MOLECULAR PHARMACOLOGY, 7, 147–154

Model Experiments on the Molecular Mechanism of Action of 6-Hydroxydopamine

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(Received September 19, 1970)

SUMMARY

6-Hydroxydopamine is rapidly oxidized to its *p*-quinone and indoline derivatives at pH 7.4, as shown by ultraviolet spectroscopy. Incubation of bovine serum albumin (0.5 mM) with ^{3}H-6-hydroxydopamine resulted in covalent binding (nonextractable with alcoholic perchloric acid) of radioactivity to the protein. The amount of radioactivity bound was concentration-dependent. At pH 7.4 and 38°, saturation was reached at a concentration of 70 mM ^{3}H-6-hydroxydopamine. The radioactivity bound at this concentration corresponds to the equivalent of 11 moles of ^{3}H-6-hydroxydopamine per mole of albumin. If oxidation of ^{3}H-6-hydroxydopamine was prevented by $Na_2S_2O_5$, the binding of radioactivity to bovine albumin was virtually completely abolished. Acetylation of albumin reduced the bound radioactivity to 19% of that of controls, whereas heat denaturation reduced it to only 75%, indicating that not denaturation as such, but the blockade of nucleophilic groups, is the main cause for the reduced binding of radioactivity.

The results of the present experiments are compatible with the hypothesis put forward recently [H. Thoenen, J. P. Tranzer and G. Häusler, *in* "New Aspects of Storage and Release Mechanisms of Catecholamines" (H. J. Schümann and G. Kroneberg, eds.), p. 130. Springer-Verlag, Berlin, 1970] that the selective destruction of adrenergic nerve terminals by 6-hydroxydopamine results from the covalent binding of its oxidation products to nucleophilic groups of biological macromolecules. The reaction seems to be nonspecific, and the high gelectivity of the destructive effect results from the efficient uptake of 6-hydroxydopamine into the adrenergic nerve terminals.

INTRODUCTION

6-Hydroxydopamine produces an efficient and very long-lasting depletion of norepinephrine from peripheral sympathetically innervated organs (1–4). Originally it was suggested that 6-hydroxydopamine may damage the amine storage sites of the adrenergic nerve terminals (2, 4) or act as a false adrenergic transmitter with an extremely long biological half-life resulting from a high affinity for the amine stores (5). However, electron microscopic studies have revealed that high doses of 6-hydroxydopamine produce selective destruction of adrenergic nerve terminals (6–8), providing a useful tool for chemical sympathectomy (8–10).

As to the mechanism of the selective destruction of adrenergic nerve terminals, it has been shown that 6-hydroxydopamine is efficiently transported by the neuronal membrane pump (1, 8) and that this uptake into the neuron is a prerequisite for the destructive effect (9, 11). However, its accumulation in the storage vesicles of the adrenergic nerve terminals does not seem to be of primary importance, since prior treatment

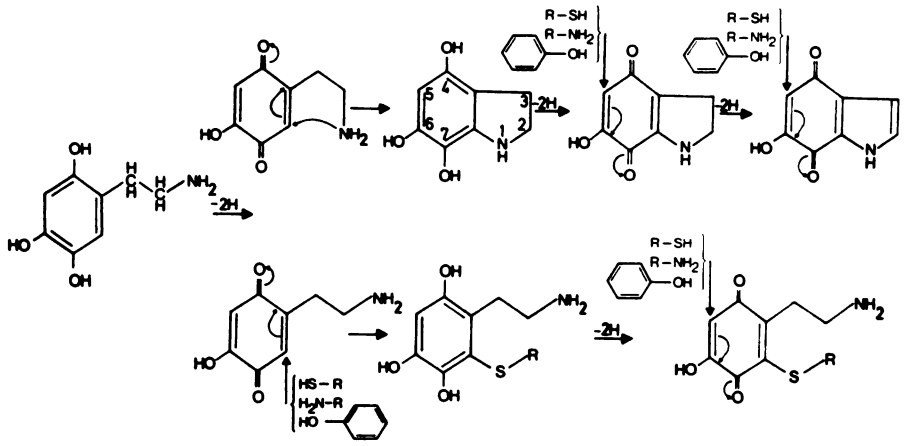

FIG. 1. *Hypothetical mechanism of action of 6-hydroxydopamine*

6-Hydroxydopamine is oxidized to its *p*-quinone derivative, which can undergo further transformation to indoline and indole derivatives. Both the *p*-quinone and the indoline indole derivatives can form covalent bonds with nucleophilic groups of biological macromolecules.

with reserpine prevented neither the characteristic ultramorphological nor the biochemical effects of 6-hydroxydopamine (1).

The information available so far permits no conclusions as to the mechanism of action at the molecular level. However, preliminary experiments with rats have shown that, 24 hr after intravenous administration of large doses of ^{3}H-6-hydroxydopamine, 30–35% of the total radioactivity present in the heart could not be extracted by homogenization in 0.4 N $HClO_4$ (8). Since it is known that oxidation products of catecholamines can undergo covalent binding with nucleophilic groups of amino acids (12–15), we have put forward the hypothesis that oxidation products of 6-hydroxydopamine may undergo covalent binding with nucleophilic groups of biological macromolecules (Fig. 1), that this reaction is nonspecific, and that the high selectivity of the destructive effects results from the efficient accumulation of 6-hydroxydopamine in the adrenergic nerve terminals (1). It is the purpose of the present experiments to evaluate whether the proposed oxidation products are formed *in vitro* and whether they undergo covalent binding with nucleophilic groups of bovine serum albumin, which is used as a representative macromolecular model.

METHODS

Spectrophotometric Analysis

The changes in the ultraviolet absorption spectra of aqueous solutions of 6-hydroxydopamine, norepinephrine, and dopamine were studied at room temperature over a period of 24 hr. The amine concentrations amounted to 50–140 μM, and the pH was varied from 3.0 to 7.4 with 0.15 M citric acid–sodium phosphate buffers. The spectra were recorded with a Beckman DB-G spectrophotometer.

Covalent Binding of Oxidation Products of ^{3}H-6-Hydroxydopamine, ^{3}H-Norepinephrine, and ^{3}H-Dopamine

The tritiated amines were purified by preparative chromatography on Whatman No. 3MM paper, using a solvent system of 1-butanol–0.5 N HCl (1:1 by volume). Stock solutions of each amine, 0.1–1.0 M in ice-cold 0.1 N HCl saturated with argon, were prepared immediately before use. The ^{3}H-amines were added to 1 ml of 0.5 mM fraction V bovine serum albumin (Nutritional Biochemicals Corporation) adjusted to the desired final pH with citric acid–sodium phosphate buffers. The final concentrations of the ^{3}H-amine varied between 1.0 and 300

mM. The ^{3}H-amine–albumin solutions were incubated in unstoppered test tubes for 12 min to 24 hr. The reaction was stopped with 4 ml of aqueous 0.4 N $HClO_4$. The precipitate was homogenized in ground-glass homogenizers. After centrifugation for 8 min at 21,000 × *g*, the supernatant fluid was decanted and the pellet was homogenized again in 2 ml of alcoholic 0.4 N $HClO_4$. This procedure was repeated nine times, until the radioactivity in the supernatant fluid had decreased to background levels. Alcoholic $HClO_4$ was used, since it is a good solvent for 6-hydroxydopamine and its first oxidation products. Furthermore, it prevents their adsorption to albumin by electrostatic forces, hydrogen bonding, or formation of charge transfer complexes. The final pellet was dissolved with 1.0 ml of a protein solubilizer solution ("NCS," Amersham, Searle). The radioactivity of the dissolved pellet was determined in a liquid scintillation counter (mark I, Nuclear-Chicago), using a solution of 6% 2-(4′-*tert*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole (Ciba, Basle) in toluene. The counting efficiency was determined with an internal standard of ^{3}H-hexadecane and varied between 35 and 45%.

Acetylation and Heat Denaturation of Bovine Albumin

Bovine serum albumin (33.5 mg) was dissolved in 1 ml of 0.3 N NaOH. Under vigorous shaking, 0.3 ml of acetic anhydride was added in drops. The stoppered test tube was then incubated for 24 hr at 35°. The acetylated albumin was dried under vacuum and then homogenized in 1 ml of 0.15 M citric acid–sodium phosphate buffer, pH 7.4.

For heat denaturation, 33.5 mg of bovine albumin were dissolved in 0.3 ml of citric acid–sodium phosphate buffer, pH 7.4, and incubated for 2 hr in a stoppered test tube at 100°. The denatured albumin was homogenized in a final volume of 1 ml of citric acid–sodium phosphate buffer, pH 7.4.

To study the effects of acetylation and heat denaturation on the covalent binding of ^{3}H-6-hydroxydopamine or of its oxidation products, the homogenates of acetylated and heat-denaturated albumin were incubated with ^{3}H-6-hydroxydopamine in the same way as native bovine albumin.

Drugs

2,4,5-Trihydroxyphenethylamine hydrobromide (6-hydroxydopamine hydrobromide) was synthesized by Dr. A. Langemann of the Chemical Department, and ^{3}H-6-hydroxydopamine hydrobromide (960 mCi/mg of base), labelled at the α- and β-carbon atoms of the side chain, was synthesized by Dr. A. Krassó of the Physical Department of Hoffmann-La Roche, Basle. 3,4-Dihydroxyphenethylamine hydrochloride (dopamine hydrochloride, Fluka), and *l*-2-hydroxy-2-(3,4-dihydroxyphenyl)ethylamine (*l*-norepinephrine, Hoechst) were obtained commercially. ^{3}H-Dopamine hydrochloride (29.2 mCi/mg of base), labeled at the α- and β-carbon atoms of the side chain, and ^{3}H-dl-norepinephrine hydrochloride (22.5 mCi/mg of base, [7-^{3}H]-dl-norepinephrine hydrochloride) were supplied by Radiochemical Centre, Amersham, England.

RESULTS

Changes in Ultraviolet Absorption of Aqueous Solutions of 6-Hydroxydopamine: Time Course and pH Dependence

It is well known that 6-hydroxydopamine is extremely unstable at neutral and alkaline pH (4, 8). Immediately after dissolution a red color appeared, indicating the rapid formation of a quinone derivative (16). However, at lower pH, 6-hydroxydopamine is much more stable and at pH 3 the ultraviolet spectrum remained unaltered for at least 2 hr, exhibiting the characteristics of unchanged 6-hydroxydopamine (Fig. 2). At this pH the first distinct changes in the absorption spectrum occurred after 10–20 hr. There was a shoulder at 265 nm and a broad peak at about 480 nm, indicating the formation of the first *p*-quinone oxidation product (Fig. 1) (16). At higher pH this first oxidation product was much more rapidly formed. However, it could not be detected since it was immediately transformed by self-condensation into 4,6,7-trihydroxyindoline, which was further oxidized

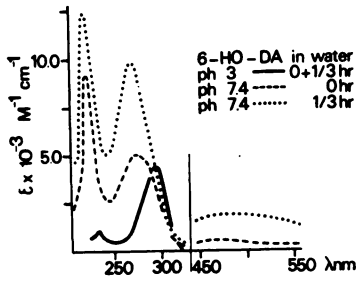

FIG. 2. *pH dependence of changes in ultraviolet spectra of aqueous solutions of 6-hydroxydopamine*

The pH was adjusted with 0.15 M citric acid–sodium phosphate buffers. The concentration of 6-hydroxydopamine (6-HO-DA) amounted to 50 μM at pH 7.4 and to 135 μM at pH 3.0. At pH 3.0, the absorption spectrum did not change during 20 min. The absorption maxima are at 292 and 235 nm. At pH 7.4, the absorption maxima are at 460 (approximately), 274, and 220.5 nm immediately after dissolution of 6-hydroxydopamine and at 485 (approximately), 270, and 220 nm 20 min later.

to the 6-hydroxyindoline *p*-quinone. The absorption characteristics of the latter compound appeared a few seconds after 6-hydroxydopamine was dissolved at pH 7.4 (Fig. 2). The absorption peaks reached their maximal levels (490 nm, ϵ = 2100 M^{-1} cm^{-1}; 270 nm, ϵ = 10,000 M^{-1} cm^{-1}; and 220 nm, ϵ = 13,200 M^{-1} cm^{-1}) after 1 hr and correspond to the absorption characteristics of 6-hydroxyindoline *p*-quinone described by Senoh and Witkop (16).

The transitory formation of the intermediate 4,6,7-trihydroxyindole can be deduced from the appearance of a shoulder at 283 nm without any absorption at 490 nm, occurring only at pH 5 and 6. This pH range also favored the transformation of the peak at 270 nm into a double peak at 293 and 275 nm (Fig. 3). This absorption pattern suggests that a rearrangement of 6-hydroxyindoline *p*-quinone to 4,6,7-trihydroxyindole took place with subsequent oxidation to 6-hydroxyindole *p*-quinone (Fig. 1). That this further oxidation took place at pH 5 and 6 but not at pH 7.4 is in accordance with the previous observation of Senoh and Witkop (16) that transformation of 6-hydroxyindoline *p*-quinone to the corresponding indole does not occur at alkaline pH.

Spectroscopic Changes of Aqueous Solutions of Norepinephrine and Dopamine at pH 7.4

In contrast to 6-hydroxydopamine, the absorption spectrum of norepinephrine was relatively stable at room temperature and pH 7.4. No major changes in the absorption characteristics occurred apart from a rise in the baseline, which most probably resulted from the rapid formation of melanin-like polymerization products from the oxidation derivatives of norepinephrine. These oxidation products did not reach a high enough concentration to change the absorption

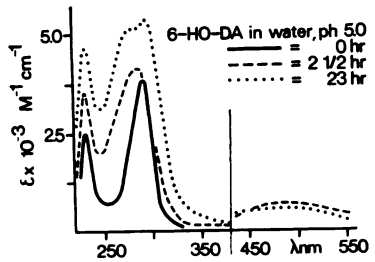

FIG. 3. *Time course of changes in ultraviolet spectra at pH 5.0*

The concentration of 6-hydroxydopamine (6-HO-DA) was 133 μM, the pH was adjusted with 0.15 M citric acid–sodium phosphate buffer, and the solution was stored at room temperature. Immediately after 6-hydroxydopamine was dissolved, the absorption maxima are at 291.5 and 231 nm; after 2.5 hr, at 485 (approximately), 287, and 231 nm; and after 23 hr, at 485 (approximately), 293.5, 275.5 (shoulder), and 231 nm.

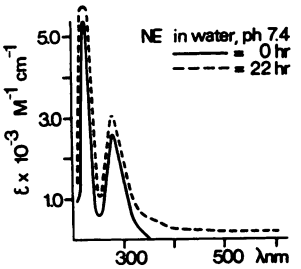

FIG. 4. *Time course of changes in ultraviolet spectra of norepinephrine (NE)*

The concentration of norepinephrine was 114 μM, the pH was adjusted with 0.15 M citric acid–sodium phosphate buffer, and the solution was stored at room temperature. The absorption maxima are at 278 and 223 nm.

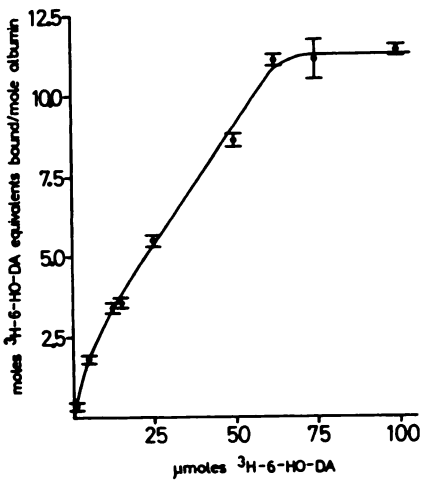

FIG. 5. *Concentration dependence of covalent binding of 6-hydroxydopamine to bovine albumin*

Bovine albumin (0.5 μmole) and ^{3}H-6-hydroxydopamine (^{3}H-6-HO-DA) (1.0–100 μmoles) were dissolved in 1 ml of 0.15 M citric acid–sodium phosphate buffer, pH 7.4, and incubated for 6 hr at 38°. The reaction was stopped with 4 ml of 0.4 N $HClO_4$. After repeated extraction with alcoholic 0.4 N $HClO_4$ (until the supernatant fluid had reached background levels), the final precipitate was dissolved in "NCS" protein solubilizer. The radioactivity bound is expressed in equivalents of ^{3}H-6-hydroxydopamine per mole of protein. The values given represent the means ± standard errors of five experiments.

characteristics (Fig. 4). The results obtained with dopamine were very similar to those with norepinephrine.

Covalent Binding of Oxidation Products of 6-Hydroxydopamine to Bovine Albumin

Rate of binding and saturation. Incubation of bovine albumin with ^{3}H-6-hydroxydopamine resulted in the binding of radioactivity which could not be extracted from the precipitated albumin by repeated homogenization in alcoholic $HClO_4$.

At pH 7.4 and an incubation temperature of 38°, the binding rate decreased progressively, and a plateau was reached after 6 hr. The level of this plateau depended on the initial ^{3}H-6-hydroxydopamine concentration, and saturation was reached at approximately 70 mM (Fig. 5). Neither a further increase in the initial concentration nor the addition of further doses of ^{3}H-6-hydroxydopamine after 6 and 12 hr could raise the amount of radioactivity bound per mole of albumin. The amount of bound radioactivity per mole of albumin equaled approximately 11 moles of ^{3}H-6-hydroxydopamine.

Dependence of binding on pH, and effect of $Na_2S_2O_5$. At a given concentration of ^{3}H-6-hydroxydopamine, the rate and extent of the binding of radioactivity to bovine albumin were pH-dependent. At lower pH values, the rate and extent of binding of nonextractable radioactivity decreased (Fig. 6). The fact that both the rate of oxidation and the rate of covalent binding of 6-hydroxydopamine are pH-dependent suggests that oxidation of 6-hydroxydopamine is a

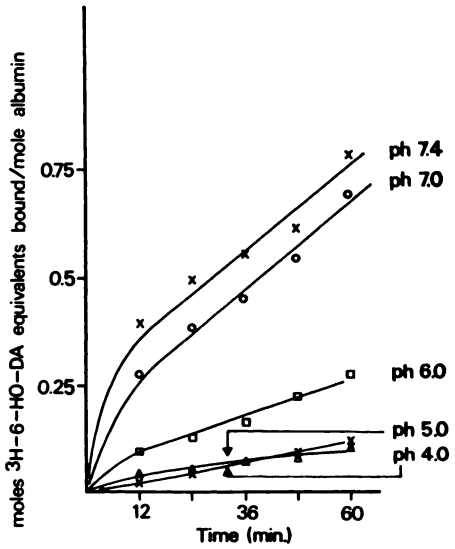

FIG. 6. *pH dependence of binding rate of 6-hydroxydopamine to bovine albumin*

Bovine albumin (0.5 μmole) and ^{3}H-6-hydroxydopamine (^{3}H-6-HO-DA) (7.5 μmoles) were dissolved in 1 ml of 0.15 M citric acid–sodium phosphate buffer and incubated for 12–60 min at 38°. The reaction was stopped with 4 ml of 0.4 N $HClO_4$. After repeated extraction with alcoholic 0.4 N $HClO_4$ (until the supernatant fluid had reached background levels), the final precipitate was dissolved in "NCS" protein solubilizer and the radioactivity was determined by liquid scintillation counting. The radioactivity bound is expressed in equivalents of ^{3}H-6-hydroxydopamine per mole of protein.

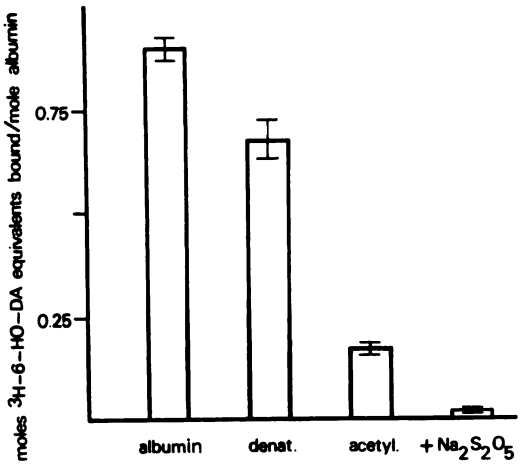

FIG. 7. *Effect of acetylation and heat denaturation of albumin on binding of 6-hydroxydopamine; effect of $Na_2S_2O_5$*

Heat-denaturated, acetylated, or native bovine albumin (0.5 μmole) was incubated together with 15 μmoles of ^{3}H-6-hydroxydopamine (^{3}H-6-HO-DA) in 1 ml of 0.15 M citric acid–sodium phosphate buffer, pH 7.4, at 38°. The concentration of $Na_2S_2O_5$ was 0.15 M. The reaction was stopped after 30 min with 4 ml of 0.4 N $HClO_4$. After repeated extraction with alcoholic 0.4 N $HClO_4$ (until the supernatant fluid had reached background levels), the final precipitate was dissolved in "NCS" protein solubilizer and the radioactivity was determined by liquid scintillation counting. The radioactivity bound is expressed in equivalents of ^{3}H-6-hydroxydopamine per mole of protein. The values given represent the means ± standard errors of six experiments.

prerequisite for the subsequent binding to the protein molecule. This assumption is further supported by the observation that the presence of the antioxidant $Na_2S_2O_5$ almost completely abolished the binding of radioactivity to albumin under experimental conditions (pH 7.4 and 38°) which otherwise favor this binding (Fig. 7).

Effect of acetylation and heat denaturation of bovine albumin. In our working hypothesis, we have suggested that the covalent binding of 6-hydroxydopamine to biological macromolecules results from the addition of the quinone oxidation products of 6-hydroxydopamine to nucleophilic groups of the macromolecule. If this assumption were correct, the acetylation of the nucleophilic groups of bovine albumin would reduce the amount of nonextractable radioactivity retained by the protein after incubation with ^{3}H-6-hydroxydopamine. As shown in Fig. 7, the radioactivity retained by acetylated albumin was 19% of that retained by the original bovine albumin. To exclude the possibility that denaturation of albumin accompanying acetylation is responsible as such for the reduced retention of radioactivity, we compared the effect of acetylation with that of heat denaturation. The latter reduced the retention of radioactivity much less than acetylation (Fig. 7), demonstrating that denaturation as such is not the main cause of the reduced binding of radioactivity.

Comparison between Covalent Binding of Oxidation Products of 6-Hydroxydopamine and Those of Norepinephrine and Dopamine

In further experiments, we compared the rate and extent of binding to albumin of radioactivity originating from ^{3}H-norepinephrine and from ^{3}H-6-hydroxydopamine. Figure 8 shows that the radioactivity bound to protein after incubation with ^{3}H-norepinephrine was much smaller than that after incubation with corresponding concentrations of ^{3}H-6-hydroxydopamine. In contrast to ^{3}H-6-hydroxydopamine, no plateau was reached within 6–8 hr at pH 7.4 and 38°, and there was a nearly linear increase up to 24 hr, the longest incubation time studied. Similar results were obtained with dopamine. The slower binding rates of norepinephrine and dopamine show close agreement with the slower rates of oxidation observed in the spectroscopic studies.

DISCUSSION

The ultraviolet spectroscopic studies of the present investigation provide good evidence that the oxidation products proposed in our working hypothesis are formed *in vitro* at a physiological pH of 7.4 (Fig. 1). In the lower pH ranges, however, 6-hydroxydopamine is relatively stable and oxidation proceeds very slowly.

The first oxidation product of 6-hydroxydopamine is its *p*-quinone derivative. In

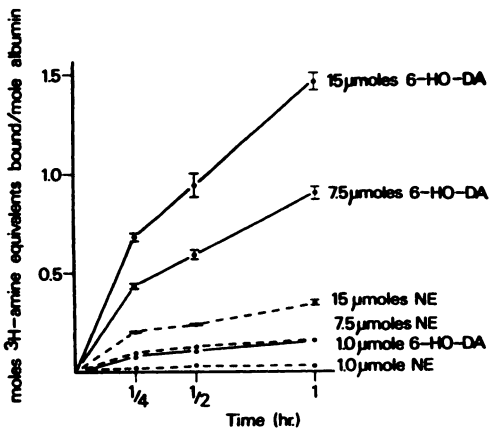

FIG. 8. *Comparison between rates of binding of 6-hydroxydopamine and norepinephrine to bovine albumin*

Bovine albumin (0.5 μmole) and ^{3}H-6-hydroxydopamine (^{3}H-6-HO-DA) (1.0–15.0 μmoles) or ^{3}H-norepinephrine (^{3}H-NE) (1.0–15.0 μmoles) were dissolved in 1 ml of 0.15 M citric acid–sodium phosphate buffer, pH 7.4, and incubated for 15–60 min at 38°. The reaction was stopped with 4 ml of 0.4 N $HClO_4$. After repeated extraction with alcoholic 0.4 N $HClO_4$ (until the supernatant fluid had reached background levels), the final precipitate was dissolved in "NCS" protein solubilizer and the radioactivity was determined by liquid scintillation counting. The radioactivity bound is expressed in equivalents of ^{3}H-amines per mole of protein. The values given represent the means ± standard errors of six experiments.

aqueous solution *in vitro*, it represents only a short-lived, transitory product, which is rapidly transformed to the corresponding trihydroxyindoline by self-condensation. At a further stage of oxidation, the trihydroxyindoline is transformed to its *p*-quinone derivative. Both the initial *p*-quinone and the indoline *p*-quinone are reactive derivatives which tend to undergo covalent binding with nucleophilic groups, as depicted in Fig. 1. In aqueous solution *in vitro*, the *p*-quinone formed by oxidation of 6-hydroxydopamine is rapidly converted to the corresponding trihydroxyindoline by 1,4-addition of the side chain amino group to the *p*-quinone. Under physiological conditions *in vivo*, however, the amino group of 6-hydroxydopamine could be fixed by the surrounding macromolecules and thus be prevented from forming an indole derivative by self-condensation. Under these conditions, the first oxidation product could already undergo covalent binding with nucleophilic groups of macromolecules and thus change their functional properties. The difference between 6-hydroxydopamine, on the one hand, and norepinephrine and dopamine, on the other, with respect to their denaturating properties is due mainly to differences in the speed of the formation of the first oxidation products.

It could be assumed that the relatively small amounts of bound radioactivity present after incubation with ^{3}H-norepinephrine result from a loss of tritium during rearrangement of the aminochrome to the indoxyl derivative of ^{3}H-norepinephrine (17). However, this seems to be rather improbable, since the amounts of radioactivity bound after incubation with both ^{3}H-norepinephrine and ^{3}H-dopamine are very similar. A loss of tritium during rearrangement of the aminochrome to the indole derivative of ^{3}H-dopamine is unlikely, since to each carbon atom of the side chain of dopamine labeled with tritium there is also bound a hydrogen atom, which would be preferentially released during rearrangement (18).

The experiments with bovine albumin have shown that derivatives of 6-hydroxydopamine are covalently bound to protein. The measurement of bound 6-hydroxydopamine derivatives might be in error because of the presence of oxidation products of 6-hydroxydopamine, which are insoluble in alcoholic 0.4 N $HClO_4$. These melanin-like products, however, were not formed to a significant extent under the present experimental conditions.

The binding of 6-hydroxydopamine can be prevented by interference with its oxidation, either by lowering the pH or by adding an antioxidant such as $Na_2S_2O_5$. This shows that oxidation of 6-hydroxydopamine is a prerequisite for its binding to protein. Lowering the pH also reduces the nucleophilicity of the bovine albumin and thereby delays the addition to the bovine albumin of the oxidation products, which are more slowly formed from 6-hydroxydopamine at lower pH.

The fact that acetylation of the nucleo-

philic groups of bovine albumin greatly reduces the binding of oxidation products of 6-hydroxydopamine supports the view that a nucleophilic addition takes place. The relatively small reduction in the binding of radioactivity to bovine albumin after heat denaturation excludes the possibility that denaturation as such, accompanying acetylation of albumin, is the main factor in reducing the binding of radioactivity to acetylated albumin.

The results obtained in our model system are consistent with the view that oxidation products of 6-hydroxydopamine undergo covalent binding with nucleophilic groups of biological macromolecules. The reaction is nonspecific, and the high selectivity of the destructive effect of 6-hydroxydopamine is due to the efficient transport of this amine into peripheral and central adrenergic neurons by the neuronal amine pump.

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