experimental Section

**Chemical Studies.** The 6-OHDA quinone was kindly supplied by Dr. P. A. Wehrli of Hoffman-La Roche. Alternatively, the *p*-quinone was generated by air oxidation of 6-OHDA. All other chemicals were reagent grade.

The liquid chromatographic separations and UV and NMR spectra, as well as electrochemical measurements, were described previously.[9] The mass spectra were obtained with a Varian-MAT

CH-5 spectrometer using an electron beam potential of 70 eV and a sample inlet temperature of 190 °C. The elemental analyses were carried out by Micro-Tech Laboratories, Inc., Skokie, Ill.

The product of oxidized 6-OHDA and GSH was prepared and isolated in the following fashion. An equimolar ratio of the quinone (64 mg of free base) and the GSH (117 mg) was placed in a small vacuum flask. Deaerated, double distilled water (10 mL/100 mg) was added and the flask quickly attached to the vacuum line to prevent air oxidation. The flask was then placed in a warm water bath (35–40 °C) until the solution became completely colorless (ca. 20 min). The product was then precipitated by adding 3–4 vol of 0.01 M citric acid in acetone. The acidity retarded further air oxidation. The sticky residue was separated by decantation, washed with pure acetone, and dried in vacuo. About a 70% yield of gray-brown product was typically obtained in several preparations.

**Animal Studies.** Male Sprague–Dawley rats (300–350 g) were anesthetized with Nembutal and a stereotaxic injection of 6-OHDA was made via a Hamilton 50-μL syringe with a 30-gauge needle. The coordinates for hypothalamus (A, 6.0; L, 0.05; D, -3.7) were obtained from the atlas by Pellegrino and Cushman.[11] Experimental rats received 100 or 200 μg of 6-OHDA (free base) dissolved in a saline–ascorbate (1 mg/mL) vehicle. The injection volumes were 5 and 10 μL for the two doses, respectively. All injections were made slowly over a 1-min period. Following a 30-s wait, the needle was withdrawn, the hole closed with a jeweler's screw, and the animal placed back in its cage until time of sacrifice.

To check the validity of the injections, similar injections of crystal violet dye solution were made in several rats. One hour after injection the animals were sacrificed and the brains examined histologically. In all cases the dye had diffused into an area ca. 4 mm in diameter around the needle tip and very little appeared to back up the injection tract. Thus it was verified that the entire hypothalamus (HT) was reasonably bathed with this style of injection.

Rats were sacrificed at 1- and 3-h intervals after injection. The brains were rapidly removed and placed on a glass plate over dry ice. The HT was dissected out and immediately frozen until analyzed. The HT from three animals were pooled and weighed. The pooled HT was homogenzied by sonication in 300 μL of cold 0.2 M perchloric acid and then centrifuged at 10 000 rpm for 15 min at 0 °C. A portion of the supernatant was used for GSH and GSSG analyses according to the method of Halprin[12] using glutathione reductase and NADPH. Other aliquots were injected directly into a LC column for detection of the reaction product of 6-OHDA and GSH.

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## References and Notes

(1) A. Saner and H. Thoenen, *Mol. Pharmacol.*, 7, 147 (1971).
(2) R. N. Adams, E. Murrill, R. McCreery, L. Blank, and M. Karolczak, *Eur. J. Pharmacol.*, 17, 287 (1972).
(3) R. L. McCreery, R. Dreiling, and R. N. Adams, *Brain Res.*, 73, 15 (1974).
(4) D. C. S. Tse, R. L. McCreery, and R. N. Adams, *J. Med. Chem.*, 19, 37 (1976).
(5) C. R. Creveling, A. Rotman, and J. W. Daly in "Chemical Tools in Catecholamine Research", Vol. I, G. Jonsson, T. Malmfors, and Ch. Sachs, Ed., North-Holland Publishing Co., Amsterdam, 1975, p 23.
(6) G. Jonsson and Ch. Sachs in ref 5, p 41.
(7) H. McIlwain and H. S. Bachelard, "Biochemistry and the Central Nervous System", Williams and Wilkins, Baltimore, Md., 1971, p 180.
(8) A. Meister in "Metabolism of Sulfur Compounds", D. M. Greenberg, Ed., Academic Press, New York, N.Y., 1975, pp 102, 103, 145.
(9) Y-O. Liang, R. M. Wightman, P. Plotsky, and R. N. Adams in ref 5, p 15.
(10) C. L. Blank, P. T. Kissinger, and R. N. Adams, *Eur. J. Pharmacol.*, 19, 391 (1972).
(11) L. J. Pellegrino and A. J. Cushman, "A Stereotaxic Atlas of the Rat Brain", Appleton, Century & Crofts, New York, N.Y., 1967.
(12) K. M. Halprin and A. Ohkawara, *J. Invest. Dermatol.*, 48, 149 (1967).

# Correction of Prior Estimate of the Biological Activity of an *N*-Trifluoroacetyl Analogue of D-*threo*-Chloramphenicol Relative to Chloramphenicol

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The inhibitory rate constants, $k$, for the inhibition of the rate of *Escherichia coli* generation at various concentrations, $C$, of chloramphenicol and its *N*-trifluoroacetyl analogue were determined in vitro in accordance with $k_{app} = k_0 - kC$ where $k_{app}$ is the apparent first-order generation rate constant at a drug concentration $C$ and $k_0$ is that constant in the absence of drug. The activity of the analogue was one-tenth that of chloramphenicol in contrast to a previously reported value.

Previously[1] our laboratories provided rate constants, $k_{app}$, for the generation of *Escherichia coli* in peptone broth USP affected by various concentrations, $C$, of various analogues of chloramphenicol by monitoring the total number of organisms, $N$, with time, $t$, in accordance with

$$\ln N = k_{app}t + \ln N_o \quad (1)$$

where $N_o$ was the total number of organisms at a time $t_0$ taken as zero. Linear plots were obtained when these $k_{app}$ values were plotted against the concentration of the substituted chloramphenicol, $C$, in accordance with

$$k_{app} = k_0 - kC \quad (2)$$

where $k_0$ is the generation rate constant in the absence of drug and $k$ is the inhibitory rate constant.

Recently,[2] it was brought to my attention that the action of the *N*-trifluoroacetyl analogue of D-*threo*-chloramphenicol (**1**) obtained from the same source[3] was not as inhibitory as chloramphenicol (**2**) in several systems. This was in contrast to the apparent results we had obtained. The compounds were restudied by the procedures given previously[1] and a systematic search was made of the original data to find a probable source of error.

## Results and Discussion

Additional **1**, obtained from the same source,[3] and the original material used in our previous studies were eval-