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Oxygen-Dependent Reaction of 6-Hydroxydopamine, 5,6-Dihydroxytryptamine, and Related Compounds with Proteins *in Vitro*: a Model for Cytotoxicity

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SUMMARY

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An oxygen-dependent, covalent interaction of radioactive 6-hydroxydopamine, 5,6-dihydroxtryptamine, and related compounds with several model proteins *in vitro* is described. 6-Hydroxydopamine and 5,6-dihydroxytryptamine react with bovine serum albumin in the presence of oxygen to yield a total of about 12 moles of amine bound per mole of protein. The nature of the oxidation product which reacts rapidly has not been defined, but in the case of 6-hydroxydopamine the amine moiety of the side chain, and hence cyclization to a dihydroxyindole, is not required. 5,7-Dihydroxytryptamine, norepinephrine, and dopamine react much more slowly, and tyramine, serotonin, and nonphenolic amines, not at all. The reaction of 6-hydroxydopamine and 5,6-dihydroxytryptamine appears to occur almost exclusively with sulfhydryl groups of proteins, but both amines appear capable of generating additional free sulfhydryl groups by reduction of disulfide bonds in proteins. Denaturation of proteins such as albumins or hemoglobins with 6 M urea results in a marked increase in the amount of radioactive amine bound per mole of protein. This binding approaches the theoretical number of potential free sulfhydryl groups, suggesting that in the native proteins certain sulfhydryl and/or disulfide bonds are inaccessible to the amine. In proteins which do not contain disulfide bonds, blockade of free sulfhydryl groups with *N*-ethylmaleimide renders the protein resistant to interaction with 6-hydroxydopamine. Polylysine reacts only slowly with 6-hydroxydopamine. The reaction product of radioactive 6-hydroxydopamine and bovine serum albumin consists primarily not of monomeric protein but of a series of polymeric proteins as determined by gel filtration and polyacrylamide gel electrophoresis. The cross-linking of bovine serum albumin to form polymers which occurred with both 6-hydroxydopamine and 5,6-dihydroxytryptamine was irreversible. Incorporation of more than 2 moles of amine per mole of protein appeared to be required for cross-linking. Acid hydrolysis of 6-hydroxydopamine–cross-linked bovine serum albumin yielded two radioactive, ninhydrin-positive products, which are suggested to be cysteine derivatives. The covalent binding and induction of cross-linking of proteins *in vitro* by 6-hydroxydopamine and 5,6-dihydroxytryptamine is discussed from a mechanistic standpoint and as a possible model for the mechanism of the cytotoxic actions of these compounds *in vivo*.

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INTRODUCTION

Autoxidizable phenethylamines, of which 6-hydroxydopamine is the best known example, have been shown to have a selective cytotoxic action upon catecholamine-containing neurons in both the peripheral and central nervous system (1). A similar cytotoxic action upon central serotonergic neurons has been shown for 5,6- and 5,7-dihydroxtryptamine (2–4), and upon peripheral noradrenergic neurons, by 5,7-dihydroxytryptamine (5). The selectivity of the cytotoxic effect of these amines is derived from the specificity of their active uptake at the plasma membrane of the nerve terminal (6). The cytotoxic action takes place when a critical intraneuronal concentration of the amine is achieved through the action of the transport mechanism in the plasma membrane. The phenethylamine derivatives which induce this cytotoxicity are uniformly capable of undergoing rapid autoxidation (5). However, the specific molecular events resulting in cytotoxicity are not understood. Two possible mechanisms have been proposed: one relates to the rapid generation during autoxidation of the amines of hydrogen peroxide, superoxide ion, and hydroxyl radical (7, 8); the other suggests that the covalent interaction of one or more of the oxidation products of the amine with intraneuronal proteins is responsible for the cytotoxicity (9–11).

In the present communication the oxygen-dependent, covalent interaction of 6-hydroxydopamine, 5,6-dihydroxytryptamine, and a series of related compounds with bovine serum albumin and other model proteins is reported. A comparison of the ability of such agents to induce extensive cross-linking of model proteins and a partial analysis of the chemical nature of the cross-linking reaction are described. Cross-linking of proteins by 6-hydroxydopamine in cultured neuroblastoma cells has been observed (10). The cross-linking of proteins by 6-hydroxydopamine and other compounds is discussed as a basis for the cytotoxic mechanism of these agents *in vivo*. Certain aspects of these studies have been reported in preliminary form (11).

MATERIALS AND METHODS

Materials. [^{3}H]3,4-Dihydroxyphenethylamine (8.0 Ci/mmole), [^{14}C]5-hydroxytryptamine bioxalate (25 mCi/mole), [^{14}C]tryptamine bisuccinate (40 mCi/mmole), and sodium [^{3}H]borohydride (more than 1.0 mCi/mmole) were purchased from New England Nuclear Corporation. [^{3}H]6-Hydroxydopamine HCl (90 mCi/mmole) and [^{3}H]β-phenethanolamine HCl (30 mCi/mmole) were prepared according to Rotman *et al.* (12). [^{3}H]5,7-Dihydroxytryptamine (45 mCi/mmole) was prepared according to the procedure of Lee *et al.* (13), modified for use with lithium aluminum [^{3}H]hydride. [^{3}H]5,6-Dihydroxytryptamine (5.6 mCi/mmol) was prepared by the following modification of the method of Lee *et al.* (13). 5,6-Dibenzyloxy-3-(β-nitrovinyl)indole (10 mg) was added to a suspension of $LiAl^3H_4$ (1.4 mg, 5 mCi) and $LiAlH_4$ (5 mg) in dry tetrahydrofuran, and the mixture was stirred with refluxing for 18 hr. After cooling, H_2O was added, and the tetrahydrofuran was evaporated under vacuum. The residue was extracted into ether, and the extract was dried over Na_2SO_4. The product, 5,6-dibenzyloxytryptamine, was precipitated from the ether as the hydrochloride salt (yield, 45%). Final conversion to [^{3}H]5,6-dihydroxytryptamine was accomplished by hydrogenation at atmospheric pressure for 6 hr with palladium on barium sulfate as catalyst in ethanol, followed by filtration.

6-Hydroxydopamine HCl, 5,6- and 5,7-dihydroxytryptamine creatinine sulfate, and 5,6-dihydroxy-α-methyltryptamine oxalate were obtained from Regis Chemical Company under Research Contract SA 43-pH-3021 and provided by Dr. A. A. Manian, Psychopharmacology Service Center, National Institute of Mental Health. 5,6-Dihydroxyindole was prepared by catalytic debenzylation of 5,6-dibenzyloxyindole (Sigma Chemical Company) with 10% pallodium on charcoal in ethanol at 45 p.s.i. for 24 hr. 2,3,4,5-Tetrahydroxyphenethylamine HCl was supplied by Dr. J. Lundstrom, Astra Läkemedal AB, Sweden. 6-Hydroxynorepinephrine HCl (14) and β-(2,4,5-trihydroxyphenyl)nitroethane

(12) were prepared as previously described. Bovine serum albumin (recrystallized six times) and ovalbumin (recrystallized six times) were obtained from Armour Pharmaceutical Company. Human hemoglobin (recrystallized twice) and bovine hemoglobin (recrystallized twice) were obtained from Mann Research Laboratories. Aldolase and dextran blue 2000 were obtained from Pharmacia. Rabbit IgG[2] was obtained from Dr. Cornelius Glaudemans, Laboratory of Chemistry, and horse hemoglobin, from Dr. Richard Simpson, Laboratory of Biophysical Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases. Rat liver catechol *O*-methyltransferase was prepared as described previously (15). All other materials were obtained from standard commercial sources.

Determination of binding of amines to proteins. BSA, ovalbumin, and the hemoglobins were prepared as 1.0% solutions in 6.7 mM sodium phosphate buffer, pH 7.2, containing 0.85% NaCl, and clarified by passage through a "sandwich" filter of glass fibers (Whatman GF/C) and Millipore (HAWPO4700). When necessary, the proteins were further purified by chromatography on Sephadex G-200 (0.5 × 50 cm) in the same buffer, and the fraction containing the monomeric protein was reduced to an appropriate volume by filtration through an Amicon pm-10 or -20 filter at 50 p.s.i. Unless otherwise stated, binding studies were carried out in reaction mixtures containing 0.16 mM protein and 8 mM 6-hydroxydopamine or other amines as indicated, for 0–48 hr in buffer (above) in a final volume of 0.05–0.1 ml, at 37° in small (10 × 75 mm) capped tubes. Isotopically labeled amine was added to the reaction mixture so that the final specific activity was 5–15 μCi/μmole. Reactions were initiated by addition of the amine. Reactions were also carried out in the presence of 6 M urea. Anaerobic reactions were carried out in amino acid hydrolysis tubes equipped with a vacuum stopcock; protein solutions were first flushed with oxygen-free argon and frozen, the solution of amine was added, and the tube was again flushed with argon, evacuated, sealed, and allowed to come to 37°. The reaction was stopped by the addition of carrier protein (25 mg of BSA) and 5 ml of 0.4 N perchloric acid, and the mixture was homogenized with a Teflon pestle and centrifuged at 20,000 × *g* for 10 min. This washing procedure was repeated five times, until no acid-soluble radioactivity could be detected in the acid supernatant. The washed protein was dissolved in 1 N acetic acid (1 ml), and the radioactivity was measured. No protein-bound radioactivity was detected in the carrier protein when the model protein was omitted from the reaction mixture. In all cases radioactivity was measured by scintillation spectrophotometry with a xylene-based scintillation fluid (Aquasol). Values were corrected for quenching.

Polymerization studies were performed with nonisotopically labeled amines in the same reaction mixture described above, except that the volume was 0.5 ml. For purposes of calculating binding, molecular weights of 64,000 for BSA, 40,000 for ovalbumin, and 62,000 for the hemoglobins were used.

Acetylated BSA was prepared as described by Saner and Thoenen (9), except that the product was purified by chromatography on Sephadex G-25 (2.5 × 100 cm). The *N*-ethylmaleimide derivatives of BSA and horse hemoglobin were prepared by allowing a solution (2 ml) of BSA (12.8 mg) or hemoglobin to react with *N*-ethylmaleimide (6.3 mg) in buffer for 7 days at 4° with stirring. The product was isolated by chromatography on Sephadex G-25 (2.5 × 100 cm).

Chromatographic analysis of amine-induced protein polymerization. The extent of cross-linking, the molecular size of the various cross-linked proteins, and the incorporation of isotopically labeled amine into the proteins were estimated by gel filtration or polyacrylamide gel electrophoresis.

For gel filtration, reaction mixtures were centrifuged at 105,000 × *g* for 60 min,

[2] The abbreviations used are: IgG, immuno-γ-globulin; BSA, bovine serum albumin.

the insoluble sediment obtained was washed twice with buffer, and the protein content was determined by a micro-Kjeldahl procedure. Aliquots of the supernatant fraction from the reaction mixtures described above, containing 0.05–5 mg of protein, were loaded on a column of Sephadex G-200 (superfine, 1 × 50 cm) and eluted with buffer, and 0.25–0.5-ml fractions were collected. Protein was estimated by absorbance at 280 nm in a flow cell or, when indicated, by the method of Lowry *et al.* (16). Radioactivity was measured in each fraction. The column was initially equilibrated with buffer and calibrated with a mixture of rabbit IgG (mol wt 153,000), BSA (64,000), aldolase (40,000), catechol *O*-methyltransferase (23,000), and dextran blue 2000 (200,000).

For polyacrylamide gel electrophoresis, aliquots of the reaction mixtures, containing 50–100 μg of protein, were incubated with sodium dodecyl sulfate (1%) in 0.1 M phosphate buffer, pH 7.0, for 30 min at 37° and chromatographed on 7% and 11% polyacrylamide gels, 5 cm in length, with a 3% stacking gel, according to the procedure of Neville (17). The gels were stained with Coomassie brilliant blue and destained against activated charcoal with a mixture of acetic acid, methanol, and water (70:75:900) for 2–5 days. The linear density of stain distribution was determined with a spectrophotometer equipped with a gel-scanning device (Beckman Acta V). Gels were cut into 1-mm sections, digested with 0.2 ml of a 2:1 (v/v) mixture of H_2O_2 (30%) and perchloric acid (60%) in a closed counting vial at 70° for 4 hr, and cooled to −20°, and scintillation fluid was added.

RESULTS

Binding of amines to protein. The maximum rate of irreversible binding of 6-hydroxydopamine to native BSA at pH 7.2 was achieved with a 50-fold excess of amine (8.0 mM) with respect to the concentration of BSA (0.16 mM). The reaction was a relatively slow process, exhibiting an initial rate of 0.05 mole of amine bound per mole of BSA per minute. Half-maximal binding was achieved in approximately 3 hr, with apparent saturation at 18 hr and 12.4 moles of amine per mole of BSA (Fig. 1). Amine concentrations greater than 8 mM or reaction times as long as 7 days failed to increase the total binding. The reaction with acetylated BSA exhibited a somewhat slower rate of 0.03 mole of amine bound per minute. However, as shown in Fig. 1, the maximum binding achieved was 29.5 moles of amine per mole of protein. The time course of irreversible binding of 6-hydroxydopamine to ovalbumin and horse, bovine, and human hemoglobin was similar to that of BSA, except that the maximum binding

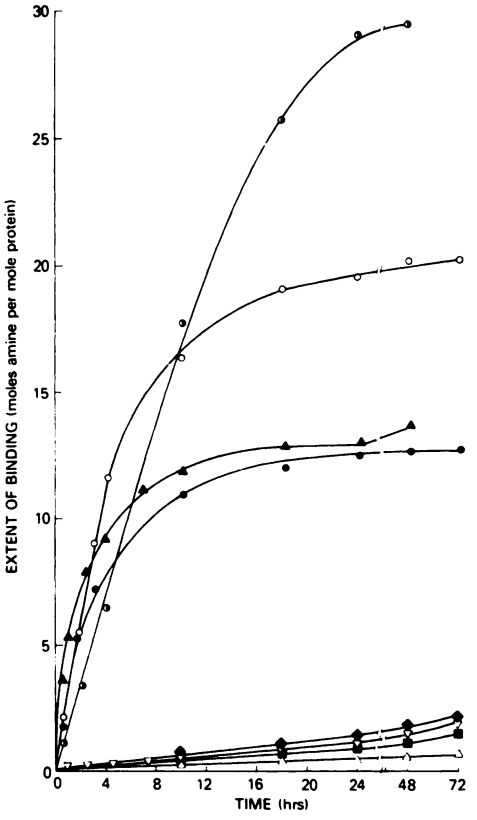

FIG. 1. *Time course of irreversible building of amines to proteins*

△——△, norepinephrine; ■——■, dopamine; ●——●, 6-hydroxydopamine; ▲——▲, 5,6-dihydroxytryptamine; ▽——▽, 5,7-dihydroxytryptamine binding to native BSA; ◑——◑, 6-hydroxydopamine binding to acetyl-BSA; ◆——◆, 6-hydroxydopamine binding to *N*-ethylmaleimide-treated BSA; ○——○, 6-hydroxydopamine binding to BSA in the presence of 6 M urea. Conditions are described in MATERIALS AND METHODS.

TABLE 1

Irreversible binding of [^{3}H]6-hydroxydopamine to proteins

Protein	Extent of binding				
	Native	6 M urea	Anaerobic	−SH residues	−S−S− residues[a]
	moles amine/mole protein				
Hemoglobin (horse)	3.5	3.9	<0.1	4	0
NEM-hemoglobin[b] (horse)	0.1	0.3		0	0
Hemoglobin (bovine)	3.8	4.1	<0.2	4	0
Hemoglobin (human)	5.2	6.2	<0.2	6	0
NEM-hemoglobin[b] (human)	0.2	0.4		0	0
Ovalbumin	2.8	7.3	<0.1	4	1
Serum albumin (bovine)	12.4	20.4	<0.2	1	17
Acetylated serum albumin[b]	29.2	30.3		1	17
NEM-serum albumin[b]	2.3	9.0		0	17
Polylysine[c]	0.7			0	0

[a] Values taken from refs. 18–23.

[b] *N*-Ethylmaleimide (NEM) and acetylated derivatives were prepared as described in MATERIALS AND METHODS.

[c] Polylysine treated in the same manner as the model proteins. Mean molecular weight = 150,000.

achieved was 2.8, 3.5, 3.8, and 5.2 moles of amine per mole of protein, respectively. However, when the reaction was carried out in the presence of 6 M urea (Table 1), the total binding to ovalbumin and BSA increased to 7.3 and 20.4 moles/mole of protein, respectively, while the binding to the other proteins remained nearly unchanged. Virtually no binding of 6-hydroxydopamine to BSA occurred in the absence of oxygen or following incubation of the amine at pH 7.2 in air for 8–16 hr prior to the addition of BSA. A polymer of 6-hydroxydopamine, isolated by gel filtration (Table 2), failed to bind irreversibly to BSA. The binding of 6-hydroxydopamine to horse hemoglobin following derivatization of the protein with *N*-ethylmaleimide was markedly reduced (Table 1). A similar derivatization of BSA rendered the BSA molecular strongly resistant to the binding reaction. The addition of a 2-fold excess of mercaptoethanol with respect to the amine concentration at various times during the binding reaction completely blocked the reaction of 6-hydroxydopamine to BSA (Fig. 2). However, mercaptoethanol failed to reverse the binding which had occurred prior to its addition to the mixture, even when the reaction was continued for as long as 23 hr. Similar results were obtained on addition of dithiothreitol, reduced glutathione, cysteine, and sodium bisulfite. The binding of 6-hydroxydopamine to BSA in the presence of ascorbate was variable, ranging from 2 to 5 moles/mole of BSA, even with a 5-fold excess of

TABLE 2

Irreversible binding of amines to bovine serum albumin

The reaction time was 24 hr.

Amine	Extent of binding
	moles amine/mole protein
Phenethylamine	<0.01
Tyramine	<0.1
Phenethanolamine	<0.01
Dopamine	0.9 ± 0.3[a]
dl-Norepinephrine	0.5
6-Hydroxydopamine	12.5 ± 2.3[a]
Oxidized 6-hydroxydopamine[b]	<0.1
Polymerized 6-hydroxydopamine[c]	<0.1
α-Methyl-6-hydroxydopamine	9.8
Tryptamine	<0.01
5-Hydroxytryptamine	<0.1
5,6-Dihydroxytryptamine	11.9 ± 2.1[a]
5,7-Dihydroxytryptamine	1.9 ± 0.5[a]

[a] Mean ± standard deviation for four samples.

[b] [^{3}H]6-Hydroxydopamine was incubated in air for 18 hr before the addition of BSA.

[c] Polymer derived from [^{3}H]6-hydroxydopamine and isolated by chromatography on Sephadex G-200 (Fig. 4).

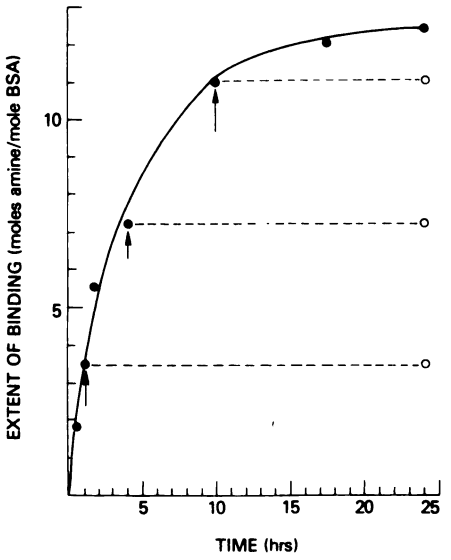

FIG. 2. *Effect of mercaptoethanol on irreversible binding of 6-hydroxydopamine to native BSA*

Mercaptoethanol to a final concentration of 16 mM was added to duplicate reaction mixtures at 1, 4, and 10 hr as indicated by the arrows and the reaction was terminated at 24 hr. Similar results (not shown) were obtained on addition to a final concentration of 16 mM of dithiothreitol, reduced glutathione, cysteine, and sodium bisulfite.

ascorbate with respect to the amine.

The rate of binding of 5,6-dihydroxytryptamine to BSA was somewhat faster than that of 6-hydroxydopamine, with half-maximal binding attained in 1.5 hr and apparent saturation at 18 hr and 12.6 moles of amine per mole of BSA (Fig. 1). The binding of α-methyl-6-hydroxydopamine was also nearly equivalent to that obtained with 6-hydroxydopamine (Table 2). In marked contrast, the irreversible binding of dopamine, norepinephrine, and 5,7-dihydroxytryptamine to BSA was much slower than observed with 6-hydroxydopamine and failed to reach any apparent saturation (Fig. 1). As shown in Table 2, the maximum binding achieved in 24 hr for these amines was 1.2, 0.5, and 0.8 moles of amine per mole of BSA, respectively. No irreversible binding was observed with tyramine, phenethylamine, phenethanolamine, tryptamine, or 5-hydroxytryptamine (Table 2).

Cross-linking of proteins by amines. Examination of the reaction products of 6-hydroxydopamine with proteins by chromatography on Sephadex G-200 or electrophoresis on polyacrylamide gels indicated that two oxygen-dependent processes were occurring. As indicated above, the irreversible binding of 6-hydroxydopamine to protein is clearly oxygen-dependent (Table 1). When the concentration of 6-hydroxydopamine was 1 μM irreversible binding to BSA occurred, with an end point of 0.2–1 mole of amine per mole of BSA. Electrophoresis of the reaction products on polyacrylamide gels (Fig. 3A) yielded a single

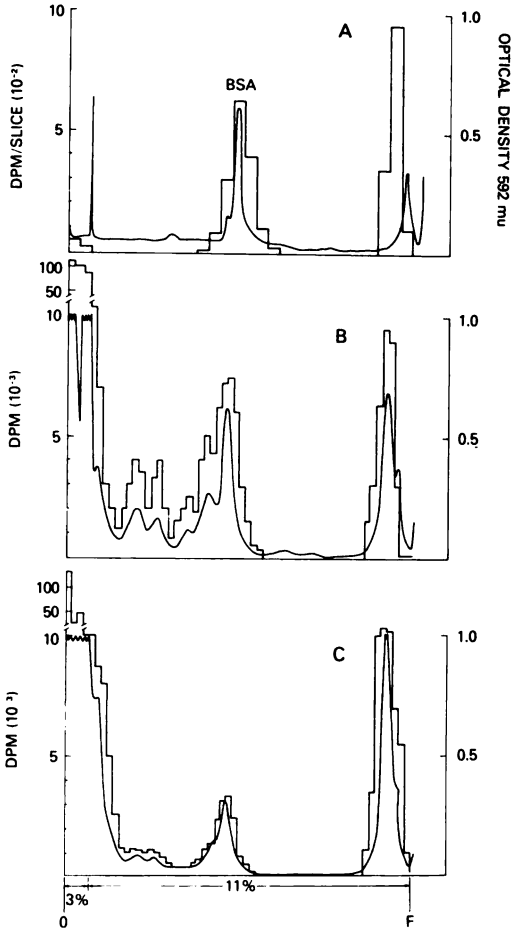

FIG. 3. *Electrophoresis of reaction products of native BSA and [³H]6-hydroxydopamine on sodium dodecyl sulfate-polyacrylamide gels*

——, optical density (592 nm) of Coomassie blue protein stain. Bar gram indicates the tritium content per slice (40–55 slices were obtained per 5-cm gel). The concentrations of 6-hydroxydopamine were 0.08 mM (A), 0.8 mM (B), and 8.0 mM (C). The concentration of BSA was 0.16 mM, and the reaction time was 24 hr.

labeled protein species identical with authentic BSA. However, when the concentration of amine was increased to 100 μM or greater, a dark brown precipitate appeared in the reaction mixture. As revealed by the chromatographic separations illustrated in Figs. 3 and 4, a series of protein species of increasing molecular size was present. 6-Hydroxydopamine was irreversibly bound to all the intermediate polymers, ranging from 0.2 to 1.0 mole of amine per mole of BSA in the monomeric form and progressively increasing to 12–15 in the highest molecular weight polymer. As the reaction proceeded, the intermediate forms with elution volumes greater than the void volume (Fig. 4) disappeared, and 80–90% of the protein was present as a complex polymer which eluted with the void volume on gel filtration (Fig. 4) or remained at the origin on 11% polyacrylamide gel electrophoresis (Fig. 3C). A variable amount of protein, ranging from 2% to 28%, was present after 24 hr as a complex polymer which was completely insoluble in buffer and was removed prior to chromatography (MATERIALS AND METHODS). Neither the soluble nor insoluble forms of the cross-linked protein could be dissociated by dialysis against buffer or treatment with mercaptoethanol, sodium bisulfite, urea, or detergents. The 6-hydroxydopamine bound to the cross-linked protein could not be removed by prolonged washing with perchloric acid (0.4 N) or trichloroacetic acid (6%). As shown in Fig. 3, approximately 20% of the BSA was present as unpolymerized monomer after reaction with 6-hydroxydopamine for 24 hr. A major fraction of the non-protein-bound radioactivity derived from 6-hydroxydopamine was eluted at the salt front on gel filtration. In experiments with larger amounts of unlabeled 6-hydroxydopamine, this material was visible as a brown band on chromatography on Sephadex G-200 (Fig. 4) or polyacrylamide gel electrophoresis (Fig. 3B and C). This material, isolated by chromatography on Sephadex G-200, contained no protein (Fig. 4) and failed to bind to BSA or to induce the cross-linking process. The formation of this apparent polymer derived from 6-hydroxydopamine increased with the time of incubation and appeared to be partially oxygen-dependent (Fig. 4). As illustrated for 6-hydroxydopamine in Fig. 4, chromatography of the reaction mixture after 24 hr under anaerobic conditions gave no indication of larger protein species, except for a slight increase in the amount of dimeric BSA. A similar dependency of the cross-linking reaction on oxygen was demonstrated for 5,6-dihydroxytryptamine (Table 3). As illustrated in Table 3, a variety of compounds, under aerobic conditions, can promote the polymerization of BSA. Extensive cross-linking occurred not only

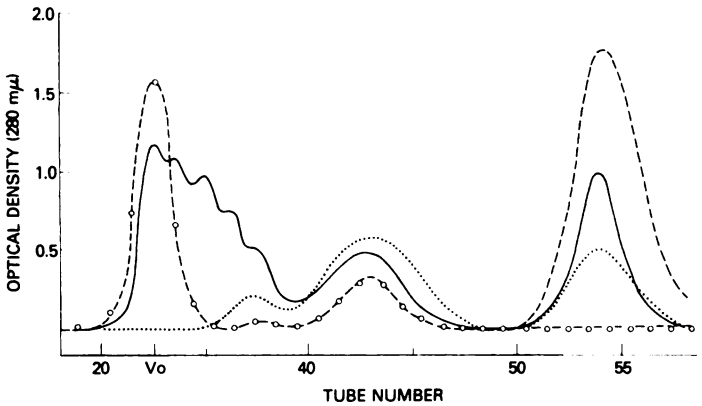

FIG. 4. *Chromatography of reaction products of native BSA and 6-hydroxydopamine on Sephadex G-200*
——, absorption pattern (280 nm) after reaction time of 2 hr; - - -, 24 hr; · · · ·, 24 hr under anaerobic conditions (see MATERIALS AND METHODS); O——O, Lowry protein content of the fractions obtained from a 24-hr reaction (normalized so that 1.0 mg/ml equaled an absorption at 280 nm of 1.0). The material eluted between tubes 50 and 58 was a visible brown in color and represents polymerized amine.

TABLE 3

Oxygen-dependent polymerization of bovine serum albumin by various agents

Agent	Polymer[a]	Monomer
	%	%
Tyramine	0	100
Dopamine	19	81
2-(2,4,5-Trihydroxyphenyl)nitroethane	79	21
6-Hydroxydopamine	83	17
α-Methyl-6-hydroxydopamine	62	38
2,3,4,5-Tetrahydroxyphenethylamine	33	67
6-Hydroxynorepinephrine	59	41
6-Hydroxydopa	65	35
5-Hydroxytryptamine	0	100
5,6-Dihydroxyindole	67	33
5,6-Dihydroxytryptamine	94	6
α-Methyl-5,6-dihydroxytryptamine	75	25
5,7-Dihydroxytryptamine	11	89
Polymerized 6-hydroxydopamine[b]	0	100

[a] Sum of protein present as an insoluble complex and that eluted at the void volume from Sephadex G-200 (see Fig. 4 and MATERIALS AND METHODS).

[b] Polymer derived from 6-hydroxydopamine and isolated by chromatography on Sephadex G-200 (Fig. 4).

with 6-hydroxydopamine but also with 2-(2,4,5-trihydroxyphenyl)nitroethane, a derivative in which the terminal amino group of 6-hydroxydopamine is replaced with a nitro group. α-Methyl-6-hydroxydopamine and 6-hydroxynorepinephrine also effectively catalyzed the cross-linking. The extent of cross-linking of BSA with 2,3,4,5-tetrahydroxyphenethylamine and dopamine was only 33% and 20%, respectively, after 24 hr. 5,6-Dihydroxytryptamine was the most effective compound tested, resulting in nearly complete cross-linking, with a half-time of 4.5 hr. α-Methyl-5,6-dihydroxytryptamine and 5,6-dihydroxyindole were also effective cross-linking agents. 5,7-Dihydroxytryptamine produced only 11% cross-linking. 5-Hydroxytryptamine and tyramine were inactive. The addition of a 2-fold excess (1.6 mM) of mercaptoethanol or dithiothreitol stopped both the binding of 6-hydroxydopamine (Fig. 2) and the cross-linking process (Table 3).

Amino acid analysis. Following induction of cross-linking of BSA by [^{3}H]6-hydroxydopamine for 18 hr, the high molecular weight fraction, which was eluted at the void volume of Sephadex G-200, was rechromatographed a second time and then dialyzed against 6 liters of water for 2 days to yield a light brown protein containing approximately 1 μCi of tritium per milligram. This preparation was subjected to acid hydrolysis (6 N HCl, 110°) for 24 and 72 hr, with recoveries of tritium in the amino acid residue of 95% and 85%, respectively. Examination of the amino acid composition by the two-dimensional paper chromatographic system of Irreverre and Martin (24) revealed the presence of one major and one minor ninhydrin-positive substance which still retained tritium and behaved as basic amino acids. A third radioactive material was present at the origin following hydrolysis for 24 hr and was still present in the 72-hr hydrolysate. A similar but larger preparation of BSA cross-linked with unlabeled 6-hydroxydopamine was subjected to acid hydrolysis. Standard amino acid analysis (Beckman model 120C) indicated the presence of two new ninhydrin-positive peaks, the larger of which was eluted between histidine and ammonia, and a smaller peak between serine and glutamic acid.

DISCUSSION

The oxygen-dependent binding of 6-hydroxydopamine to protein and the subsequent cross-linking of the protein are clearly relevant to the mechanism of the cytotoxic action of 6-hydroxydopamine *in vivo*. The absolute requirement for oxygen and the diminished binding in the presence of reducing agents clearly indicate that the initial event in the binding reaction is oxidative formation of the 1,4-*p*-quinone derivative. This quinone is an electron-deficient species (24) and is highly reactive with respect to nucleophilic groups. The preferred site of reaction of 1,4-*p*-quinone with protein appears to be at free sulfhydryl groups, in confirmation of an earlier report (8). The equivalency between the binding of 6-hydroxydopamine and the number of free sulfhydryl groups

in horse (18), bovine (19), and human hemoglobin (20), the loss of binding when the sulfhydryl groups of horse hemoglobin are blocked with *N*-ethylmaleimide, the approximate equivalency between the 6-hydroxydopamine bound and the total number of potential sulfhydryl groups present, both free and in disulfide bonds, in ovalbumin (21) and in acetylated BSA (22, 23), the failure of blocking other nucleophilic moieties in acetylated BSA to reduce the binding, and the lack of reaction with polylysine strongly support this view (Table 1). The rapid termination of the binding reaction of 6-hydroxydopamine with BSA which results from the addition of mercaptoethanol, dithiothreitol, or reduced glutathione (Fig. 2) suggests a rapid interaction of the 1,4-*p*-quinone species with the more accessible free sulfhydryl groups of these compounds. Since the extent of binding of 6-hydroxydopamine exceeds the number of free sulfhydryl groups available in native BSA (23), acetylated BSA, and both native BSA and ovalbumin in the presence of 6 M urea, and approaches the number potentially available from reduction of disulfide bonds (Table 1), it is suggested that a reduction of disulfide bonds can accompany the oxidation of 6-hydroxydopamine. Thus it is surprising that derivatization of the free sulfhydryl group in BSA with *N*-ethylmaleimide resulted in a protein resistant to reaction with 6-hydroxydopamine, since disulfide bonds should still have been capable of generating free sulfhydryl groups.

The irreversible binding of amines such as phenethylamine, phenethanolamine, or tryptamine is insignificant compared with the binding of 6-hydroxydopamine or 5,6-dihydroxytryptamine (Table 2). The presence of a β-hydroxy moiety in phenethanolamine did not increase the binding, whereas the introduction of an aromatic phenolic group, as in tyramine and 5-hydroxytryptamine, increased the irreversible binding approximately 10-fold. However, the binding of these phenolic amines is still quantitatively insignificant with respect to that observed with 6-hydroxydopamine and 5,6-dihydroxytryptamine (Table 2). Dopamine and norepinephrine bind to proteins, under the conditions described, 50–100 times more effectively than the parent phenethylamine structure. Both these catechols are readily converted to *o*-quinones and, as such, can react with nucleophilic sites on proteins (25, 26). More important, both dopamine (27) and probably norepinephrine under autoxidation form their respective 6-hydroxy derivatives and thus give rise to reactive species. However, since the extent of binding of dopamine or norepinephrine is relatively slow compared to that observed with 6-hydroxydopamine, it is evident that this mechanism is at least quantitatively distinct from that of 6-hydroxydopamine (Fig. 1 and Table 2). The same distinction applies to the possibility that the irreversible binding of catecholamines is a consequent of intramolecular cyclization to reactive indoleamine structures (26).

A schematic representation of the proposed reaction of 6-hydroxydopamine with sulfhydryl groups on protein and the ensuing polymerization is shown in Fig. 5. Following an initial oxidation of 6-hydroxydopamine to the 1,4-*p*-quinone, a covalent reaction between an available sulfhydryl group and one of the electron-negative centers on the aromatic ring ensues. The protein-amine conjugate then oxidizes and reacts with a sulfhydryl on another protein to form a dimer, and this continues until a complex protein lattice is generated. The polymerization reaction with model proteins appears to require the binding of more than 2 moles of ligand per mole of protein before efficient cross-linking can occur. Uncross-linked BSA containing 0.2–1.0 mole of 6-hydroxydopamine persists after 90% of the BSA has polymerized. This may indicate that the binding of 6-hydroxydopamine fails to reach the critical level in these unpolymerized molecules. The marked increase in binding of 6-hydroxydopamine following unfolding of the tertiary structure in acetylated BSA and in BSA and ovalbumin in 6 M urea indicates that some nucleophilic sites are sterically hindered with respect to attack by the quinone species in the native protein. The absence of any significant increase in binding with the hemoglobins, proteins

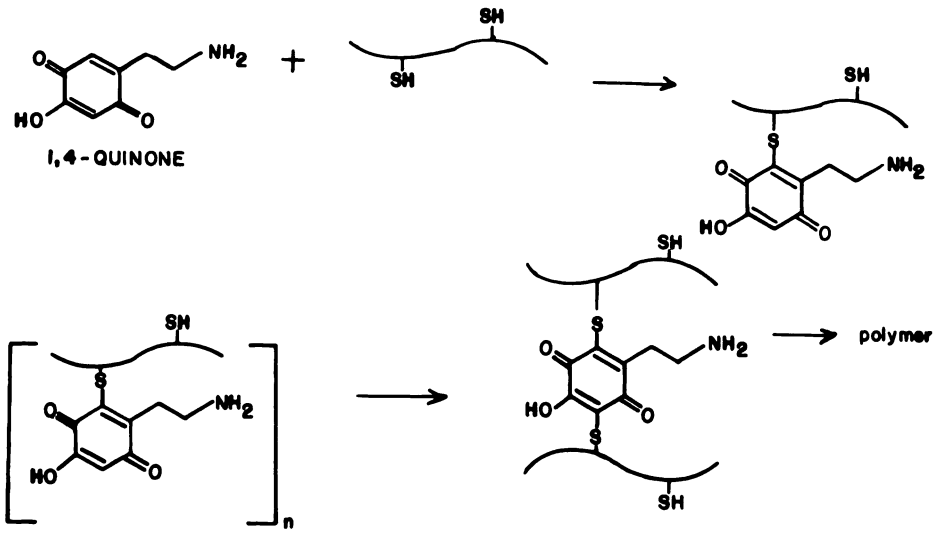

FIG. 5. *Schematic representation of cross-linking of proteins by 6-hydroxydopamine*

devoid of disulfide bonds (18), on urea denaturation suggests that the potential nucleophilic sites exposed in BSA and ovalbumin are disulfide bonds. In this regard, the proposed generation of hydrogen peroxide and/or superoxide radical ion during the oxidation of 6-hydroxydopamine (8) might increase the reaction rate of the quinone species through denaturation of proteins and concomitant exposure of disulfide bonds and nucleophilic sites. Efficient cross-linking of proteins *in vitro* is seen only at concentrations of 6-hydroxydopamine in excess of approximately 100 μM which is in accord with the observation that a critical concentration threshold must be achieved intraneuronally before the neurodegenerative effect is initiated (23). The uptake mechanism at the plasma membrane of the adrenergic nerve terminal can certainly yield concentrations of 6-hydroxydopamine in this order of magnitude and greater.

Intramolecular cyclization of the 1,4-*p*-quinone from 6-hydroxydopamine occurs readily *in vitro* and gives rise to an intermediate aminochrome which spontaneously rearranges to form 5,6-dihydroxyindole (26, 28). 5,6-Dihydroxyindole, 5,6-dihydroxytryptamine, and α-methyl-5,6-dihydroxytryptamine all irreversibly bind to BSA and induce the cross-linking process under aerobic conditions (Table 3). Thus it is conceivable that the neurodegenerative action of 6-hydroxydopamine could be mediated through intramolecular cyclization to 5,6-dihydroxyindole after uptake of 6-hydroxydopamine by the nerve terminal. However, the nitro analogue of 6-hydroxydopamine (Table 3), which can undergo oxidation to a 1,4-*p*-quinone but not the intramolecular cyclization to an indole, cross-links protein as effectively as 6-hydroxydopamine. Thus, in this case, the initial formation of the 1,4-*p*-quinone is sufficient for cross-linking. Additional support for the primary role of the 1,4-*p*-quinone as a cross-linking agent is suggested by electrochemical studies, which indicate that the rate of nucleophilic attack by cysteine and other sulfhydryl compounds on the 1,4-*p*-quinone is substantially faster than the intramolecular cyclization (29, 30). Thus it appears that the 1,4-*p*-quinone derived from 6-hydroxydopamine and the aminochrome derived by oxidation of 5,6-dihydroxytryptamine are the active cross-linking agents, and that the primary site for the reaction is at free sulfhydryl groups. The presence of two radioactive, ninhydrin-positive substances derived from [^{3}H]6-hydroxydopamine in acid hydrolysates of extensively cross-linked BSA indicates the formation of at least two stable amino acid derivatives. The strongly basic nature of the major

component is consistent with the structure illustrated in Fig. 6A. The retention of the ethylamine side chain of 6-hydroxydopamine would account for the basic character of this amino acid derivative. The chromatographic behavior of the minor radioactive component in the hydrolysate was intermediate between that of serine and glutamic acid and is consistent with the structure illustrated in Fig. 6B, where the basic character of the amine has been lost through intramolecular cyclization to an indole. Since the interaction of the 1,4-*p*-quinone from 6-hydroxydopamine with sulfhydryl groups on protein appears to occur more rapidly than the intramolecular cyclization, it can be presumed that the minor component (Fig. 6B) is formed following the cross-linking reaction. Further efforts are in progress to define the structure of the products obtained with both 6-hydroxydopamine and 5,6-dihydroxytryptamine. It is of interest that a naturally occurring amino acid, 2,5-*S*,*S*-dicysteinyldopa (31), is structurally similar to the proposed *S*,*S*-dicysteinyl derivative (Fig. 6A). In addition, *S*-cysteinylcatechol derivatives are known to be involved in the structure of phaeomelanine (32).

The mechanism for the cytotoxic action of 5,7-dihydroxytryptamine appears to differ from that of 6-hydroxydopamine, for, unlike 6-hydroxydopamine, the neurotoxicity of 5,7-dihydroxytryptamine in peripheral noradrenergic neurons is prevented by prior inhibition of monoamine oxidase (5). Furthermore, the α-methyl derivative of this amine is devoid of neurotoxicity even though it is accumulated within noradrenergic neurons (5). Thus it was suggested that one or more of the metabolic products derived from 5,7-dihydroxytryptamine might account for the cytotoxicity. It is unlikely that the failure of 5,7-dihyroxytryptamine to effectively induce cross-linking of protein (Fig. 1 and Table 3) can be attributed to a reduced rate of autoxidation, since both 5,6- and 5,7-dihydroxytryptamine undergo autoxidation at nearly the same rate (5). Thus, if the cross-linking of proteins is operant in the cytotoxic action of 5,7-dihydroxytryptamine, some further explanation is required. To induce the proposed cross-linking, an agent must have at least two active sites. All three of the resonance forms of 5,6-dihydroxytryptamine are activated toward nucleophilic attack in both positions 4 and 7 of the aromatic ring, whereas 5,7-dihydroxytryptamine has only two resonance forms, both of which carry negatively charged oxygens on the aromatic ring (Fig. 7). The only possible positions for nucleophilic attack on the aromatic ring are therefore positions 4 and 6. However, position 6 is partially masked by the negative charge on the oxygens at positions 5 and 7. A reduced capacity for nucleophilic attack may explain the failure of 5,7-dihydroxytryptamine to cross-link proteins *in*

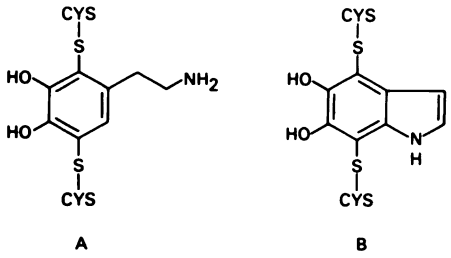

FIG. 6. *Proposed structures of reaction products of cysteine residues in proteins with 6-hydroxydopamine*

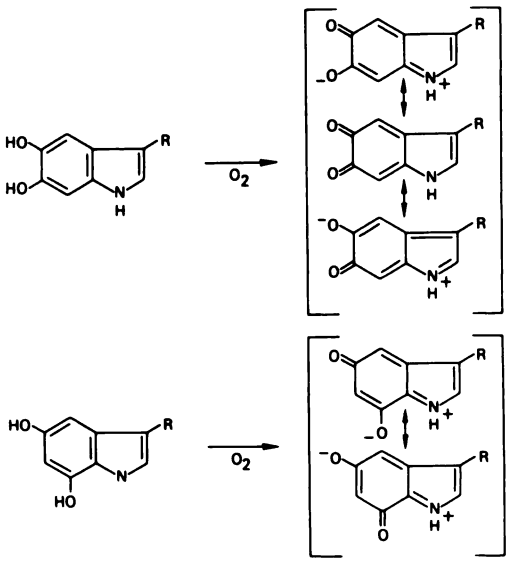

FIG. 7. *Resonance forms of 5,6- and 5,7-dihydroxytryptamine*

vitro. Conversion of this amine to an aldehyde through the action of monoamine oxidase appears to be an obligatory step for the induction of degeneration in noradrenergic neurons in mouse heart *in vivo* (5). The introduction of an aldehyde function on the molecule would provide the additional electrophilic site and thus might lead to the cross-linking of proteins. Synthesis of this aldehyde is currently being pursued in order to determine whether or not it can effectively induce cross-linking of proteins *in vitro*.

Recently it was reported that 6-hydroxydopamine not only bound covalently to the intracellular proteins of a clonal line of cultured neuroblastoma cells, but resulted in the formation of high molecular weight proteins containing irreversibly bound isotope derived from radioactive 6-hydroxydopamine (10). This evidence strongly suggests that 6-hydroxydopamine can induce the cross-linking of proteins in a living cell, and is similar in many respects to observations with model proteins *in vitro*. Preliminary results from our laboratory and elsewhere (33) have demonstrated that the decrease in neuronal uptake of norepinephrine in mouse atrium which follows the administration of 6-hydroxydopamine *in vivo*, or in isolated atrial preparations, is accompanied by covalent binding of the amine. The observed blockade of uptake and binding of 6-hydroxydopamine in atrial preparations *in vitro* is a time-dependent, irreversible process and is compatible with the hypothesis that the loss of function results from the cross-linking of proteins. While the precise nature of the cytotoxic mechanism generated by the amines *in vivo* is not completely understood, the present evidence indicates that the cross-linking of proteins essential for the functional survival of the neuronal terminal is one possible alternative. The contribution to cytotoxicity of the formation of toxic substances such as hydrogen peroxide, the superoxide ion, and the hydroxyl radical (8, 34) during the intraneuronal autoxidation of these amines must also be considered.

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