Br. J. Pharmac. Chemother. (1968), **32**, 46–56.

CATECHOLAMINE AND ACETYLCHOLINESTERASE DISTRIBUTION IN RELATION TO NORADRENALINE RELEASE. AN ENZYME HISTOCHEMICAL AND AUTORADIOGRAPHIC STUDY ON THE INNERVATION OF THE CAT NICTITATING MUSCLE

BY

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(*Received August* 16, 1967)

Langley (1900) originally suggested that the postganglionic sympathetic nerves which innervate the cat nictitating membrane are derived from cell bodies in the superior cervical ganglion. This contention was supported by Thompson (1961) who demonstrated that sympathetic nerves are conveyed from the ganglion by the carotid nerve and thence via orbital branches of the trigeminal nerve to the inferior and medial nictitating muscles. Investigating the nature of the autonomic innervation of the nictitating membrane, Gardiner, Hellman & Thompson (1962) described a plentiful supply of acetylcholinesterase-positive nerves to the Harderian gland but a very sparse distribution of the same type of fibres within the nictitating muscles. More recently Jacobowitz & Koelle (1965) have demonstrated the presence of catecholamines and acetylcholinesterase in relation to nerves within cat nictitating muscle. Because none of these previous investigations on the innervation of the nictitating membrane was conducted at a fine structural level we have investigated the distribution of adrenergic and acetylcholinesterase-positive nerves in relation to the cat nictitating muscle by both light and electron microscopy, with a view to testing further the important hypothesis of Burn & Rand (1959) that adrenergic nerves contain a cholinergic mechanism which is functionally related to the release of noradrenaline.

METHODS

A total of nine young adult cats of either sex was used in this study. In two cats right superior cervical ganglionectomy was performed under ether anaesthesia with recovery ; the animals were killed respectively 2 days and 28 days after sympathectomy.

The nictitating membrane of the cat is retracted by two smooth muscle sheets—the inferior and the medial muscles—both arising from the peribulbar fascia (Acheson, 1938). Samples of smooth muscle can be readily obtained via an incision through the orbital fascia after fully exhibiting the nictitating membrane by drawing it laterally across the eye. Specimens from intact and sympathectomized animals were processed as follows.

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Formol-fluorescence

Muscle pieces were frozen on to metal cryostat chucks and sectioned at 18 μ. Controlled formaldehyde–catecholamine condensation was performed according to the method of Spriggs, Lever, Rees & Graham (1966).

Electron microscopy per se

Pieces of muscle were fixed in 1% osmium tetroxide buffered to *p*H 7.5 with veronal acetate and embedded in Araldite. Fine sections were stained with lead citrate (Reynolds, 1963) and examined in a Siemens Elmiskop I electron microscope.

Autoradiography

Freshly excised nictitating muscle was immersed in McEwen solution (McEwen, 1956) gassed with 95% O_2 and 5% CO_2 containing 81.3 μc/ml. ^{3}H-*dl*-noradrenaline (1.2×10^{-5} g/ml.) for 25 min. Control specimens were immersed in oxygenated McEwen solution containing 0.6×10^{-5} g/ml. unlabelled *l*-noradrenaline. Excess noradrenaline was removed from the tissues by washing with several changes of McEwen solution during the next 60 min. Some specimens were then " stained " for acetylcholinesterase and all specimens were processed for high-resolution autoradiography according to Lever, Spriggs & Graham (1967).

Demonstration of acetylcholinesterase

Specimens were fixed in glutaraldehyde and incubated for 4 hr at pH 5.5 with acetylthiocholine in the presence of 2×10^{-4} M ethopropazine (Lewis & Shute, 1966). As controls, specimens of other tissues—sympathetic ganglia and pancreas—were always simultaneously processed.

RESULTS

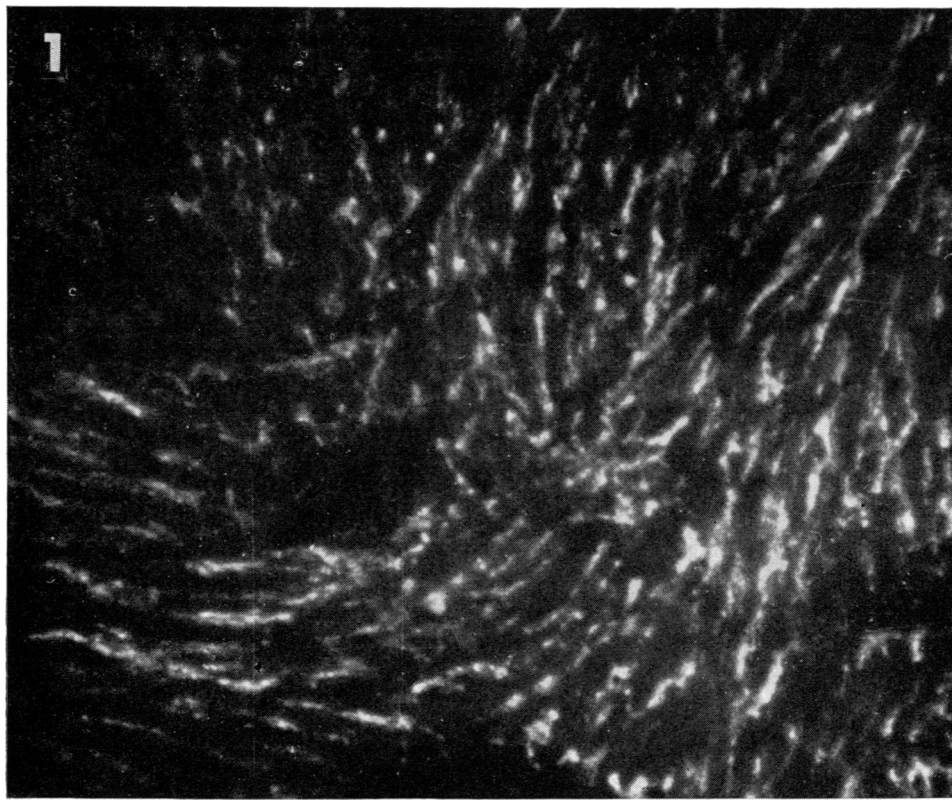
Formol-fluorescence

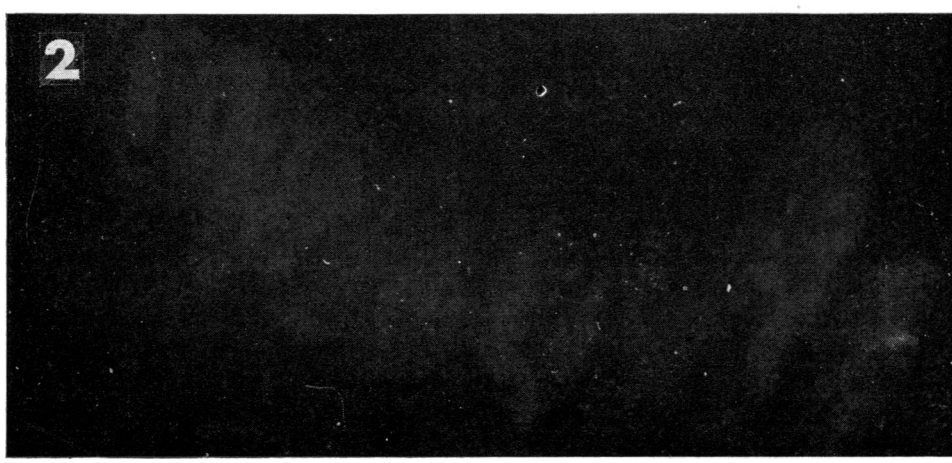
A dense adrenergic innervation to both medial and inferior muscles can be demonstrated after treating muscle sections with formaldehyde gas (Fig. 1). Two days after right superior cervical ganglionectomy only a few beaded fluorescent fibres were observed in the denervated muscle and after 28 days no fluorescent nerves were found (Fig. 2); the fluorescent nerve distribution on the contralateral (intact) side did not differ from that of unoperated controls.

Elecron microscopy per se

The histology of the cat nictitating membrane at a fine structural level has been described by Lever & Graham (1964) and in both medial and inferior muscles the smooth muscle cells are orientated in groups between which are extensive irregular spaces containing connective tissue, neural elements and blood vessels. Nerve bundles in these tissue spaces usually contain approximately six axons although larger and smaller bundles have been observed (Fig. 3). For the most part nerves are distributed to muscle surfaces bordering on these tissue spaces but nervous penetration into intermuscular spaces has been observed (Fig. 4).

Nerve terminal areas (Lever, Graham, Irvine & Chick, 1965) are covered only by basement membrane while elsewhere axons have a variable covering of Schwann-cell processes. Terminal nerves exhibit beaded expansions which may coincide with the nerve terminal areas. These expansions contain concentrations of mitochondria and





Figs. 1 and 2. Fluorescence micrographs of cat nictitating membrane muscle (×*ca.* 218).

Fig. 1. Adrenergic nerve plexus in innervated muscle.

Fig. 2. Absence of discrete fluorescence in denervated muscle 28 days after superior cervical ganglionectomy.

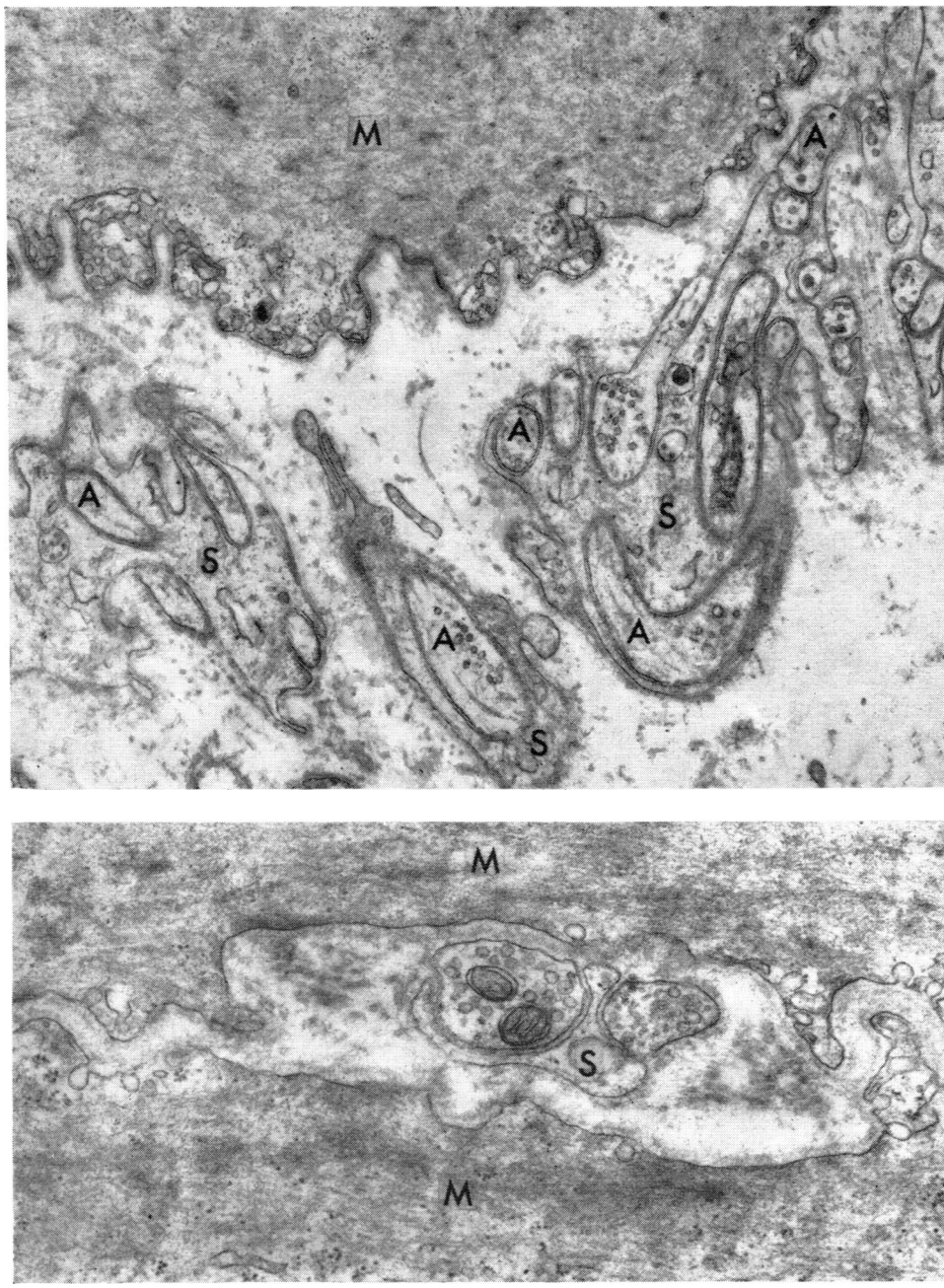


Fig. 3. Unmyelinated axons (A) enclosed to varying extents by Schwann-cell processes (S) in a tissue space within the nictitating muscle. M, Smooth muscle cell (×*ca.* 21,000).

Fig. 4. Two axons partly covered by Schwann-cell (S) in an expansion of a tissue space between two muscle cells (M) (×35,000).

microvesicles—some of which are dense-cored (Fig. 5). Vesico-mitochondrial and inter-vesicular connexions were observed and have been described previously by Spriggs, Lever & Graham (1967).

Neuro-muscular intervals may be as small as 200Å but may measure 600Å or more. The smaller intervals were often found between " pedicle "-like muscle cell processes and juxtaposed axons (Fig. 6). These processes often contain profiles of the endoplasmic reticulum, mitochondria and glycogen particles and in cross-section may superficially resemble transected axons. Because they do not possess dense-cored vesicles or neuro-fibrillae, however, and can often be traced through serial sections to their parent muscle cells, their identity can usually be established.

Two days after superior cervical sympathectomy, nerves in relation to the nictitating muscle exhibited axoplasmic disorganization—for example, irregular clumping of inclusions many of which appeared fragmented. Axolemmal sheaths also showed varying degrees of fragmentation (Fig. 7).

Twenty-eight days after sympathectomy the overall picture is one of complete denervation of the nictitating muscle although rarely axons of normal appearance were encountered. In the intact control, axon counts per grid square (100 μ side) average 69.5 ($\pm$7.1 S.E.).

Autoradiography

In electron autoradiographs of muscle incubated with ^{3}H-noradrenaline concentrations of silver grains were located over about 65% of axon profiles (Fig. 8). The number of silver grains per unit area is significantly greater ($P<0.001$) over Schwann-axon bundles 9.4 $\pm$ 1.8 S.E.) than over non-neural elements and extracellular spaces (0.7 $\pm$ 0.1 S.E.).

In autoradiographs of nictitating muscle excised 2 days after superior cervical ganglionectomy and incubated with ^{3}H-noradrenaline, no accumulations of silver grains were found over the degenerative axons.

Demonstration of acetylcholinesterase

Throughout all blocks of the control tissues (sympathetic ganglia and pancreas) clear and consistent staining for acetylcholinesterase was obtained. In specimens of nictitating muscle stained for acetylcholinesterase few axons exhibited a positive reaction. As depicted in Fig. 9 the reaction product is readily visualized and seems to be associated with the axolemma. In twelve sections of muscle selected at random from twelve blocks from three cats a differential axon count revealed means of 69.5 ($\pm$7.1 S.E.) acetylcholinesterase-negative axons and only 0.3 ($\pm$0.2 S.E.) acetylcholinesterase-positive axons per 100 μ^2 area of muscle.

In most cases each acetylcholinesterase-positive axon shared its investing Schwann-cell with acetylcholinesterase-negative axons (Fig. 9). Of the few positively stained axons none approached the surface of a smooth muscle cell closer than 1400Å.

In specimens of muscle incubated with ^{3}H-noradrenaline and subjected to both acetylcholinestrase " staining " and autoradiography, no axons with accumulations of silver grains exhibited any acetylcholinesterase reaction product.

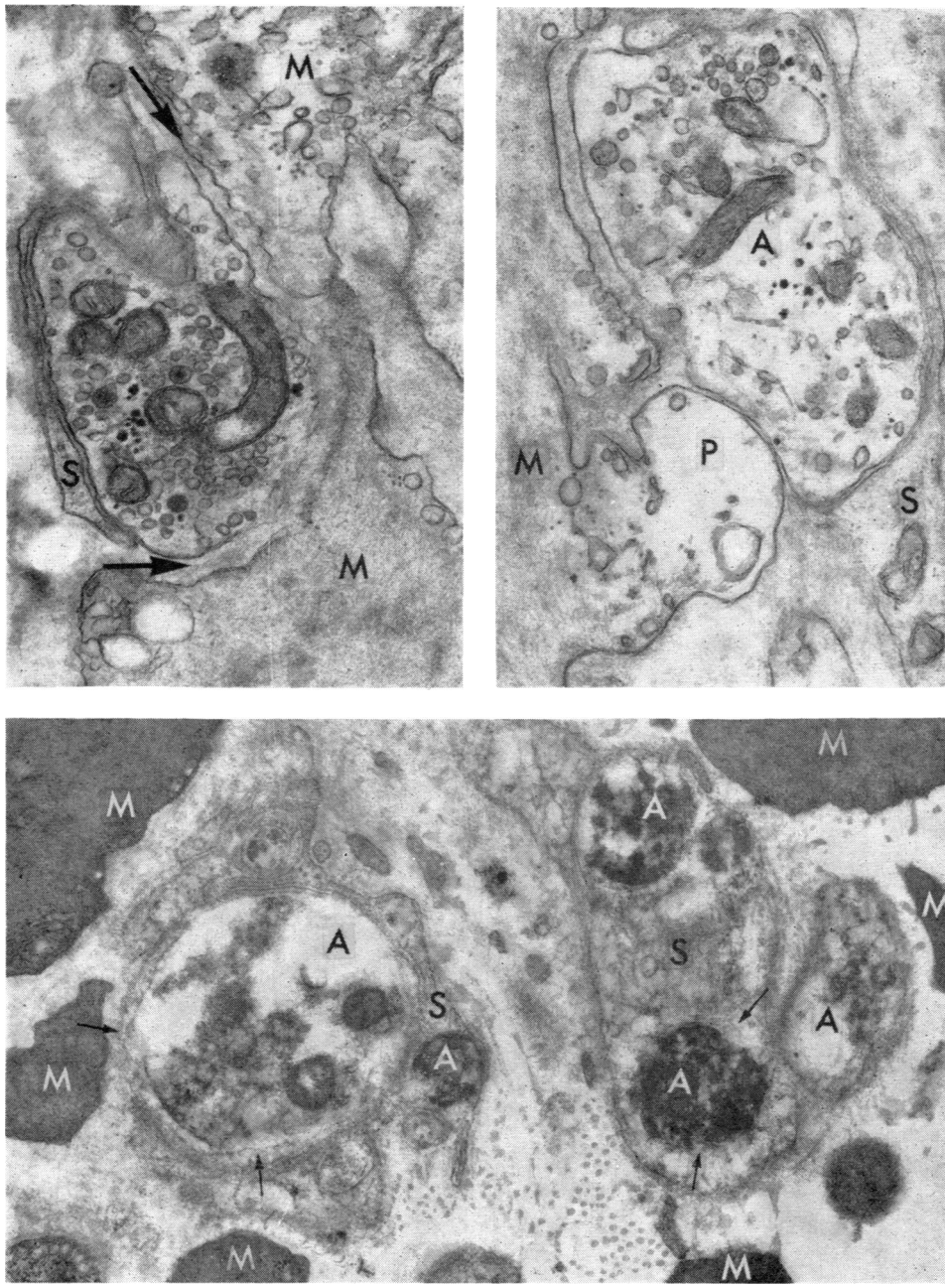
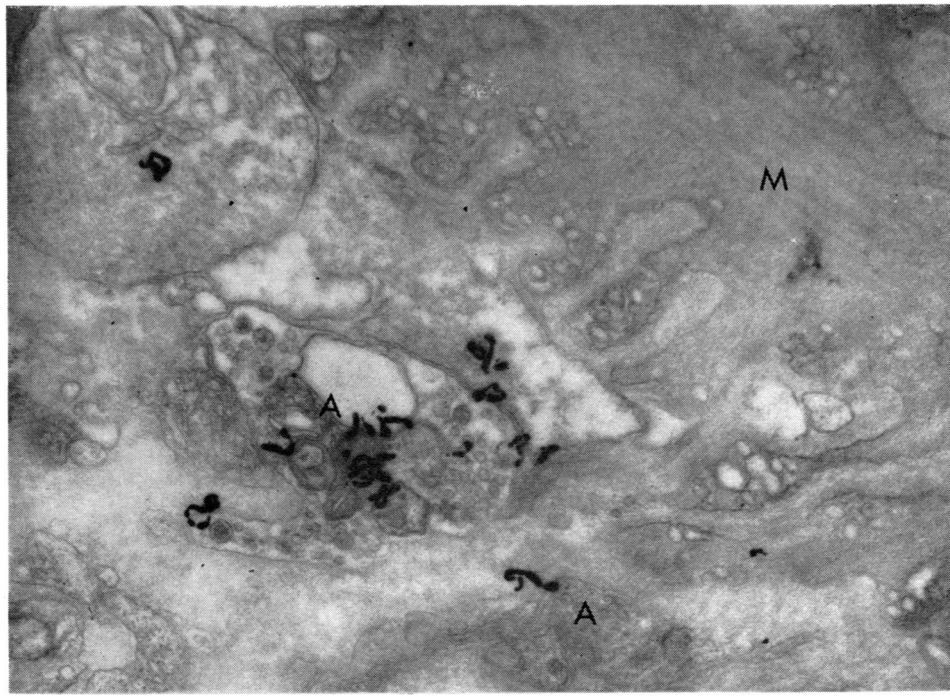


Fig. 5. Axon in synaptic relationship to smooth muscle (M). Note the concentrations of mitochondria and microvesicles, some of which possess an electron-dense content. The intra-synaptic interval (→←) measures approximately 500Å and contains only basement membrane. S, Schwann cell (×35,000).

Fig. 6. Axon (A) related to a " pedicle-like " process (P) of a muscle cell (M). The synaptic interval at this point is approximately 200Å. S, Schwann cell (×35,000).

Fig. 7. Axons (A) exhibiting evidence of degeneration 2 days after superior cervical ganglionectomy. Note clumping of axoplasm and its inclusions and fragmentation of the axolemma (↑). S, Schwann cell ; M, muscle cell (×20,000).



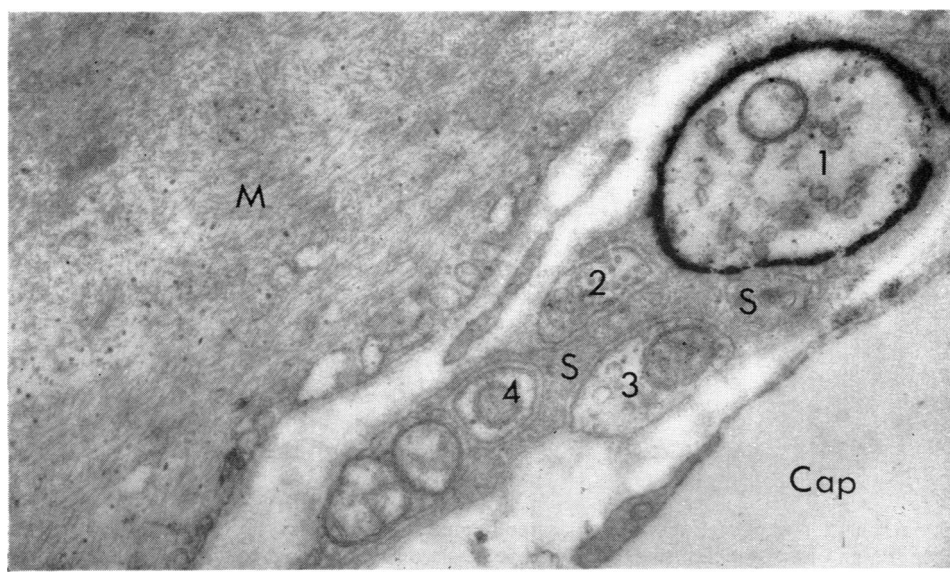


Fig. 8. Electron autoradiograph of nictitating muscle incubated with ^{3}H-noradrenaline. Note the concentration of silver grains over axon profiles (A). M, Smooth muscle (×30,000).

Fig. 9. Electron micrograph of nictitating muscle incubated with acetylthiocholine. The axolemma of axon 1 is obscured by the electron dense acetylcholinesterase reaction product. Note that the unstained axons 2, 3 and 4 are invested by processes of the same Schwann cell (S) as axon 1. M, Muscle cell ; Cap, capillary (× 35,000).

DISCUSSION

The sympathetic nature of the nerve supply to the nictitating muscles in the cat, originally suggested by Langley (1895, 1900), has since been confirmed by other workers (Zernick, 1928; Rosenblueth & Bard, 1932; Thompson, 1961). It is now generally agreed that the postganglionic sympathetic nerves are derived from cell bodies in the superior cervical ganglion and conveyed to the orbit by the carotid nerve and then via branches of the trigeminal nerve (Thompson, 1961).

Using a formol-fluorescence technique the nictitating muscles have been shown to possess a dense plexus of catecholamine-containing nerve fibres. Electron microscopy reveals a plentiful distribution of unmyelinated axons. Two days after superior cervical ganglionectomy catecholamine-fluorescence was greatly reduced and electron microscopy showed extensive axoplasmic degeneration. Twenty-eight days after sympathectomy an overall picture of complete denervation of the muscle was obtained both by electron and fluorescence microscopy. Jacobowitz & Koelle (1965) have also reported the disappearance of catecholamine fluorescence in nictitating muscle 2–4 weeks after superior cervical ganglionectomy.

We may conclude therefore that the nerves in the nictitating muscle as observed with the electron microscope corresponding to those revealed by the formol-fluorescence technique and that these nerves are adrenergic and sympathetic in nature. Further confirmation of the adrenergic identity of these axons was obtained in our autoradiographic studies. Noradrenaline is selectively accumulated by adrenergic nerves (see Lever, Spriggs & Graham, 1967) and in autoradiographs of innervated nictitating muscles incubated with ^{3}H-noradrenaline, silver grains were concentrated specifically over some 65% of axon profiles.

Burn & Rand (1959) postulated that an impulse passing along a post-ganglionic sympathetic nerve fibre might release acetylcholine, which in turn might liberate noradrenaline, and later (Burn & Rand, 1965) described much pharmacological evidence in support of this theory. The hypothesis was, however, challenged in its application to the innervation of the cat nictitating muscle by Gardiner & Thompson (1961) who failed to inhibit with hemicholinium (HC3) contractions of the muscle *in vitro* in response to post-ganglionic nerve stimulation. Wilson & Long (1959) had previously reported the inability of hemicholinium to block sympathetic transmission to the nictitating muscle *in vivo*. Burn, Rand & Wien (1963) found that eserine potentiated contractions of cat nictitating muscle induced by postganglionic nerve stimulation, an effect which was prevented or abolished by atropine or hyoscine, and interpreted their findings in favour of the hypothesis of Burn & Rand (1959). Because acetylcholinesterase activity has been found to correlate well with choline-acetylase activity and acetylcholine content in many regions of the central and peripheral nervous systems (MacIntosh, 1941; Feldberg & Vogt, 1948; Burgen & Chipman, 1951; Nachmansohn, 1959), it is reasonable to assume that a system utilizing acetylcholine may be detected through its acetylcholinesterase activity. The distribution of acetylcholinesterase in the nictitating muscle is therefore of paramount importance in this controversy.

Burn & Philpot (1953) and Gardiner, Hellman & Thompson (1962), using manometric methods, found small amounts of acetylcholinesterase activity in nictitating muscle. In

addition histochemical observations with the light microscope have indicated the presence of a few acetylcholinesterase-positive nerves in nictitating muscle (Gardiner *et al.*, 1962; Jacobowitz & Koelle, 1965). Our results with the electron microscope confirm the presence of acetylcholinesterase-positive axons in nictitating muscle. The number of these axons is very small, however, less than 0.5% of total axon profiles in the muscle being acetylcholinesterase-positive. None of these esterase-positive axons possessed a close synaptic relationship with the smooth muscle cells, an observation in accord with that of Gardiner *et al.* (1962) who suggested that these fibres may end on small blood vessels.

The results of our combined autoradiographic and enzyme histochemical studies show that no specifically labelled adrenergic axons display a positive acetylcholinesterase reaction.

Evidence for the efficacy, validity and completeness of the acetylcholinesterase staining in the present experiments can be cited as follows:

(*a*) Clear and consistent enzyme-staining was observed throughout all control specimens of ganglia and pancreas. (*b*) Each axon observed was either acetylcholinesterase-positive—in which event the presence of the reaction product was unequivocal—or it was acetylcholinesterase-negative—in which event no reaction product was present. (*c*) The fact that acetylcholinesterase-positive and -negative axons have been found within the same Schwann-axon bundle indicates that the reaction substrate must have been available to the "unstained" axons. (*d*) Ethopropazine has been shown specifically to inhibit pseudocholinesterase in concentrations which have little or no inhibitory effect on acetylcholinesterase (Bayliss & Todrick, 1956; Cavanagh & Holland, 1961; Lewis & Shute, 1966). We have found that 2×10^{-4} M ethopropazine inactivates the non-specific esterases of the exocrine cells of the pancreas (Gerebtzoff, 1959) but not the acetylcholinesterase of cholinergic nerve fibres which retains its ability to hydrolyse acetylthiocholine (Spriggs, Lever & Graham, unpublished).

As discussed earlier, the absence of acetylcholinesterase suggests the absence of a cholinergic mechanism. On this assumption our results are incompatible with the hypothesis that noradrenaline release is mediated by acetylcholine within the same axon. It must be added that because cholinergic and adrenergic axons have been observed occasionally within the same nerve bundle in the nictitating muscle it is possible that in these situations the acetylcholine released from the cholinergic axons is able to influence noradrenaline release from the neighbouring adrenergic axons (see also Jacobowitz & Koelle, 1965; Fray & Leaders, 1967). The occurrence of these mixed nerve bundles is so rare in the nictitating muscle, however, that their functional significance in this tissue is questionable.

SUMMARY

1. The smooth muscle from innervated and denervated nictitating membranes of cats was examined by electron and fluorescence microscopy.

2. Formol-fluorescence revealed a typical catecholamine-containing nerve plexus which was not visualized after denervation by superior cervical ganglionectomy.

3. Electron microscopy of intact and sympathectomized nictitating muscle confirmed the sympathetic nature of the nerve supply.

4. Incubation of the muscle with ^{3}H-noradrenaline and subsequent electron autoradiography revealed a specific uptake of the amine by these sympathetic nerves and affirmed their adrenergic identity.

5. Positive histochemical staining for acetylcholinesterase was found in relation to the axolemma of a few (less than 0.5%) of the axons in the muscle.

6. These acetylcholinesterase-positive fibres, which presumably possess a cholinergic mechanism, are always entirely discrete from the adrenergic fibres but the two types may co-exist in the same Schwann-axon bundle.

7. No evidence was obtained which would support the hypothesis that adrenergic terminal axons contain a cholinergic mechanism.

It is a pleasure to thank Mrs. Gillian Howells for her excellent technical assistance. This work is supported financially by the Medical Research Council. The electron microscope is on permanent loan to one of us (J. D. L.) from the Wellcome Trust. A. C. E. acknowledges research grants from the Ernest Oppenheimer Memorial Trust and the South African C.S.I.R.

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