Journal of Supramolecular Structure 9:207–217 (1978)
Transmembrane Signaling 221–231

Neural Uptake of Catecholamines and Their Molecular Structures: A Histopharmacologic Study

H. Uno and J.H. Fellman

Cutaneous Biology and Laboratory of Pathology, Oregon Regional Primate Research Center, Beaverton, Oregon 97005 (H.U.); and Department of Biochemistry, University of Oregon Health Sciences Center, Portland, Oregon 97201 (J.H.F.)

Using ultrastructural and histofluorescence methods, we investigated the uptake mechanism of catecholamines by the nerve terminals in the cutaneous smooth muscles of stump-tailed macaques (Macaca arctoides). This in vivo approach ultilized the observed cytotoxic effects of 6-hydroxydopamine on these catecholamine-containing terminals and the protective effects of simultaneous treatment with catecholamines (dopamine, norepinephrine, and epinephrine), their 3-0-methylated derivatives (metanephrine and normetanephrine), and catechol acids (3, 4-dihydroxymandelic acid and 2, 4, 5-trihydroxymandelic acid). Both catecholamines and 3-0-methylated derivatives protected these nerve terminals from destruction by 6-hydroxydopamine, but catechol acids did not. However, the 3-0-methylated derivatives were less effective than the catecholamines. The degree of protection afforded by these amines depended largely on their concentration. Only catecholamines intensified the electron density of the intravesicular mass or the fluorescence in the nerve terminals; therefore, 3-0-methylated derivatives may inhibit 6-hydroxydopamine uptake at axoplasmic membrane sites, but not inside the axon.

These observations led to the discovery that there are two sites for the catecholamine uptake process. One site is the axoplasmic membrane. The terminals are protected by catecholamines and their 3-0-methylated derivatives from 6-hydroxydopamine uptake and thus destruction. The other site is the intra-axonal compartments. Here competitive binding between the vesicular protein and both 6-hydroxydopamine and the catecholamines plays a main role.

Key words: catecholamines, 6-hydroxydopamine, metanephrine, normetanephrine, catechol acids, neural uptake, sympathetic nerve, ultrastructure, histofluorescence.

The competitive uptake between norepinephrine and various sympathomimetic amines by the cardiac sympathetic nerves was extensively studied by Iversen [1] and by Burgen and Iversen [2] with the isolated heart preparation and perfusion of ^{3}H-norepinephrine with other amines.

Received May 9, 1978; accepted July 26, 1978.

0091-7419/78/0902-0207$02.30

Our study was designed to observe the sequential morphological changes in the cutaneous sympathetic nerve terminals that were induced by intradermally injected 6-hydroxydopamine (6-OHDA) mixed with various catecholamines and their analogues. 6-OHDA is widely used as a specific cytotoxic agent on sympathetic and catecholaminergic neurons. The fact that 6-OHDA was highly concentrated in the synaptic vesicles of the terminal axons shortly after injection was revealed by a significant increase in the vesicular dense-cored granules (as seen in electron micrographs). Whether the subsequent cytotoxic effect of 6-OHDA on the terminal axons proceeded to the vesicular structures or to entire axon structures depended on the dose given. A small dose caused swelling, partial disruption of the vesicles, and disappearance of the granules (degranulation); a large dose induced complete degeneration (coagulation necrosis) of the terminal axons [3–7]. Thus, the observed destructive changes of the nerve terminals by 6-OHDA could be utilized as a semiquantitative index of its uptake.

Our previous studies had revealed that the piloarrector muscles of stump-tailed macaques (Macaca arctoides) are abundantly innervated with catecholamine-containing nerve terminals (adrenergic) and very sparsely with nonadrenergic terminals (probably cholinergic). The muscles located in dense dermal collagen have no direct vascular supply inside the muscle bundles. After local (intradermal) injection of 6-OHDA, we observed that most of the terminal axons in the muscles degenerated and that catecholamine in the nerve plexuses was completely depleted [8]. Moreover, when 6-OHDA was injected simultaneously with other amines, the degree of destructive change and the depletion of catecholamine in the nerve terminals were significantly less than when the same dose of 6-OHDA was used alone [9].

We employed this experimental model and procedure in an in vivo approach to examine the competitive uptake between 6-OHDA and various catecholamine analogues by the sympathetic nerve terminals. Using the histofluorescence method of revealing the terminal catecholamine content and electron microscopic observation of the structural changes in the terminal axons, we examined the inhibitory effect of several compounds on the cytotoxicity of 6-OHDA. The compounds were catecholamines (norepinephrine, epinephrine, and dopamine) and their metabolites (metanephrine, normetanephrine, and 3,4-dihydroxymandelic acid). We also examined the effects of 2,4,5-trihydroxyphenylacetate (6-hydroxydopac), the deaminated analogue of 6-OHDA.

This report is a summary of the results of our studies and a discussion of the mechanism of axonal membrane transport of catecholamines and related compounds in sympathetic nerve terminals.

MATERIALS AND METHODS

Twenty-four adult stump-tailed macaques (Macaca arctoides) were used. The drugs – ie, 6-OHDA hydrobromide, 3-hydroxytyramine hydrochloride (dopamine), norepinephrine hydrochloride, epinephrine bitartrate, metanephrine hydrochloride, normetanephrine hydrochloride, 3,4-dihydroxymandelic acid (all purchased from Sigma Chemical Co.), and 6-hydroxydopac [10] – were prepared fresh each time by dissolution in 0.001 N hydrochloride or 0.2% ascorbic acid saline solution. For single injections, 1 mg of each drug was dissolved in 0.1 ml of vehicle. For mixed injections, two groups of different concentrations of 6-OHDA and each drug were used; one group of solutions contained 1 mg of 6-OHDA plus 1 mg of each drug in 0.1 ml of vehicle, and the other group of solutions contained 1 mg of 6-OHDA plus 5 mg of each drug in 0.1 ml of vehicle. Each solution was injected intradermally with a 27-gauge needle, which made a wheal about 1 cm in diameter.

Skin specimens (1 × 0.5 cm) from the injected areas of all animals were obtained 24 h after injection under anesthesia (ketamine hydrochloride, 5 mg per kilogram of body weight, intramuscularly). Various regions of the scalp, nucha, and back were used as injection sites. Skin specimens from at least two and as many as six animals were used for each single- or mixed-drug injection.

Each skin specimen was divided into two sections; one was immediatelly frozen in liquid nitrogen for histofluorescence study, and the other was fixed with glutaraldehyde and osmium for electron microscopic study. Frozen samples 2 mm thick were freeze-dried over phosphorus pentoxide at -40° at 10^{-3} mm Hg for 1 week. The tissues were then exposed to formaldehyde vapor in 70% relative humidity at 80° for 1 h and embedded in paraffin. Sections were cut 10 μm thick, deparaffinized by xylene, and mounted with paraffin oil. Fluorescence was observed in approximately 20 piloarrector muscles in each animal by means of a Zeiss universal microscope with a BG-12 ultraviolet filter and a "50" fluorescence emission filter.

For electron microscopic study, glutaraldehyde-fixed tissues were refixed with 1% osmium solution and embedded in epoxy resin.

Thin sections were cut with an LKB ultramicrotome and stained with lead citrate and uranium acetate for observation in a Philips 200 electron mciroscope. The ultrastructure of the nerve terminals was examined in at least 10 piloarrector muscles from each injected skin sample.

RESULTS

Histologically, the piloarrector muscles in the skin of stump-tailed macaques are located in the upper dermis; the muscle bundle reaches to both the hair follicle and the epidermal ridge. Each muscle bundle is surrounded by dense, collagenous tissue of the subepidermal layer (Fig 1).

When viewed by means of fluorescence microscopy, the muscle in the skin of control animals (saline injection) always contained dense plexuses of nerve terminals that fluoresced with the strong greenish-yellow light characteristic of catecholamines (Fig 2).

In the electron microscope we observed that the nerve plexuses in the muscles consisted of numerous varicose terminal axons, mostly oriented parallel to the long axis of the muscle bundles and dispersed in the intercellular spaces between the smooth muscle cells (Fig 3a). The axons were partially ensheathed with Schwann cells and were not in direct contact with the muscle cells. A small number of fine collagenous fibers was always present between the terminal nerves and the smooth muscle cells. In the muscles of control animals, the terminal axons contained numerous synaptic vesicles; most of them were small dense-cored vesicles, but a few were large dense-cored ones. Generally, the small vesicles contained a tiny speck of electron-dense material (Fig 3b). Muscle tissue into which 1 mg of 6-OHDA had been injected (a dose sufficient to destroy most of the terminals in the area examined) exhibited total loss of fluorescent catecholamine in the terminal plexuses (Fig 4). Ultrastructurally, a majority of the terminal axons in the muscles showed severe degenerative change (Figs 5 and 6). The entire axoplasma of the degenerated axons appeared as an amorphous, electron-dense mass, and the necrotic axons were totally surrounded by Schwann cells (Fig 5).

The results of histofluorescence and electron microscopic studies in the animals given either a single injection of various compounds or a mixed injection of 6-OHDA with each compound are summarized in Table I. The granular density of the small dense-cored vesicles (see Fig 3) corresponded to that of the controls having normal granularity.

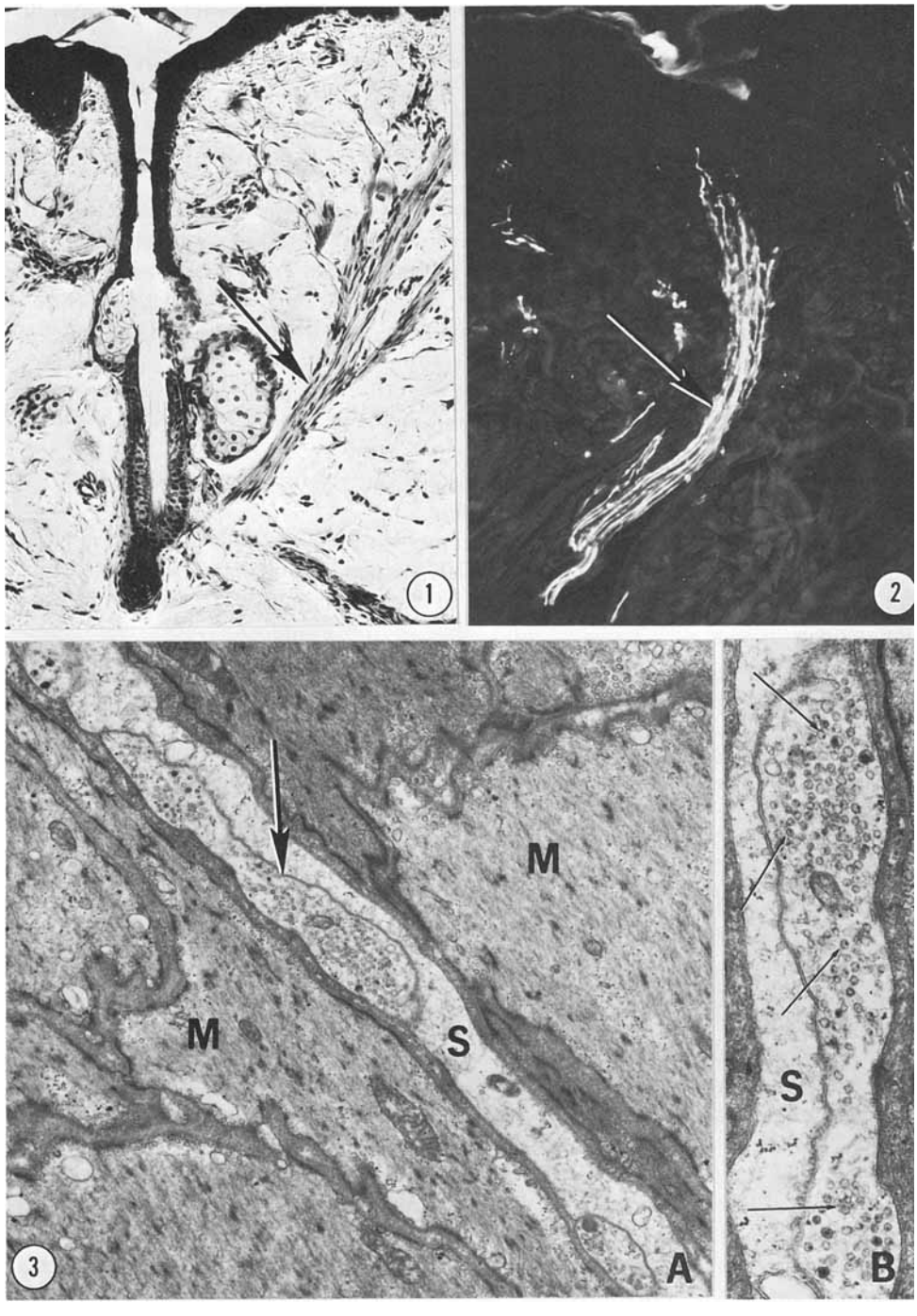

Fig 1. Micrograph of piloarrector muscle (arrow) in dense collagenous tissue in upper dermis of macaque skin (original magnification, × 100).

Fig 2. Control (saline injection); dense nerve plexuses of catecholamine fluorescence (arrow) innervating the entire muscle bundle (original magnification, × 100).

Fig 3. Control. a. Varicose terminal axons (arrow) and Schwann cells (S) located between smooth muscle cells (M) (original magnification, × 20,000). b. High-power view of axon. Note the many small dense-cored vesicles containing tiny electron-dense granules (arrows) and a few large dense-cored vesicles (original magnification, × 35,000).

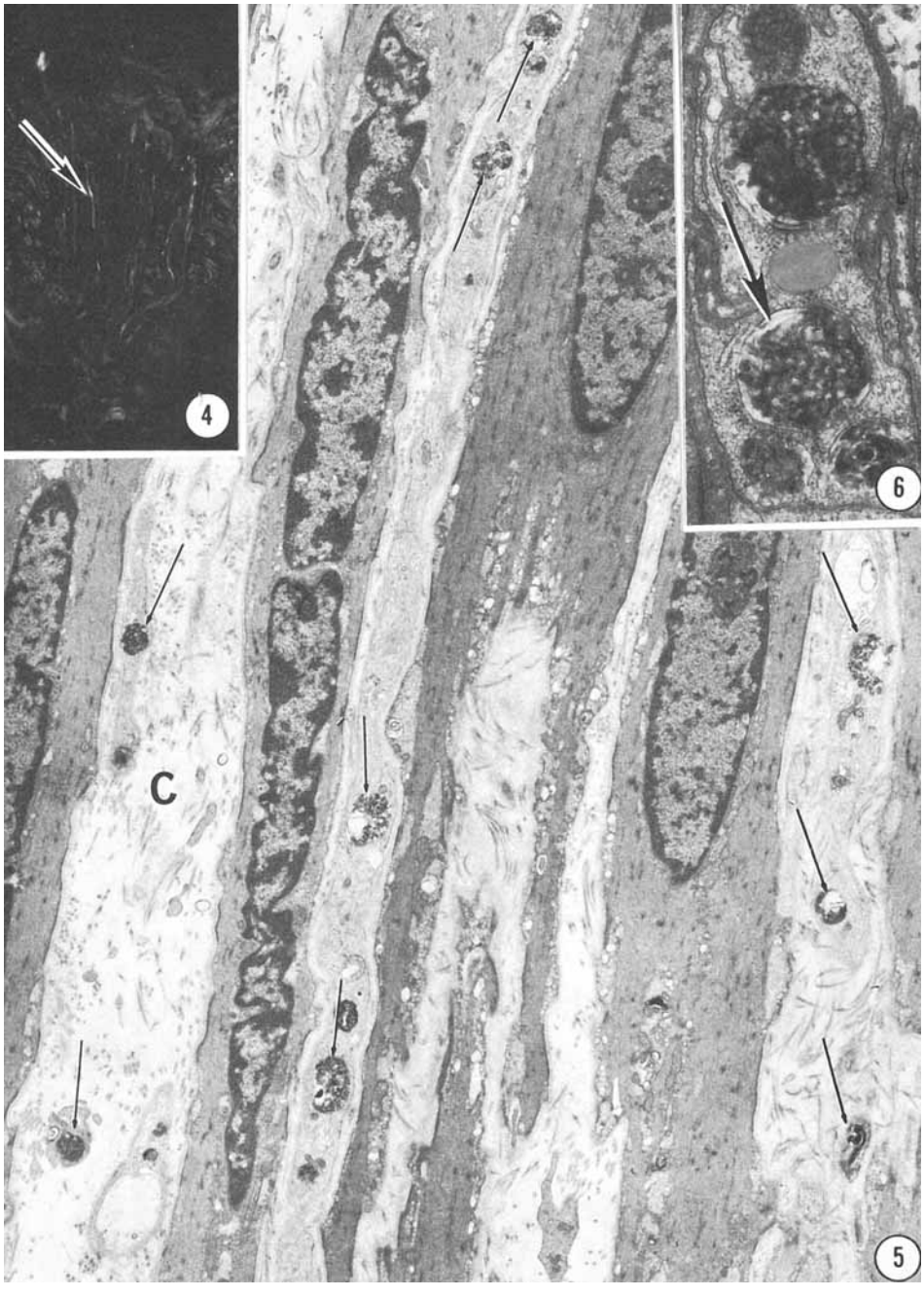

Fig 4. Fluorescence micrograph after injection of 1 mg of 6-hydroxydopamine. There is complete disappearance of catecholamine fluorescence from the nerve fibers and nonspecific yellow fluorescence of the collagen fibers (arrow) in the muscle bundle (original magnification, × 200).

Fig 5. Many degenerated terminal axons (arrows) appeared in the intercellular spaces between muscle cells after injection of 1 mg of 6-hydroxydopamine. Collagen fibers (C) are seen in the intercellular spaces (original magnification × 10,000).

Fig 6. Degenerated axons replaced the amorphous, electron-dense mass after injection of 1 mg of 6-hydroxydopamine. Note that the axons are totally surrounded by Schwann cells (original magnification, × 30,000).

TABLE I. Intensity of Catecholamine Fluorescence and Ultrastructural Findings in the Nerve Terminals

	Type of injection					
			Mixed injection			
	Single injection		1 mg mixed with 1 mg of 6-OHDA		5 mg mixed with 1 mg of 6-OHDA	
Compound	Fluorescence[a]	Ultrastructure	Fluorescence[a]	Ultrastructure	Fluorescence[a]	Ultrastructure
Saline	++ (2)	Normal (3)				
6-OHDA (1 mg)	– (4)	Degeneration (5,6)				
Catechols (1 mg)						
Norepinephrine	++	Normal	+ (7)	Degranulation (8)	++ (9)	Increased Granularity (10)
Epinephrine	++	Normal	+	Degranulation	++	Increased Granularity
Dopamine	++	Normal	+	Degranulation	++	Increased Granularity
Metanephrine	++[b]	Normal	±[b]	Degranulation	++	Normal
Normetanephrine	++[b]	Normal	± (11)[b]	Degeneration (12)	±	Degranulation (13)
Dihydroxymandelate	++[b]	Normal	–	Degeneration	–	Degeneration
6-Hydroxydopac	++[b]	Normal	–	Degeneration	–	Degeneration (14)

Note: The numbers in parentheses indicate the relevant figures.

[a] ++ = strong fluorescence (similar to that seen in control specimens); + = weak fluorescence; ± = most muscles showed weak fluorescence but some showed no fluorescence; – = no fluorescence.

[b] Fluorescence of endogenous catecholamine.

In the group that received single-drug injections (1 mg each), all catecholamines (norepinephrine, epinephrine, and dopamine), their metabolites (metanephrine, normetanephrine, and dihydroxymandelic acid), and 6-hyroxydopac failed to induce a significant change in the intensity of fluorescence or the granular content of the small dense-cored vesicles in the terminal axons. By the technique of formaldehyde-induced fluorescence, we revealed that the chemical conversion of catechol compounds to a fluorescent, active substance (dihydroisoquinoline) occurs only in catecholamines (norepinephrine, epinephrine, and dopamine), and not in the 3-0-methylated compounds and catechol acids [Uno, unpublished data].

Therefore, the fluorescense that appeared in the tissue specimens treated with 3-0-methylated compounds or catechol acids was considered to be that of endogenous catecholamines in the nerve terminals, probably norepinephrine.

The disruptive effects of 6-OHDA were prevented by simultaneous injection of sufficient amounts of catecholamines. A weak fluorescence of the terminals remained in all muscles from animals given mixed injections of equal amounts (1 mg of each) of 6-OHDA and each catecholamine (norepinephrine, epinephrine, or dopamine) (Fig 7), and the granular density of the vesicles decreased significantly. This degranulation, accompanied by swelling of the vesicles, appeared in both small and large dense-cored vesicles (Fig 8).

After injection of 1 mg of 6-OHDA with 5 mg of either norepinephrine, epinephrine, or dopamine, intense fluorescence was observed in the nerve plexuses of all muscles (Fig 9), and the granular density increased greatly in many small dense-cored vesicles (Fig 10).

Injection of equal amounts (1 mg each) of metanephrine and 6-OHDA caused a marked weakening of fluorescence in the nerve plexuses of the muscles (Fig 11) and complete degranulation of the vesicles in the terminal axons. Normetanephrine plus 6-OHDA (1 mg each) also induced a weakening of fluorescence and caused severe degenerative change in most of the terminal axons (Fig 12).

After injecting 5 mg of metanephrine and 1 mg of 6-OHDA, we observed strong fluorescence in the nerves. Ultrastructurally, the granular density in the vesicles was similar to that in control specimens.

With 5 mg of normetanephrine and 1 mg of 6-OHDA, a weak fluorescence of the nerves was observed in most of the muscles; a few muscles had no fluorescent nerves. Ultrastructurally, most of the terminals contained many degranulated vesicles, and some terminals showed degeneration (Fig 13).

Injection of 3,4-dihydroxymandelic acid or 6-hydroxydopac with an equal amount of 6-OHDA (1 mg each) resulted in complete disappearance of the fluorescent nerves in the muscles. Ultrastructurally, most of the terminals in the muscles degnerated in each sample.

After injecting either 3,4-dihydroxymandelic acid or 6-hydroxydopac (5 mg each) with 1 mg of 6-OHDA, we observed the complete disappearance of fluorescent nerves in the muscles and degneration of most terminal axons (Fig 14).

DISCUSSION

Uptake of 6-OHDA by the cutaneous sympathetic nerve terminals was inhibited by simultaneous injection of 6-OHDA with catecholamines and 3-0-methylated catecholamines. Deaminated compounds (catechol acids: 3,4-dihydroxymandelic acid and 6-hydroxydopac) had no effect on the terminals or on the uptake of 6-OHDA. The degree of inhibition depended largely on their concentration in the local environment. Because the piloarrector muscles were totally surrounded by dense, dermal collagen and had no vascular

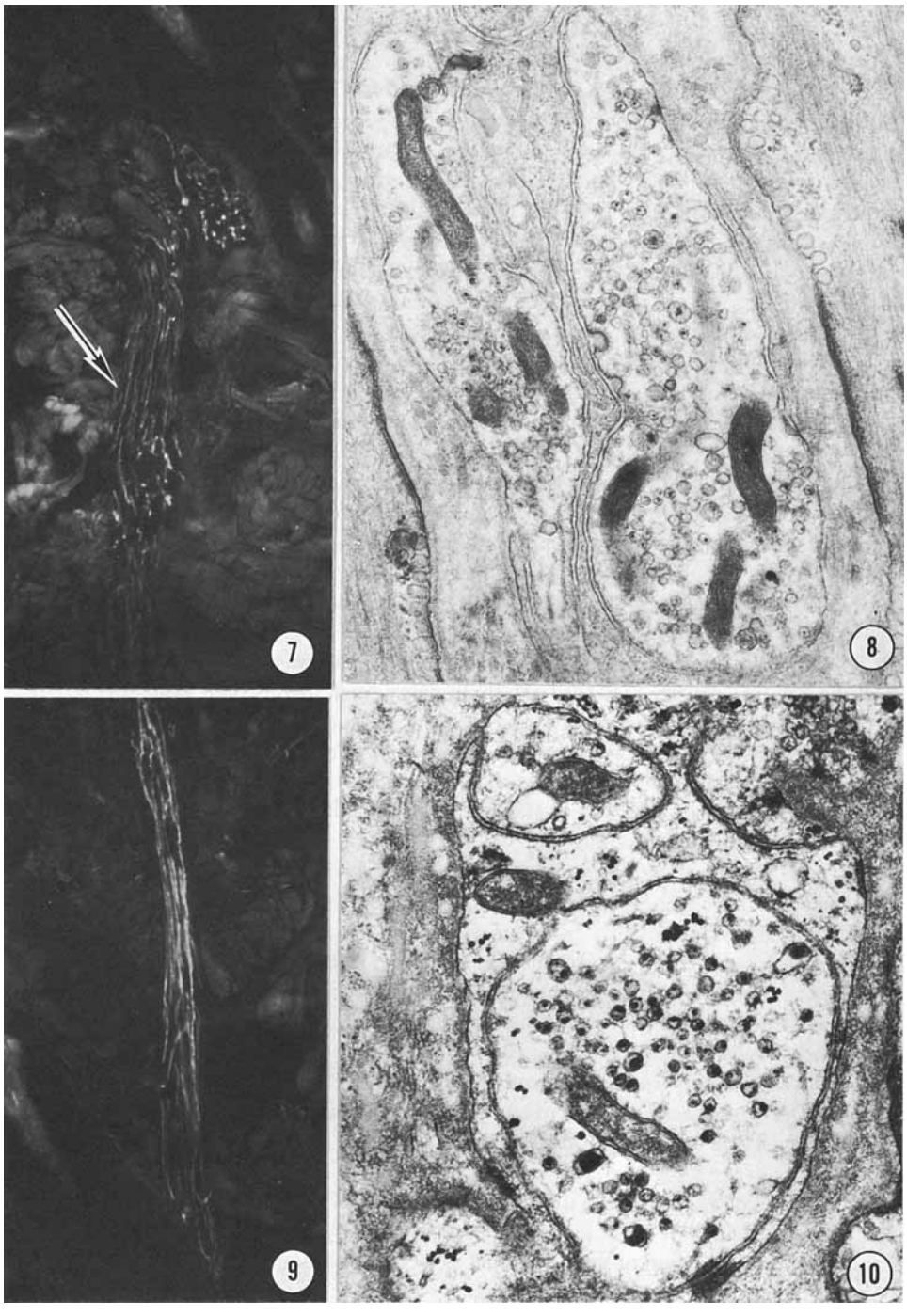

Fig 7. There is still a weak catecholamine fluorescence in the nerve fibers in muscle (arrows) after injection of 1 mg of 6-hydroxydopamine with 1 mg of norepinephrine (original magnification, × 200).

Fig 8. Vesicles in terminal axons show swelling and partial disruption after injection of 1 mg of 6-hydroxydopamine with 1 mg of norepinephrine. Dense-cored granules have disappeared from most of the vesicles (degranulation) (original magnification, × 45,000).

Fig 9. Strong catecholamine fluorescence appeared in the nerve fibers in muscle after injection of 1 mg of 6-hydroxydopamine with 5 mg of dopamine (original magnification, × 200).

Fig 10. There is a marked increase in dense-cored granules in many vesicles after injection of 1 mg of 6-hydroxydopamine with 5 mg of dopamine (original magnification, × 45,000).

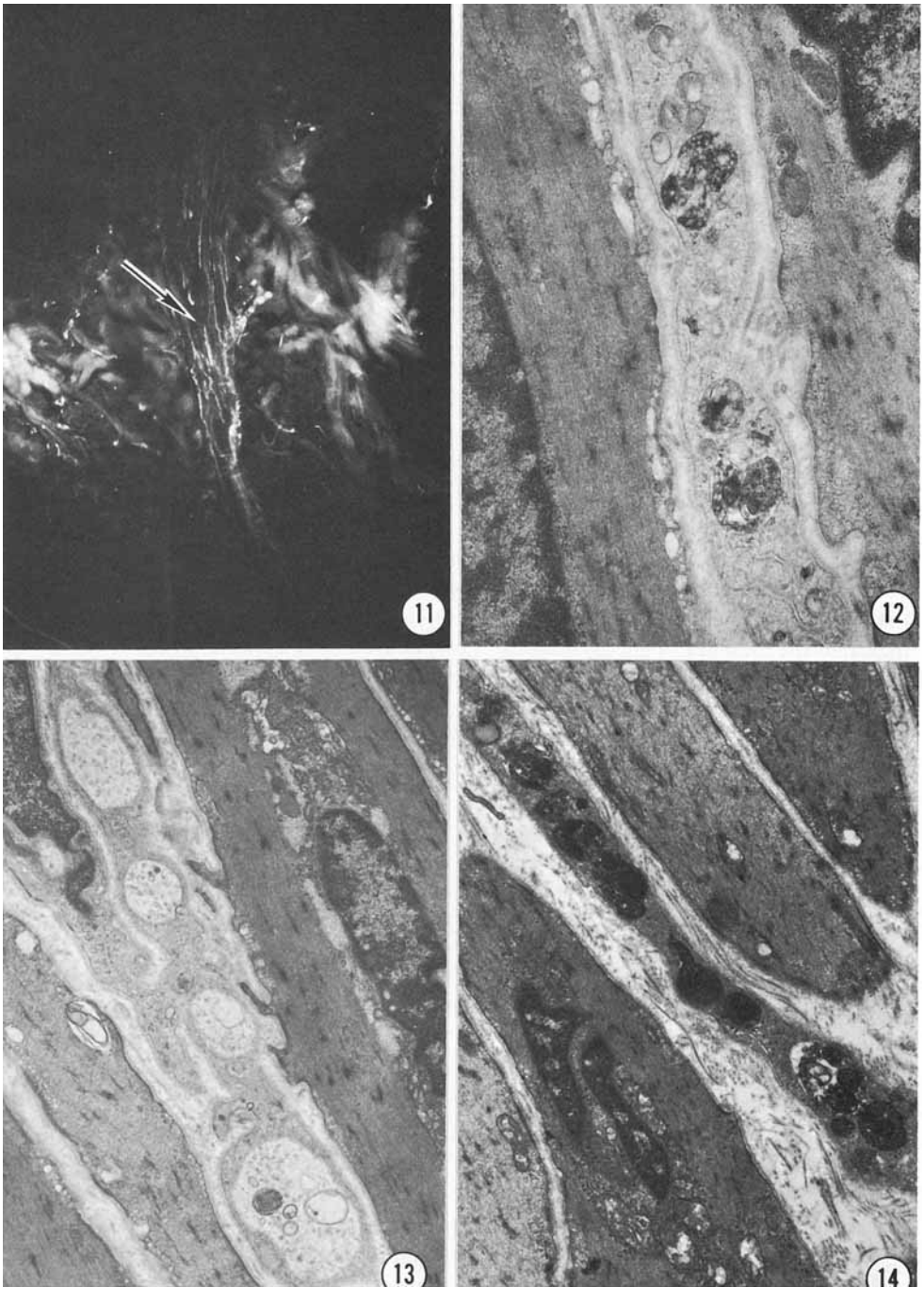

Fig. 11. There is a marked weakening of catecholamine fluorescence in the nerve fibers in muscle (arrow) after injection of 1 mg of 6-hydroxydopamine with 1 mg of normetanephrine (original magnification, × 200).

Fig 12. Degenerated axons appeared in most of the muscle fibers after injection of 1 mg of 6-hydroxydopamine with 1 mg of normetanephrine (original magnification, × 5,000).

Fig 13. Terminal axons after injection of 1 mg of 6-hydroxydopamine with 5 mg of normetanephrine. Vesicles in most of the axons show degranulation (original magnification, × 5,000).

Fig 14. Terminal axons after injection of 1 mg of 6-hydroxydopamine with 5 mg of 6-hydroxydopac. All terminal axons show severe degeneration (original magnification, × 5,000).

innervation, the injected drugs were homogeneously and slowly absorbed by the muscle bundles. These muscles were abundantly innervated with sympathetic terminals. Therefore, this experimental model and procedure provided a suitable tool for the observation of neuronal uptake of catecholamines and their analogues by the terminals. The degree of the cytotoxic effect of 6-OHDA on the nerve terminals was known to be dose-dependent, and the induced changes were clearly visible by both histofluorescence and electron microscopy.

The nature of the molecular structures of the different groups of catecholamines and their metabolites obviously influenced the inhibition. The effective order of inhibition was norepinephrine, epinephrine, and dopamine > metanephrine > normetanephrine. Because the deaminated compounds did not appear capable of neuronal uptake, we concluded that the presence of an amino group in the molecular structure is necessary for axonal membrane transport of catecholamines.

Although Burgen and Iversen [2] used different approaches, they found that 3-0-methylated compounds have a weaker inhibitory effect on norepinephrine uptake by the cardiac nerves than do other amines. Their results also revealed that normetanephrine has a much weaker effect than metanephrine.

The current technique (formaldehyde-induced fluorescence of catecholamine) cannot reveal 3-0-methylated compounds. Therefore, fluorescence in the nerve terminals in tissue treated with metanephrine or normetanephrine, either alone or with 6-OHDA, indicated the presence of endogenous catecholamine (norepinephrine) in the terminals. Ultrastructurally, 3-0-methylated compounds, unlike all catecholamines, do not increase the granular density of the vesicles. These facts suggest that 3-0-methylated compounds are not taken up by the vesicles or probably even by the axoplasma. Nevertheless, both metanephrine and normetanephrine apparently inhibit the uptake of 6-OHDA by the terminals.

Although we need further information on the fate of 3-0-methylated compounds in the terminal-receptor junction, the results of our studies suggest that both metanephrine and normetanephrine inhibit 6-OHDA uptake by the axonal membrane; they do bind with sites on the membrane that transport the catecholamines, including 6-OHDA, into the axoplasma.

Thus, we propose a two-site model for the catecholamine uptake process by the sympathetic nerve terminals. One site, just mentioned above, locates at the axoplasmic membrane; all monamine compounds used in this study competitively bind with the membrane parts (receptor), and catecholamines and 6-hydroxydopamine further transport into the axon, but 3-0-methylated compounds may remain at the membrane. The other site occurs in the intra-axonal compartments; transported catecholamines and 6-hydroxydopamine reach to the vesicles through the axon membrane where they competitively bind with the vesicular protein.

All three catecholamines acted similarly to inhibit 6-OHDA uptake and showed a strong tendency to bind with proteins in the synaptic vesicles. When a highly concentrated amount was injected into the dermal milieu, the storage capacity of the vesicles for these catecholamines appeared far greater than that in control specimens, and there was a remarkable increase in electron-dense granules in the vesicles. Although we need a precise method for identification of each of these amines inside the vesicles, the possibility that epinephrine is stored in the sympathetic nerves has not been completely ruled out by our results, a fact that may suggest the release, uptake, and storage of neuronal norepinephrine are influenced by humoral catecholamines.

Although 6-hydroxydopac has reactive properties similar to those of 6-OHDA in that it is spontaneously and rapidly oxidized to a reactive quinone and reacts with proteins to form covalent bonds [11, 12], it does not disrupt synaptic terminals, a fact that underscores

the requirement of a cationic amine functional group for uptake. The anionic character of the carboxylate group apparently prevents its uptake by the membrane site responsible for catecholamine retrieval.

The histofluorescence method of revealing catecholamines and electron microscopic observation of the synaptic vesicles are useful in discerning rather precisely the nature of the molecular requirements for neurohumoral uptake.

ACKNOWLEDGMENTS

Publication No. 986 of the Oregon Regional Primate Research Center. Supported in part by grants RR 00163, NS 01572, and RR 05694 from the National Institutes of Health and grant 7624 from the Medical Research Foundation of Oregon.

We are deeply grateful to Tsutomu Yoshida, Tsunekazu Fuse, Douglas E. Kligman, and Daniel L. Toyooka for their excellent technical assistance.

REFERENCES

1. Iversen LL: Br J Pharmacol 25:18, 1965.
2. Burgen ASV, Iversen LL: Br J Pharmacol 25:34, 1965.
3. Thoenen H, Tranzer JP: Arch Pharmakol Exp Pathol 261:271, 1968.
4. Bennett T, Burnstock G, Cobb JLS, Malmfors T: Br J Pharmacol 38:809, 1970.
5. Kostrzewa RM, Jacobowitz DM: Pharmacol Rev 26:199, 1974.
6. Uno H, Montagna W: Cell Tiss Res 148:1, 1975.
7. Uno H: Neurosci Abstr 2:476, 1976.
8. Uno H, Montagna W: Anat Rec 185:49, 1976.
9. Uno H, Fellman JH: Neurosci Abstr 1:408, 1975.
10. Wada GH, Fellman JH: Biochemistry 12:5212, 1973.
11. Rotman A, Daly JW, Creveling CR: Molec Pharmacol 12:887, 1976.
12. Liang WD, Platsky PM, Adams RN: J Med Chem 20:581, 1977.