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AN EXAMINATION OF ADRENERGIC AXONS AROUND PANCREATIC ARTERIOLES OF THE CAT FOR THE PRESENCE OF ACETYLCHOLINESTERASE BY HIGH RESOLUTION AUTORADIOGRAPHIC AND HISTOCHEMICAL METHODS

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The hypothesis that a cholinergic mechanism mediates the release of noradrenaline from postganglionic sympathetic adrenergic nerve terminals was proposed by Burn & Rand in 1959 and the evidence in favour of this hypothesis was reviewed recently (Burn & Rand, 1965 ; Burn, 1967). Evidence exists, however, which is incompatible with the hypothesis (Iversen, 1967 ; Fray & Leaders, 1967 ; Esterhuizen, Graham, Lever & Spriggs, 1967, 1968). Histochemical studies have revealed some situations in which acetylcholinesterase (ACHE) may be demonstrated in association with adrenergic nerves and others in which it may not (Jacobowitz & Koelle, 1965). Light microscopy fails to resolve the individual axons which compose a nerve trunk in such a situation and must necessarily fail to distinguish between (a) a nerve containing a mixture of discrete adrenergic and cholinergic axons and (b) a nerve containing axons which might possess a dual adrenergic and cholinergic mechanism.

The aim of the present electron microscopic study was to distinguish between these two possibilities and thereby to provide evidence for or against a hypothesis of an intraneuronal cholinergic link in adrenergic transmission. The nerves examined in this work were periarteriolar nerves in the pancreas of the cat.

METHODS

Two intact and two sympathectomized cats were used in this study. Specimens from several different parts of the pancreas of each were examined.

Sympathectomy

Under anaesthesia with ether the pancreas was denervated by bilateral resection of the coeliac ganglia and careful stripping of the perivascular tissue surrounding the anterior mesenteric and coeliac arteries for a distance of 5 mm from the abdominal aorta. The animals were allowed to recover and were used 6 months later.

Perfusion

Atropine sulphate 1 mg/kg was injected subcutaneously 30 min before etherization. The abdomen was opened widely in the midline and splenectomy performed. The pancreas was mobilized by section of the duodenum between ligatures, section of vascular connections between pancreas and stomach, pancreas and ileum and pancreas and liver: a length of abdominal aorta extending from the level of the diaphragm to the renal arteries was removed together with the pancreas and duodenum. The organ complex with its vascular pedicle was then transferred to a 100 ml. bath of nutrient solution (McEwen, 1956) gassed with 5% carbon dioxide in oxygen and maintained at 37° C. The distal end of the cut aorta was cannulated and perfused with the same solution at a rate of 4 ml./min delivered by a roller pump (Palmer & Co.) the perfusate escaping freely from the cut end of the portal vein. After 40 min the perfusion was continued with McEwen's solution to which ^{3}H-*dl*-noradrenaline (^{3}H-NA) had been freshly added to make a concentration of 6.9 μc/ml.) (10^{-6} g/ml. of base). This perfusion continued for 25 min and was followed by normal McEwen's solution for 60 min, during which period the bath was flushed repeatedly. The tissue was then "fixed" by perfusion with glutaraldehyde solution according to the method of Lewis & Shute (1966).

Staining for acetylcholinesterase

Specimens were incubated with acetylthiocholine in the presence of the specific pseudocholinesterase-inhibitor ethopropazine according to the method of Lewis & Shute (1966) in order to demonstrate the presence of functional acetylcholinesterase.

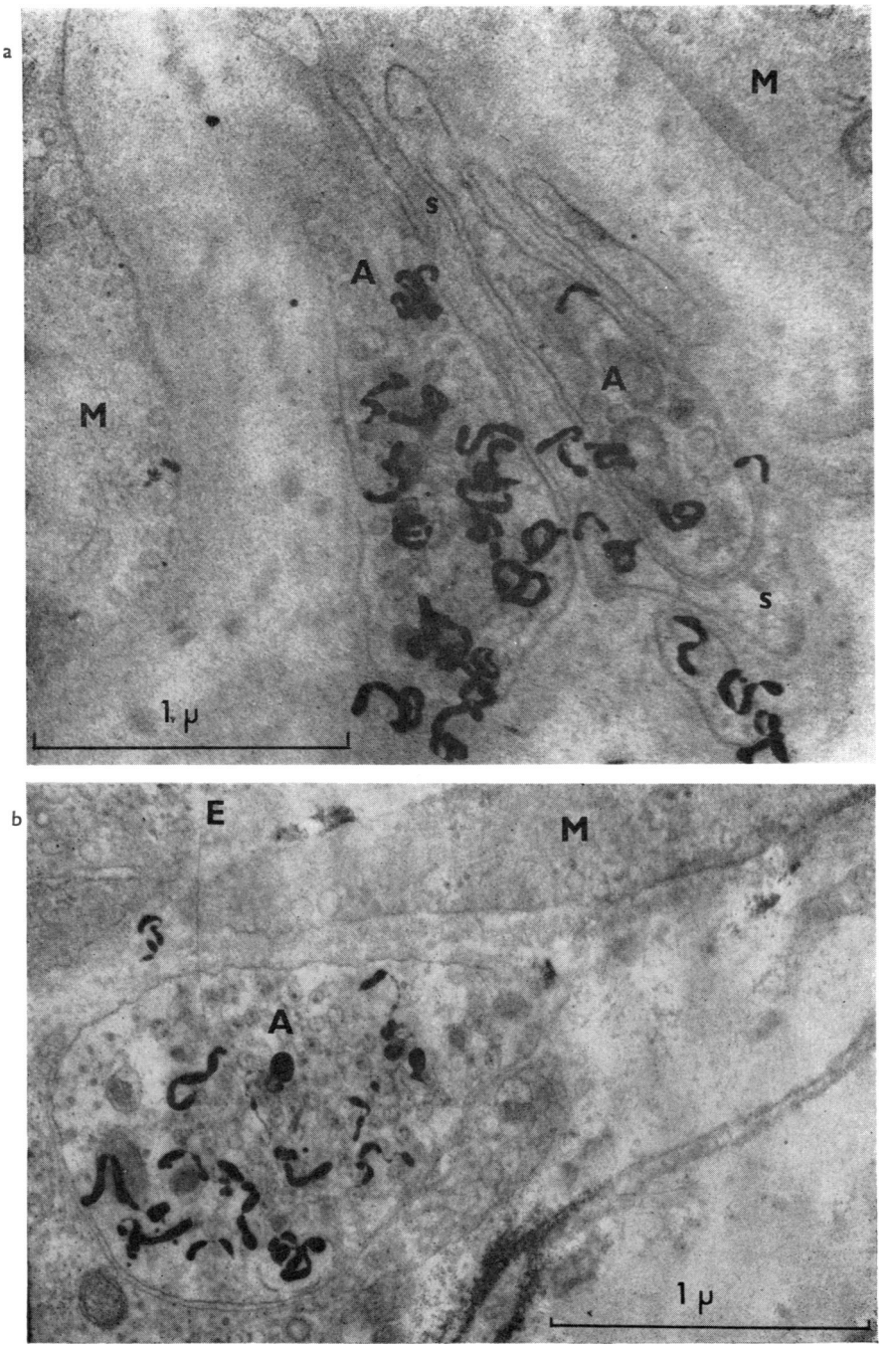
Autoradiography

The acetylcholinesterase-"stained" specimens were postosmicated and embedded in Araldite. Fine sections were cut and processed for high resolution autoradiography according to the method of Lever, Spriggs & Graham (1968). The autoradiographs were developed photographically after four weeks and examined with a Siemens' Elmiskop 1 electron microscope.

RESULTS

Intact pancreas

Accumulations of silver grains (indicating the location of molecules of ^{3}H-NA were found over 50–80% of profiles of vasomotor axons related to pancreatic arterioles (Plate 1). The number of silver grains per unit area of these axons was 3.8 ± 0.26 (S.E.) in contrast to the background distribution of silver grains which was 0.22 ± 0.10 grains per unit area (in electron micrographs; $\times 42{,}000$). No vasomotor axons exhibited the electron-dense deposit characteristic of acetylcholinesterase reaction product (Plate 1). In addition to vasomotor fibres in close relationship to arteriolar muscle, many bundles of axons were found distant to the muscle cells, lying superficial to the arteriolar adventitial fibroblast sheath: because of their situation at this ultimate level of the arterial tree, these nerves were not considered to be vasomotor. In this superficial situation some axons exhibited accumulations of silver grains and no ACHE "staining" but most axons were devoid of superimposed silver grains and possessed an intense ACHE "stain" associated with the axolemma (Plate 2a) as is characteristic of cholinergic fibres. Nerve bundles consisting exclusively of adrenergic axons or cholinergic axons were found and in addition some bundles were observed to contain a mixture of discrete adrenergic and cholinergic axons (Plates 2b and 3). In such "mixed" bundles adrenergic and cholinergic axons were sometimes juxtaposed, apparently without any Schwann cell cytoplasm intervening (Plate 2b).



Plates 1–3. Electron autoradiographs of specimens from cat pancreas perfused with ^{3}H-NA and "stained" for ACHE. Scale 1 μ.

Plate 1a and b. Accumulations of silver grains superimposed over profiles of adrenergic vasomotor axons (A). Notice the absence of ACHE reaction product in relation to the axons (cf. Plate 2a). M, Smooth muscle of arteriole ; E, endothelium ; S, Schwann cell.

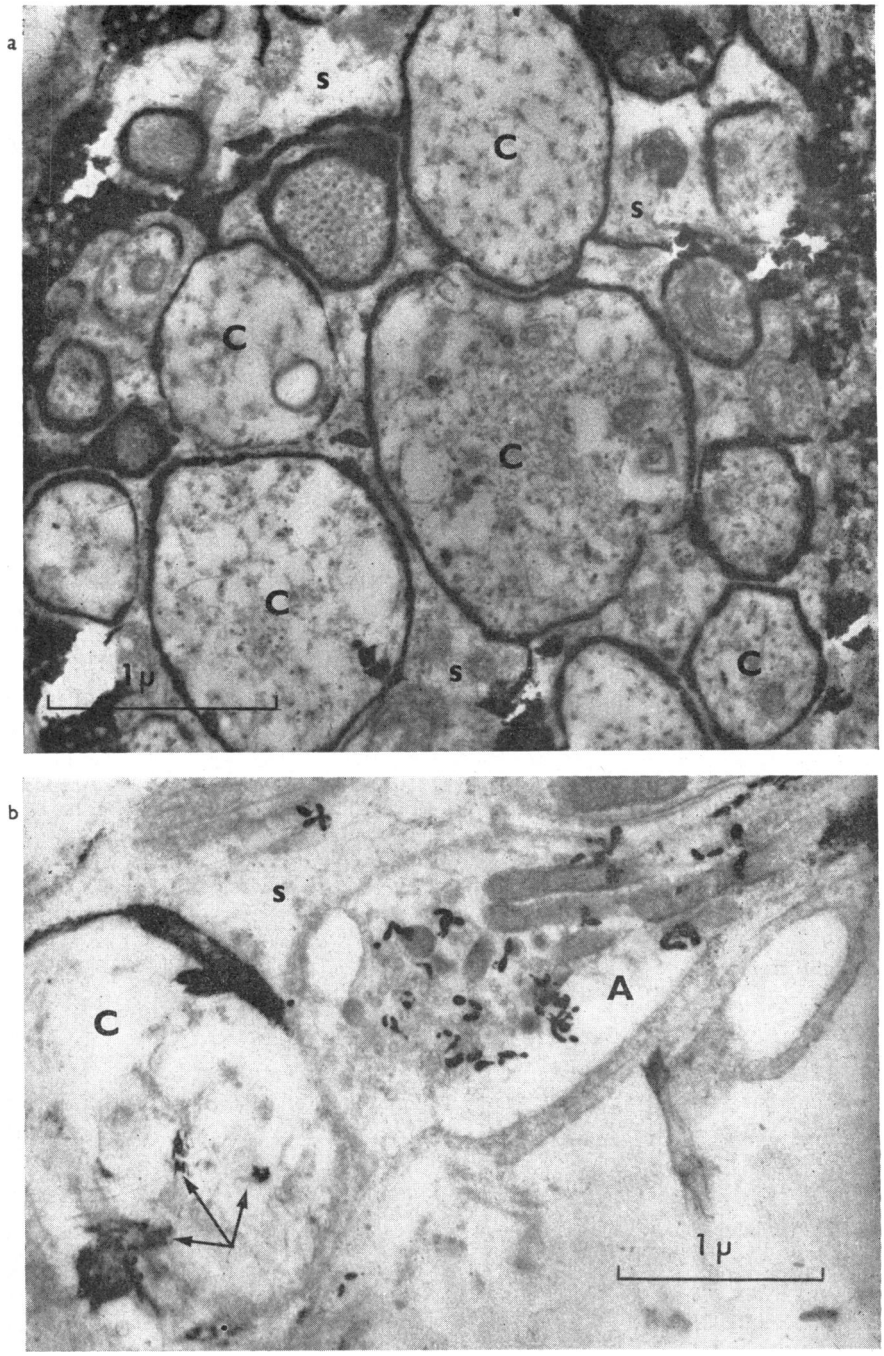


Plate 2a: Cholinergic axons (C) showing electron-dense ACHE reaction product associated with the axolemmae. These axons form part of a large nerve bundle located superficially to the adventitial sheath of an arteriole. Notice absence of superimposed silver grains. S, Schwann cell. b: An adrenergic axon (A) and cholinergic axon (C) juxtaposed in a "mixed" nerve bundle lying external to the adventitial sheath of an arteriole. Notice the superimposed silver grains and absence of ACHE "stain" on the adrenergic axon and the intense ACHE "stain" and absence of silver grains over the cholinergic axon. The deposits arrowed are probable ACHE reaction product and clearly not silver grains, which possess a characteristic form with smooth contours. S, Schwann cell.

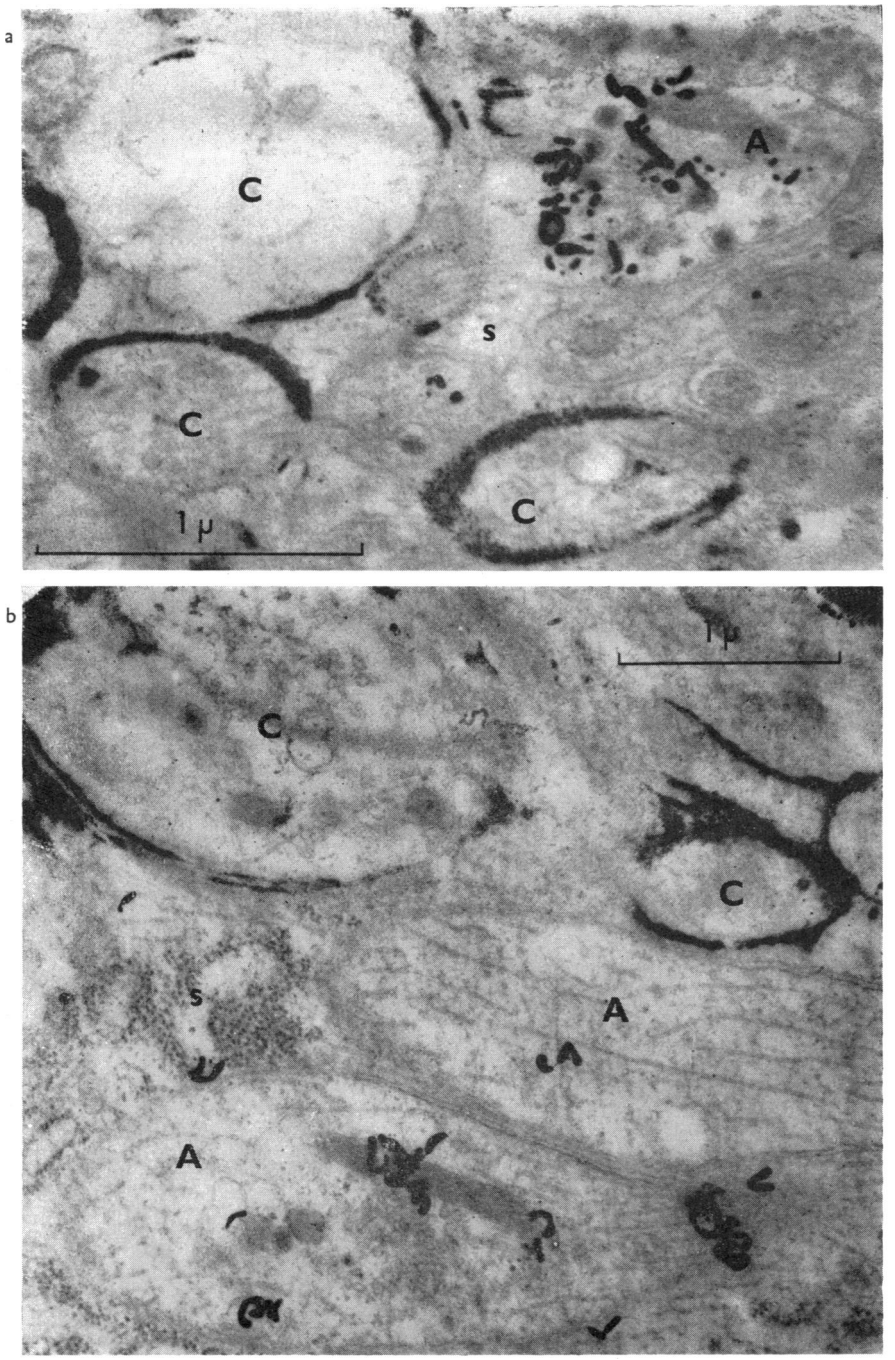


Plate 3a and b. Adrenergic (A) and cholinergic (C) axons in " mixed " nerve bundles found superficial to the adventitial sheaths of two arterioles. Note the superimposed silver grains and absence of ACHE " stain " on adrenergic axons and the intense ACHE " stain " and absence of silver grains over the cholinergic axons. S, Schwann cell.

Considering the periarteriolar nerves as a whole, of 234 axons exhibiting ACHE "staining," 95.7% were devoid of accumulations of superimposed silver grains; of 119 axons showing accumulations of silver grains 91.7% were ACHE-negative.

Sympathectomized pancreas

Only occasional vasomotor fibres were found in relation to arteriolar smooth muscle cells in the pancreas 6 months after sympathectomy. All these axons were ACHE-negative and the majority were silver grain-positive. However, remote from the muscle cells and superficial to the adventitial fibroblast sheath ACHE-positive axons were present, although mixed bundles of discrete cholinergic and adrenergic axons were only rarely observed. The ACHE-positive axons showed no accumulations of silver grains.

DISCUSSION

It is widely accepted from biochemical and pharmacological evidence that adrenergic nerves selectively accumulate noradrenaline (Iversen, 1967). Morphological evidence backed by statistical analysis has recently been presented in support of this concept (Lever *et al.*, 1968).

Vasomotor nerves to the pancreatic arterioles are sympathetic in nature, most of them showing short-term degenerative changes and eventually disappearing after pancreatic sympathectomy (Lever *et al.*, 1968). The presence of occasional adrenergic axons in the sympathectomized pancreas reflects the difficulty of obtaining and maintaining complete denervation. After sympathectomy, however, the number of cholinergic fibres lying superficial to the arteriolar adventitial sheath was not greatly reduced in comparison with intact controls; these are probably of vagal origin. Certainly the dorsal gastric nerve contributes branches which accompany the coeliac and anterior mesenteric arteries which supply the pancreas. These cholinergic fibres of non-sympathetic origin probably represent the 25% of the total periarteriolar axons which were found to remain after sympathectomy (Lever *et al.*, 1968). Therefore, when the pancreas is perfused with ^{3}H-NA and specimens are processed for high resolution autoradiography we can confidently assume that vasomotor axons which exhibit accumulations of superimposed silver grains are adrenergic and sympathetic in nature.

In this study the presence of ACHE has been used as the criterion for a functional cholinergic mechanism. The tenability of this concept in the context of the technique used for ACHE "staining" in this work has been presented earlier (Esterhuizen *et al.*, 1968). The most important criticism of this "staining" is the possibility of the substrate being hydrolysed by non-specific esterases. This is minimized by the presence in the incubating medium of ethoproprazine, a selective inhibitor of pseudocholinesterase. There is no evidence that the method fails to detect ACHE where it is present. Burn, Rand & Wien (1963) state that "The histochemical method cannot prove the absence of cholinesterase" but this statement seems to be an opinion based on the notorious difficulty of proving a negative and is unsupported by evidence. It is pertinent to emphasize that much of the evidence for the hypothesis of a cholinergic link (Burn & Rand, 1965; Burn, 1967) has been obtained by using drugs which inhibit ACHE activity; the distribution of this enzyme in the vicinity of terminal adrenergic nerves therefore provides the fundamental evidence essential to the evaluation of this hypothesis.

The present results indicate two distinct categories of terminal axons. One is silver grain-positive and ACHE-negative—that is, adrenergic fibres which show no evidence of an intrinsic cholinergic mechanism. The other is ACHE-positive and silver grain-negative—that is, cholinergic fibres which show no evidence of an intrinsic adrenergic mechanism. This evidence is therefore incompatible with the concept (Burn & Rand, 1959; Burn, 1967) that a nerve impulse arriving at adrenergic nerve terminal areas mobilizes ACh which then plays an essential part in the release of noradrenaline from the terminal areas of these same axons.

The small number of axons apparently showing both accumulations of silver grains and ACHE " staining "—less than 2.5% of the total axons surveyed—may be accounted for by the following factors. Some diffusion of ACHE reaction product occurs during the incubation with acetylthiocholine. Thus an adrenergic axon juxtaposed to a cholinergic axon may appear to be ACHE-positive due to diffusion of reaction product from the cholinergic axolemma across the small interval to the axolemma of the adrenergic axon. Similarly, if the section of nerve passes through an adrenergic axon and just cuts through the axolemma of an underlying juxtaposed cholinergic axon the appearance would resemble an adrenergic axon with ACHE staining on part of its axolemma. In addition the resolution of the autoradiographic method is such that a silver grain may be reduced by the β-emission from a molecule of ^{3}H-NA situated at a distance of 100 mμ (Lever *et al.*, 1968). Hence it is possible for silver grains to appear over a cholinergic axon profile if, within 100 mμ distance, there is an adrenergic axon containing ^{3}H-NA molecules.

Vasomotor nerves related to the smooth muscle cells of pancreatic arterioles appear to be exclusively adrenergic in that they accumulate noradrenaline and do not stain for ACHE. Outside the arteriolar adventitial fibroblast sheath nerve bundles may contain only cholinergic or only adrenergic axons or a mixture of discrete adrenergic and cholinergic axons. The adrenergic axons in the " mixed " nerve bundles may dissociate from the cholinergic axons to penetrate the arteriolar adventitia more peripherally and thus become vasomotor, or they may continue the association and ultimately provide the adrenergic innervation of the endocrine cells of the islets of Langerhans (Esterhuizen, Spriggs & Lever, 1968).

Similar " mixed " nerve bundles have been found in the toad bladder (Robinson & Bell, 1967) and, although rarely, in the nictitating muscle of the cat (Esterhuizen *et al.*, 1967, 1968), and if they occur in other autonomically innervated tissues may provide an alternative explanation for results which have hitherto been interpreted as evidence of a cholinergic link in adrenergic transmission. Catecholamine-containing cell bodies devoid of ACHE and ACHE-rich cell bodies devoid of catecholamines have been described in several sympathetic ganglia of the cat (Hamberger, Norberg & Sjöqvist, 1965) and the hypogastric nerve ganglion supplying the vas deferens of the guinea-pig (Bell & McLean, 1967). In the pancreatic " mixed " nerve, the two types of axon may be juxtaposed without the intervention of insulating Schwann cell processes (Plate 2b). Clearly electrical stimulation of such a nerve would excite both adrenergic and cholinergic axons and result in the release of noradrenaline and ACh from the terminal areas of the respective nerve. Furthermore, in areas of close apposition, ACh released from a cholinergic axon may depolarize a juxtaposed adrenergic axon, thus reinforcing adrenergic activity. Ferry (1963) and Cabrera, Torrance & Viveros (1966) have shown that ACh can depolarize

adrenergic axons. Such cholinergic intervention would be more readily detected in the presence of an anticholinesterase drug and when the frequency of stimulation is low, and this has been shown experimentally by Burn and others (see Burn, 1967). A local cholinergic-adrenergic interaction has been postulated from pharmacological evidence by Leaders (1963). The ultimate vasomotor innervation of pancreatic arterioles seems to be exclusively adrenergic, however, as does the innervation of the cat nictitating muscles (Esterhuizen *et al.*, 1967, 1968). We must, therefore, question the validity of stimulating large autonomic nerve trunks which may contain adrenergic and cholinergic axons—especially in the presence of anticholinesterases—and interpreting end-organ responses in terms of physiological events. In normal conditions, when discrete autonomic nerves are activated from the central nervous system, it is likely that the cholinergic fibres in a " mixed " nerve trunk will not be excited at a time when are the adrenergic fibres, and vice versa. In addition ACHE demonstrated along the length of the axolemma of cholinergic axons may be present to protect adjacent adrenergic nerves from ACH which might otherwise leak from the stimulated cholinergic fibres.

The results detailed in this paper on the vasomotor nerves to pancreatic arterioles, and in earlier papers on the innervation of nictitating muscle (Esterhuizen *et al.*, 1967, 1968) show that the terminal adrenergic nerves related to these muscles do not possess intrinsic cholinergic mechanisms and must cast doubt on the physiological significance of any cholinergic modulation induced experimentally by stimulating the more proximal reaches of the adrenergic axons where there may exist anatomical association with cholinergic axons.

SUMMARY

1. The isolated pancreas of the cat was perfused *in vitro* with ^{3}H-NA and specimens subsequently " stained " for ACHE and processed for high resolution autoradiography.

2. Electron autoradiographs revealed the nature of axons accompanying pancreatic arterioles as (a) adrenergic—indicated by accumulation of silver grains superimposed over axon profiles, or (b) cholinergic indicated by ACHE " staining " of the axolemmae.

3. Adrenergic axons were devoid of ACHE " staining " and cholinergic axons showed no superimposed accumulations of silver grains. This evidence militates against the presence of an intrinsic cholinergic mechanism within terminal adrenergic axons.

4. Vasomotor axons to arterioles were exclusively adrenergic. Axons superficial to the arteriolar adventitial sheath were either adrenergic or cholinergic, the latter predominating, and some nerve bundles contained a mixture of discrete adrenergic and cholinergic axons.

5. After pancreatic sympathectomy, only occasional adrenergic vasomotor nerves were found. Many cholinergic axons remained superficial to the arteriolar adventitial sheath and these were probably derived from the dorsal gastric nerve (parasympathetic).

6. The present findings are discussed in the context of present knowledge of post-ganglionic sympathetic nerves.

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