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An ultrastructural and histochemical study of the short-term effects of 6-hydroxydopamine on adrenergic nerves in the domestic fowl

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Summary

1. The effects of 6-hydroxydopamine (6-OHDA) on adrenergic nerves in the domestic fowl have been investigated with ultrastructural and fluorescence histochemical methods.

2. 6-OHDA depletes the nerves of catecholamine, initially by displacing it from the storage vesicles. 6-OHDA enters large as well as small vesicles, indicating that large granular vesicles in adrenergic nerves are sites of amine storage.

3. Doses of 6-OHDA, insufficient to cause degeneration, still cause loading of the vesicles.

4. The effects of various drugs on the action of 6-OHDA indicate that this drug must be taken up by the nerves and reach a critical extragranular axoplasmic concentration before degeneration will occur; 6-OHDA bound in the vesicles plays no part in the degenerative process.

Introduction

It has been known for some time that 6-hydroxydopamine depletes the catecholamine content of sympathetically innervated tissues (Porter, Totaro & Stone, 1963; Laverty, Sharman & Vogt, 1965; Porter, Totaro & Burcin, 1965). It is only recently, however, that this drug has been shown to cause specific degeneration of adrenergic nerves (Tranzer & Thoenen, 1967; 1968a; Thoenen & Tranzer, 1968; Malmfors & Sachs, 1968; Jonsson & Sachs, 1970). Previous ultrastructural investigations of the effects of 6-hydroxydopamine on adrenergic nerves have dealt only with the appearance of the degenerating nerves 24 h or more after the last injection of a series of repetitive doses of the drug (Tranzer & Thoenen, 1967; 1968a; Thoenen & Tranzer, 1968). The present study deals with the ultrastructural changes in adrenergic nerves of the domestic fowl during the first 24 h following single doses of 6-hydroxydopamine, and these alterations have been correlated with changes in the nerves as seen with the fluorescence histochemical technique of Falck and Hillarp for the localization of biogenic monoamines. An examination has also been made of the changes in adrenergic nerves following treatment with 6-hydroxydopamine in conjunction with drugs that interfere with catecholamine uptake and storage in order to investigate the mechanism of action of 6-hydroxydopamine.

Methods

Two hundred white leghorn chicks were used in this study ; they were 1–2 weeks old and weighed between 60–90 g. The following drugs were administered intravenously by injection into the wing vein: 6-hydroxydopamine hydrochloride (H 88/32 Hässle, Sweden) ; reserpine (Serpasil, Ciba) ; (+)-amphetamine bitartrate ; desmethylimipramine (Pertofran, Geigy) ; and metaraminol bitartrate (Aramine, Merck, Sharp and Dohme). Doses were calculated as the salts ; 6-hydroxydopamine (6-OHDA) was dissolved in saline solution containing 0·2 mg/ml ascorbic acid. Control experiments were carried out in which the carrier solution was injected without 6-OHDA ; the carrier had no effect.

The expansor secundariorum—a smooth muscle from the wing (see George & Berger, 1966)—was used in the present study, for this tissue is densely innervated by post-ganglionic adrenergic nerves only (see Buckley & Wheater, 1968 ; Bennett & Malmfors, 1970). The muscle from one wing was processed for fluorescence microscopy while that from the other wing was prepared for electron microscopy. Control experiments showed that injection into the wing vein did not produce a differential effect in the muscles from opposite sides. Animals were killed by an overdose of ether, and the preparations made as described below.

Fluorescence histochemical localization of catecholamines

The technique used was that of Falck & Hillarp (Falck, Hillarp, Thieme & Torp, 1962 ; Falck & Owman, 1965 ; see Corrodi & Jonsson, 1967). The expansor secundariorum was frozen in liquid propane cooled with liquid nitrogen and freeze-dried. It was then incubated at 80° C with formaldehyde gas, generated from paraformaldehyde in equilibrium with air of 70% relative humidity, for 1 h. Following vacuum embedding and sectioning, the tissue was examined with the fluorescence microscope. The specificity of the fluorescence was tested using the accepted procedures (see Corrodi & Jonsson, 1967).

Electron microscopy

The expansor secundariorum muscle was exposed *in situ* by removal of the skin, immediately after the animal had been killed. The wing was then cut off and pinned to a cork board in such a way that the muscle was prevented from contracting. Fixative (2% osmium tetroxide buffered to pH 7·4 with veronal acetate) was pipetted on to the tissue and left for 15 min at room temperature. The muscle was then removed, fixed for a further 30–45 min in buffered osmium tetroxide, washed in 20% acetone, dehydrated in acetone and embedded in Araldite. Thin transverse sections were cut, stained with lead citrate and uranyl acetate and examined at 50 kV in an Hitachi HU 11b electron microscope.

Results

Fluorescence microscopy

One hour after injection of a low dose (1 to 2 mg/kg) of 6-OHDA there was a slight reduction in the fluorescence intensity of terminal fibres. This reduction persisted for at least 6 h, but 24 h after administration of the drug, the fluorescence intensity and numbers of terminal fibres appeared normal. Non-terminal axon bundles were

unaffected by low doses of 6-OHDA and showed no increase in fluorescence intensity indicative of catecholamine accumulation due to degeneration of terminal fibres (Malmfors & Sachs, 1968 ; Jonsson & Sachs, 1970).

Following doses of 10 to 25 mg/kg 6-OHDA, terminal fibres showed a reduction in fluorescence intensity within the first 1 to 2 h after injection of the drug. After 24 h the fluorescence of the terminal fibres was still below normal intensity and, following the higher dose (25 mg/kg), there were fewer terminal fibres (about 50 to 80% remaining), indicating that some were degenerating. At this time, non-terminal axon bundles showed increased fluorescence intensity due to catecholamine accumulation.

There was a marked decrease in fluorescence intensity of terminal fibres within 1 h after injection of 6-OHDA 50 mg/kg, and there were indications of catecholamine accumulation in non-terminal axon bundles within 6 h. By 24 h after this dose only about 30–50% of the terminal fibres were detectable and their fluorescence was much below the normal intensity ; there were large accumulations of catecholamines in the otherwise normal non-terminal axon bundles.

Following a higher dose (100 mg/kg) of 6-OHDA the initial reduction in fluorescence intensity of terminal fibres was rapid ; after 15 min there was a perceptible decrease, and after 30 min this reduction was marked. By 1 h the terminal fibres that were detectable were only very faintly fluorescent and remained so for the next 24 h. Non-terminal axon bundles showed slightly increased fluorescence intensity due to catecholamine accumulation as early as 2 h after 100 mg/kg doses of 6-OHDA ; the accumulations were marked after 24 h. At this time the few (about 5–10%) terminal fibres that remained were only faintly fluorescent.

Many of the drugs used in conjunction with 6-OHDA caused a decrease in fluorescence intensity, so it was not always possible to assess their effects on the action of 6-OHDA. For example, following reserpine (20 mg/kg) terminal fibres were undetectable in all tissues examined after 2 h, and there was a marked reduction in the fluorescence intensity of non-terminal axon bundles.

Injection of desmethylimipramine (20 mg/kg) 30 min before treatment with 6-OHDA (5 to 50 mg/kg) prevented the effects of the latter drug. However, with higher doses of 6-OHDA (100 mg/kg) there was a slight decrease in fluorescence intensity of terminal and non-terminal axon bundles, but there was no disappearance of terminal fibres or signs of catecholamine accumulation in non-terminal fibres up to 24 h after treatment.

Electron microscopy

In the animals examined the smooth muscle cells of the expansor secundariorum were 3–4 μm in diameter. On average, one axon profile was associated with three or more muscle cells ; occasionally, axon profiles naked of a Schwann cell sheath were seen lying embedded in grooves in the muscle cells. About 20% or more of the axon profiles were 1·5–2 μm in diameter and had Schwann cell sheaths around them. The distribution of axon profiles was even throughout the muscle bundles. Small groups of nerve fibres were seen running in the collagen tracts that separated the muscle bundles. There were up to twenty unmyelinated fibres in these axon bundles. Such non-terminal axon bundles running between the muscle bundles of the expansor secundariorum have been demonstrated with fluorescence microscopy (Bennett & Malmfors, 1970). Further evidence that they

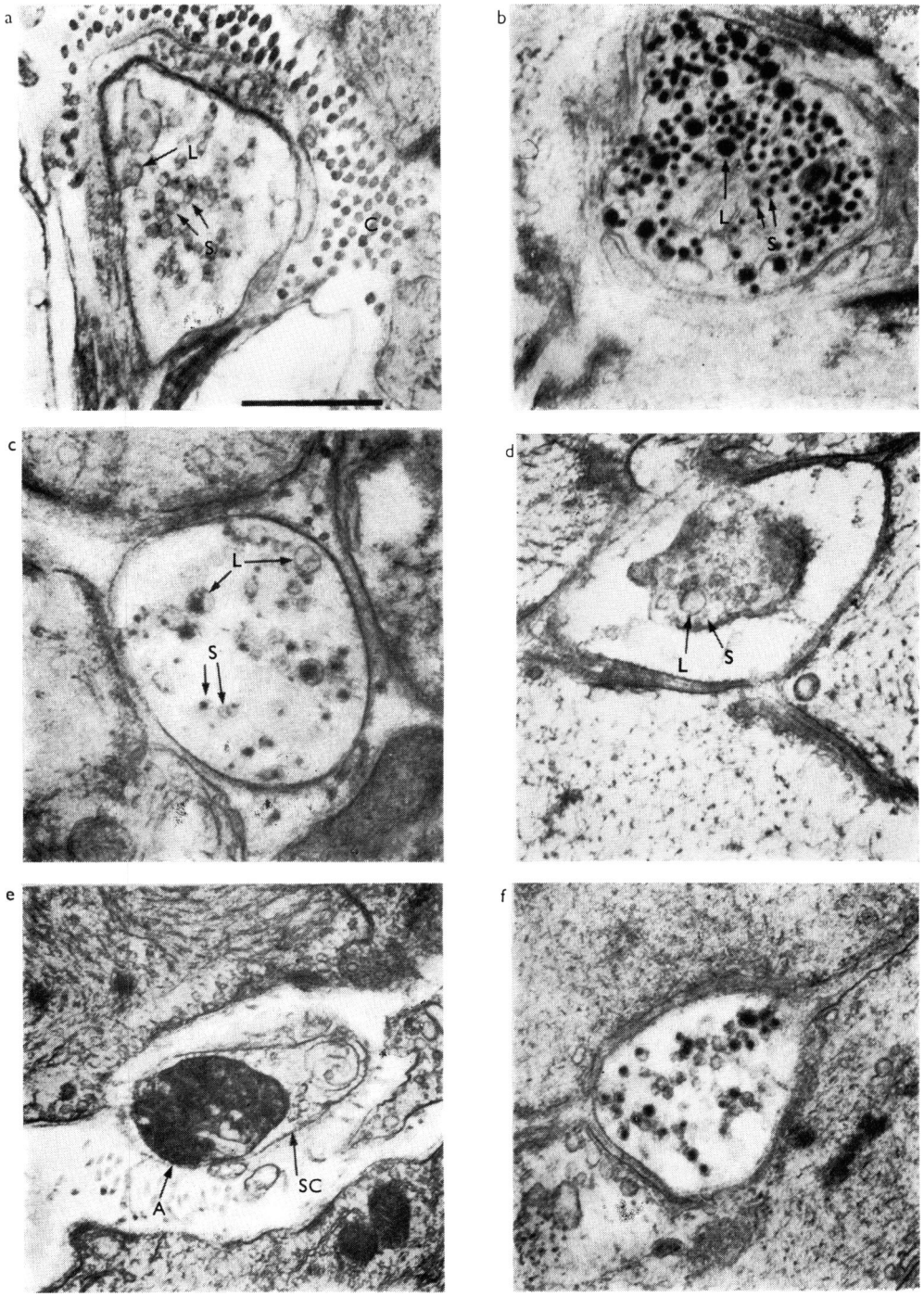

FIG. 1. Electron micrographs. (a) Axon profile in the expansor secundariorum muscle from a normal animal. L = large, apparently agranular vesicle. S = small vesicles showing various degrees of granulation. C = collagen. (b) Similar axon profile in an animal 1 h after injection of 6-OHDA (100 mg/kg). Note that all the large (L) and small (S) vesicles are almost filled with a very electron-opaque deposit. The axon profile otherwise appears normal. (c) Axon profile 2 h after injection of 6-OHDA (100 mg/kg). The large (L) and small (S) vesicles show less dense granulation than in (b), and in some cases the vesicles have completely degranulated. The axon profile appears swollen. (d) Axon profile 4 h after injection of 6-OHDA (100 mg/kg). The axoplasm and degranulated large (L) and small (S) vesicles are clumped together within the disrupted axon profile. (e) Axon profile 24 h after injection of 6-OHDA (100 mg/kg) showing electron-opaque coagulation of axoplasm and organelles (A). The Schwann cell (SC) remains intact. (f) Axon profile 24 h after injection of 6-OHDA (25 mg/kg). In this case there are no obvious signs of degeneration, but many vesicles are degranulated. Calibration: 1 μm in (a), (b), (c), (d) and (f); 2 μm in (e).

were non-terminal was that, in the present study, axon profiles in such bundles very rarely contained vesicles; those present were irregular and only faintly granular. This was in contrast to the axon profiles lying within the muscle bundles which contained large numbers of vesicles. The vesicles were either large (800 to 1,200 Å in diameter) or small (400 to 600 Å in diameter). About 50% of the total vesicle population had cores of variable, but generally low, density. The majority of vesicles in any one profile were small, but most profiles contained three or more large vesicles (Fig. 1a).

Effects of 6-OHDA

With the electron microscope it was easier to observe indications of the effects of 6-OHDA at lower doses than with the fluorescence histochemical technique. Thus, there was an obvious granulation of the large vesicles in axon profiles 1 h after a dose of 1 mg/kg 6-OHDA. The granulation was much more marked following a dose of 2 mg/kg 6-OHDA, but there was still no indication of loading of the small vesicles. With a higher dose (5 mg/kg) there was loading of large and small vesicles after 1 h, and after the same time with a dose of 10 mg/kg 6-OHDA, the cores of the large and small vesicles appeared very dense. The density did not change over the next 6 h, but 24 h after administration of the drug the vesicles were normal in appearance. Generally speaking, the higher the dose of 6-OHDA administered, the denser and more extensive were the cores of the loaded vesicles. Thus 1–6 h after a dose of 25 mg/kg 6-OHDA, most vesicles had cores that almost filled them. Following injection of higher doses of 6-OHDA, the loading of the vesicles was seen earlier; after treatment with 100 mg/kg 6-OHDA all vesicles were loaded after 5–10 min. While the loading of vesicles appeared to be a transitory change, certain other alterations were seen in axons following treatment with high doses of 6-OHDA (25 to 100 mg/kg), and appeared to be degenerative changes. A systematic study was made of these changes following a dose of 100 mg/kg 6-OHDA. At 1 h all vesicles were loaded (Fig. 1b), but by 2 h most vesicles had degranulated (Fig. 1c), that is, the cores that remained were less dense than those seen after 1 h. Most axon profiles were swollen and some axon membranes were ruptured; some abnormal necrotic mitochondria were also present in the axon profiles. After 3 h, rupture of axon membranes was more extensive, but the degranulated vesicles present were still dispersed as normal; by 4 h the axoplasm and agranular vesicles had coagulated and formed an electron-dense mass in the damaged axon profile (Fig. 1d). After 24 h many axon profiles had disappeared, only the Schwann cell sheath remaining. However, some damaged axon profiles with coagulated contents were seen (Fig. 1e); and occasionally swollen axon profiles containing degranulated vesicles were observed.

The time course of degeneration differed depending on the dose of 6-OHDA used. Thus, 4 h after a dose of 50 mg/kg 6-OHDA the majority of axon profiles contained degranulated vesicles as seen 2 h after injection of 100 mg/kg 6-OHDA (Fig. 1c). Twenty-four hours after a dose of 50 mg/kg 6-OHDA most axon profiles were at the same stage of degeneration as those seen 4 h after an injection of 100 mg/kg 6-OHDA. The same time after an injection of 25 mg/kg 6-OHDA, vesicles in most profiles were degranulated, and the membranes of some axons showed signs of rupturing. However, axon profiles still contained some loaded vesicles (Fig. 1f).

Effect of drugs on the action of 6-OHDA

In an attempt to investigate the mode of action of 6-OHDA, various drugs were employed that are known to interfere with the uptake and storage of noradrenaline by adrenergic nerves.

Desmethylimipramine

Desmethylimipramine is known to be an inhibitor of the membrane uptake mechanism of adrenergic nerves (see Iversen, 1967). Following treatment with desmethylimipramine (20 mg/kg) 20 min previously, a medium dose of 6-OHDA (25 mg/kg) had no detectable effect after 1 h. With the same pre-treatment, however, injection of a higher dose of 6-OHDA (100 mg/kg) caused loading of large and small vesicles after 1 h. No degenerative changes were seen even after 4 h.

Reserpine

Reserpine specifically blocks the ATP-Mg^{2+} dependent uptake mechanism of the amine storage granules, and thereby causes loss of the amine into the axoplasm (see Carlsson, 1965). Injection of reserpine (20 mg/kg) 30 min or 1 h before 6-OHDA (25 mg/kg) prevented the loading of vesicles usually seen after 1 h. Pre-treatment with the same dose of reserpine (20 mg/kg) 1 h before injection of higher doses of 6-OHDA (100 mg/kg) did not prevent the degenerative changes. However, the changes seen were less extensive than those following injection of 6-OHDA (100 mg/kg) alone.

Treatment with reserpine (20 mg/kg) 1 h after injection of 6-OHDA (10 mg/kg) caused degranulation of the vesicles. Thus 2 h after the treatment with 6-OHDA there was only a medium level of loading of the vesicles, and after 6 h there was only slight granulation of some vesicles. Six hours after a higher dose (25 mg/kg) of 6-OHDA followed by the same reserpine treatment, the axon profiles were degenerating and were comparable in appearance with those seen 4 h after treatment with 100 mg/kg 6-OHDA alone. This point is of particular interest since 6 h after an injection of 25 mg/kg 6-OHDA alone, no signs of degeneration were seen.

Amphetamine

Amphetamine has no effect on the membrane uptake mechanism, but causes an increased release of extra-granularly located noradrenaline (Hamberger & Malmfors, 1967). Pre-treatment with amphetamine (20 mg/kg) 15 min before 6-OHDA (10 to 50 mg/kg) had no effect on the granulation of the vesicles seen 1 h later. Pre-treatment with amphetamine (20 mg/kg) 15 min before injection of 6-OHDA (100 mg/kg) prevented the degeneration usually seen 4 h later, although at this time all the vesicles were still heavily loaded. Similarly, injection of 6-OHDA (100 mg/kg) followed 10–15 min later by amphetamine (10 to 20 mg/kg) still caused loading of vesicles after 1 h, but there were no signs of axon degeneration 4–6 h later, and the vesicles still remained loaded. However, following the same combination of drugs injected 1 h apart, the vesicles were degranulated after 6 h and most axon profiles showed signs of degeneration.

Following treatment with 6-OHDA (25 mg/kg), reserpine (20 mg/kg, 1 h later) and amphetamine (20 mg/kg, 15 min after reserpine), some axon profiles were seen to be degenerating after 6 h, but the degeneration was less marked than that seen after treatment with 6-OHDA and reserpine together (see above). Thus the majority of axon profiles appeared normal but contained weakly granular or degranulated vesicles. When the order of drug injection was changed (reserpine 20 mg/kg; 6-OHDA 100 mg/kg 30 min later; amphetamine 20 mg/kg, 15 min after 6-OHDA) all axon profiles contained only weakly granular vesicles after 6 h, but there were no signs of degeneration. As mentioned above, following injection of reserpine and 6-OHDA there was some degeneration after 6 h.

Metaraminol

Metaraminol is a "false transmitter"; it is taken up, by the membrane mechanism, into the axoplasm, and from there is accumulated in the amine storage granules (see Thoenen, 1969). In this way it displaces noradrenaline from the extragranular and intragranular compartments (Shore, Busfield & Alpers, 1964). Metaraminol (20 mg/kg) injected 15 min before a medium dose (25 mg/kg) of 6-OHDA suppressed the granulation of the vesicles; thus, after 1 h about 50% of the vesicles were granulated, but the cores were less electron-dense than after treatment with 6-OHDA alone. Similar treatment with metaraminol before a higher dose (100 mg/kg) of 6-OHDA prevented degeneration, all vesicles only showing heavy loading after 4 h. Injection of 6-OHDA (25 to 100 mg/kg) followed 15 min later by metaraminol (20 mg/kg) caused no increased granulation 4 h later, and even after the highest dose (100 mg/kg) of 6-OHDA there were no signs of degeneration after 4 h.

Discussion

Earlier work (Porter *et al.*, 1963; Porter *et al.*, 1965; Laverty *et al.*, 1965) indicated that 6-OHDA caused long-lasting depletion of noradrenaline in sympathetically innervated tissues, and it was suggested that 6-OHDA or a metabolite displaced the noradrenaline (Porter *et al.*, 1965). Furthermore Porter *et al.* (1963) suggested that 6-OHDA "destroys or alters the binding sites (for noradrenaline) in such a way that they must be replaced". More recently it has been demonstrated that 6-OHDA causes degeneration of adrenergic nerves in mammals (Tranzer & Thoenen, 1967, 1968a; Thoenen & Tranzer, 1968; Malmfors & Sachs, 1968; Jonsson & Sachs, 1970).

In the present study, 6-OHDA was seen to have similar effects on adrenergic nerves in the bird. It was found, however, that low doses (1 to 5 mg/kg) of 6-OHDA generally caused only a decrease in fluorescence intensity of nerves in the expansor secundariorum and this was correlated with a loading of the intra-axonal vesicles with 6-OHDA. These changes were rapidly reversed and after 24 h the nerves appeared almost normal. The depleting effect of low doses of 6-OHDA was blocked by desmethylimipramine, but, as would be expected with a competitive inhibitor, there was some uptake of 6-OHDA after injection of higher doses. However, under these conditions axon degeneration was never seen, thus indicating that 6-OHDA must be taken up by the nerves in order to cause degeneration, as pointed out earlier (Malmfors & Sachs, 1968; Jonsson & Sachs, 1970). Further-

more, it appears that the 6-OHDA must reach a certain critical intraneuronal concentration before degeneration will occur. That the concentration of 6-OHDA in the axoplasm is the important factor in causing degeneration is indicated by the observation that pre-treament with reserpine, which caused block of uptake of 6-OHDA into the vesicles (as reflected by the lack of vesicle granulation), did not prevent degeneration. However, in these conditions, degeneration was less extensive, suggesting that the 6-OHDA located in the vesicles normally leaks out and thereby increases the concentration in the axoplasm. A more direct demonstration that the axoplasmic concentration of 6-OHDA is the critical factor in degeneration is that an increase in 6-OHDA in the axoplasm, caused by treatment with reserpine after injection of 6-OHDA, prompted degeneration when it would not normally have occurred. This finding further indicates that the 6-OHDA located in the vesicles only plays a part in causing degeneration when it is released. A further support for this conclusion is indicated by the experiments with amphetamine, a drug which causes only an increased release of extra-granularly located noradrenaline (Hamberger & Malmfors, 1967). In the present study it was found that after treatment with amphetamine, degeneration was always less extensive than it would have been with 6-OHDA alone, and furthermore, degeneration was often prevented. However, it was clear that amphetamine little affected the uptake of 6-OHDA into the vesicles, since they always appeared heavily loaded after treatment with 6-OHDA and amphetamine together.

Pre-treatment with metaraminol appeared to suppress the loading of vesicles by 6-OHDA and to prevent degeneration. This finding confirms that of Porter *et al.* (1963), who showed that pre-treatment with metaraminol prevented the longer lasting depletion of noradrenaline caused by 6-OHDA. From the present study it seems feasible that pre-treatment with metaraminol suppressed uptake of 6-OHDA by the membranes of the adrenergic nerves and also the uptake of 6-OHDA from the axoplasm into the vesicles. Injection of metaraminol after 6-OHDA appeared to cause degranulation of the vesicles, as did reserpine, but metaraminol prevented rather than prompted degeneration. It seems probable that the metaraminol taken up by the nerves displaced the 6-OHDA from both the vesicles and the axoplasm, and thus kept the axoplasmic concentration of 6-OHDA at a level below that necessary to cause degeneration.

A particular point of interest in the present study is that with the lowest doses (1 to 2 mg/kg) of 6-OHDA used, the drug loaded, preferentially, the large granular vesicles. The uptake into the large vesicles was blocked by reserpine, as was the uptake into the small vesicles. This is a direct indication that large granular vesicles in adrenergic nerves are part of the noradrenaline storage system. Previous studies with 5-hydroxydopamine (Tranzer & Thoenen, 1968b) have indicated that this substance may also be taken up into large granular vesicles in adrenergic nerves.

From the present work it seems that 6-OHDA affects adrenergic nerves in the bird and mammal in a similar way. The suitability of this drug for producing complete adrenergic denervation in the bird is now being investigated.

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