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Autoxidation versus Covalent Binding of Quinones as the Mechanism of Toxicity of Dopamine, 6-Hydroxydopamine, and Related Compounds toward C1300 Neuroblastoma Cells *in Vitro*

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SUMMARY

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The mechanism of cytotoxicity of 6-hydroxydopamine, 2,4,5-trihydroxyphenylalanine, dopa, dopamine, norepinephrine, and epinephrine was explored by asking whether cytotoxicity was a reflection of the potential for autoxidation of each polyphenol or of the sulfhydryl reactivity of its quinone products. The cytotoxicity of the polyphenols, as measured by inhibition of [^{3}H]thymidine incorporation into DNA by C1300 neuroblastoma cells in tissue culture, correlated with the rate of autoxidation, as measured spectrophotometrically or by oxygen electrode studies. Polarographic determinations of the oxidation potentials of the polyphenols were also predictive of cytotoxicity; the most cytotoxic compounds had the most negative half-wave potentials and thus were the most readily oxidized. By contrast, the sulfhydryl reactivity of the quinone oxidation products of the polyphenols, as measured by inhibition of purified calf thymus DNA polymerase α, exhibited an inverse relationship to the cytotoxicity of the polyphenols; the most toxic compounds, 6-hydroxydopamine and 2,4,5-trihydroxyphenylalanine, were oxidized to the least reactive quinone products. An alternative mechanism of toxicity was observed with *N*-acetyldopamine, which was oxidized to 4-(2-*N*-acetylaminoethyl)-1,2-benzoquinone, a potent sulfhydryl reagent. *N*-Acetyldopamine was more toxic than predicted by its half-wave potential or its rate of autoxidation. Furthermore, while norepinephrine completely neutralized 6-hydroxydopamine and 2,4,5-trihydroxyphenylalanine as cytotoxic agents, the toxicity of *N*-acetyldopamine was minimally affected. Thus we conclude that 6-hydroxydopamine and 2,4,5-trihydroxyphenylalanine kill cells through the production of H_2O_2, O_2^-, and OH·, while for dopamine and dopa the reaction of quinone oxidation products with nucleophiles probably also contributes to their cytotoxicity.

INTRODUCTION

Controversy persists over whether the toxicity of 6-hydroxydopamine for catecholamine neurons is mediated by superoxide (O_2^-) and hydroxyl (OH·) radicals and hydrogen peroxide (H_2O_2) (1-3) or whether this effect results from covalent binding of the quinone oxidation products of 6-hydroxydopamine with sulfhydryl or other nucleophilic groups within the cell

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(2–8). Persistence in the pursuit of this question is prompted by the possible role of 6-hydroxydopamine in the pathogenesis of Parkinson's disease, manic-depressive illness, and schizophrenia (9, 10).

Previous studies have established that oxidation products of 6-hydroxydopamine are covalently bound to proteins of the cytoplasm and axonal membranes of neurons in the course of 6-hydroxydopamine toxicity (7, 8). There is evidence that the hydroxy *p*-quinone product of 6-hydroxydopamine (**15**, Fig. 1) (11) is covalently bound to cysteinyl residues, e.g., of glutathione, *in vivo* (12). Other studies inculpate the cyclized aminochrome, **14**, and/or the indole quinone, **25** ($R_1 = R_2 = H$, $n = 1$), as the quinone bound to nucleophilic groups in effecting toxicity (13). While a correlation exists between cytotoxicity and the amount of 6-hydroxydopamine bound (7), this observation does not prove that it is the binding itself that results in cell death or axonal degeneration.

In addition to the generation of quinones, the process of autoxidation results in the generation of H_2O_2 by the reaction

$$RH_2 + O_2 \rightarrow R + H_2O_2$$

Another product of autooxidation is $O_2^{\cdot-}$, resulting from the fact that autoxidation of polyphenols proceeds via 1-electron transfer reactions:

$$O_2 + e^- \rightarrow O_2^{\cdot-}$$

The final species in this toxic triad, the hydroxyl radical (OH·), is thought to be formed by the Haber-Weiss reaction (14, 15):

$$H_2O_2 + O_2^{\cdot-} \rightarrow OH\cdot + OH^- + O_2$$

An appreciation of the toxicity of H_2O_2 has existed for some time, with a role in hemolysis after exposure to 8-aminoquinolines as a general model for this effect (16). The superoxide radical, $O_2^{\cdot-}$, may be toxic through its effects as an oxidant (15, 16) or through its role in the production of OH·.

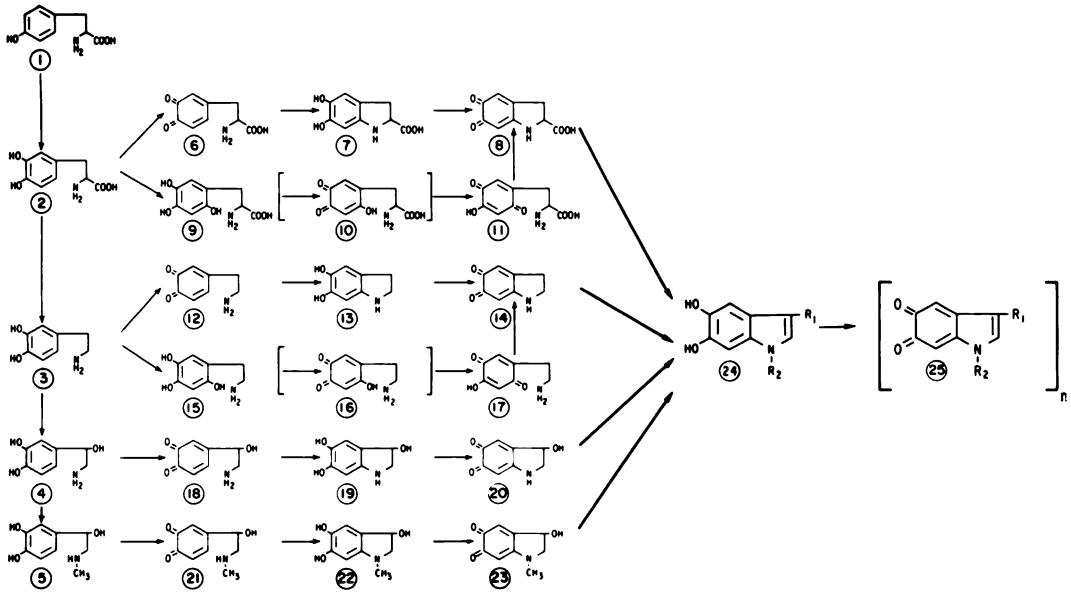


FIG. 1. *Proposed oxidative pathways for dopa and the catecholamines (11)*

1, tyrosine; **2**, dopa; **3**, dopamine; **4**, norepinephrine; **5**, epinephrine; **6**, dopaquinone; **7**, leukodopachrome; **8**, dopachrome; **9**, topa; **10**, *o*-quinone of topa; **11**, *p*-quinone of topa; **12**, dopamine *o*-quinone; **13**, leukoaminochrome; **14**, aminochrome; **15**, 6-hydroxydopamine; **16**, *o*-quinone of 6-hydroxydopamine; **17**, *p*-quinone of 6-hydroxydopamine; **18**, norepinephrine *o*-quinone; **19**, leukonoradrenochrome; **20**, noradrenochrome; **21**, epinephrine *o*-quinone; **22**, leukoadrenochrome; **23**, adrenochrome; **24**, dihydroxyindole (and derivatives); **25**, indole quinone (and derivatives). *n* refers to the likelihood that this species, perhaps along with other quinones in these pathways, polymerizes to form the various melanins.

The hydroxyl radical, well known for its part in irradiation-induced injury, notably damages DNA and membranes (15, 17). Thus the products of the univalent reduction of oxygen seem likely mediators of 6-hydroxydopamine cytotoxicity.

In cell-free systems superoxide dismutase and catalase speed the removal of O_2^- and H_2O_2, and thus these enzymes would be expected to protect the cell from the injurious effects of these species (15, 18). O_2^- can be quenched by epinephrine and norepinephrine (15, 19), while the highly reactive OH· can be absorbed by agents such as ethanol, benzoate, mannitol, or dimethyl sulfoxide (15, 17, 19) as well as by the electrophilic catecholamines (2). Suggestive of a major role for free radical species in 6-hydroxydopamine toxicity, experiments *in vivo* have shown that increased concentrations of norepinephrine will protect axon terminals from 6-hydroxydopamine toxicity (19).

In these studies 6-hydroxydopamine, topa,[1] dopamine, norepinephrine, and epinephrine and the related catechols *N*-acetyldopamine and isoproterenol have been evaluated as cytotoxic agents for C1300 neuroblastoma cells. These experiments asked whether the relative toxicity of these agents is correlated with the rate of autoxidation and the production of H_2O_2, O_2^-, and OH·, or with the reactivity of their quinone products as measured by inhibition of a representative sulfhydryl enzyme, purified calf thymus DNA polymerase α.

MATERIALS AND METHODS

Preparation of polyphenols and quinones. Dopamine, norepinephrine, epinephrine, isoproterenol, 6-hydroxydopamine, topa, and *N*-acetyldopamine (Sigma Chemical Company) and dopa (Calbiochem) were used immediately after being dissolved in deionized water. Dopachrome, **8**, was prepared by incubating dopa at 0.5 mg/ml with mushroom tyrosinase (*o*-phenol:O_2 oxidoreductase, EC 1.10.3.1; Worthington Biochemicals), 0.25 mg/ml in 0.025 M potassium phosphate buffer, pH 6.8, for 30 min at 25°. One-milliliter volumes were loaded onto 1 × 25 cm columns of Sephadex G-25 (fine) (Pharmacia) equilibrated and eluted with water. Topa and 6-hydroxydopamine were oxidized in the presence of tyrosinase to yield the *p*-quinones **11** and **17** (11, 20). Aminochrome, **14**, noradrenochrome, **20**, and adrenochrome, **23**, were produced through oxidation of dopamine, norepinephrine, and epinephrine with 2 Eq of sodium metaperiodate ($NaIO_4$). In like manner the yellow *o*-quinone, 4-(2-*N*-acetylaminoethyl)-1,2-benzoquinone, was obtained by oxidizing *N*-acetyldopamine with 1 Eq of $NaIO_4$. The quinone products were isolated by Sephadex chromatography. The instability of the quinones was minimized through conducting chromatography at 4° in the dark, using deaerated, demineralized water, and by assaying each for inhibition of calf thymus DNA polymerase immediately after isolation.

Autoxidation of polyphenols. The rate of autoxidation of polyphenols was determined spectrophotometrically, using a Beckman DU spectrophotometer with Gilford accessories, and by the rate of oxygen consumption as determined with a Gilson K-1C Oxygraph.[2]

Electrochemical measurements of the oxidation potentials of the polyphenols were made using voltametry, the measurement of current as a function of linearly changing applied potential. A rotating glassy carbon disc electrode (21) was chosen instead of a dropping mercury electrode, since most of the polyphenols oxidized at potentials anodic of the potential where mercury itself is oxidized, and also instead of a rotating platinum electrode, since the carbon electrode provided less overlap of the oxidation signals of polyphenols and solvent (water). The glassy carbon disc electrode was rotated using a Sargent 1800-rpm synchronous motor. A saturated calomel electrode was the reference electrode, and a Sargent model XV polarograph was utilized for voltage application and current measurement. Voltammetric measurements were started at −0.60 V (vs. the saturated calomel elec-

[1] The abbreviations used are: topa, 2,4,5-trihydroxyphenylalanine (6-hydroxydopa); TCA, trichloracetic acid.

[2] We thank Dr. R. B. Jennings and Ms. Sonnie Hill for the use of this instrument.

trode) and varied in the anodic direction at the rate of 0.3 V/min. As oxidation occurred, a wavelike signal with a plateau current was obtained for each. The voltage at the midpoint of the deflection was determined graphically and taken as the half-wave potential. Polyphenols were dissolved in nitrogen-saturated 0.1 M sodium phosphate, pH 6.8, at concentrations of 0.5–1 mM. Four to eight determinations were performed at 22° with each polyphenol.

Inhibition of [^{3}H]thymidine incorporation by C1300 neuroblastoma cells. The C1300 neuroblastoma was obtained from Jackson Laboratory as a subcutaneous tumor in A/J mice. The tumor was carried through serial subcutaneous passages of 1 mm^3 at 2-week intervals. Tumors were removed using a sterile technique, minced with scissors, and dissociated by trypsin (0.4%) at room temperature for 10 min. Cells were isolated by centrifugation and resuspended in RPMI 1640 medium (Grand Island Biological Company) with glutamine (2 mM) and 20% fetal calf serum (Reheis Chemical Company). Cells were diluted to a concentration of 10^6 living cells/ml, using trypan blue exclusion as the index of viability. Triplicate 100-μl volumes of the cell suspension were pipetted into 5-ml Falcon tubes and exposed to 30-μl volumes of water or polyphenol (freshly dissolved in water and sterilized by Nalgene filtration) for 15 min at room temperature. Then 1 μl of [^{3}H]thymidine (1 μCi, 40–60 Ci/mmole) was added, and the cells were incubated at 37° in 95% air–5% CO_2 for 3 hr. The incubation was terminated by placing the tubes on ice and adding 5 ml of cold 5% TCA. Labeled DNA was collected on GF/C glass fiber filters in a Millipore manifold, washed with 15 ml of cold 1% TCA and 3 ml of 95% ethanol, dried, and digested with 0.5 ml of "NCS" (Amersham) by the procedure described by Schrier and Wilson (22). The samples were counted in 15 ml of toluene with 2,5-diphenyloxazole (4 g/liter) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.1 g/liter), using a Beckman LS150 scintillation spectrometer. Counting efficiency averaged 48%.

Inhibition of calf thymus DNA polymerase α by quinone sulfhydryl reagents. DNA polymerase α was isolated from calf thymus by the method of Yoneda and Bollum (23) as described previously (24), using glands obtained from the Randolf Packing Company, Ashboro, N. C. The purified enzyme was stored at −20° in 50% glycerol–50 mM Tris-HCl, pH 7.2, with 1 mM dithioerythritol and 1 mM EDTA. Prior to exposure to sulfhydryl reagents, the enzyme was dialyzed against two 1000-fold volumes of 50% glycerol buffer without dithioerythritol at −20°. The enzyme was assayed as described previously (24). A final volume of 50 μl was constituted on ice and contained 50 mM Tris (pH 7.5), 7 μM [^{3}H]TTP (12,600 dpm/pmole, New England Nuclear), 80 μM dATP, dGTP, and dCTP (Sigma), 4 mM $MgCl_2$, 2.5 μg of activated calf thymus DNA [Sigma, activated with pancreatic deoxyribonuclease as described earlier (25)], 5% glycerol, and sufficient enzyme to result in the incorporation of 2–3 pmoles of [^{3}H]TTP into DNA after 30 min of incubation at 37°. Quinones were evaluated as inhibitors of DNA polymerase α by including them in the reaction mixture, without previous incubation of the quinones with the enzyme. The reaction was terminated by placing the tubes on ice and adding 0.1 ml of 0.1 M sodium pyrophosphate, 1 drop of heat-denatured salmon sperm DNA (Sigma, 30 A_{260} units/ml), and 5 ml of cold 5% TCA, and the labeled DNA was collected on GF/C filters and washed, digested, and counted as described above.

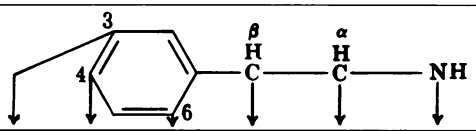
RESULTS

Polyphenolic derivatives of dopamine were evaluated as cytotoxic agents for C1300 neuroblastoma cells, utilizing inhibition of [^{3}H]thymidine incorporation into DNA as the index of cell injury. In Table 1 the effects of increasing concentrations of each on the incorporation of [^{3}H]thymidine are presented. Dopamine was found to be much more toxic than the β-hydroxylated catecholamines norepinephrine, epinephrine, and isoproterenol, suggesting either that dopamine was more readily oxidized within the cell or that its quinone products were more reactive. In like fashion, dopamine was more toxic than dopa, but less toxic than its *N*-acetylated derivative. By

TABLE 1

Effects of catecholamine derivatives on [^{3}H]thymidine incorporation by C1300 neuroblastoma cells

C1300 neuroblastoma cells (10^5) were exposed to the concentrations of polyphenol given, and the incorporation of [^{3}H]thymidine into DNA was determined as described in MATERIALS AND METHODS.

Inhibitor							Concentration	[^{3}H]Thymidine incorporation	Inhibition
							μM	*dpm ± SEM*	%
None								811,558 ± 12,934	0
Dopamine	OH	OH	H	H	H	H	50	656,418 ± 54,948	19
							100	604,418 ± 3,512	26
							150	444,367 ± 30,040	45
Norepinephrine	OH	OH	H	OH	H	H	50	776,661 ± 15,256	4
							100	776,176 ± 12,347	5
							150	774,354 ± 21,210	5
							1,000	596,878 ± 18,676	26
Epinephrine	OH	OH	H	OH	H	CH_3	50	906,647 ± 16,374	0
							100	873,576 ± 25,963	0
							150	877,286 ± 6,005	0
							1,000	573,454 ± 21,331	29
Isoproterenol	OH	OH	H	OH	H	C_3H_7	50	849,311 ± 27,668	0
							100	734,230 ± 15,378	10
							150	766,992 ± 13,401	6
							1,000	54,294 ± 4,208	93
6-Hydroxydopamine	OH	OH	OH	H	H	H	50	385,040 ± 4,176	53
							100	207,091 ± 16,378	75
							150	82,875 ± 2,660	90
Topa	OH	OH	OH	H	COOH	H	50	259,839 ± 7,036	68
							100	93,464 ± 9,540	88
							150	61,211 ± 2,777	92
Dopa	OH	OH	H	H	COOH	H	50	756,347 ± 54,338	7
							100	675,389 ± 2,181	17
							150	574,426 ± 22,056	29
N-Acetyldopamine	OH	OH	H	H	H	$COCH_3$	50	569,424 ± 5,366	30
							100	296,088 ± 8,405	64
							150	157,501 ± 9,461	81

far the most toxic species were the trihydroxyphenols, 6-hydroxydopamine and topa.

The rates of autoxidation of the polyphenols could be compared by observing the production of aminochrome or hydroxy-*p*-quinone products in atmosphere-equilibrated buffers at room temperature. Autoxidation was accelerated at higher pH values, and the rates of autoxidation of 6-hydroxydopamine and topa were much greater than those of the other polyphenols (Table 2).

The initial rates of oxygen consumption by the various polyphenols are presented in Table 3. Again the greater rates of autoxidation of 6-hydroxydopamine and topa are apparent, along with the observation that dopamine is oxidized more rapidly than dopa, *N*-acetyldopamine, or norepinephrine. These data correlate with previously published observations by Creveling and his colleagues regarding the rate of oxygen consumption by 6-hydroxydopamine, dopamine, and analogous compounds (5, 26). The O_2 consumption data correlated with the ease of oxidation as evaluated by polarography. The half-wave potentials ($E_{1/2}$) against a saturated calomel electrode were found to be most negative for the most readily oxidized polyphenols. The slope of the change in current vs. applied potential suggested a degree of irreversibility for the oxidation of all the polyphenols tested.

TABLE 2

Autoxidation of polyphenols

A 0.2 mM concentration of each polyphenol was allowed to oxidize in 40 mM buffer (sodium phosphate for pH 6.2–7.2, sodium carbonate for pH 9.0–10.5), and initial rates were calculated from tangents drawn to the recording of absorbance at 480 nm vs. time.

Polyphenol	pH 6.2	pH 6.8	pH 7.2	pH 9.0	pH 9.5	pH 10.0	pH 10.5
				$\Delta A_{480}/min$			
6-Hydroxydopamine	0.0189	0.0586	0.0759	>1			
Topa	0.0156	0.0363	0.0559	>1			
Dopamine	0.0001	0.0002	0.0003	0.0054	0.0137	0.0284	0.0493
Norepinephrine				0.0008	0.0025	0.0062	0.0215
Dopa					0.0019	0.0045	0.0211
Epinephrine					0.0023	0.0063	0.0151

TABLE 3

Autoxidation and cytotoxicity of polyphenols and sulfhydryl reactivity of their quinones

Oxygen consumption was determined at 30° by dissolving polyphenols at 0.16 mM in 0.05 M sodium carbonate buffer, pH 10.5, and recording initial rates of O_2 utiliziation. Values are means ± standard errors of at least four determinations. Half-wave potentials were determined as described under MATERIALS AND METHODS. The polyphenol concentrations required for 50% inhibition of [^{3}H]thymidine incorporation are the means of four or more values obtained graphically; variation among experiments was seen, particularly with 6-hydroxydopamine and topa, depending on the amount of autoxidation that had taken place before pipetting. Similarly, by choosing concentrations above and below those producing 50% inhibition of calf thymus DNA polymerase α, the concentrations yielding this degree of inhibition were determined graphically three or more times. For the *p*-quinone of topa, concentrations up to 500 μM resulted in only 30% inhibition of the enzyme.

Polyphenol inhibitor	O_2 consumed by 0.5 μM polyphenol	E_1	[Polyphenol] for 50% inhibition of [^{3}H]-thymidine incorporation	Resulting quinone	[Quinone] for 50% inhibition of DNA of polymerase α
	μmoles/min	*V*	μM		μM
6-Hydroxydopamine	15.90 ± 2.16	−0.09 ± 0.01	37	**17**	1700
Topa	14.76 ± 1.88	+0.09 ± 0.01	45	**11**	>500
Dopamine	1.67 ± 0.31	+0.24 ± 0.01	162	**14**	150
N-Acetyldopamine	0.28 ± 0.05	+0.28 ± 0.01	64	**(12)**[a]	13
Dopa	0.80 ± 0.14	+0.30 ± 0.03	196	**8**	155
Norepinephrine	0.33 ± 0.09	+0.37 ± 0.02	>1000	**20**	130

[a] The quinone resulting from the oxidation of *N*-acetyldopamine is an analogue of **12**, 4-(2-*N*-acetylaminoethyl)-1,2-benzoquinone.

Thus $E_{1/2}$ would not be the same as E^0, the standard or equilibrium potential, whereas E^0 and $E_{1/2}$ are equivalent in reversible systems (27). The slopes of current vs. voltage were roughly the same for all the polyphenols, however, so that a comparison of half-wave potentials appeared to be a valid reflection of the relative reducing powers of these compounds.

The O_2 consumption by *N*-acetyldopamine was less than would be predicted by its $E_{1/2}$. Since the acetyl group renders the amino nitrogen non-nucleophilic, the *o*-quinone product could not cyclize to leukoaminochrome, **13**, and thus a second equivalent of O_2 was not utilized.

The rate of autoxidation was found to correlate with the cytotoxicity of the polyphenols, a relationship documented previously (26). The cytotoxicity for each is presented as the concentration required to achieve 50% inhibition of [^{3}H]thymidine incorporation into DNA by C1300 neuroblastoma cells in tissue culture (Table 3). The trihydroxyphenols, 6-hydroxydopamine and topa, effected 50% inhibition at concentrations less than 50 μM, while 160–200 μM was required for dopamine and dopa. Nor-

epinephrine and epinephrine (Table 1) displayed little toxicity, requiring greater than 1 mM concentrations to reach this end point. One compound was more toxic than predicted by its half-wave potential or its rate of autoxidation: *N*-acetyldopamine caused 50% inhibition of [^{3}H]thymidine incorporation at 64 μM.

A possible explanation for the greater-than-predicted toxicity of *N*-acetyldopamine is provided in Table 3. The reactivity of its quinone oxidation product, 4-(2-*N*-acetylaminoethyl)1,2-benzoquinone, against calf thymus DNA polymerase α was much greater than that of aminochrome, **14**, dopachrome, **8**, or noradrenochrome, **20**. The *o*-benzoquinone from *N*-acetyldopamine produced 50% inhibition of this enzyme at 13 μM [the corresponding concentration for *p*-chloromercuribenzoate was 4 μM (24)], while 130–155 μM concentrations were required for the latter quinones and also for adrenochrome, **23**. The hydroxy-*p*-quinone products of 6-hydroxydopamine (**17**) and topa (**11**) were exceedingly weak sulfhydryl reagents by comparison, the latter quinone effecting less than 30% inhibition with concentrations in excess of 500 μM.

Table 4 shows not only that norepinephrine is minimally toxic to C1300 neuroblastoma cells but that its presence provides protection against the toxicity of 6-hydroxydopamine or topa. A 2-fold molar excess of norepinephrine over 6-hydroxydopamine or topa significantly reduced the inhibition of [^{3}H]thymidine incorporation by these two polyphenols, and a 20-fold molar excess of norepinephrine completely neutralized their toxic effects. By contrast, the toxicity of *N*-acetyldopamine was affected little if any. The reduction of toxicity of dopamine or dopa by norepinephrine was less striking than that of 6-hydroxydopamine or topa.

TABLE 4

Effect of norephinephrine on polyphenol toxicity

C1300 neuroblastoma cells (10^5) were incubated with polyphenols at the concentrations shown, and [^{3}H]thymidine incorporation into DNA was determined after 3 hr. Cells exposed to water in medium without norepinephrine displayed a control incorporation of 562,839 ± 17,271 (SEM) dpm, while cells in medium containing 0.1 mM norepinephrine incorporated 641,529 ± 8,404 dpm and cells in medium containing 1 mM norepinephrine incorporated 345,000 ± 21,166 dpm.

Inhibitor	Concentration	Inhibition of [^{3}H]thymidine incorporation		
		Control	0.1 mM norepinephrine	1 mM norepinephrine
	μM	%	%	%
6-Hydroxydopamine	50	59	15	1
Topa	50	57	6	0
Dopamine	200	74	32	47
Dopa	200	37	8	30
N-Acetyldopamine	100	68	60	69

DISCUSSION

These studies provide evidence for two clearly separate modes of cytotoxicity by the polyphenols included in these experiments: (a) the production of H_2O_2, O_2^-, and $OH\cdot$ as the result of autoxidation and (b) the production of quinone species, which kill cells through inhibition of sulfhydryl enzymes and reaction with other nucleophilic groups within the cell. The following evidence suggests that the first mechanism largely accounts for the cytotoxicity of 6-hydroxydopamine and topa: (a) the correlation between the rate of autoxidation and the cytotoxicity of the polyphenols tested, (b) the observation that the hydroxy-*p*-quinone products **11** and **17** of topa and 6-hydroxydopamine oxidation are exceedingly weak as sulfhydryl reagents, and (c) neutralization of the toxicity of these two species by a slight excess of norepinephrine, an efficient scavenger of the O_2^- and $OH\cdot$ produced during autoxidation (15, 16, 19).

It is noteworthy that while O_2^- can act as a chain-propagating radical in the autoxidation of polyphenols (15), autoxidation of polyphenols would continue in spite of the reaction of O_2^- with norepinephrine. Thus the quenching of O_2^- (and $OH\cdot$) by norepinephrine would be beneficial in removing these toxic species from solution rather than through an effect on the rate of autoxidation per se. Norepinephrine would be oxidized in the process to the *o*-quinone,

18, which would be more likely to cyclize to form the weakly reactive noradrenochrome, 20, than react with external nucleophiles (11, 28, 29).

Whereas 6-hydroxydopamine and topa appear to be toxic through the production of free radical species, these studies show that the cytotoxicity of *N*-acetyldopamine is largely mediated by the reaction of its quinone product with nucleophilic groups within the cell. *N*-Acetyldopamine results in greater inhibition of [^{3}H]thymidine incorporation by C1300 neuroblastoma cells than would be predicted from its half-wave potential or its rate of autoxidation. Furthermore, its *o*-quinone product is a much more potent inhibitor of calf thymus DNA polymerase α than are quinones of the aminochrome group (8, 14, 20, and 23) and is particularly more effective as an inhibitor than the hydroxy-*p*-quinones, 11 and 17. The observations of Borchardt (13) confirm the weak sulfhydryl reactivity of hydroxy-*p*-quinones with another enzyme sensitive to sulfhydryl reagents, catechol *O*-methyltransferase. Consistent with the supposition that *N*-acetyldopamine kills neuroblastoma cells through reactivity with nucleophiles, such as cysteinyl residues of enzymes in DNA synthesis and energy production, its cytotoxicity was not significantly affected by adding norepinephrine.

The effects of norepinephrine on the cytotoxicity of dopamine and dopa were intermediate in degree, suggesting that the autoxidation of these compounds may result in cytotoxicity through both mechanisms. The production of H_2O_2, $O_2^{\cdot-}$, and OH· could result from the autoxidation of catechols 2 and 3 to *o*-quinones 6 and 12 and of leukoaminochrome, 13, and leukodopachrome, 7, to aminochrome, 14, and dopachrome, 8. The *o*-quinones, 6 and 12, would be reactive with external nucleophiles, such as sulfhydryl, amino, or phenolic hydroxyls, as an alternative to cyclization, and would produce cell injury by this mechanism (28). The aminochrome and dopachrome products of internal cyclization would have lesser degrees of reactivity with external nucleophiles.

The greater cytotoxicity of dopamine compared with norepinephrine or epinephrine appears to derive from two major factors. Dopamine undergoes autoxidation more readily than the other catecholamines, thus generating more toxic products. As previously demonstrated, the oxidation of epinephrine or norepinephrine to the *o*-quinones is followed by more rapid cyclization than is seen with dopamine (11, 29). Tse *et al.* (30) observed that the reaction of dopamine *o*-quinone with sulfhydryl groups was more rapid than internal cyclization by a factor of 10^3. Thus the dopamine *o*-quinone, 12, represented in this study by the oxidation product of *N*-acetyldopamine, has a finite lifetime within the cell in which to react with external nucleophiles such as critical cysteinyl residues of enzymes. If, as suggested by Senoh *et al.* (31), the oxidation of dopamine also results in 6-hydroxydopamine, an even more toxic species would be produced. Adams, however, considered this last reaction unlikely, since the rate of cyclization of 12 is much greater than its rate of reaction with water (10, 30).

A variable not tested in these studies was the relative rate of uptake of the polyphenols by C1300 cells. The greater lipid solubility of *N*-acetyldopamine over dopamine may result in accelerated entry into the cell. The ensuing higher intracellular concentration of *N*-acetyldopamine would then be expected to result in greater toxicity. Similarly, the possible competition by norepinephrine for polyphenol entry into C1300 cells has not yet been explored. This may differ among the various analogues and must remain a possible explanation for the effects of norepinephrine reported here.

An important consideration in attempting to apply these observations to the intact nervous system is the composition of the intracellular milieu, particularly with regard to ascorbate and GSH. The re-reduction of quinones to their parent polyphenols by ascorbate (2, 30) would certainly reduce the quantity of catecholamine carbon atoms incorporated into the neuromelanin polymer in catecholamine neurons (11). Whether this would be protective or harmful for the neuron with regard to 6-hydroxydopamine and dopamine toxicity would depend upon which are the most important toxic species, the products of ox-

ygen reduction or the quinones resulting from 6-hydroxydopamine and dopamine oxidation. Re-reduction of the *p*-quinone, **17**, and of dopamine *o*-quinone, **12**, would again generate the autoxidizable species **3** and **15**, with the resulting additional production of $O_2^{\cdot-}$, OH·, and H_2O_2, while denying reactive quinones to surrounding nucleophiles.

Through arylation of its cysteinyl residue, GSH protects the cell from the toxicity of 6-hydroxydopamine and related compounds, with different affinities for each of the quinone species (12, 13). Whether the *p*-quinone, **17**, can react with two sulfhydryl groups is a matter of dispute (6, 8, 12). Reaction with 2 cysteinyl residues by **17** or its cyclized forms, **14** and **25** ($R_1 = R_2 = H$, $n = 1$), would be necessary to explain the covalent cross-linking of bovine serum albumin and other proteins observed by Rotman *et al.* (6, 8).

Whether calf thymus DNA polymerase α, as reported in this study, or catechol *o*-methyltransferase (13) is truly representative of the sulfhydryl-sensitive enzymes within the cell, it is noteworthy that *p*-hydroxyquinones like **11** and **17** were much less effective inhibitors of both enzymes than quinones of the aminochrome class (**8**, **14**, **20**, and **23**). As demonstrated by Liang *et al.* (12), however, 6-hydroxydopamine *p*-quinone, **17**, does react with GSH, and ostensibly other cysteinyl residues, both *in vitro* and *in vivo*. Thus, while statements of relative reactivity may be valid, if the *p*-quinone, **17**, is present within the cell in high enough concentration it is likely to react with a sufficient quantity of sulfhydryl groups to result in cell injury or death. Thus if, as suggested by these studies, the primary cytotoxic species in 6-hydroxydopamine toxicity are $O_2^{\cdot-}$, OH·, and H_2O_2, the sulfhydryl reactivity of the resulting quinones still probably makes some contribution to 6-hydroxydopamine cytotoxicity. The work reported by Borchardt *et al.* (3) supports this conclusion; methylated derivatives of 6-hydroxydopamine capable of generating both reactive quinones and free radicals were slightly more toxic than those derivatives which resulted only in the genesis of H_2O_2, $O_2^{\cdot-}$, and OH· (3).

Several additional speculations come to mind as the result of the experiments reported here. An abnormality in dopamine metabolism or transport that led to increased levels of dopamine within the cytosol could result in autoxidation to reactive quinones and the liberation of free radical species. Such injury over the course of time could explain the premature degeneration of dopaminergic neurons seen in idiopathic Parkinson's disease, a likely example of Gowers' concept of abiotrophy (32). The treatment of parkinsonism with L-dopa might have harmful as well as beneficial consequences according to the data presented in this paper. Oxidation of dopa to dopaquinone, **6**, or topa, **9**, or of dopamine to its products would present the neuron with additional toxic species.

The demonstrated cytotoxicity of 6-hydroxydopamine for neuroblastoma cells *in vivo* (33) as well as *in vitro* (34, 35) suggests that this or similar compounds may have potential application in the treatment of this neoplasm in humans. The specificity of this agent for neuroblastoma cells is more a product of selective entry into neuroblastoma cells (34) than of any specific metabolic transformation within the tumor cell. These observations hold promise for an agent that could be concentrated by neuroblastoma cells and metabolized to a trihydroxyphenol such as 6-hydroxydopamine, thus resulting in cytotoxicity through autoxidation and the production of toxic free radicals.

REFERENCES

1. Cohen, G. & Heikkila, R. E. (1974) *J. Biol. Chem.*, **249**, 2447–2452.
2. Sachs, C. & Jonsson, G. (1975) *Biochem. Pharmacol.*, **24**, 1–8.
3. Borchardt, R. T., Burgess, S. K., Reid, J. R., Liang, Y. O., & Adams, R. N. (1977) *Mol. Pharmacol.*, **13**, 805–818.
4. Saner, A. & Thoenen, H. (1971) *Mol. Pharmacol.* **7**, 147–154.
5. Creveling, C. R., Lundstrom, J., McNeal, E. T., Tice, L. & Daly, J. W. (1975) *Mol. Pharmacol.*, **11**, 211–222.
6. Rotman, A., Daly, J. W. & Creveling, C. R. (1976) *Mol. Pharmacol.*, **12**, 887–899.
7. Jonsson, G. (1976) *Med. Biol.*, **54**, 406–420.
8. Rotman, A., Daly, J. W., Creveling, C. R. & Breakefield, X. O. (1976) *Biochem. Pharmacol.*,

25, 383–388.

9. Stein, L. & Wise, C. D. (1971) *Science,* **171,** 1032–1036.
10. Adams, R. N. (1974) *Bull. Menninger Clin.,* **38,** 57–69.
11. Graham, D. G. (1978) *Mol. Pharmacol.,* **14,** 633–643.
12. Liang, Y. O., Plotsky, P. M. & Adams, R. N. (1977) *J. Med. Chem.,* **20,** 581–583.
13. Borchardt, R. T. (1975) *Mol. Pharmacol.,* **11,** 436–449.
14. Haber, F. & Weiss, J. (1934) *Proc. R. Soc. Lond., Ser. A,* **147,** 332–351.
15. Fridovich, I. (1975) *Annu. Rev. Biochem.,* **44,** 147–159.
16. Goldberg, B. & Stern, A. (1977) *Arch. Biochem. Biophys.,* **178,** 218–225.
17. Morgan, R. A., Cone, R. L. & Elgert, T. M. (1976) *Nucleic Acids Res.,* **3,** 1139–1149.
18. Cohen, G. & Hochstein, P. (1964) *Biochemistry,* **3,** 895–900.
19. Sachs, C., Jonsson, G., Heikkila, R. & Cohen, G. (1975) *Acta Physiol. Scand.,* **93,** 345–351.
20. Graham, D. G. & Jeffs, P. W. (1977) *J. Biol. Chem.,* **252,** 5729–5734.
21. Stulikova, M. & Vydra, F. (1972) *J. Electroanal. Chem.,* **38,** 349–357
22. Schrier, B. K. & Wilson, S. H. (1973) *Anal. Biochem.,* **56,** 196–207.
23. Yoneda, M. and Bollum, F. J. (1965) *J. Biol. Chem.,* **240,** 3385–3391.
24. Graham, D. G., Tiffany, S. M. & Vogel, F. S. (1978) *J. Invest. Dermatol.,* **70,** 113–116.
25. Graham, D. G., Tye, R. W. & Vogel, F. S. (1977) *Cancer Res.,* **37,** 436–439.
26. Lundstrom, J., Ong, H., Daly, J. & Creveling, C. R. (1973) *Mol. Pharmacol.,* **9,** 505–513.
27. Heyrovsky, J. (1966) in *Principles of Polarography,* pp. 121–145, 205–266, Academic Press, New York.
28. Scheulen, M., Wollenberg, P., Bolt, H. M., Kappus, H. & Remmer, H. (1975) *Biochem. Biophys. Res. Commun.,* **66,** 1396–1400.
29. Hawley, M. D., Tatawawadi, S. V., Piekarski, S. & Adams, R. N. (1967) *J. Am. Chem. Soc.,* **89,** 447–450.
30. Tse, D. C. S., McCreery, R. L. & Adams, R. N. (1976) *J. Med. Chem.,* **19,** 37–40.
31. Senoh, S., Creveling, C. R., Udenfriend, S. & Witkop, B. (1959) *J. Am. Chem. Soc.,* **81,** 6236–6240.
32. Gowers, W. R. (1902) *Lancet,* **1,** 1003–1007.
33. Chelmicka-Szorc, E. & Arnason, B. G. W. (1976) *Cancer Res.,* **36,** 2382–2384.
34. Angeletti, P. U. & Levi-Montalcini, R. (1970) *Cancer Res.,* **30,** 2863–2869.
35. Prasad, K. N. (1971) *Cancer Res.,* **31,** 1457–1460.