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Fluorescent Histochemical Studies on the Effects of 6-Hydroxydopamine on Adrenaline-Containing Nerves in the Toad

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Summary. Fluorescence histochemistry has been used to study the effects of 6-hydroxydopamine (6-OHDA) (100 mg/kg injected into the dorsal lymph sac) on adrenaline-containing nerves in the large intestine, mesentery, lung, bladder and heart atria of the toad *Bufo marinus*. A gradual decrease both in fluorescence intensity and in number of detectable fibres during the first 4 hours after 6-OHDA was accompanied by a build-up of fluorescence in the nonterminal regions. These phenomena have been discussed in relation to the time course of the degeneration produced by 6-OHDA in noradrenergic nerves of higher vertebrates. Almost complete "chemical sympathectomy" was seen after one day, and it was not till 13 days that regenerating nerve fibres were seen in any organ. In the large intestine, however, re-innervation was slower, being first noted after 39 days. The time course of regeneration has been compared with that following sympathectomy in various mammalian organs.

Key-Words: 6-Hydroxydopamine — Adrenergic innervation — Amphibian — Fluorescence histochemistry.

Introduction

In mammals, 6-hydroxydopamine (6-OHDA) has been shown to affect noradrenergic nerves of both the peripheral autonomic and the central nervous systems (see e.g. Thoenen and Tranzer, 1968; Uretsky and Iversen, 1970). It is apparently taken up by the same mechanism as is noradrenaline (NA) and displaces NA from its intraneuronal storage sites. Bennett, Burnstock, Cobb and Malmfors (1970) have shown that when the concentration of 6-OHDA in the axoplasm reaches a critical level the adrenergic nerve terminal undergoes degeneration. The ultrastructural features of 6-OHDA action involve firstly an increase in the granularity of the vesicles followed by typical degenerative changes (Tranzer and Thoenen, 1968; Thoenen and Tranzer, 1968; Bennett *et al.*, 1970). Fluorescent histochemical observations show a decrease in NA after 6-OHDA treatment of the animal. 6-OHDA itself does not form a fluorescent compound with formaldehyde, since ring closure in the initial condensation reaction cannot occur because of the -OH substituent in the 6 position. Thus the intensity of fluorescence observed in the adrenergic nerves after 6-OHDA treatment reflects the level of endogenous NA.

The present study on the toad was undertaken, since it was thought to be of interest to compare the responses of amphibian adrenergic nerves, which are known to contain predominantly adrenaline (A) rather than NA (see e.g. Falck, Häggendal and Owman, 1963; Angelakos, Glassman, Millard and King, 1965),

with those of mammals, which contain NA (von Euler, 1956) and which have been much more extensively studied. With this aim in view, adrenergic nerves in various viscera of the toad have been examined. During the preparation of this manuscript, Siggins and Bloom (1970) published an account of the effects of 6-OHDA on the periarteriolar nerves of the frog (*Rana pipiens*) retrolingual membrane. They correlated their fluorescent histochemical studies with electron microscope autoradiography and observations on the occurrence of vasoconstriction and/or vasodilatation following electrical stimulation of the periarteriolar nerves in the anaesthetized animal.

Materials and Methods

Male and female toads (*Bufo marinus*) of between 70 and 140 g were used in this study. 6-Hydroxydopamine (6-OHDA) (H 88/32 AB Hässle, Göteborg, Sweden), 100 mg/kg, was injected into a dorsal lymph sac of each animal. The 6-OHDA was dissolved in a saline containing ascorbic acid (100 ml saline contained NaCl, 0.65 g; KCl, 0.014 g and ascorbic acid, 0.020 g) to reduce the rate of oxidation of the amine. At various times after the 6-OHDA injection the animals were killed by pithing and tissue was removed for processing by the fluorescent histochemical method, mainly according to Falck and Owman (1965). Whole mount preparations of the mesentery, lung and bladder were dried at room temperature over phosphorus pentoxide. Whole mount preparations of large intestine were freeze-dried and, after subsequent formaldehyde treatment and infiltration *in vacuo* with paraffin oil, delaminated to display Auerbach's plexus and the muscle coats, as previously described (Read and Burnstock, 1968a, 1969). Small portions of anterior large intestine and heart atria were freeze-dried and, after formaldehyde treatment and vacuum embedding in paraffin wax, sectioned. Results of preliminary experiments with whole mounts of large intestine dried over phosphorus pentoxide and with freeze-dried whole mounts of atrium showed that these tissues could not be satisfactorily prepared like this because of the thickness of the tissue and/or the high levels of autofluorescence present. After being dried, the tissue was exposed to formaldehyde vapour (which was generated from paraformaldehyde previously stored in a sealed desiccator containing sulphuric acid of concentration sufficient to produce a relative humidity of 70%—see Hamberger, Malmfors and Sachs, 1965) for 3 hours at 80° C. This procedure is adequate for the histochemical localization of A, as discussed by Falck (1962).

In some animals, noradrenaline (NA; Levophed, Winthrop, 0.1% solution), 10 mg/kg, or α-methylnoradrenaline (α-methyl NA; Neocobefrin, Sterling), 10 mg/kg, was injected into a dorsal lymph sac, usually $^1/_2$–1 hour before killing the animal, and the tissue was then given only 1 hour formaldehyde treatment. This procedure of loading the adrenergic nerves with exogenous amine was, of course, not used in those animals where the earliest effects of 6-OHDA (i.e. the gradual decrease in fluorescence intensity over the first few hours) were examined. It was, however, useful in later stages (after 1 day). Tissue from untreated animals was processed together with that from experimental animals.

Since toads are known to exhibit marked seasonal changes in their levels of catecholamines (Donoso and Segura, 1965; Segura, Biscardi and Apelbaum, 1967) and in their responses to pharmacological and physiological stimuli (G. D. Campbell, unpublished observations), this series of experiments was carried out over a relatively short part of the year (June-September, i.e. Winter—early Spring).

Results

Exposure of control tissue to formaldehyde vapour for 3 hours resulted in the demonstration of green fluorescence in the adrenergic nerves whereas very little fluorescence was detectable after only 1 hour formaldehyde except in those animals injected with NA or α-methyl NA. After loading the nerves with exogenous amine, the fluorescence was generally more stable and of higher intensity than

that observed in adrenergic nerves of untreated animals after 3 hours formaldehyde. In none of the tissues examined, however, was the number or distribution of fluorescent nerves altered by previous injection of the amine.

1. Large Intestine

Bundles of fluorescent nerve fibres (but no pericellular networks of these about the nonfluorescent ganglion cells) in Auerbach's plexus and numerous fine varicose fibres in the circular (but not the longitudinal) muscle (Fig. 1a, e), as described by Read and Burnstock (1968a, 1969), were seen in the large intestine. The only other fluorescence was in perivascular nerves, especially of the submucosa, yellow fluorescent enterochromaffin cells in the mucosal epithelium, and scattered autofluorescent cells (see also Read and Burnstock, 1968b).

One hour after the 6-OHDA injection, bundles of varicose nerves, with lower than normal fluorescence intensity, were demonstrable in Auerbach's plexus (Fig. 1b) and a few fibres of exceedingly low intensity could still be seen in the circular muscle. Weakly fluorescent perivascular nerves occurred in the submucosa and lamina propria. Two hours after 6-OHDA some fluorescent nerves were again detected at these three sites, but their intensity was extremely low. The number of nerves detectable and their fluorescence intensity was even lower at $3^3/_4$ hours (compare Fig. 1b, c) and fluorescent fibres in the muscle could now be identified only in sections and not in whole mount preparations.

One day after 6-OHDA the only fluorescent nerves detected were occasional isolated fibres in Auerbach's plexus (Fig. 1d) and in sparse submucosal perivascular plexuses. No changes in their number, fluorescence intensity, or morphological appearance were noted after NA (and 1 instead of 3 hour formaldehyde). Because of the almost complete absence of fluorescent nerves now, autofluorescence in nerve cell bodies of Auerbach's plexus and in scattered cells particularly of the submucosa was more conspicuous than usual.

Between 3 and 25 days after 6-OHDA there was exceedingly little fluorescence in the large intestine (Fig. 1f). In one animal 4 days after 6-OHDA (NA; 1 hour formaldehyde) a few weakly fluorescent fibres were seen in perivascular plexuses in the lamina propria. The only other catecholamine fluorescence in this period was in a few isolated morphologically abnormal fibres, which were probably degenerating adrenergic nerves, in Auerbach's plexus 6 days after 6-OHDA. Although there were still no fluorescent nerves in the large intestine, fluorescent nerve trunks were seen in the adjacent mesentery (Fig. 1g) 18 days after 6-OHDA.

The first fluorescent nerves had reappeared in the intestine by 39 days after 6-OHDA. Bundles of fluorescent fibres, with growth cones and some indication of irregular varicosities, were observed in Auerbach's plexus. These were not numerous, their distribution was patchy and their fluorescence intensity was very low, even after NA and 1 hour formaldehyde. Occasional weakly fluorescent perivascular nerves were seen in the submucosa.

In all animals injected with 6-OHDA, enterochromaffin cells in the mucosa and intensely green or yellow-green fluorescent cells, which are sometimes seen along large nerve trunks and blood vessels in the region of Auerbach's plexus (Read and Burnstock, 1968a) appeared normal.

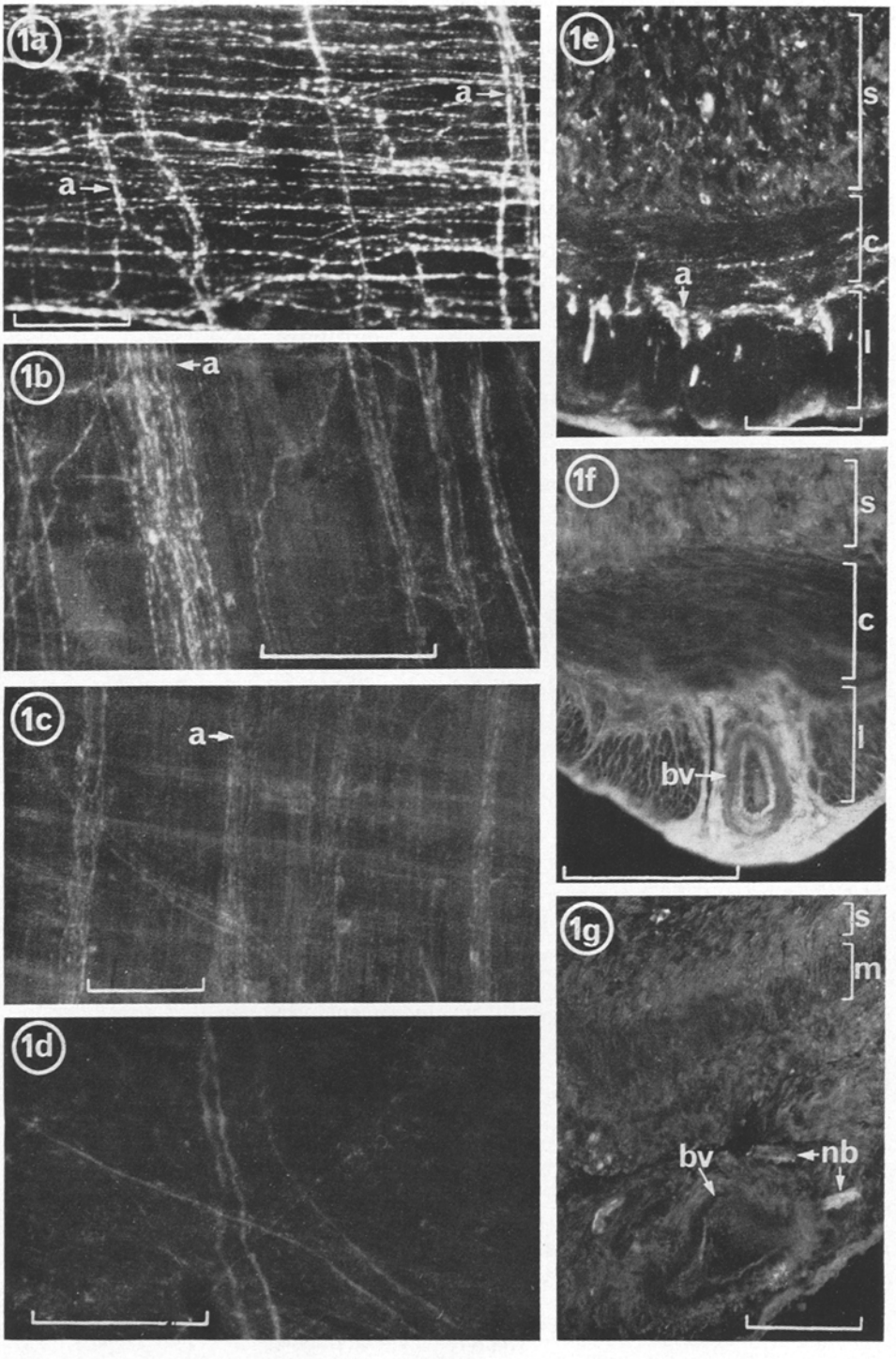

Fig. 1 a-g

2. *Mesentery*

Most fluorescent nerves in the mesentery were associated with blood vessels. Many of the major arteries had prominent plexuses of fluorescent varicose nerves at their media-adventitial borders and larger bundles of fluorescent fibres (chiefly smooth nonterminal axons) parallel with them. There were fewer fluorescent nerves associated with veins and none with venules or capillaries. Fluorescent nerves followed the smaller arterial branches towards the gut, although some small nerve bundles, and perhaps also single fluorescent fibres, left these perivascular plexuses but were not associated with any detectable structures in the mesentery. Intensely green to yellow-green fluorescent cells, similar to (although more numerous than) those in the large intestine, were scattered along some of the nerve trunks and large blood vessels.

One to 2 hours after 6-OHDA, fluorescent nerves were observed in their usual locations in the mesentery but their intensity was generally reduced (Fig. 2a). Occasional nerves, however, had a higher fluorescence intensity than usual (Fig. 2b). The intensity of all fibres detectable $3^3/_4$ hours after 6-OHDA was much less.

One to 4 days after 6-OHDA, no specific fluorescence was detected, except in a few distorted fibres (Fig. 2c), most of which were in large nerve trunks, although some were in perivascular plexuses about major arteries. Smaller vessels had no fluorescent perivascular nerves.

The first regenerating nerves were seen at 13 days, when fluorescent fibres were detected in the adventitia of some major arteries (Fig. 2d) and in nerve trunks some distance from blood vessels (Fig. 2e). They had a lower fluorescence intensity (even after NA) than in normal adrenergic nerve terminals but were not smooth like typical preterminal axons. Instead, they had irregular swellings, like large, poorly demarcated varicosities, alternating with their thinner intervaricose regions and larger swellings or growth cones at their tips (Fig. 2e).

Fig. 1a–g. Large intestine. a–d: Whole mount preparations. e–g: Sections. Calibration 100 μ
a Control toad. α-methyl NA, 10 mg/kg, $3^1/_2$ hr; 1 hr formaldehyde. Small bundles of fluorescent nerves in Auerbach's plexus (*a*), and numerous fine varicose fibres in the circular muscle coat. b 6-OHDA, 100 mg/kg, 1 hr; 3 hr formaldehyde. Bundles of weakly fluorescent varicose nerve fibres in Auerbach's plexus (*a*), but none in the circular muscle coat. c 6-OHDA, 100 mg/kg, $3^3/_4$ hr; 3 hr formaldehyde. Varicose nerves of very low fluorescence intensity in Auerbach's plexus (*a*). No fluorescent nerves are detectable in the muscle. d 6-OHDA, 100 mg/kg, 1 day; 3 hr formaldehyde. Isolated weakly fluorescent and rather diffuse fluorescent nerves in Auerbach's plexus. e Control toad (same animal as in Fig. 1a). Transverse section (T.S.). Bundles of fluorescent nerves in Auerbach's plexus (*a*) and fine varicose fibres in the circular muscle (*c*). There is no specific fluorescence in the submucosa (*s*) here and very little in the longitudinal muscle (*l*). f 6-OHDA, 100 mg/kg, 4 days, and NA, 10 mg/kg, 25 min; 1 hr formaldehyde. T.S. There is no specific fluorescence in the submucosa (*s*), circular (*c*) or longitudinal (*l*) muscle, nor in association with the artery (*bv*) in the longitudinal muscle. Autofluorescent connective tissue surrounding this vessel is particularly pronounced because of the long photographic exposure. g 6-OHDA, 100 mg/kg, 18 days, and NA, 10 mg/kg, 30 min; 1 hr formaldehyde. Oblique section. There are no fluorescent nerves in the submucosa (*s*) or muscle (*m*; longitudinal and circular coats are not distinguishable here) in the intestinal wall, but there are bundles of weakly fluorescent nerves (*nb*) alongside the artery (*bv*) in the mesentery

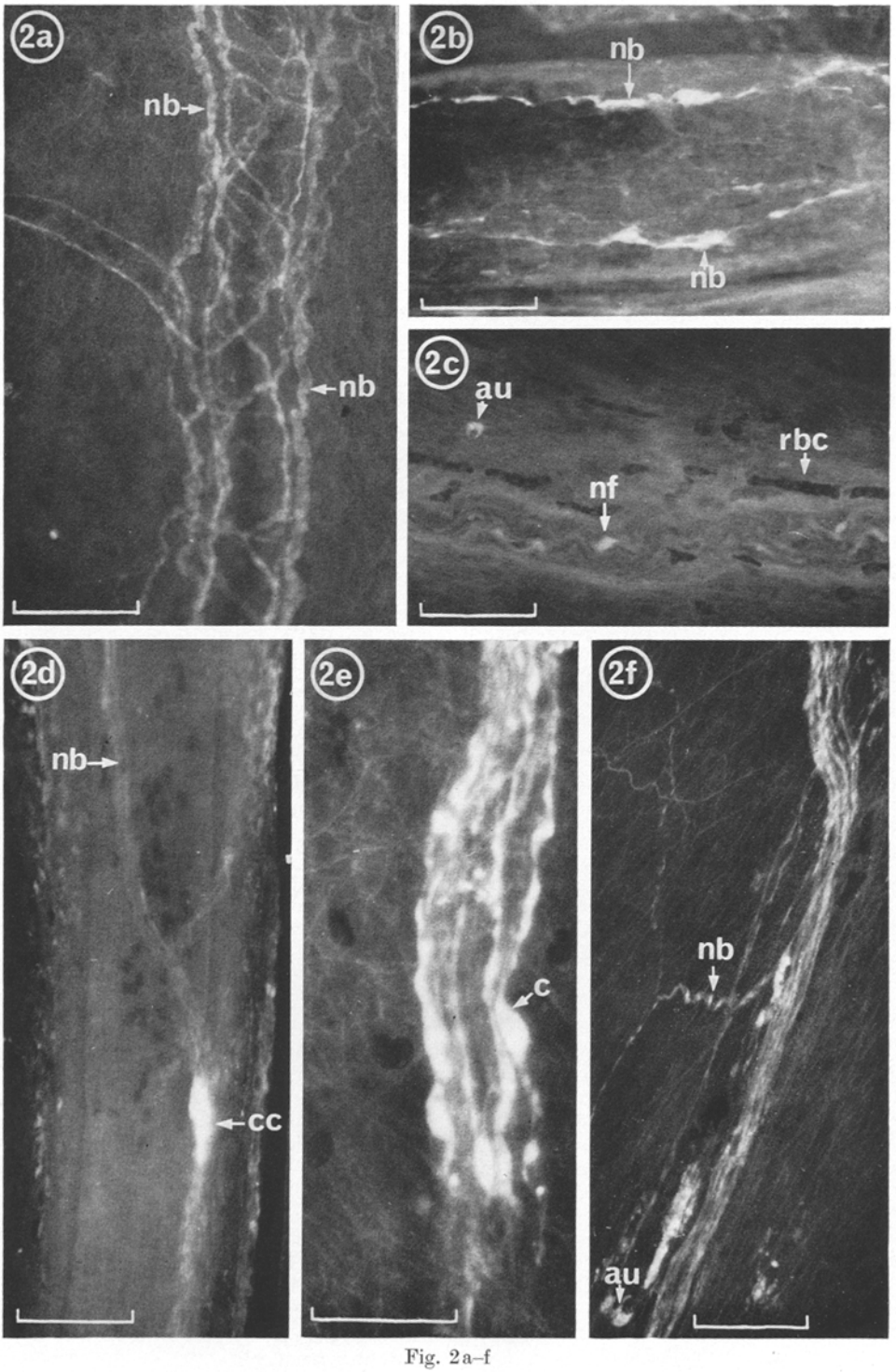

Fig. 2a–f

These fibres were most prominent in nerve trunks diverging from blood vessels. Fluorescent nerves were not yet seen in smaller nerve bundles nor in perivascular plexuses about the smaller blood vessels.

Fluorescent nerves were progressively more numerous in all succeeding stages (18 to 39 days) but, even at 39 days, they were not as common, particularly in association with arterioles, as in controls. Furthermore, fluorescent fibres with growth cones, similar to those at 13 days, were still detectable at 39 days although these were now less markedly restricted to the large nerve trunks and perivascular plexuses about only the largest arteries (Fig. 2f).

In all animals intensely green to yellow-green fluorescent chromaffin cells of normal appearance and distribution were seen after 6-OHDA (Fig. 2d).

3. Lung

The adrenergic innervation of the toad lung has been described in detail by McLean and Burnstock (1967) and their observations have been confirmed here. Fluorescent nerves were thus observed in perivascular plexuses and in small bundles without any definite relationship to detectable effector structures, but in only a small proportion of the muscle bands. No specifically fluorescent ganglion cells were observed in any animals here, although orange autofluorescent cytoplasmic granules were common in most cell bodies.

Fluorescent nerves were present in their usual locations and in about normal number 1–2 hours after 6-OHDA (Fig. 3a, b), but their intensity was less than in controls. $3^3/_4$ hours after 6-OHDA, the number of nerves detectable was considerably reduced and their intensity was even lower.

A few fluorescent cells, which were unlike any described by McLean and Burnstock (1967), were observed along nonfluorescent nerve bundles in one animal $3^3/_4$ hours after 6-OHDA (3 hour formaldehyde). Four of these (Fig. 3c) were bright yellow-green fluorescent and elongated in shape and at least one had processes from its cell body. They were thus similar to, but smaller than, the chromaffin cells observed along large blood vessels and nerve trunks in the abdominal viscera examined. Two other cells in the same animal were rounder

Fig. 2a–f. Mesentery. a 6-OHDA, 100 mg/kg, 2 hr; 3 hr formaldehyde. Nerve fibres, of lower fluorescence intensity than in control animals, in a perivascular plexus about a blood vessel and smaller branch thereof. Larger bundles of fluorescent nerves (*nb*) run parallel with the vessel. Calibration 100 μ. b Same animal as in Fig. 2a. Particularly intensely fluorescent nerve bundles (*nb*) alongside a large blood vessel. Calibration 100 μ. c 6-OHDA, 100 mg/kg, 3 days; 3 hr formaldehyde. Very weakly fluorescent degenerating nerve fibres (*nf*). Note also the rows of (dark) red blood corpuscles (*rbc*) in capillaries near the nerve bundle and the small granular autofluorescent cell (*au*). Calibration 100 μ. d 6-OHDA, 100 mg/kg, 13 days, and NA, 10 mg/kg, 35 min; 1 hr formaldehyde. A large artery in the mesentery is accompanied by bundles of very weakly fluorescent nerves (*nb*) and scattered intensely fluorescent chromaffin type cells (*cc*). Calibration 100 μ. e Same animal as in Fig. 2d. A small bundle of regenerating fluorescent nerves with very prominent growth cones (*c*). Scattered non-fluorescent red blood corpuscles are also visible. Calibration 50 μ. f 6-OHDA, 100 mg/kg, 39 days, and NA, 10 mg/kg, 25 min; 1 hr formaldehyde. Smaller bundles of fluorescent nerves (*nb*) branch out from a larger trunk. Note also the autofluorescent cells (*au*). Calibration 50 μ

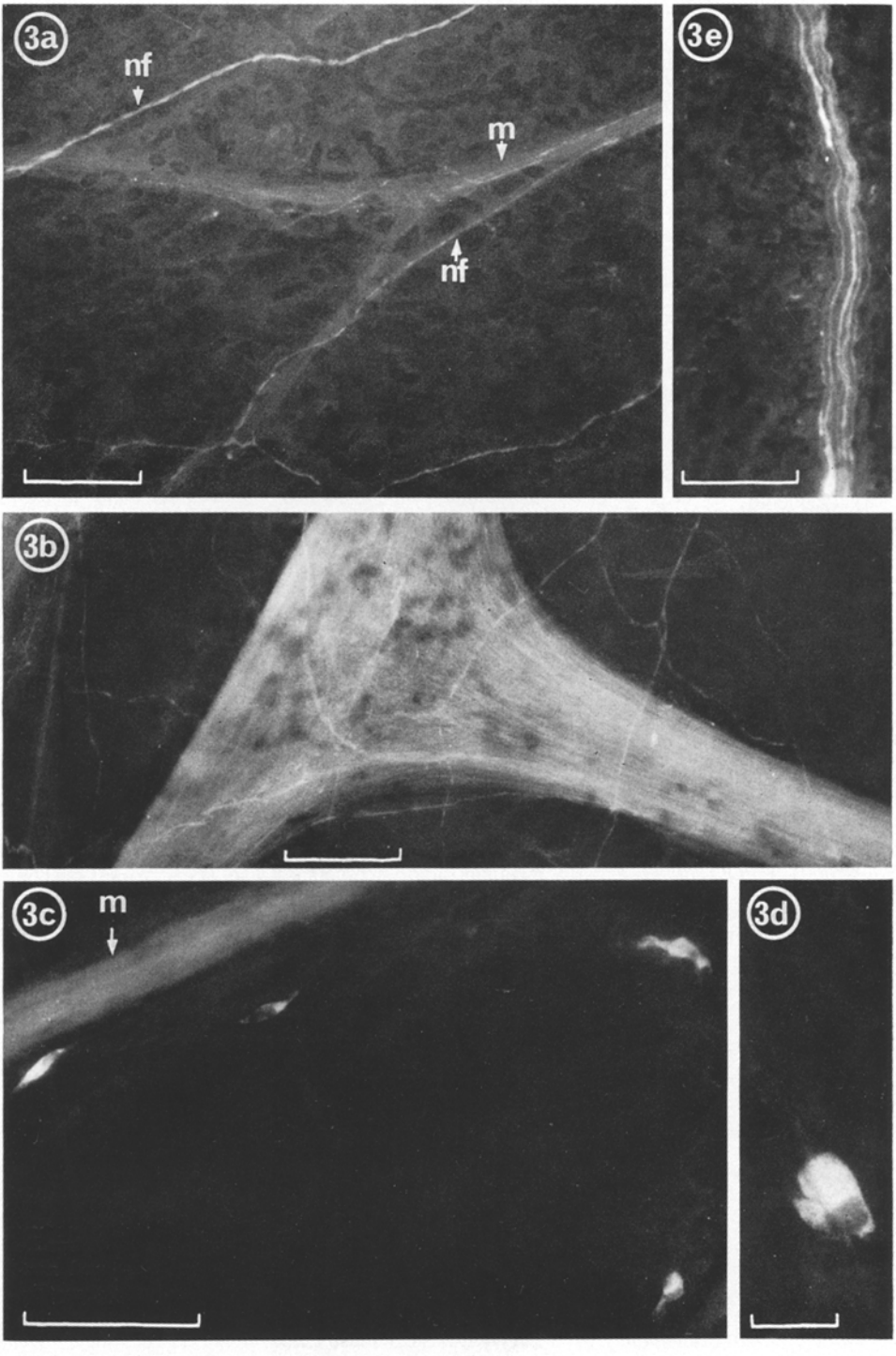

Fig. 3a–d

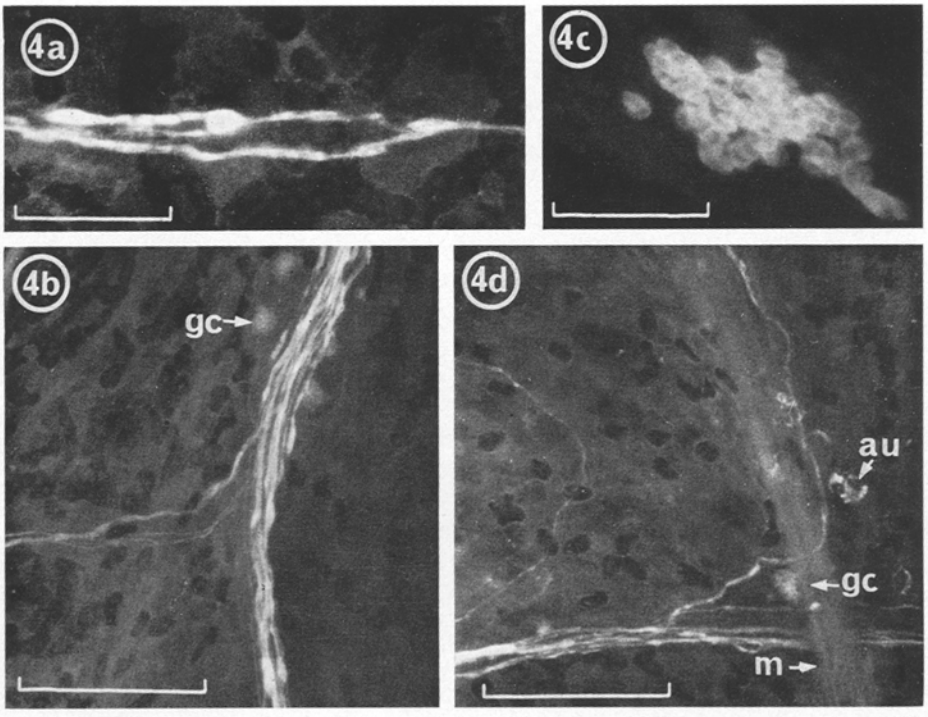

Fig. 4a–d. Lung. 6-OHDA, 100 mg/kg, 13 days, and NA, 10 mg/kg, 35 min; 1 hr formaldehyde. Small bundle of fluorescent nerve fibres with growth cones. Red blood corpuscles are visible in the background. Calibration 50 μ. b 6-OHDA, 100 mg/kg, 18 days, and NA, 10 mg/kg, 30 min; 1 hr formaldehyde. Bundle of fluorescent regenerating nerve fibres, some with prominent growth cones, and several autofluorescent ganglion cells (*gc*) along the nerve trunk. Calibration 100 μ. c 6-OHDA, 100 mg/kg, 18 days; 3 hr formaldehyde. Group of small intensely green fluorescent cells along a muscle band. Calibration 50 μ. d 6-OHDA, 100 mg/kg, 39 days, and NA, 10 mg/kg, 25 min; 1 hr formaldehyde. Small bundles of fluorescent nerves leave a larger trunk. There is no indication of adrenergic innervation of the muscle band (*m*) shown here. Along the nerve trunk one autofluorescent ganglion cell (*gc*) is visible, and there are several smaller more intensely autofluorescent cells (*au*). Calibration 100 μ

Fig. 3a–e. Lung. a 6-OHDA, 100 mg/kg, 2 hr; 3 hr formaldehyde. Relatively weakly fluorescent nerve fibres (*nf*), some of them running with a muscle band (*m*). Numerous dark, red blood corpuscles are visible in the background. Calibration 100 μ. b Same animal as in Fig. 3a. Weakly fluorescent nerve fibres form a sparse meshwork in the lung but are not associated with the large muscle band here. Calibration 100 μ. c 6-OHDA, 100 mg/kg, $3^3/_4$ hr; 3 hr formaldehyde. Four brightly fluorescent cells along a small (nonfluorescent) nerve bundle. Part of a muscle band (*m*) is visible but no fluorescent nerves are detectable in the lung here. Calibration 100 μ. d Same animal as in Fig. 3c. Unusual green fluorescent cells on a small nonfluorescent nerve bundle. Calibration 25 μ. e 6-OHDA, 100 mg/kg, 4 days, and NA, 10 mg/kg, 25 min; 1 hr formaldehyde. Nerve bundle containing several fluorescent nerve fibres, one of which is particularly bright. Calibration 100 μ

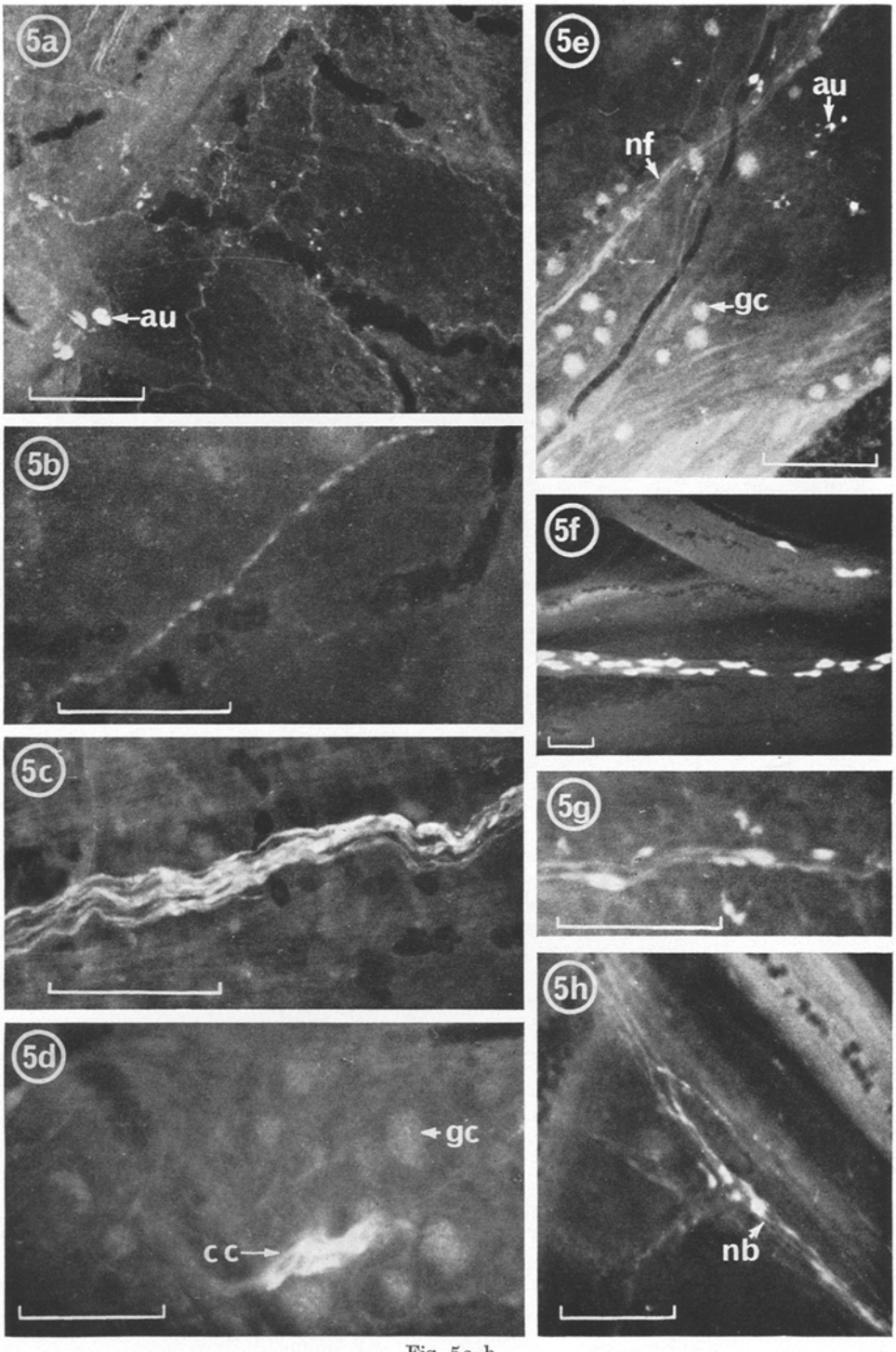

Fig. 5a–h

in shape, had a weaker, greener fluorescence, and had no distinguishable processes (Fig. 3d).

Fluorescent nerves were not detected 3–4 days after 6-OHDA except in one animal (NA; 1 hour formaldehyde), where a few large nerve trunks had some distorted fibres (Fig. 3e). Some of these were more brightly fluorescent than the nerve fibres seen in similar locations in controls.

The first regenerating fluorescent nerves were seen at 13 days (Fig. 4a). They were similar to those in the mesentery, although less closely associated with blood vessels. Some of the largest nerve trunks, along which autofluorescent ganglion cells are distributed, now had one or two regenerating fibres. Fluorescent nerves were commoner after 13 days, but growth cones at their tips (Fig. 4b) were still present at 39 days. Fewer fluorescent fibres were detectable than in controls and at 39 days there was still no indication of any varicose nerves associated with the muscle bands in a manner indicative of adrenergic innervation of the muscle (Fig. 4d).

Intensely fluorescent chromaffin cells, similar to those in the abdominal viscera, were sometimes seen along large nerve bundles and blood vessels in the lung but were apparently unaffected by 6-OHDA. The small rounded bright green or yellow-green fluorescent cells located at the junctions of the septal muscle bands (see also McLean and Burnstock, 1967) were also unchanged by 6-OHDA (Fig. 4c).

4. Bladder

Fluorescent nerves in periarteriolar plexuses and fine fluorescent varicose fibres amongst the smooth muscle cells in a small proportion of the muscle bands, as reported by McLean and Burnstock (1966), were observed in the bladder. Ganglion cells, which were located along some of the largest nerve trunks, were not specifically fluorescent but often contained autofluorescent granules.

Fig. 5a–h. Bladder. a 6-OHDA, 100 mg/kg, 2 hr; 3 hr formaldehyde. Weakly fluorescent nerve fibres follow the course of some of the small blood vessels here. Several granular autofluorescent cells (*au*) are visible. Calibration 100 μ. b Same animal as in Fig. 5a. Very weakly fluorescent varicose nerve fibre near a small blood vessel. Calibration 100 μ. c 6-OHDA, 100 mg/kg, 4 days, and NA, 10 mg/kg, 25 min; 1 hr formaldehyde. Bundle of weakly fluorescent nerve fibres. Some red blood corpuscles in nearby capillaries are also visible. Calibration 100 μ. d 6-OHDA, 100 mg/kg, 4 days; 3 hr formaldehyde. Cluster of ganglion cells (*gc*), with autofluorescent granules in their cell bodies, and intensely fluorescent chromaffin cells (*cc*) located along a large nonfluorescent nerve trunk. Calibration 50 μ. e 6-OHDA, 100 mg/kg, 21 days; 3 hr formaldehyde. Numerous autofluorescent ganglion cells (*gc*) are located along large nerve trunks which contain only a few (weakly) fluorescent nerve fibres (*nf*). Also visible are several small autofluorescent cells (*au*) and a dark line of red blood corpuscles in a blood vessel. Calibration 100 μ. f 6-OHDA, 100 mg/kg, 21 days; 3 hr formaldehyde. Large number of intensely fluorescent chromaffin cells along a large nonfluorescent nerve trunk and three of these cells along a large blood vessel. Calibration 100 μ. g Same animal as in Fig. 5e. Bundle of weakly fluorescent regenerating nerve fibres. Note the growth cones. Calibration 100 μ. h 6-OHDA, 100 mg/kg, 25 days; 3 hr formaldehyde. Smaller bundles of fluorescent nerves branch out from a larger trunk (*nb*). Calibration 100 μ

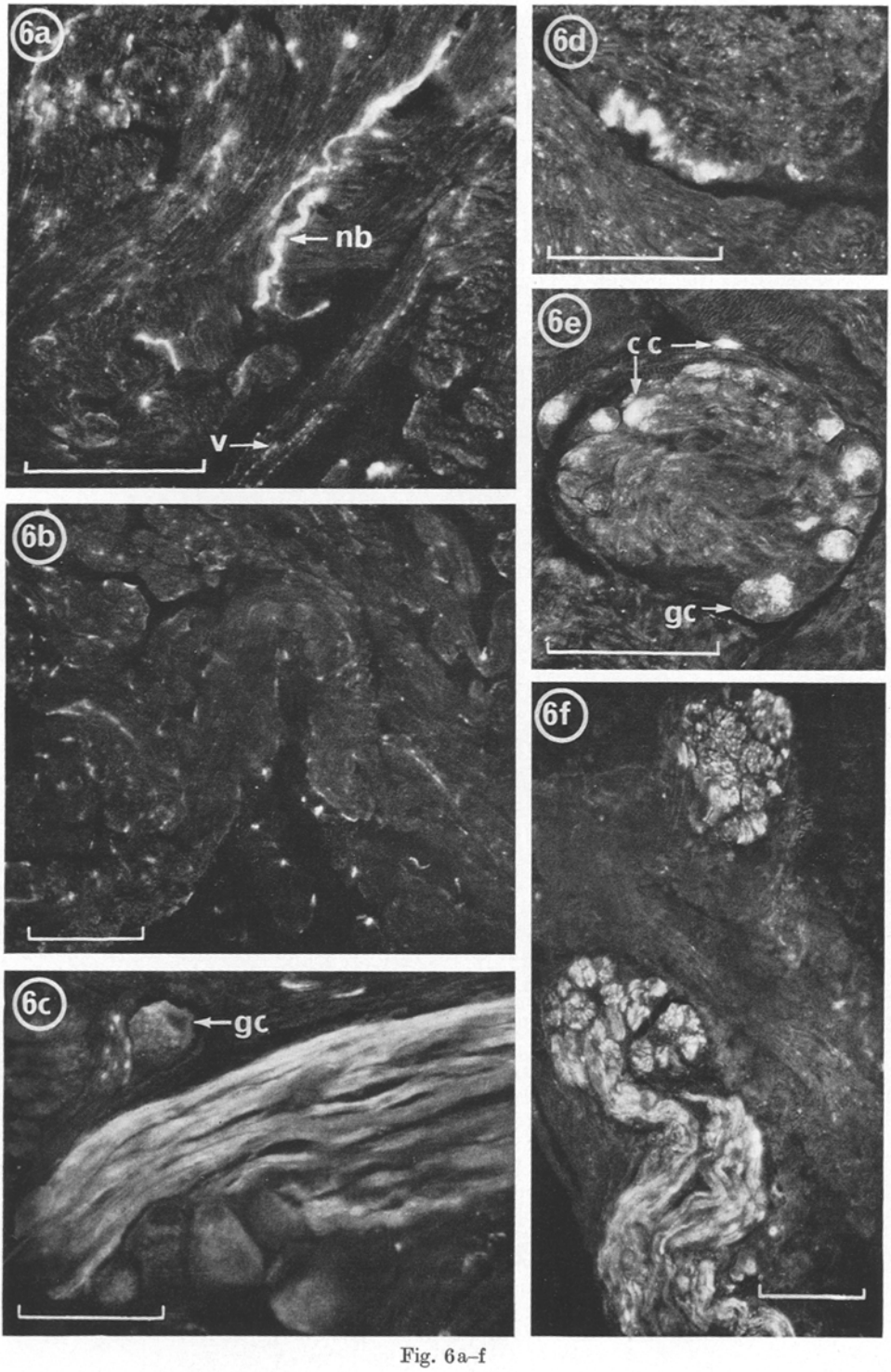

Fig. 6a–f

Between one and $3^3/_4$ hours after 6-OHDA, fluorescent nerve fibres, of progressively lower intensity, were detected by blood vessels (Fig. 5a, b), but none were clearly associated with any muscle bands. Apart from occasional very weakly fluorescent distorted fibres in a few nerve bundles (Fig. 5c), no fluorescent nerves were seen 1–4 days after 6-OHDA.

Thirteen days after 6-OHDA, however, some fluorescent regenerating nerves were observed in large trunks at the neck of the bladder. Such fibres were less numerous than those seen at the same time in the mesentery and lung, and their fluorescence was too weak (3 hours formaldehyde; no NA) for them to be photographed satisfactorily. Eighteen to 39 days after 6-OHDA there were still few fluorescent nerves in the bladder, and growth cones were still visible at their tips (Fig. 5e, g). They were located in major nerve trunks in the neck region but not in close association with muscle bands (Fig. 5h) nor, in contrast with the intestine, mesentery and lung, in perivascular plexuses.

Intensely fluorescent cells, similar to those in the large intestine, mesentery and lung, were noted along some large vessels and nerve trunks at the neck of the bladder of both control and experimental animals (Fig. 5d, f).

5. Heart Atrium

Numerous fine fluorescent varicose nerve fibres were observed in the cardiac muscle (Fig. 6a) and larger bundles of more intensely fluorescent fibres were present, especially in the epicardium.

In favourable sections the two large nerve trunks from the ramus cardiacus (i.e. the dorsal and ventral septal nerves—Ábrahám, 1963; Robb, 1965) were seen in the atrial septum. These contained some relatively strongly fluorescent smooth axons as well as occasional small bright green fluorescent cells and autofluorescent ganglion cells. As in the other organs examined, there were no specifically fluorescent ganglion cell bodies.

One to $3^3/_4$ hours after 6-OHDA, fluorescent nerves in the atrial muscle (compare Figs. 6a, b) and in the two large nerve trunks in the atrial septum (Fig. 6c) became increasingly less intensely fluorescent. Scattered small bundles of fluorescent nerves in the muscle, however, still retained a relatively high intensity.

Fig. 6a–f. Heart atrium. a Control toad. 3 hr formaldehyde. Fine fluorescent varicose nerve fibres (*v*) amongst the cardiac muscle cells and a small bundle of fluorescent fibres (*nb*). Calibration 100 μ. b 6-OHDA, 100 mg/kg, 2 hr; 3 hr formaldehyde. Weakly fluorescent varicose nerve fibres in the muscle. Calibration 100 μ. c Same animal as in Fig. 6b. Group of six ganglion cells (*gc*), with orange autofluorescent granules in their cytoplasm, beside a large weakly fluorescent nonterminal nerve trunk. Calibration 50 μ. d 6-OHDA, 100 mg/kg, 3 days; 3 hr formaldehyde. One of the few small weakly fluorescent nerve bundles seen in the cardiac muscle. There is no other specific fluorescence here. Calibration 100 μ. e Same animal as in Fig. 6d. T.S. large nerve trunk containing several autofluorescent ganglion cells (*gc*) and two intensely fluorescent cells (*cc*; only the tips of these cells are visible in this section but their identity was checked in serial sections) but no fluorescent nerve fibres. Calibration 100 μ. f 6-OHDA, 100 mg/kg, 21 days; 3 hr formaldehyde. Two large trunks of fluorescent nerves, seen here in T.S. and L.S. Calibration 100 μ

After 3 days, extremely few fluorescent nerves were present in the atrial muscle (Fig. 6d) and none were detectable in the two septal nerve trunks (Fig. 6e).

Thirteen to 18 days after 6-OHDA, the first fluorescent regenerating nerve fibres were seen in the larger trunks, but it was impossible to study these in further detail in the sectioned material. Fluorescent nerve fibres in the two large nerve trunks (Fig. 6f), and some small bundles of fluorescent nerve fibres in the atrial muscle were clearly detectable at 21 days. Single varicose adrenergic nerve fibres in intimate relationship with the cardiac muscle cells were not seen yet.

Small, intensely fluorescent cells, similar to those in controls, were sometimes observed after 6-OHDA (Fig. 6e).

Discussion

In all organs studied here (toad large intestine, mesentery, lung, bladder and heart) virtually no specific fluorescence could be seen in the adrenergic nerves for a period of between 1 and 13 days after 6-OHDA. A single injection of 6-OHDA (100 mg/kg) into the dorsal lymph sac, therefore, appears to produce almost complete "chemical sympathectomy" (Thoenen and Tranzer, 1968) of the toad viscera.

The lack of histochemically detectable fluorescent nerves within 24 hours of an injection of 6-OHDA has been correlated ultrastructurally with degeneration of adrenergic fibres in birds (Bennett *et al.*, 1970) and mammals (Furness, Campbell, Gillard, Malmfors, Cobb and Burnstock, 1970). These authors observed a rapid disappearance of the fluorescence (and hence, presumably, of the NA) in the sympathetic nerves of chicken expansor secundariorum muscle and rat and mouse vas deferens within the first few hours of administration of 6-OHDA. Twenty-four hours after 6-OHDA, however, the fluorescence intensity again appeared normal although the number of fluorescent fibres now detectable was reduced compared with normal. From these observations they concluded that NA is probably displaced from the adrenergic nerve fibres in the first 4–6 hours and that those fibres, where the axoplasmic concentration of 6-OHDA is not sufficient to initiate degeneration, then begin to reaccumulate NA, so that by the end of the first day any surviving fibres show normal fluorescence intensity.

The present results indicate that a similar phenomenon of initial displacement of adrenergic transmitter by 6-OHDA also occurs in amphibians. In all tissues the intensity of the fluorescence was reduced after 1 hour, although the distribution of adrenergic nerves still appeared normal. However, in all toad tissues examined, as also in the mouse vas deferens (Furness *et al.*, 1970), the number of fibres detectable, and their fluorescence intensity, was reduced 4 hours after an injection of 6-OHDA, 100 mg/kg. Little sign of the reversible depletion of adrenergic transmitter by 6-OHDA described by Thoenen and Tranzer (1968) for the rat heart was seen in any tissue here, probably because of the high dose (100 mg/kg) used.

Reappearance of fluorescent nerve fibres was first seen in toad heart, lung and mesentery after 13 days. In contrast, however, regenerating adrenergic

nerves were very uncommon in the bladder at 13 days and were not seen in the large intestine until 39 days after 6-OHDA. A parallel pattern of sensitivity has also been seen in mammals, where cardiovascular adrenergic innervation is re-established more quickly after denervation with 6-OHDA than is that of either the gastrointestinal tract or the urinogenital system (Gillard, unpublished). Furthermore, as noted also for the corresponding mammalian systems (Gillard, unpublished), the initial regenerating fibres were never very closely associated with the muscle bundles. They generally accompanied the blood vessels except in the atria where, in contrast with mammals (Robb, 1965), there is no coronary vascular system. Regenerating adrenergic nerves were seen associated with the larger arteries earlier than with the smaller vessels. This is presumably because the axons growing out from the nerve cell bodies usually follow the arterial tree to the effector organs.

The morphology of the reappearing fluorescent fibres closely parallelled that of regenerating sympathetic nerves in rat and rabbit sciatic nerve (Dahlström, 1965; Blümcke and Niedorf, 1965; Olson, 1969) and of sympathetic fibres re-innervating rat iris transplanted into the anterior eye chamber (Malmfors and Olson, 1967). Typical growth cones or drop-like enlargements at the growing nerve tips, similar to those originally described from silver-stained preparations by Ramon y Cajal (1928) and, more recently, with the fluorescent histochemical method by Olson (1969), for regenerating mammalian sympathetic sciatic fibres, were also seen here in amphibian adrenergic nerves.

In all of the organs examined here, some of the fluorescent nerves demonstrable 39 days after the injection of 6-OHDA (100 mg/kg) were still morphologically similar to regenerating fibres. A normal adrenergic innervation pattern was, therefore, not re-established within 39 days after 6-OHDA. In this period, no fluorescent varicose nerve fibres were observed to be in close association with either cardiac or smooth muscle, indicating a prolonged absence of functional direct adrenergic innervation of the musculature.

Falck *et al.* (1963) and Woods (1970b), in comparing the adrenergic innervation of frog (*Rana temporaria*) and mammalian heart, noted that non-varicose adrenergic nerves are relatively more intensely fluorescent (compared with the varicose terminals) in amphibians than in mammals. This observation was confirmed here for the toad, where the non-varicose preterminal adrenergic fibres in the nerve trunks of the atrial septum were found to be relatively strongly fluorescent in control animals. In mammals and birds, however, non-terminal (i.e. non-varicose) axons have been reported to show stronger fluorescence shortly after (8 hours after 6-OHDA, 20 mg/kg, in mouse iris; 2 or more hours after 6-OHDA, 100 mg/kg, in chick expansor secundariorum) the intravenous injection of 6-OHDA due to the accumulation of NA (Malmfors and Sachs, 1968; Bennett *et al.*, 1970). Furthermore, the evidence of Dahlström and Häggendal (1966) regarding axonal flow and rate of transport of NA above ligations of the sciatic nerves of rat and cat would lead to the expectation of a continual build-up of fluorescence back along the nonterminal axon, as the varicose terminal regions begin to degenerate. A similar accumulation of fluorescence has now been noted in toad nonterminal adrenergic nerves a few hours after 6-OHDA (e.g. Fig. 2b).

At this point it is of interest to recall that Woods (1970b) noted a build-up of fluorescence in adrenergic nerves of the frog heart after transection of the vagosympathetic trunks. The increase in fluorescence was seen first in the nerve terminals 1 hour after axotomy and then, successively, in the less terminal parts of the adrenergic fibres. Such a sequential build-up of fluorescence was not seen here in the toad after 6-OHDA, presumably because the 6-OHDA actually displaces the A from the terminals. However, a sequential disappearance of fluorescence from first the terminals and then the nonterminal fibres, similar to that (after the initial build-up of fluorescence) after surgical sympathectomy of the frog heart (Woods, 1970b) was seen in the toad. In both the frog and the toad (after surgical and chemical sympathectomy respectively) the loss of detectable fluorescence closely parallelled the morphological degeneration of adrenergic fibres (note, for example, the striking similarity between the weakly fluorescent degenerating nerves illustrated in Fig. 4 of the paper by Woods, 1970b, and in Fig. 2c here).

No adrenergic nerve cell bodies were seen in any of the tissues examined. No evidence of catecholamine uptake by any of the ganglion cells in the lung was ever seen here after NA or α-methyl NA (10 mg/kg) treatment, in contrast with the observation of McLean and Burnstock (1967) of NA uptake in some ganglion cells after pretreatment of toads with high doses of nialamide. Fluorescent ganglion cells were also not seen in the toad heart, an observation which is in agreement with the reported absence of adrenergic ganglion cells from the frog (*Rana pipiens*, *R. temporaria*) heart (Angelakos *et al.*, 1965; Woods, 1970a). In contrast, Falck *et al.* (1963) found, near the atrioventricular groove of *R. temporaria*, fluorescent structures that they interpreted as adrenergic ganglion cells. Woods (1970a) has recently presented evidence that these structures are actually gross dilatations, possibly caused by long-lasting mechanical constriction at the atrioventricular ring, of adrenergic nerve fibres in the region of Bidder's ganglia. This particular part of the toad heart was not examined in detail here.

All of the fluorescent non-neuronal cells seen in the toad tissues appeared to be unaffected by treatment with 6-OHDA. Thoenen and Tranzer (1968) have also reported the absence of any significant effect of 6-OHDA on the amine content of rat adrenal gland. The inability of 6-OHDA to reduce the amine level in both mammalian adrenal and amphibian chromaffin cells may be related to the capacity of some of these cells to take up amino acid precursors (which are then decarboxylated to the amines), rather than the amines themselves (see Pearse, 1966, 1968, for discussion of amino acid precursor uptake by various cells). Similarly, the absence of effect of 6-OHDA on the enterochromaffin cells is probably because these cells are specialized for uptake of indoles rather than catecholamines.

Siggins and Bloom (1970) studied the response to 6-OHDA of terminal arterioles of the retrolingual membrane of the frog, *Rana pipiens*. 6-OHDA produced extensive ultrastructural degeneration in the adrenergic perivascular nerves and an inability of these nerves to take up or bind catecholamines for a period of at least 3 weeks (longer time intervals not examined). The vasoconstrictor response to electrical stimulation of the perivascular nerves was

reduced and eventually abolished by topical applications of 6-OHDA, although the nerve-mediated vasodilatation persisted. The dysfunction of these adrenergic nerves was still evident, although a slight recovery was also noted in some animals at 21 days. This finding thus correlates well with the single weakly fluorescent nerve seen by Siggins and Bloom (1970) at this time, and also with the regenerating adrenergic nerve fibres observed here in toad viscera from day 13 onwards (except in the large intestine, where re-innervation is much slower). The discrepancy between 21 days (Siggins and Bloom, 1970) and 13 days for reappearance of adrenergic nerves may be due to organ or species differences or to a more exhaustive examination by fluorescence histochemistry of the toad than of the frog tissue, or to a combination of these factors. Restoration of physiological function of the adrenergic nerves in each organ presumably occurs some time after the appearance of the first regenerating nerves, but before the full complement of nerves is established.

Haeusler, Haefely and Thoenen (1969) observed strongly reduced responses to sympathetic nerve stimulation in isolated cat heart and nictitating membrane and very low NA levels in these and other (spleen, iris) tissues for 2–3 weeks after the last of a series of injections of 6-OHDA. Thoenen and Tranzer (1968) also observed depressed levels of NA in rat heart, spleen and vas deferens after 6-OHDA, with no sign of recovery for 2–3 weeks. Haeusler *et al.* (1969) studied the recovery of NA content more extensively in the cat and correlated their results with the recovery of responses to sympathetic nerve stimulation in the heart and nicitating membrane. They reported that NA levels gradually returned to normal over approximately 14 weeks whereas responses to stimulation were almost normal in the heart after 8–9 weeks and were normal in the nictitating membrane after only 3–4 weeks. Restoration of adrenergic nerve function earlier than the NA level has also been noted after surgical denervation (about 21.5 and 26 weeks respectively for cat spleen—Kirpekar, Wakade and Prat, 1970). Hence it is apparent that function is restored before the full complement of adrenergic nerves is present in each organ.

Thus, it appears that both the morphological and functional responses to 6-OHDA are similar in adrenergic nerves of amphibians and in noradrenergic nerves of mammals.

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