# CHOLINERGIC FACTORS
# IN THE MAINTENANCE OF VASCULAR TONE

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SUMMARY

1. The aim of the study was to examine cholinergic factors which might contribute to the control of blood flow in a peripheral cutaneous artery, the central artery of the rabbit ear.

2. The study comprised morphological investigations, including histochemistry at both the light-microscope and electron-microscope levels, an enzyme assay, and functional (perfusion) studies using the isolated whole rabbit ear, and also the isolated central rabbit ear artery.

3. With light-microscope histochemical techniques, the enzymes cholinesterase (pseudocholinesterase) and acetylcholinesterase were demonstrated in transverse sections of rabbit ear artery, near the medial-adventitial border. The number of areas stained for the enzymes was greatly reduced after sympathetic nerve degeneration. Comparison with the results of fluorescence microscopy for the demonstration of catecholamines in adjacent tissue sections, and within single sections, indicated an extremely close morphological relation between the enzymes and adrenergic fibres in the artery.

4. The general ultrastructural morphology of the rabbit ear artery was studied with the electron microscope, with particular attention to the neural elements in the artery wall. On the

basis of the morphology of the neuronal vesicles, and their ability to take up 5-hydroxydopamine, all axons in the artery wall were classified as adrenergic. In sections of artery treated to demonstrate the enzymes the adrenergic axons exhibited weak acetylcholinesterase activity in association with the axon membrane, and more intense cholinesterase activity was associated with the Schwann cells.

5. No choline acetylase activity could be demonstrated in the rabbit ear artery, using either an enzyme assay technique or a histochemical method.

6. In perfusion studies using the isolated whole rabbit ear, exogenous acetylcholine (ACh) was shown to have a potent dilator action when the perfusion pressure was raised with the synthetic octapressin analogue POR8. The dilator effect of ACh was prevented by atropine.

7. In the isolated perfused central artery of the rabbit ear, exogenous ACh had a potent dilator action when the perfusion pressure was raised by periarterial electrical stimulation. The action of ACh was prevented by antimuscarinic agents, and enhanced by cholinesterase inhibitors. No evidence was seen for a facilitatory role of exogenous ACh, and there was no indication of an action of endogenous ACh in the artery's constrictor response.

8. The dilator action of ACh was further studied by examining the effects of the ester on other constrictor stimuli, namely -

   (i) noradrenaline

   (ii) noradrenaline in the presence of cocaine

   (iii) noradrenaline in sympathetically denervated arteries

   (iv) histamine

   The results indicated that the artery had cholinergic inhibitory receptors related to the sympathetic nerve terminals, and also inhibitory receptors related to the arterial smooth muscle. It appeared likely that the neuronal action of ACh was responsible, in the majority of arteries, for the depression of responses to periarterial electrical stimulation.

9. The study demonstrated pharmacological actions of ACh on the rabbit ear artery which may be of importance in other vascular systems. Although cholinergic elements were demonstrated histochemically within the artery wall, the suggestion of functional, endogenous cholinergic mechanisms in the artery was not confirmed.

# DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge contains no material previously published by another person, except where due reference is made in the text.

WYATT RODERIC HUME

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# CHAPTER I

## GENERAL INTRODUCTION

The probable role of peripheral vascular function in the aetiology of essential hypertension has inspired a large body of work concerning the mechanisms which control the diameter of blood vessels, and hence the peripheral flow resistance. Most of these studies have been directed towards the mechanisms by which vessels constrict, and the agents and nerves which control constriction, while in recent years relatively little attention has been given to dilator mechanisms. The present work, a study of cholinergic influences on vascular function in a peripheral vessel, is related to both constriction and dilatation. Acetylcholine has been recognised as a vasodilator substance since the observations of Dale in 1914; more recently it has been implicated in several roles in constrictor responses, as will be outlined later in this Chapter. As an introduction to the studies described in this thesis it is useful to trace briefly the origins of concepts of neurohumoral transmission, many of which resulted from the study of the blood vascular system, and then to outline the known and postulated roles for acetylcholine in peripheral vascular function.

### Neurohumoral transmission - historical outline.

In 1869 Schmiedeberg and Koppe found that the alkaloid muscarine, from the mushroom *Amanita Muscaria*, depressed cardiac activity in the

frog, having an effect similar to that of vagal stimulation. Like the vagus, muscarine did not affect the sensitivity of the heart to electrical or mechanical stimulation, and thus was not acting as a muscle poison. Atropine, which had earlier been shown to prevent the effect of vagal stimulation, also prevented the depressor effect of muscarine. These observations were probably the first showing similarities between the action of a drug and the effects of nerve stimulation. Later Dixon (1907), again using the frog, demonstrated a muscarine-like compound in alcoholic extracts of heart. The degree of activity of the extracts was determined on the live heart, and was directly related to vagal stimulation of the donor heart before extraction. Dixon proposed that the action of the vagus might therefore be mediated chemically, by an 'inhibitory hormone' which had properties in common with muscarine.

A similar sequence of discovery took place in the other arm of the autonomic nervous system. Langley (1901) described the action of extracts of dog adrenal glands on anaesthetised cats, and noted that virtually all of the effects of the extracts were the same as could be produced by sympathetic nerve stimulation. Since the extracts were effective after postganglionic sympathetic nerve degeneration, Langley suggested that they acted directly on the tissues, rather than via the nerves. It was on the basis of these and other experiments that Langley proposed the use of the terms *sympathetic* and *parasympathetic* for thoraco-lumbar and cranio-sacral efferents, and

*autonomic* for the whole. However Elliott (1904) was impressed by Langley's description of the similarity of effects of adrenalin and of stimulation of the sympathetics. Elliott noted that in moribund animals suffering the effects of adrenal ablation the tissues innervated by the sympathetics lost their tone, while the sensory and somatic nerves appeared to retain their efficiency. He stated, '...*the facts suggest that the sympathetic axons cannot excite peripheral tissues except in the presence, and perhaps through the agency, of the adrenaline or its immediate precursor... Adrenaline might then be the chemical stimulant liberated on each occasion when the impulse arrives at the periphery.*'

Partly because of its chemical similarity to muscarine, and partly because it was known to be present in the adrenal gland, Hunt and Taveau (1906) studied the effects of choline and some choline derivatives on the circulation of the anaesthetised rabbit. They found that acetylcholine was extremely effective in depressing the blood pressure, noting responses to doses of five and ten nanograms of the drug in some animals. Dale (1914) also examined choline derivatives in the hope of determining the chemical structures of muscarine. He did not achieve this aim, since the compounds were all dissimilar from muscarine in having additional nicotine-like actions. But Dale noted parallels between the actions of the compounds and the effects of nerve stimulation in a variety of organs, and drew further attention to the potency of acetylcholine. He refrained from

making any concrete suggestions regarding acetylcholine and nerve action, partly because the compound could not be demonstrated within the body.

Although Elliott (1904) and Dixon (1907) had suggested that humoral transmission from nerves might exist, it required the elegant experimental system of Loewi (1921) to demonstrate the phenomenon. He used two isolated frog hearts containing Ringer solution; the experiment is best described in his own words (Loewi, 1960). *'The vagus nerve of the first heart was stimulated for a few minutes. Then the Ringer solution that had been in the first heart during stimulation of the vagus was transferred to the second heart. It slowed and its beats diminished just as if its vagus had been stimulated. Similarly, when the accelerator nerve was stimulated and the Ringer from this period transferred, the second heart speeded up and its beats increased. These results unequivocally proved that the nerves do not influence the heart directly but liberate from their terminals specific chemical substances which, in their turn, cause the well-known modifications of the function of the heart characteristic of the stimulation of its nerves'.* Later Loewi and Navratil (1926) provided strong evidence that the inhibitory substance, at first called 'Vagustoff', was the compound acetylcholine. Several other workers saw evidence of a cardio-accelerator substance in blood after peripheral sympathetic nerve stimulation, and Cannon and Bacq (1931) suggested that this substance be called 'sympathin'.

In 1937 Cannon and Rosenblueth presented a concept of the structure and action of the two arms of the autonomic nervous system basically in accord with that which is accepted today. It was recognised that the sympathetic transmitter (sympathin) had some properties in common with the adrenal hormone adrenaline, but they noted important differences. Cannon and Rosenblueth suggested in explanation of sympathin elaboration from organs, as one possibility, *'secretion from the minute nerve terminals'*. Sympathin was shown to be most probably noradrenaline by von Euler (1948), and von Euler and Hillarp (1956) demonstrated the presence of noradrenaline in the granular fraction of adrenergic nerves. There is now little doubt that noradrenaline is released from sympathetic nerves to mediate their effects, but the mechanism of release is not known. This thesis was in part concerned with a theory of acetylcholine's involvement in such release. The concept that acetylcholine is released from parasympathetic postganglionic fibres remains essentially unchallenged, although again how release occurs is not known.

Vascular cholinergic action.

Dale, in 1914, was able to compare in the discussion of his results the effects of acetylcholine administration and vasodilator nerve stimulation, but despite the passage of more than half a century the mode of action of the drug and of the nerves, and indeed the very existence of the nerves in some species (Unvas, 1966) is still in doubt. In man, as has been outlined by Whelan (1967), there is

little functional evidence for a physiological role of acetylcholine from cholinergic nerves in the overall control of peripheral circulation. The dilator response in skin during body heating may have a cholinergic component, but it may also be due to the action of bradykinin released from cholinergically controlled sweat glands, as suggested by Fox and Hilton (1958). There is some evidence for active cholinergic dilatation in human skeletal muscle vascular beds, but there is also evidence for active adrenergic dilatation in the same vessels (Whelan, 1967). Studies on the effects of administration of exogenous acetylcholine in man were cited by the same author; the drug causes a transient dilatation in skin vessels, with flushing and a temperature rise as the notable responses, but it is relatively quickly inactivated in the circulation. Vane (1969) has suggested that acetylcholine is inactivated in both the blood itself and also during passage through the pulmonary vascular bed, and is therefore unlikely to have important effects as a circulating hormone.

The actions of acetylcholine on isolated perfused vascular beds and on isolated arteries *in vitro* have been reviewed by Furchgott (1955) and more recently by Somlyo and Somlyo (1970). In general acetylcholine dilates vessels and relaxes strip or chain preparations, but in some experimental systems the ester has constrictor effects, particularly when applied in high concentrations. Two mechanisms have been proposed to explain these constrictor effects. Furchgott (1955) considers that in some cases the action is 'undoubtedly' on

cholinergic motor receptors in the smooth muscle, but that in other cases acetylcholine has a local, indirect effect by releasing noradrenaline from stores in the tissue. This latter effect will be considered in more detail below.

*Adrenergic-cholinergic interactions.*

Many autonomically controlled systems, the heart being a prime example, receive dual adrenergic and cholinergic innervation, the two types of nerves exerting mutually antagonistic effects. Bell (1968) has provided both morphological and functional evidence that such a system of dual control exists in the uterine artery of the guinea pig; Schenk and El Badawi (1968) demonstrated such a system of nerves in arteries from dogs and cats, using histochemical techniques. It is, however, questionable whether such innervation, and such control, exists in other vessels, and this study was in part concerned with seeking evidence for a system of this type. Dual control obviously constitutes one type of functional intercommunication between adrenergic and cholinergic factors; others have been proposed. Historically, perhaps the first was that mentioned above, the local, indirect effect of acetylcholine in releasing noradrenaline from peripheral stores. This action is well documented; it has been observed in a variety of systems, for example the vessels of the rabbit's ear (Kottegoda, 1953), the isolated cat heart (Haeusler et al., 1968) and the perfused cat spleen (Krauss, Kopin and Carpenter, 1970). The effect follows

application of large doses of acetylcholine or nicotine, and is not decreased and sometimes increased by atropine (Haeusler et al., 1968). These latter authors measured actual noradrenaline output from the isolated cat heart fluorimetrically.

Another type of proposed functional interaction is that acetylcholine and noradrenaline might act presynaptically, one inhibiting the release of the other. Inhibition of noradrenaline release by acetylcholine has been demonstrated in the isolated rabbit heart by Lindmar, Loffelholz and Muscholl (1968); the opposite effect has been observed in a non-vascular tissue, the isolated guinea-pig ileum, where noradrenaline and adrenaline reduced the output of acetylcholine (Paton and Vizi, 1969). These results suggest interesting possibilities in systems of control, with the particular advantage of transmitter economy. However, the proposed interaction which has stimulated the most interest in recent years is the 'cholinergic link' hypothesis, which was of particular importance in the development of the present study and will therefore be considered separately below.

*'Cholinergic link' hypothesis.*

Burn and Rand (1959, 1960) drew attention to a number of experiments in different systems in which acetylcholine and nicotine mimicked the effects of sympathetic stimulation. The effects were seen in the presence of atropine, but not after reserpine pretreatment or sympathetic nerve degeneration, so the action was nicotinic, and required

an intact noradrenaline store. The authors also presented evidence that there were cholinergic fibres in the sympathetic supply in many organs, and therefore suggested that '*...cholinergic fibres...may liberate noradrenaline from the store at the nerve endings, and thus be adrenergic in effect.*' (Burn and Rand, 1959). It should be emphasised that in these papers the main suggestion was that the acetylcholine arose from cholinergic fibres to act on a separate noradrenaline store, which was thought to be either in adrenergic nerve endings or chromaffin cells in the tissue itself. It was proposed that there might be two mechanisms for noradrenaline release, one simply from adrenergic fibres, the other by the action of acetylcholine from cholinergic fibres on the store. Figure 1.1 illustrates these concepts.

In later works the suggestion was modified, largely because of experiments using nerve blocking agents. Burn and Rand (1960) reported that choline 2, 6-xylyl ether bromide (TM 10) and bretylium blocked the response of the cat nictitating membrane to sympathetic nerve stimulation, but not to tyramine, which acts by releasing noradrenaline. Chang and Rand (1960) showed that in a number of preparations hemicholinium, which inhibits the synthesis of acetylcholine, caused failure of sympathetic nerve action. Burn (1961) therefore proposed as a working hypothesis that '*...all sympathetic post-ganglionic fibres release acetylcholine, which in turn releases noradrenaline*'. The hypothesis has been re-stated by Burn and Rand (1962, 1965) and more recently by Burn (1971) and is shown in diagrammatic form in Figure 1.2.

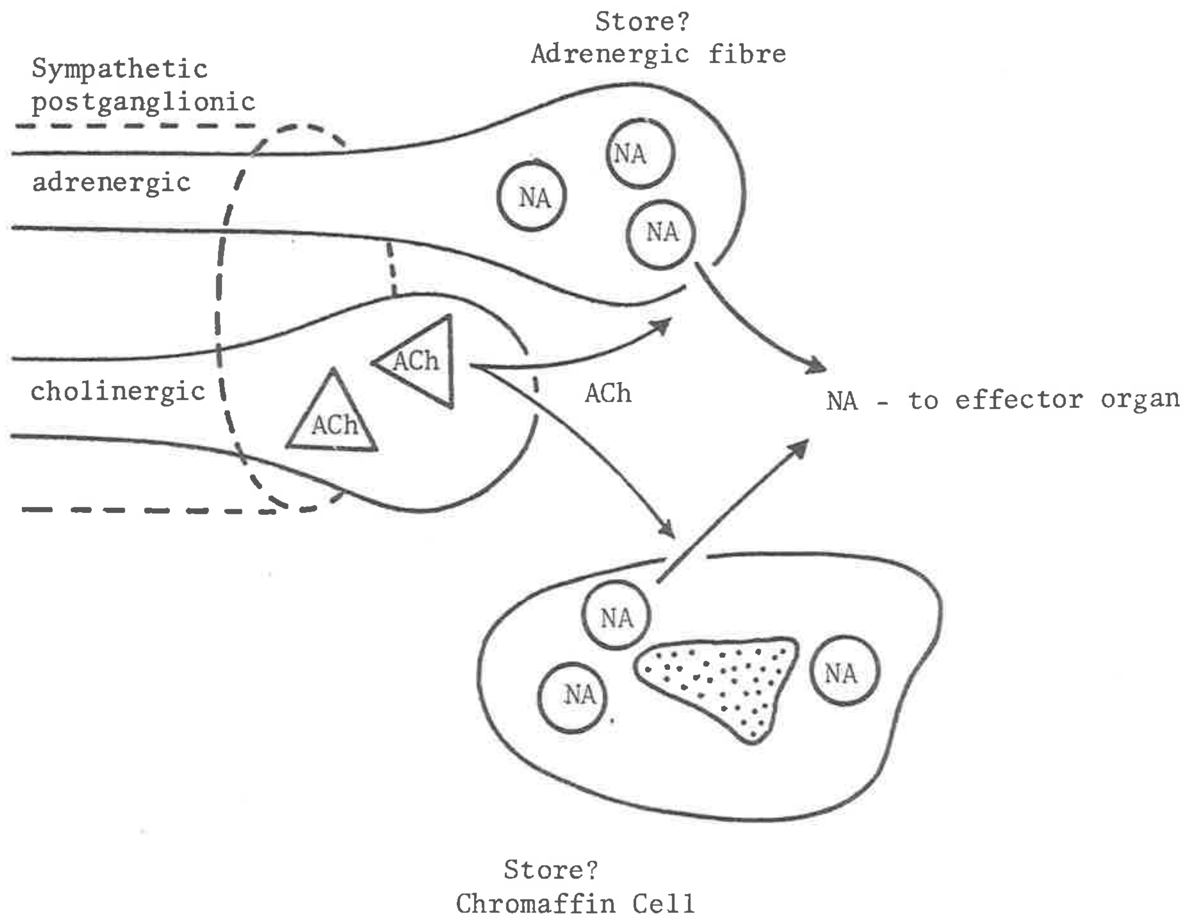



Figure 1.1

Diagrammatic representation of the suggestion of Burn and Rand (1959) that acetylcholine (ACh) from cholinergic axons in postganglionic sympathetics might release noradrenaline (NA) from stores, either in adrenergic axons or chromaffin cells.

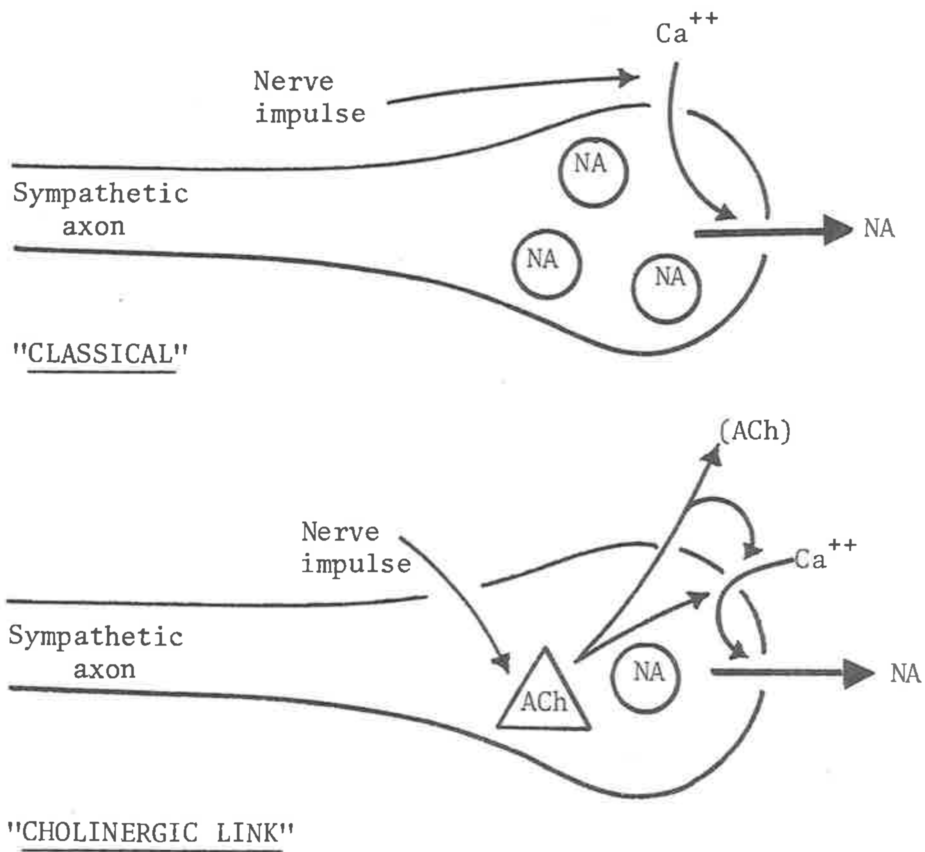



Figure 1.2

Diagrammatic representations of (above) the 'classical' (Ferry, 1966) concept of noradrenaline (NA) release from adrenergic nerve endings and (below) the mechanism as first suggested by Burn (1961). In each case a nerve impulse allows ingress of calcium ions ($Ca^{++}$) to bring about the NA release, but in the latter there is a 'cholinergic link' involving acetylcholine (ACh). From Ferry (1966).

Ferry (1966) made an extensive review of the 'cholinergic link' hypothesis, considering principally the later concept that all sympathetic axons contained an intrinsic cholinergic mechanism in their noradrenaline release systems. He proposed criteria which could be applied to test the validity of the concept, and examined the evidence in individual organs in the light of these criteria. Ferry concluded, *'A survey of all the preparations shows that in very few of them has it been established that acetylcholine is linked with the adrenergic nerves....the existence of cholinergic process in the release of the sympathetic transmitter can neither be confirmed nor denied.'* Recently, however, Malik and Ling (1969) have presented evidence that acetylcholine both facilitates and depresses the response to sympathetic nerve stimulation in isolated perfused rat mesenteric vessels, and have interpreted this evidence as being in favour of the 'cholinergic link' hypothesis. During the course of the present study Rand and Varma (1970) made similar observations in the isolated rabbit ear artery, and drew similar conclusions. These studies are considered in more detail in later Chapters of this thesis.

The known and postulated actions of ACh on vascular tissue, which formed the basis of the present study, may be summarised as follows:

(i) Direct dilatation of vessels.

(ii) Direct constriction of vessels.

(iii) Indirect constriction of vessels, by releasing transmitter from sympathetic nerve endings.

(iv) Depression of constrictor responses by inhibition of transmitter release from sympathetic nerves.

(v) Facilitation of constrictor responses by participation in the postulated 'cholinergic link' process.

(vi) Depression of constrictor responses by block of the 'cholinergic link'.

Scope of the study

The present work was an examination of arterial cholinergic mechanisms, using the central artery of the rabbit ear. The study was primarily concerned with cholinergic actions and adrenergic-cholinergic interactions in the artery, and as was outlined above this of necessity included considerations of both adrenergic and cholinergic nerve function. Much of the work was therefore related to the morphology and action of the vascular nerves, but the effects of cholinergic, anticholinergic and anticholinesterase drugs on the perfused vessel were also examined in detail.

A combination of morphological and functional studies was used during the investigation. The sequence in which these studies are described follows the general pattern of the investigation, but for the sake of clarity the functional (perfusion) studies, which in fact overlapped the morphological experiments to a large degree, are described in the latter part of the thesis. Two relatively minor investigations, which were peripherally related to the main study, are described as

Appendices. The work began with histochemical studies designed to determine the localisation of cholinergic structures in the artery wall.

Terminology and abbreviations.

The term *tone* has been described by Mellander (1970) in relation to blood vessels as '...*the average level of contractility of the numerous smooth muscles in a region or section of the circulatory system*'. This state of contractility directly influences the diameter of the blood vessels and therefore the resistance to flow. The term is used in this thesis in a slightly more general sense, to embrace these latter factors.

In general, drugs are referred to by the nonproprietary names listed in the Appendix, or by a shortened form of the name; the names *adrenaline* and *noradrenaline* are used, rather than *epinephrine* and *norepinephrine*. The nomenclature of the enzymes cholinesterase and acetylcholinesterase is clarified elsewhere (page 2.2).

Abbreviations are used consistently within single Chapters, but in some cases the full name is used in other parts of the thesis, when such use is necessary to avoid possible confusion. The frequently used abbreviations are:

| | |
|---|---|
| ACh | acetylcholine |
| NA | noradrenaline |
| AChE | acetylcholinesterase |

| | |
|---|---|
| ChE | cholinesterase |
| ChAc | choline acetylase |

Abbreviations of names of drugs, although explained in the text, may be checked by reference to the Appendix, page 7.

# CHAPTER TWO

## HISTOCHEMICAL LOCALISATION OF CHOLINESTERASES

The ideal way to localise cholinergic structures within an organ would be to demonstrate acetylcholine in tissue sections. At this time no method has been reported for such a demonstration. Sites of cholinergic function have instead been demonstrated in many tissues by the localisation of cholinesterases, particularly acetylcholinesterase, the enzyme that catalyses the hydrolytic breakdown of acetylcholine. Before describing the use of this histochemical approach in the rabbit ear artery, the nomenclature of the enzymes and some aspects of the origin of the technique warrant discussion.

Esterases are distinguished from lipases on the basis of their preference for short-chain fatty acid esters of simple alcohols, and their inhibition by bile salts. Dale (1914), who noted the brevity of acetylcholine's action in whole animals, suggested that an esterase might contribute to the rapid removal of acetylcholine from the blood. Stedman, Stedman and Easson (1932) prepared such an enzyme which they called choline-esterase, from horse serum. They considered that the enzyme was a specific esterase for choline esters, including acetyl-choline. Later, on the grounds of substrate preferences and properties related to enzyme inhibitors, the concept arose of acetylcholin-esterase, a specific enzyme for the hydrolysis of acetylcholine, as distinct from other cholinesterases. The nomenclature adopted by the

Commission on Enzymes of the International Union of Biochemistry in 1961 was that introduced by Augustinsson and Nachmansohn (1949) as follows:

(i) *Acetylcholinesterase* (acetylcholine acetyl-hydrolase, 3.1.1.7). Acetylcholinesterase (ACLE) has also been described as 'true' cholinesterase. It hydrolyses acetylcholine more rapidly than it does other choline esters, and is found at the neuromuscular junction, at ganglionic synapses and in other nervous tissues, as well as in red blood cells (Koelle, 1963).

(ii) *Cholinesterase* (acylcholine acyl-hydrolase, 3.1.1.8). Cholinesterase (ChE) has been called pseudocholinesterase, non-specific cholinesterase and butyrylcholinesterase. It hydrolyses various choline esters more readily than it does acetylcholine, and is found in many tissues including the plasma (Augustinsson, 1963). The physiological function of ChE is not known, although it may regulate the homeostasis of free choline (Funner and Oliver, 1965), or it may hydrolyse naturally formed choline esters which inhibit AChE (Lehmann and Liddell, 1969).

Chessick (1954) warned that the division of the enzymes into two groups should be viewed with caution. He stated that, 'the cholinesterases.... should therefore be viewed as a family of enzymes, forming a spectrum with multiple overlapping and differences with respect to physical properties, effects of inhibitors and substrate specificities'. In the present work this concept is borne in mind, but the division is used as

one of convenience. The abbreviations *AChE* and *ChE* will be used to signify the two groups, and the plural word *cholinesterases* to signify the two together.

The most common methods for the histochemical localisation of cholinesterases make use of the hydrolysis of compounds similar to choline esters to give characteristic products. Gomori (1948) used higher fatty esters of choline to yield the corresponding lipid at sites of activity. Koelle and Friedenwald (1949) introduced thiocholine esters as substrates. These react very similarly to their choline ester analogues, and on hydrolysis liberate thiocholine, which can be used to form a visible product. The Koelle and Friedenwald (1949) technique involved the immediate precipitation of copper thiocholine sulphate, which was then converted to black copper sulphide. The crystalline nature of the initial precipitate and the need for a second precipitation process were undesirable features of the method (Malmgren and Sylven, 1956). Karnovsky and Roots (1964) devised a technique in which enzymatically liberated thiocholine reduced ferricyanide to ferrocyanide, which then precipitated as the brown copper salt. The technique was termed 'direct coloring' since the initial precipitate was visible. The simplicity of the technique and its apparent good localisation have led to its wide use in recent years.

In this study, the technique of Karnovsky and Roots (1964) has been applied, with modifications, to cryostat sections of rabbit ear artery

in order to examine the distribution of cholinesterases in the artery wall in normal and pre-treated animals.

## MATERIALS AND METHODS

### Histochemistry.

Ear arteries from forty-one semi-lop eared rabbits of either sex, bred at the Central Animal House, University of Adelaide, were treated to demonstrate ChE or AChE. Rabbits were anaesthetised with ethyl carbamate (urethane) approximately 2 gm/Kg intraperitoneally. After removal of the overlying skin, segments of the proximal part of the central ear artery were gently dissected free and taken from the animal, or blocks 3 x 3 cm containing a segment of the artery were taken from the full thickness of the ear. The location of the central artery in the rabbit ear is shown diagrammatically in Figure 2.1.

Figure 2.2 shows the steps of the stain procedure. Specimens were frozen in liquid nitrogen or in acetone containing dry-ice (solid $CO_2$) and sectioned either immediately or after storage for up to 14 days at -50$^{o}$C. Transverse sections of artery were cut at 4-20 microns thickness on a cryostat (Cryo-cut, American Optical Co.) and thawed onto glass slides. The cryostat was set to maintain a cabinet temperature of -17$^{o}$C. The sections were dried in air for ten minutes and then fixed in formalin-sucrose-ammonia (Pearson, 1963) for 15-30 minutes. All other steps prior to dehydration were carried out in 0.1M aqueous sodium hydrogen malate (pH 6.0).

Sections were exposed to inhibitors and substrates in various com-

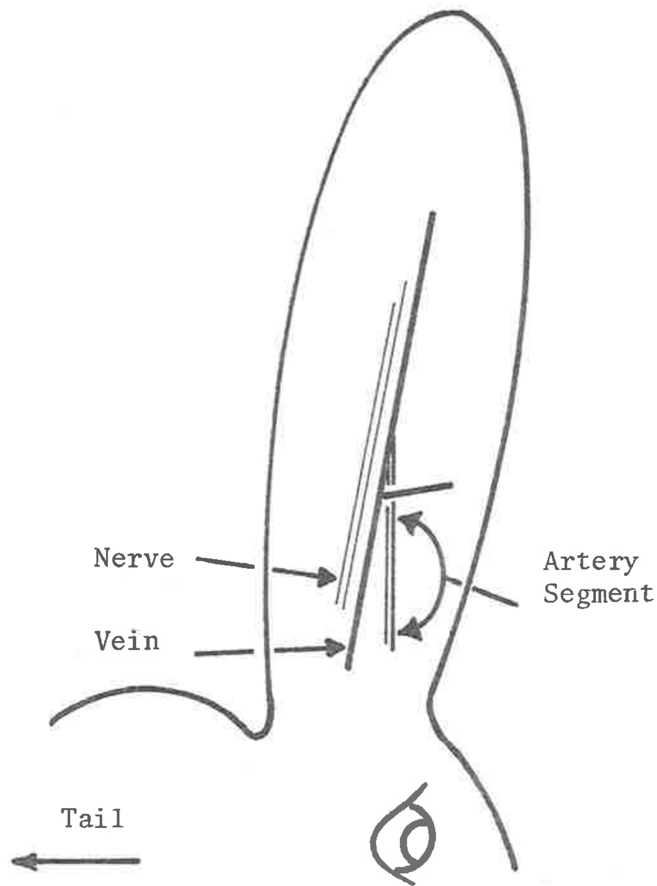



Figure 2.1

A diagram of a rabbit ear, showing the relative positions of the great auricular nerve, the central vein and the central artery. The double arrows indicate the segment of the artery which was used in the morphological experiments. The diagram represents the left ear, held erect and viewed from the right side of the animal.

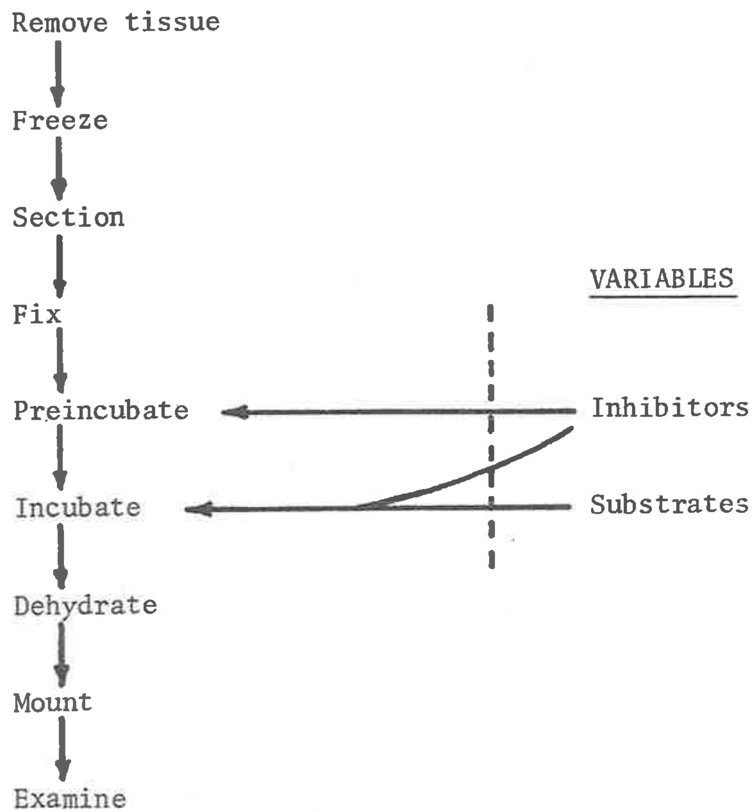



Figure 2.2

Showing the stages of treatment of tissue for the histochemical demonstration of cholinesterases, and the points of introduction of the variables in the system, the inhibitors and substrates.

binations, which are described in Results. Sections were either pre-incubated in one or two of the enzyme inhibitors described below for one hour at 37°C, or held in malate buffer under the same conditions without inhibitors. The inhibitors used were:

(i) *iso*OMPA (tetra*iso*propyl pyrophosphoramide, Koch Light),

(ii) BW284C51 (1, 5-bis-(N-allyl-n, N-dimethyl-4-ammonium phenyl) pentan-3-one dibromide, Burroughs Wellcome),

(iii) DFP (di*iso*propylfluorophosphoramide, Koch Light).

The inhibitors *iso*OMPA and BW 284C51 were freshly prepared from the solid by serial dilution, and DFP was prepared from a stock solution of $10^{-1}$M in propylene glycol. The concentrations of inhibitors used were *iso*OMPA $10^{-5}$M or 3 x $10^{-5}$M, BW 284C51 3 x $10^{-5}$M or $10^{-4}$M, and DFP $10^{-4}$M.

After a brief buffer rinse, sections were placed in an incubation medium which had been freshly prepared from stock solutions (see Appendix page 1) and either acetylthiocholine iodide (AThI) or butyrylthiocholine iodide (BuThI), 0.5 ug/ml. For those sections which had been pre-incubated in BW284C51 the compound was included in the incubation medium at the same concentration. The substrate was omitted in treating control sections. Incubation times of 30 minutes to 20 hours were employed, and at the end of incubation the sections were rinsed in malate buffer, dehydrated through graded concentrations of ethanol, and mounted with Xam (Gurr).

Sections were examined with a Zeiss S.V. microscope with ordinary

(tungsten) light using light or dark field illumination. Photomicrographs were made with a Zeiss Ikon automatic camera and Ilford Pan F film.

The above procedure, while based on that of Karnovsky and Roots (1964), differed in the following aspects:

(i) The tissue was fixed after sectioning using a formalin-sucrose-ammonia mixture, while Karnovsky and Roots fixed in 10% neutral formalin with 1% $CaCl_2$ before freezing. The present procedure was found to reduce the loss of sections during incubation.

(ii) A pre-incubation step was introduced to allow accurate application of time-dependent (cumulative) enzyme inhibitors.

(iii) The preferred concentration of $CuSO_4$ in the incubation medium was 6mM in the present study, compared with 3mM by Karnovsky and Roots. This concentration was chosen after experiments in which all components and the pH of the medium were varied to find optimal conditions for stain localisation.

<u>Pre-treatment of tissues.</u>

a. Sympathetic denervation.

Twenty rabbits were prepared by unilateral excision of a superior cervical ganglion at times between 20 hours and 21 days before sacrifice, using the technique described by de la Lande and Rand (1965). Animals

were premedicated with atropine 2.5 mg/Kg intraperitoneally, and anaesthetised with ether. The ventral surface of the neck was shaven, washed with chlorhexidine-cetrimide mixture, and the subcutaneous tissues infiltrated with lignocaine 20 mg/ml with adrenaline 12.5 ug/ml (Xylocaine, Astra Pharmaceuticals). A midline incision was made over the trachea, and was extended by blunt dissection to the trachea'a lateral aspect to expose the carotid sheath. The superior cervical ganglion was located by referring to the medial aspect of the carotid artery and the line of the angle of the mandible. The ganglion was dissected free and removed with short lengths of the pre- and post-ganglionic trunks. The incision was closed with interrupted sutures and the suture line covered with a plastic spray wound dressing. Heat sterilised instruments and aseptic technique were employed.

Early indications of the effectiveness of ganglion removal were dilatation of the ear vessels and constriction of the pupil on the operated side. Segments of ear artery were removed at sacrifice and treated to demonstrate catecholamine fluorescence using the technique of Falck and Owman (1965) as modified by Waterson and Smale (1967) in order to further test the effectiveness of denervation. Details of this treatment are found in Appendix, page 2.

b. Reserpine pre-treatment.

Five rabbits were pre-treated with reserpine (Serpasil, Ciba) 2.5 mg/Kg intraperitoneally 24 hours before sacrifice. Segments of

artery were treated for cholinesterase, and other segments were treated for catecholamine fluorescence, as described above.

c. Krebs solution.

Four arteries were stained for ChE or AChE after being perfused with Krebs bicarbonate solution for up to six hours, as described in Chapter 6. Three arteries were stained after storage in the same solution for 24-48 hours at $4^{o}C$.

RESULTS

Sections of rabbit ear artery.

a. No inhibitors.

Following both AThI and BuThI incubation, discrete heavy deposits of brown stain were observed near the medial-adventitial border of the artery. In addition, the AThI incubated sections often showed staining of red blood cells in the lumen of the artery (Figure 2.3a).

b. Inhibitor pre-incubation.

*BW284C51* $3 \times 10^{-5}M$ *or* $10^{-4}M$.

The distribution and density of staining at the medial-adventitial border was indistinguishable from that in sections treated without inhibitors. In AThI incubated sections the red blood cells did not stain.

*iso*OMPA $10^{-5}M$ *or* $3 \times 10^{-5}M$.

In incubated sections staining was not evident. AThI incubated sections showed faint but definite staining near the medial-adventitial border, and staining of red blood cells was comparable to that observed in sections not exposed to inhibitor (Figure 2.3b).

*isoOMPA and BW284C51.*

No stain was evident in sections treated with both *iso*OMPA and BW284C51.

*DFP $10^{-4}$M*

No brown stain was evident in sections treated with DFP.

c. Control incubation.

Sections treated in a medium not containing substrate showed no staining (Figure 2.3c).

The results described above are summarised in Table 2.1.

TABLE 2.1 Stain intensity near the medial-adventitial border in sections of rabbit ear artery treated with different combinations of substrate and inhibitor.

| Substrate | Inhibitor[a] | Stain intensity[b] |
|---|---|---|
| AThI | | Heavy |
| AThI | *iso*OMPA | Faint |
| AThI | BW284C51 | Heavy |
| AThI | *iso*OMPA & BW284C51 | No stain |
| BuThI | | Heavy |
| BuThI | *iso*OMPA | No stain |
| BuThI | BW284C51 | Heavy |

[a]Concentration of inhibitors as described in the text.

[b]Equivalent incubation times in the range 3-20 hours.

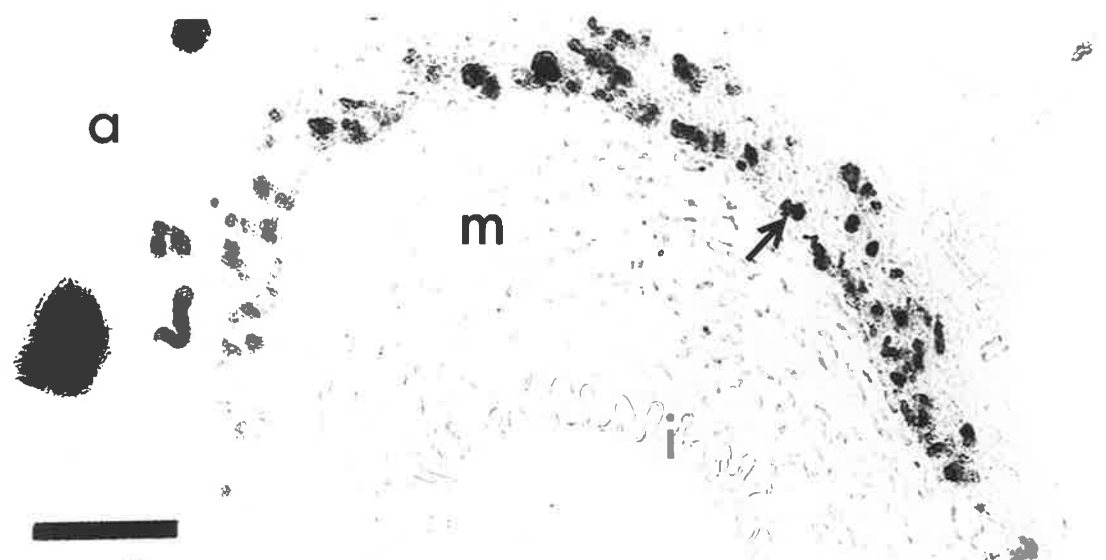
Figure 2.3.

Transverse sections of the wall of the rabbit ear artery in the region of the medial-adventitial border (arrowed). Scale, 50μ. a, adventitia; m, media; i, intima.

(upper panel) Butyrylthiocholine incubation, 3 hr. No inhibitor. Stain due to ChE at the medial-adventitial border.

(middle panel) Acetylthiocholine incubation, 3 hr. Inhibitor, *iso*OMPA $10^{-5}$M, 1 hr. Stain due to AChE at medial-adventitial border (arrowed).

(lower panel) Control incubation, 3 hr. Medium contained no substrate. No stain in the region of the medial-adventitial border (arrowed).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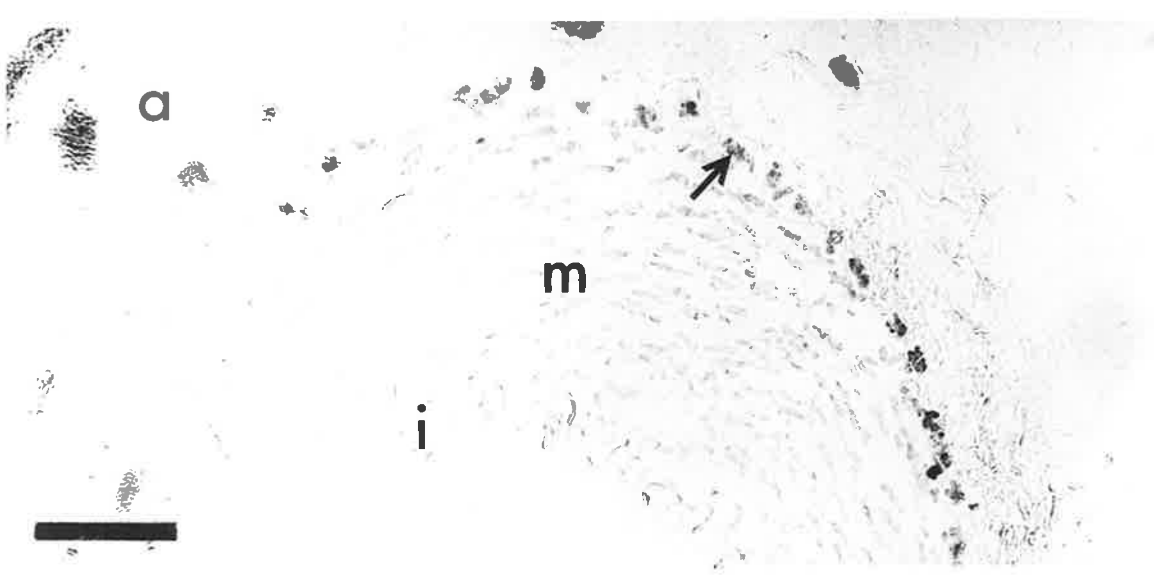
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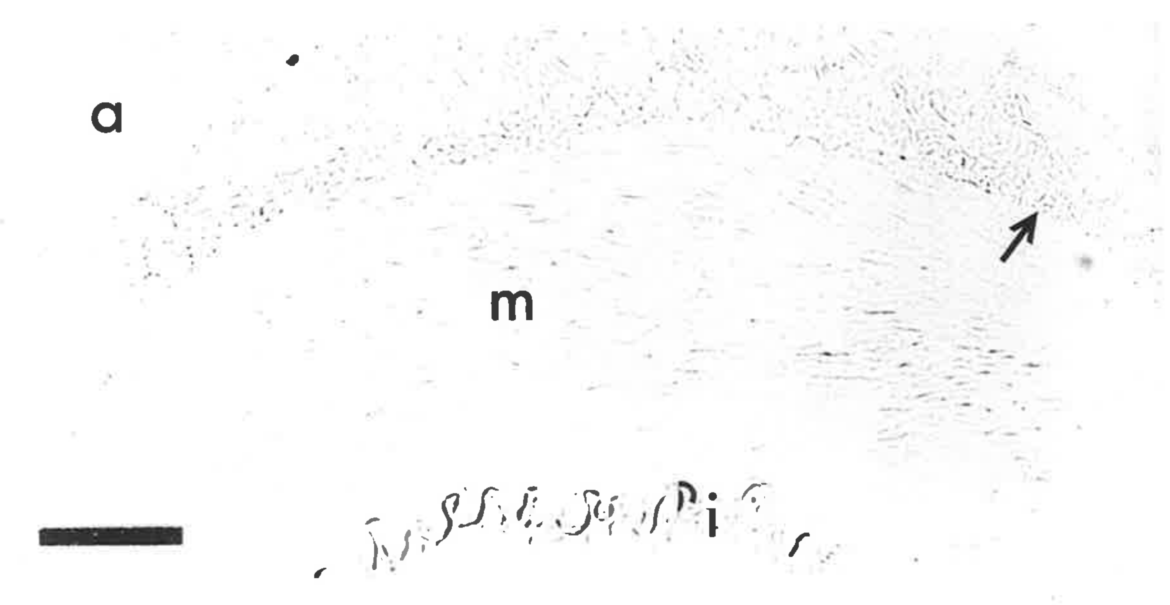
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Difference in distribution of stained structures, as well as differences in stain intensity, were sometimes noted when comparing sections treated for AChE (*iso*OMPA pre-incubation and AThI incubation) and those treated for ChE (e.g. BuThI incubation). In the former case the faint AChE stain was present in fewer areas, which were generally closer to the medial-adventitial border (e.g. Figures 2.2a and 2.2b, which are from adjacent sections of the same artery). But the difference was not observed when long incubation times were required to demonstrate AChE stain, as was often the case. Incubation times of 3 hours or less resulted in strong ChE stain in all arteries, but times of up to 20 hours were required to produce a weak AChE stain.

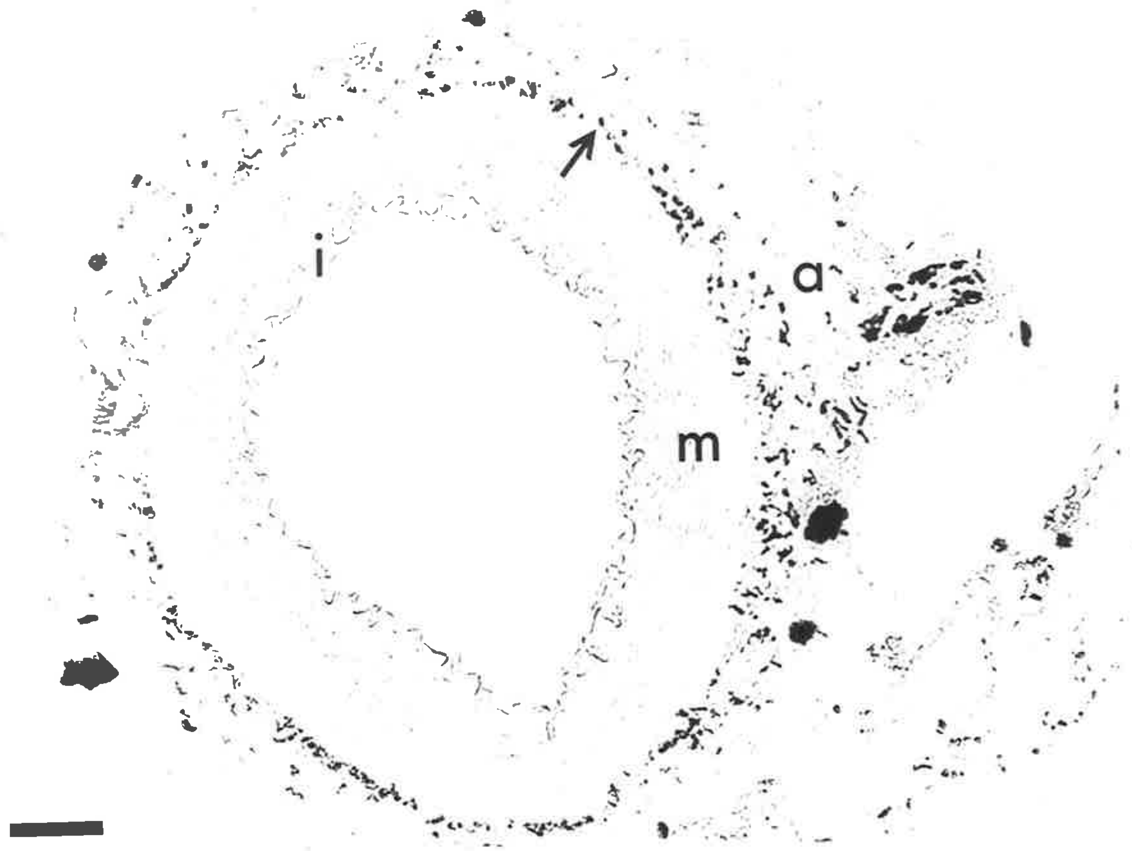
Pre-treated tissues

a. Sympathetic denervation.

The effect of extirpation of a superior cervical ganglion on perivascular ChE and AChE stain is summarised in Table 2.2 for each of twenty rabbits. When a decrease in stain was noted it was a decrease in the *number* of the discrete areas of stain, and not a decrease in stain *intensity*. In all cases of stain decrease both ChE and AChE stain were affected, in what appeared to be equivalent proportions. In those arteries where the effect on perivascular staining is described as 'abolished' only a few discrete areas of stain were seen close to the media. Figure 2.4 shows such an artery and its control.

Figure 2.4

Transverse sections of control (upper photomicrograph) and experimental (lower photomicrograph) rabbit ear arteries from an animal which had undergone unilateral superior cervical ganglionectomy 21 days before sacrifice. Butyrylthiocholine incubation, 2 hr. No inhibitors. Medial-adventitial border arrowed. Scale, 100µ. a, adventitia; m, media; i, intima. In the lower figure a few discrete areas of stain are seen in the adventitia. This was a consistent finding after ganglionectomy.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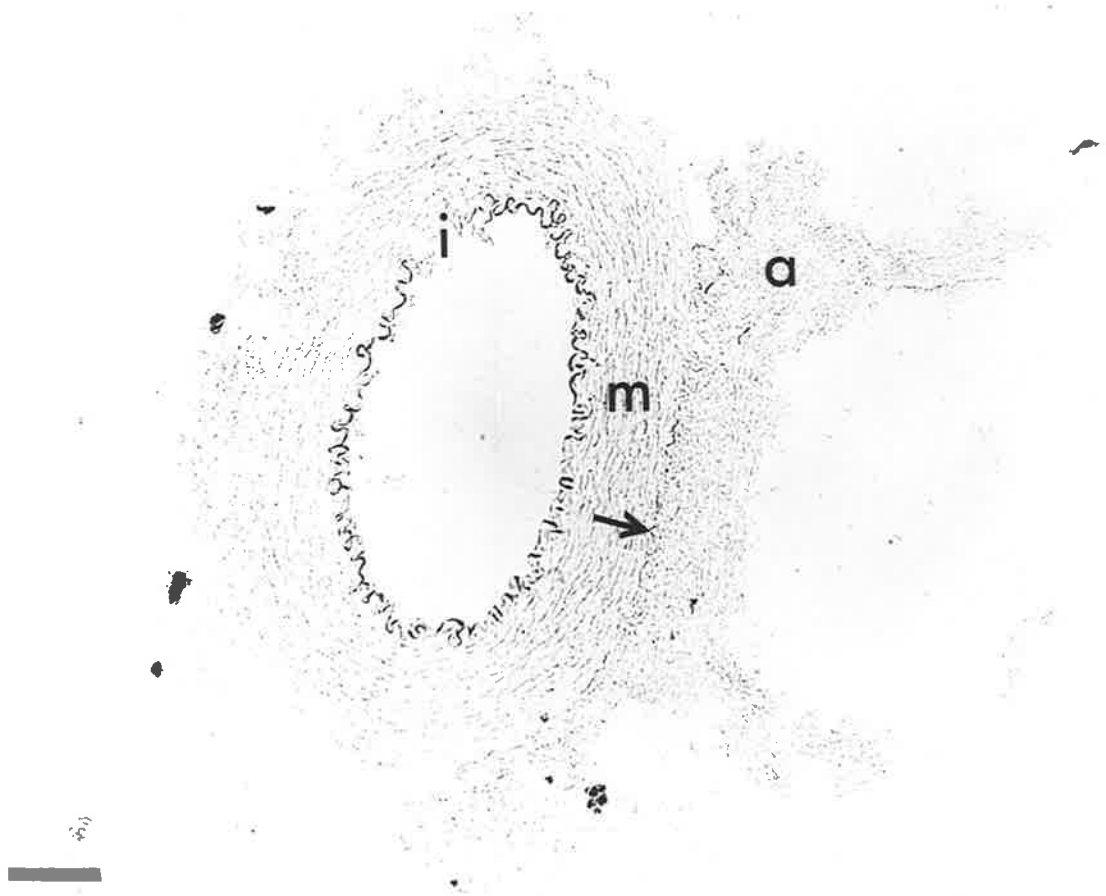
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TABLE 2.2 Number of animals showing different degrees of perivascular cholinesterase stain in the ear on the operated side at different times after unilateral superior cervical ganglionectomy.

| Time elapsed between ganglionectomy and sacrifice | Assessment of perivascular stain | | |
|---|---|---|---|
| | Not changed[a] | Decreased[b] | Abolished[c] |
| 20 hours | 1 | | |
| 24 hours | 4 | | |
| 48 hours | 3 | | |
| 72 hours | 1 | 2 | |
| 7 days | | 1 | 2 |
| 21 days | | 1 | 3 |
| 28 days | | 1 | 1 |

Note:

In all cases the experimental artery was compared with that on the control side.

[a]Not changed = indistinguishable from the control side.

[b]Decreased = noticeably less than the control.

[c]Abolished = very few areas of stain near the medial-adventitial border.

In each of the twenty rabbits the experimental side artery showed much less catecholamine fluorescence than the artery from the control side (Figure 2.5). Although the number of fluorescent areas varied between different experimental side arteries, no relation was observed between the time after ganglionectomy and the degree of fluorescence remaining in the range 20 hours to 28 days.

b. Reserpine pre-treatment.

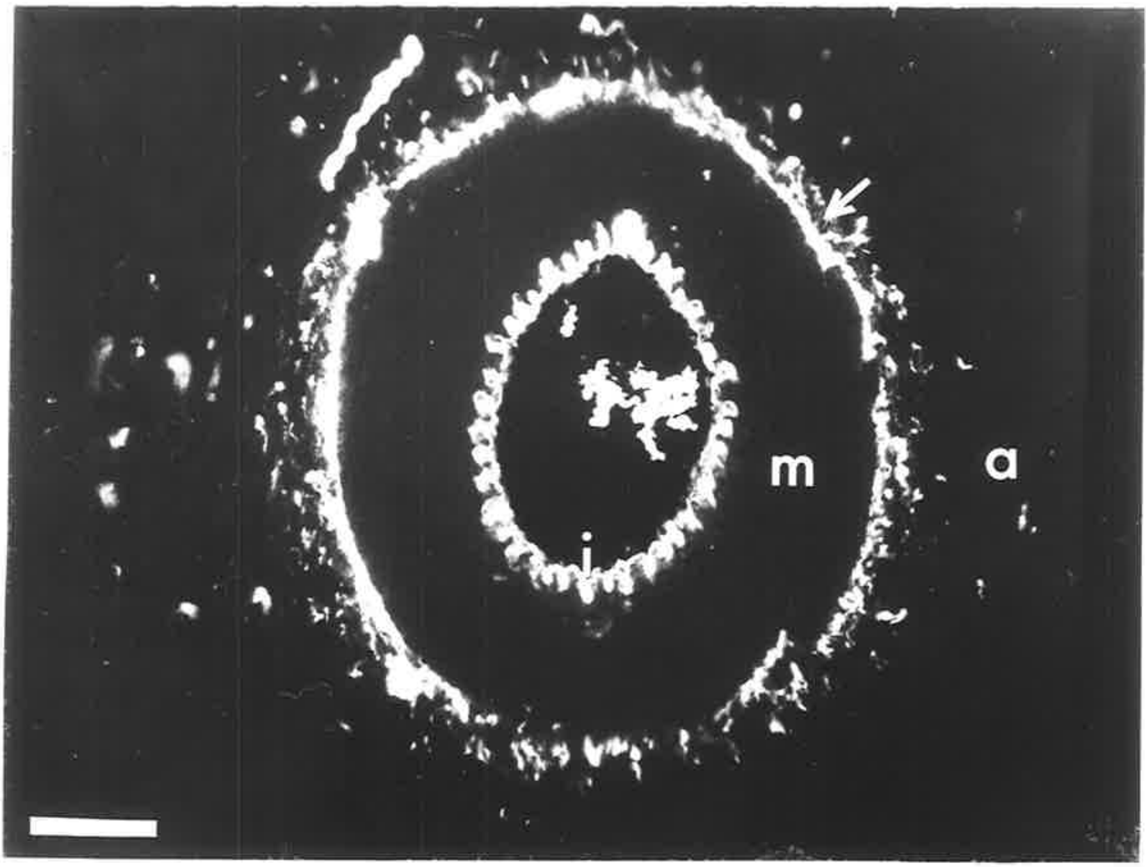
Arteries taken from rabbits pre-treated with reserpine showed a distribution and density of ChE and AChE stain indistinguishable from that seen in untreated arteries. No catecholamine fluorescence was evident in segments of these vessels.

c. Krebs solution.

Arteries perfused with or stored in Krebs bicarbonate solution showed staining which appeared identical with that seen in freshly frozen arteries.

Figure 2.5

Transverse section of control (upper photomicrograph) and experimental (lower photomicrograph) rabbit ear arteries from an animal which had undergone unilateral superior cervical ganglionectomy 28 days before sacrifice. The arteries were treated using the technique of Falck and Owman (1965), as modified by Waterson and Smale (1967), for the demonstration of catecholamine fluorescence. Illuminated with light from a mercury vapour lamp, using the optical system described in Chapter 3 (page 3.5). Scale 100μ. a, adventitia; m, media; i, intima. Medial-adventitial border arrowed.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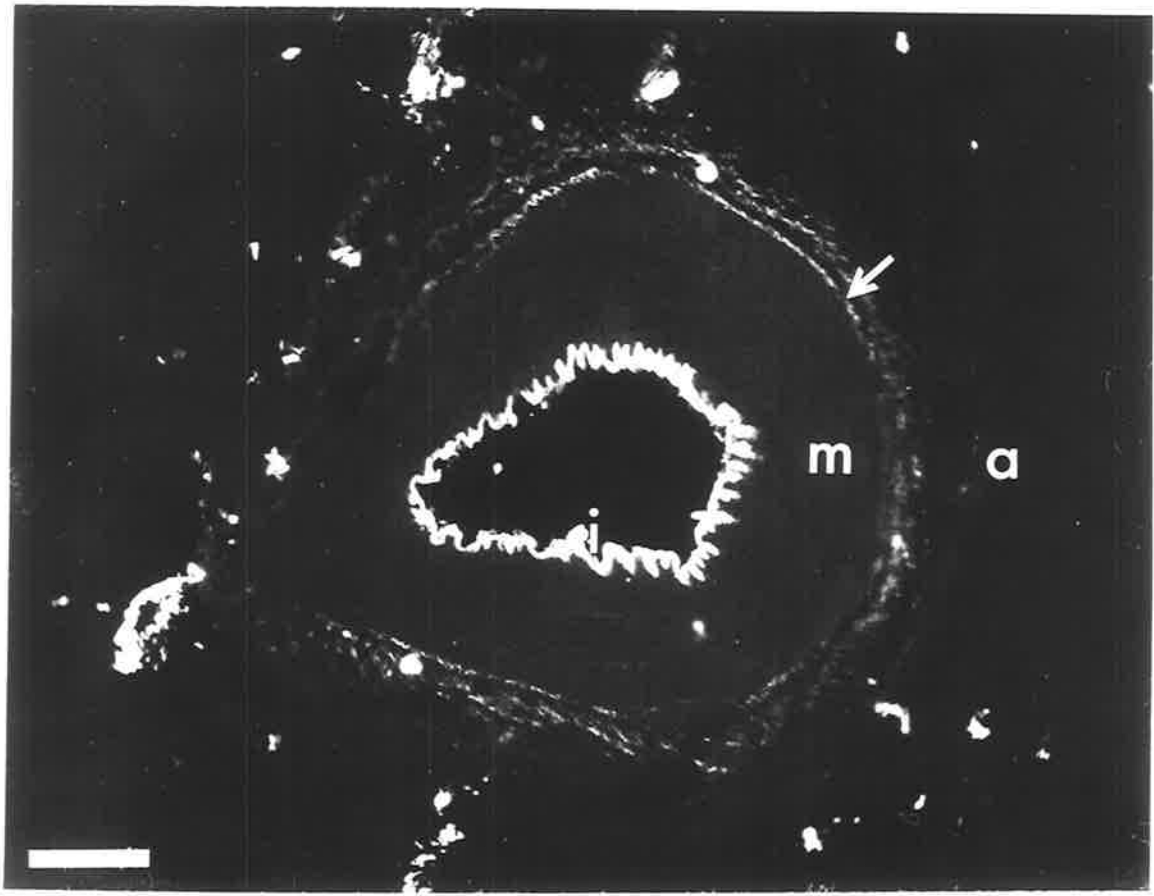
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DISCUSSION

The results described above indicated that ChE and AChE were located close to the medial-adventitial border of the rabbit ear artery, and that the enzymes were not present after degeneration of the sympathetic nerves.

Evidence for the identity of the two enzyme groups ChE and AChE was based on the effects of different combinations of substrates and inhibitors.

(i) BuThI, a substrate for ChE only (Gomori, 1952) produced heavy stain, therefore indicating the presence of ChE. Inhibition with *iso*OMPA, which is effective against 90-92% of ChE by manometric estimation (Bayliss and Todrick, 1956) at the present concentrations, prevented this stain formation, showing that any fraction of enzyme remaining was not detectable histochemically.

(ii) AThI, a substrate for both AChE and ChE (Koelle and Friedenwald, 1949) also produced heavy stain. Treatment with *iso*OMPA markedly reduced this stain formation, but a small and definite amount of stain was produced. This suggested that some AChE was present.

(iii) Direct evidence for the presence of AChE was provided by the use of the inhibitor BW284C51, which is a highly selective

inhibitor of AChE (Bayliss and Todrick, 1956). The stain attributed to AChE in (ii) above was not formed when *iso*OMPA *and* BW 284C51 were used with AThI incubation. This indicated that the stain in (ii) with *iso*OMPA alone was due to AChE. The staining of luminal red blood cells, which are known to contain AChE (Koelle, 1963), served as a useful control in this regard.

The enzymes acting under the conditions of these combinations are related to the intensity of stain in Table 2.3.

TABLE 2.3 Combinations of substrates and inhibitors used, and enzymes acting under these conditions, related to stain intensity.

| Substrate | Inhibitor | Acting enzymes[a] | Stain intensity[b] |
|---|---|---|---|
| AThI | | ChE & AChE | Heavy |
| AThI | *iso*OMPA | AChE | Faint |
| AthI | BW284C51 | ChE | Heavy |
| AthI | *iso*OMPA and BW284C51 | - | No stain |
| BuThI | | ChE | Heavy |
| BuThI | *iso*OMPA | - | No stain |
| BuThI | BW284C51 | ChE | Heavy |

[a]See text above [b]From Table 2.2.

The relative intensities of stain, and the differences in incubation times required in most cases for satisfactory staining of AChE as against ChE, suggested that more ChE than AChE was present. Such a conclusion was reached by Thompson and Tickner (1953) who assayed AChE and ChE activity in minced rabbit ear arteries using a manometric technique.

The localisation of ChE and AChE stain seen in the present study, near the medial-adventitial border, was similar to that described by Grant and Thompson (1963). They examined whole mounts of rabbit ear stained for cholinesterases by the method of Gomori (1952) and described a close association between the enzymes and the nerves which ramify in the adventitial layer of the arterial system. The examination of transverse sections of rabbit ear artery for a fluorescent noradrenaline derivative by Waterson and Smale (1967) allowed the identification and accurate localisation of noradrenergic nerve terminals near the junction of media and adventitia. The disposition of stain observed in the present study was very similar to the disposition of those terminals. A close anatomical association between the enzymes and the sympathetic nerve terminals is thus suggested.

In the rabbit, superior cervical ganglionectomy is followed by loss of the sympathetic nerves in the ear artery on the experimental side. This loss was shown functionally by de la Lande and Rand (1965) and histochemically by de la Lande, Frewin and Waterson (1967). The

disappearance of ChE and AChE after ganglionectomy reported in this Chapter showed that there was a close link between the enzymes and an intact sympathetic innervation. It should be noted that in almost all vessels a small number of stained and fluorescent areas remained after ganglionectomy, and that when this number was relatively high (so as to be classified only as 'decreased',Table 2.2) the number of stained and fluorescent areas seemed equivalent. This may be considered due to incomplete sympathetic denervation.

While the relation between the enzymes and the sympathetic terminals seemed close,their link with the nerves' catecholamine was not absolute. Reserpine treated arteries showed no catecholamine fluorescence, and were presumably depleted of noradrenaline, but the staining properties of ChE and AChE in the vessels seemed unaffected. Secondly, the time course for the disappearance of cholinesterases after superior cervical ganglionectomy differed from the time course for the disappearance of catecholamine from the same arteries, estimated using the fluorescence technique of Falck and Owman (1965) for noradrenaline. Very little catecholamine fluorescence could be seen 24 hours after ganglionectomy, whereas ChE and AChE stain were not noticeably reduced in the first 48 hours after the operation. This suggests that the enzymes were associated with a more stable structure than the amines.

It was of obvious importance to determine more clearly the relation between cholinesterases and the sympathetic nerve terminals.

Chapter 3 begins with a discussion of the anatomical possibilities which were suggested by the results described above.

## SUMMARY

1. Using a histochemical technique, ChE and AChE were demonstrated near the medial-adventitial border of the rabbit ear artery.
2. ChE stain was much more intense than AChE stain after equal incubation times.
3. The number of areas stained for AChE or ChE was greatly reduced after sympathetic nerve degeneration.

# CHAPTER THREE

## CHOLINESTERASES AND CATECHOLAMINES

In the last Chapter it was shown histochemically that the rabbit ear artery contained cholinesterases (ChE and AChE). These enzymes were closely associated with the intact sympathetic innervation, since they were greatly reduced after superior cervical ganglionectomy. Several anatomical possibilities are suggested by this relationship.

(i) The enzymes may be part of the noradrenergic sympathetic nerves.

(ii) The enzymes may be present in a system of cholinergic nerves which have their origin in the superior cervical ganglion. Such a system would be consistent with the histochemical observations of Hamberger, Norberg and Sjoqvist (1965) that sympathetic ganglia contain two populations of cell bodies, a larger adrenergic and a smaller cholinergic.

(iii) The enzymes could be part of cholinergic nerves which pass through the ganglion, without synapses, to the ear artery.

(iv) The enzymes could be associated with structures such as Schwann cells, which are related to the sympathetic nerves, and which change in some way when the nerves degenerate. ChE is known to be associated with Schwann cells (Koelle, 1963).

While the presence of cholinesterases cannot be considered to prove any functional cholinergic role, two of the anatomical possibilities outlined above are related to the two forms of the 'cholinergic link' hypothesis illustrated in Figures 1.1 and 1.2. Separate adrenergic and cholinergic sympathetic fibres may interact as suggested by Burn and Rand (1959), or one type of fibre, containing noradrenaline and with a cholinergic component, may function as suggested by Burn (1961). It is also possible that the rabbit ear artery may have separate adrenergic and cholinergic innervations as described for the uterine artery of the guinea-pig (Bell, 1968), with the provision that the cholinergic fibres pass through the superior cervical ganglion or have their cell bodies therein.

Experiments were designed to establish the morphological relationship between cholinesterases and catecholamine in the rabbit ear artery, using the latter as an indicator of the site of adrenergic nerves (Eranko, 1967). Although AChE is the enzyme classically associated with neural cholinergic function, in the present work ChE localisation was also studied, since acetylcholine can be hydrolysed by both AChE and ChE, as Kirk (1969) has emphasised in relation to arterial cholinesterases. Two types of experimental approach were used. Adjacent cryostat (frozen) sections were treated for cholinesterases and catecholamine respectively, and photomicrographs of similar fields of adjacent sections were compared. In the second method, segments of

artery were treated for cholinesterases and then for catecholamine, and then sectioned. Each section was then examined for enzyme and amine. The two approaches will be dealt with in turn for the sake of clarity, with a discussion related to the method in each case, and a brief general discussion will follow.

## PART A. DEMONSTRATION IN ADJACENT SECTIONS.

Using a modification of the method described by El-Badawi and Schenk (1967), catecholamine fluorophore was demonstrated in evenly numbered serial cryostat sections of rabbit ear artery, and the remaining sections were stained for ChE or AChE.

## MATERIALS AND METHODS

In all experiments segments of the proximal parts of rabbit central ear arteries were obtained as described on page 2.5, and were held in some cases in Krebs solution (see appendix, p.5) bubbled with 5% carbon dioxide and 95% oxygen, for up to two hours at room temperature before use.

### Sectioning

Arteries were frozen in liquid nitrogen, and mounted vertically at their base on microtome object discs which had been cooled in liquid nitrogen. Only the base of the artery was embedded in ice,

to seal the tissue to the disc, the remainder of the artery standing free. Serial transverse sections of artery, four microns in thickness, were cut with a Cryostat (American Optical Co.), with a cabinet temperature of -30°C. Each section was thawed from the cryostat knife onto a numbered glass slide at room temperature, and then immediately placed in front of a fan blowing normal room air for from five to ten minutes. This time variation in drying allowed about twenty sections to be cut. The slides had been numbered in series, and the odd-numbered slides were placed in racks at -30°C for later ChE or AChE staining. The remaining slides (even-numbered) were placed in dry glass slide racks for exposure to formalin vapour.

Catecholamine fluorophore production.

Paraformaldehyde was prepared for use as suggested by Hamberger, Malmfors and Sachs (1964), by storing in 5 gm aliquots in small beakers in an airtight container at a relative humidity of 94% (see Appendix, page 1) for between one and six weeks. Five minutes after the last section of each artery was cut, a beaker of paraformaldehyde was transferred to a one litre jar, the slide rack and slides placed in the jar above the beaker, and the jar tightly sealed and placed in a dry heat oven at 55°C. A second group of serial sections was sometimes then cut from the artery, and even-numbered slides in this group treated similarly in a separate jar. After one hour at 55°C the slides were removed and passed through two 30 second changes of fresh absolute

alcohol and then two of xylol. The sections were mounted under glass cover-slips with Entellan (Merck) at full strength. The paraformaldehyde was not re-used.

Treatment for cholinesterases.

AChE and ChE staining was carried out as described in Chapter 2. To find the optimal times for stain production without risking loss of the retained odd-numbered sections, additional test sections of each artery were cut and treated to show ChE or AChE activity. Periods of incubation ranging from 2 hours for ChE to up to 20 hours for AChE were used. Sections from 4 arteries were stained for AChE, from 2 arteries for ChE, and odd-numbered sections from 10 arteries were divided into two further groups and each group stained for one type of enzyme.

Photomicrographs.

Sections treated for catecholamine fluorescence were examined on a Zeiss S.V. microscope, using an Osram HBO 200 lamp, a 4 mm BG 38 heat filter, a 3 mm BG 12 excitation filter and 470 or 530 millimicron wavelength barrier filters. Photomicrographs were made on 35 mm Kodak Photoflure film using a Zeiss Ikon camera, with 5-30 second exposure times. Sections treated for ChE or AChE were examined using the same microscope with a tungsten lamp and no filters, and photomicrographs were made using the same Zeiss Ikon camera with Ilford Pan F film and an automatic exposure device. Photographic prints of similar fields

from adjacent sections were made at identical magnifications. Catecholamine fluorescence and ChE or AChE stain were compared by superimposition of traces.

In addition to treating alternate sections for catecholamine and cholinesterases as described above, unbroken series of sections were stained for ChE or AChE or treated for catecholamine fluorophore. Photomicrographs of these sections were made in order to gain an appreciation of the degree of correlation of stained or fluorescent structures to be expected between adjacent sections.

## RESULTS

### Catecholamine fluorescence.

Fluorescence was seen in all of the arteries examined, and the colour was assessed as bright green, which is characteristic for adrenaline and noradrenaline (Falck and Owman, 1965). The distribution of fluorescent structures was identical to that observed in sections treated by the method of Waterson and Smale (1967), as is seen by comparing Figures 2.5 and 3.2, but in most cases the catecholamine fluorescence at the medial-adventitial border was less intense using the present (cryostat) technique. Cryostat sections treated in the usual way with the exception that no paraformaldehyde was present during the heat treatment, or which were immersed in water before mounting, showed no fluorescence at the medial-adventitial border.

The intimal fluorescence and the light, thread-like fluorescence in the adventitia were seen in these sections, indicating that this was due to tissue autofluorescence, most probably of elastin.

Treatment of unbroken series.

Figure 3.1 shows corresponding areas of two adjacent 4 micron sections of rabbit ear artery, each treated for ChE, and a drawing of superimposed traces of the outlines of stain in the two sections. The degree of correlation between what were probably corresponding structures was not high in adjacent sections treated for ChE, as is seen in Figure 3.1. The structures could, however, be equated in most cases. A similar result was obtained with unbroken series treated for AChE, or for catecholamine fluorescence.

Treatment of adjacent sections for cholinesterases and catecholamines.

Photographic prints of pairs or triads of adjacent sections were grouped together, and the positions of stained and fluorescent structures near the medial-adventitial border were compared by superimposition of traces of the prints. The type and number of comparisons made are shown in Table 3.1.

TABLE 3.1 Number of arteries in which comparisons between adjacent sections were made, and type and number of comparisons.

| Number of arteries | Comparison type | Number of comparisons |
|---|---|---|
| 5 | F[a] - ChE | 14 |
| 2 | F - AChE | 9 |
| 2 | F - ChE - AChE | 4 |

[a]F = catecholamine fluorescence

Corresponding areas of two adjacent 4 micron sections of rabbit ear artery, one treated for catecholamine fluorescence and the other treated for ChE, are shown in Figure 3.2. Also shown is a drawing of superimposed traces of the outlines of the fluorescence and stain. Figure 3.3 shows two photomicrographs and a similar drawing for fluorescence and AChE in another artery.

## DISCUSSION

The main shortcoming of this technique was that, as is seen in Figure 3.1, a high degree of correlation could not be expected between adjacent sections when they were treated to show the same substance.

Figure 3.1

Two photomicrographs and a drawing of adjacent transverse sections of the wall of a rabbit ear artery in the region of the medial-adventitial border. Scale, 50μ.
a, adventitia; m, media.

(upper and middle panels) Butyrylthiocholine incubation, 2 hours. No inhibitor. Stain due to ChE.

(lower panel) Drawing of superimposed traces of the stain outlines in the two photomicrographs. The upper trace is drawn with a thin line, and the stained area is dotted; the trace of the lower photomicrograph is drawn with a thicker line, and the stained area is clear.

a

m

a

m

a

m

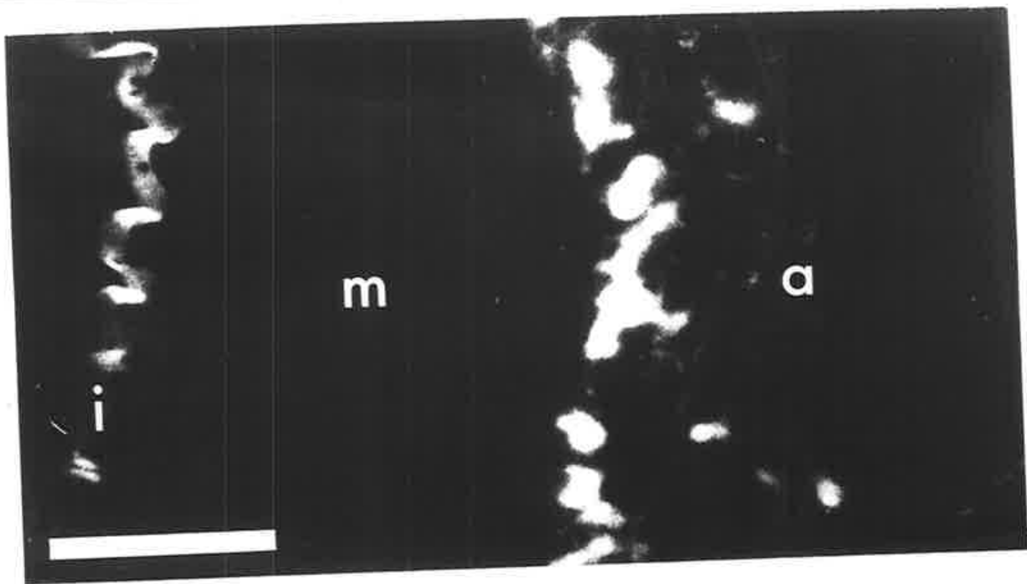
Figure 3.2

Two photomicrographs and a drawing of adjacent transverse sections of the wall of the rabbit ear artery in the region of the medial-adventitial border. Scale, 50μ.
a, adventitia; m, media; i, intima.

(upper panel) Section treated for catecholamine fluorescence by the method described in the text.

(middle panel) Adjacent section treated for ChE, butyryl-thiocholine incubation, 2 hours. No inhibitors.

(lower panel) Drawing of superimposed traces of the fluorescent and staining structures. Fluorescent structures are drawn with a heavy line, and are clear. ChE stain is represented by the dotted areas.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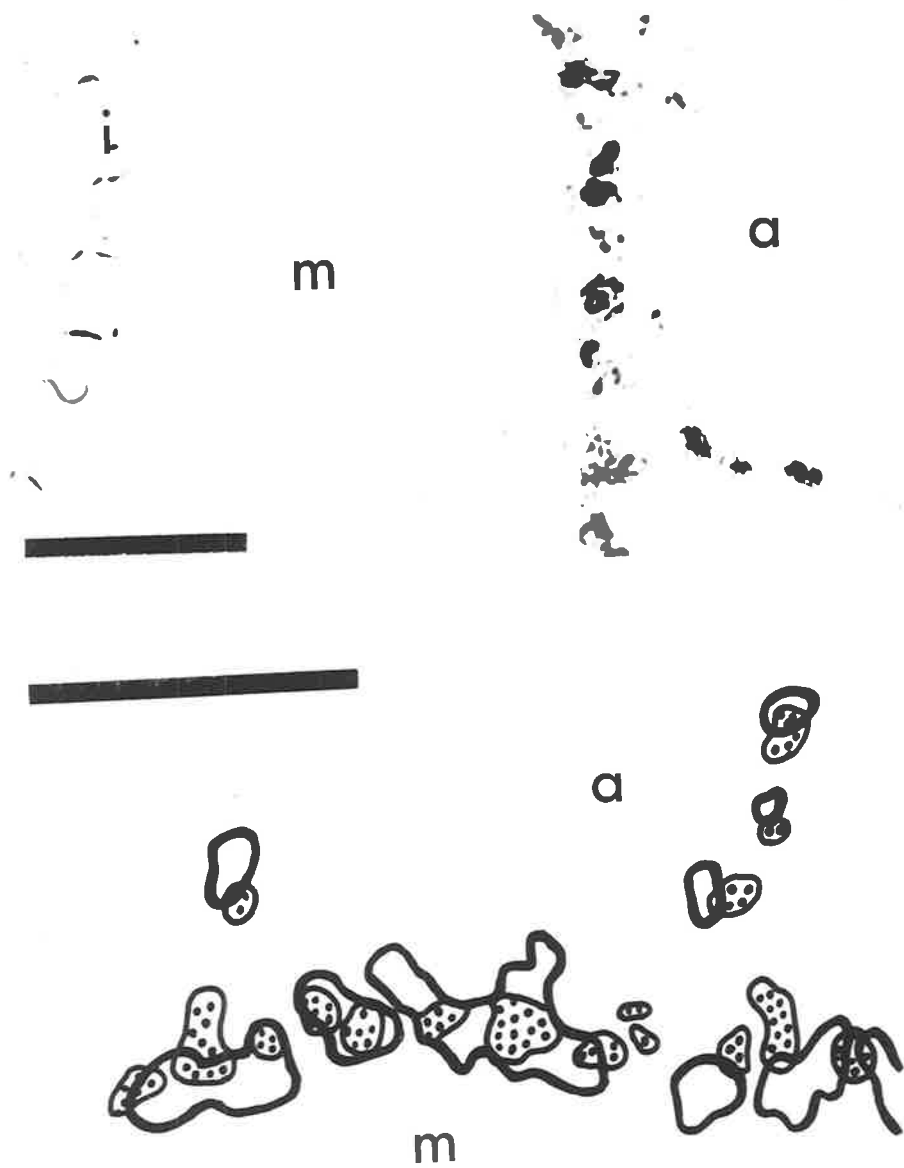
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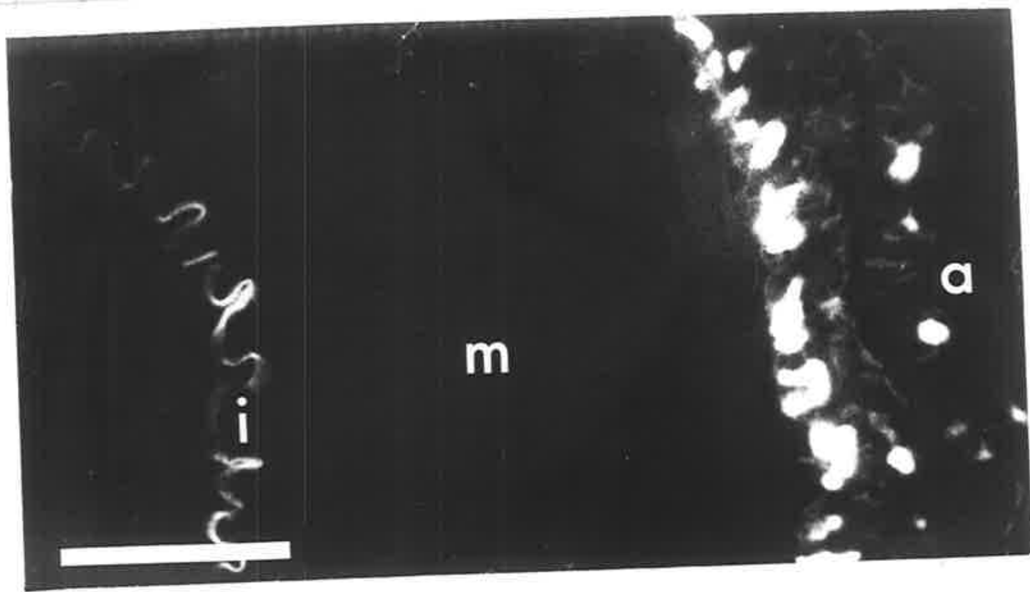
Figure 3.3

Two photomicrographs and a drawing of adjacent transverse sections of the wall of the rabbit ear artery. Scale, 50μ. a, adventitia; m, media; i, intima.

(upper panel) Section treated for catecholamine fluorescence by the method described in the text.

(middle panel) Adjacent section treated for AChE, acetylthiocholine incubation 10 hours, *iso*OMPA $10^{-5}$M one hour.

(lower panel) Drawing of superimposed traces of the fluorescent and staining structures. Fluorescent structures are drawn with a heavy line, and are clear. AChE stain is represented by dotted areas.


i
m
a

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This suggested that the course of the staining or fluorescent structures in the artery wall was in a large part oblique, rather than in the long axis of the vessel. Such an arrangement was to be expected, particularly in the light of the demonstration of the reticular nature of the nerve plexus in this vessel by Grant and Thompson (1963). Distortion of sections during cutting may also have contributed to difficulties of exact correlation.

Taking into account the degree of superimposition which could be expected using the present method in this vessel, a trend was observed in the results described above. The trend suggested that AChE, ChE and catecholamines show a similar pattern of distribution at the medial-adventitial border of the rabbit ear artery. The phrase 'similar pattern of distribution' is used to describe only the visual pattern observed, and does not imply that the enzymes and the amine were within the same nerve fibres. The structural implications of the localisation of stain and fluorescence will be discussed further at the end of this Chapter.

## PART B. SIMULTANEOUS DEMONSTRATION

The technique for sequential demonstration of catecholamines and cholinesterases originally described by Eranko (1964), and used by Jacobowitz and Koelle (1965) and Ehinger and Falck (1966) was applied without success in the present study. El-Badawi and Schenk (1967) also found it 'practically impossible' to obtain satisfactory cholinesterase stain after formalin vapour treatment, as is required by the technique. A second suggested technique for sequential demonstration, the cold formalin method of El-Badawi and Schenk (1967) was also applied without success. The comparatively thin sections used in the present work may have contributed to the failure of these methods.

A new approach to the demonstration of cholinesterase and catecholamine in single sections was developed. The approach enabled the simultaneous production of weak ChE stain and weak catecholamine fluorophore, but its value lay in the indirect demonstration that the fluorophore formed at the same sites as the ChE or AChE stain. The technique was suggested by the preliminary experimental observation that treatment of artery segments in Krebs bicarbonate, adjusted to pH 6.0 with HCl, for 24 hours or more did not prevent the subsequent development of fluorophore. pH 6.0 was found to be optimal for stain localisation using the Karnovsky and Roots (1964) method in the rabbit ear artery (see page 2.7).

## MATERIALS AND METHODS

Rabbit ear arteries were obtained and held in Krebs solution as described in Part A of this Chapter (page 3.3). Each artery was cut so that one animal yielded up to ten segments. The pieces of artery were treated in modified Krebs bicarbonate solution, pH 5.95-6.0, containing the components of the full ChE or AChE staining media (Appendix, page 1), at 4°C for 1-24 hours. Further details of this treatment are given in Results, below. The segments were then frozen and treated for the production of catecholamine fluorescence by the technique described in the Appendix, page 1, which was based on that of Falck and Owman (1965) and Waterson and Smale (1967). Sections were examined using light from a mercury vapour lamp (Osram HBO 200) and from a tungsten lamp, with a Zeiss S.V. microscope. The sub-stage optical systems for the two types of light were as described on page 3.5, and the above-stage optical system was not changed. Photomicrographs of each section with the two types of illumination were made using Kodak Tri-X-Pan film.

## RESULTS

(i) Full ChE incubation medium in Krebs pH 6.0 was used to treat segments of artery from eleven rabbits. Treatment times greater than two hours gave ChE stain near the medial-adventitial border which was similar in appearance to that described in Chapter 2, but which was more diffuse. No fluorescence was seen in these segments.

Shorter incubation times gave less ChE stain and some weak fluorescence in the same section (Figure 3.4).

(ii) Inhibition of ChE with *iso*OMPA $3.10^{-5}$M for one hour in Krebs pH 6.0 before exposure to the full ChE incubation medium in Krebs pH 6.0 for up to 12 hours resulted in strong fluorescence and no stain. The result was observed in segments of 6 arteries.

(iii) Treatment for AChE (*iso*OMPA inhibition and AThI substrate) in modified Krebs solution pH 6.0 for 4 or 8 hours gave no brown stain and weak catecholamine fluorescence (3 animals). However, in segments from 6 animals treatment for 24 hours gave diffuse brown stain near the medial-adventitial border and no fluorescence, while in each case control sections, incubated for the same time in the same solution containing BW284C51 $3 \times 10^{-5}$M, showed normal fluorescence and no stain.

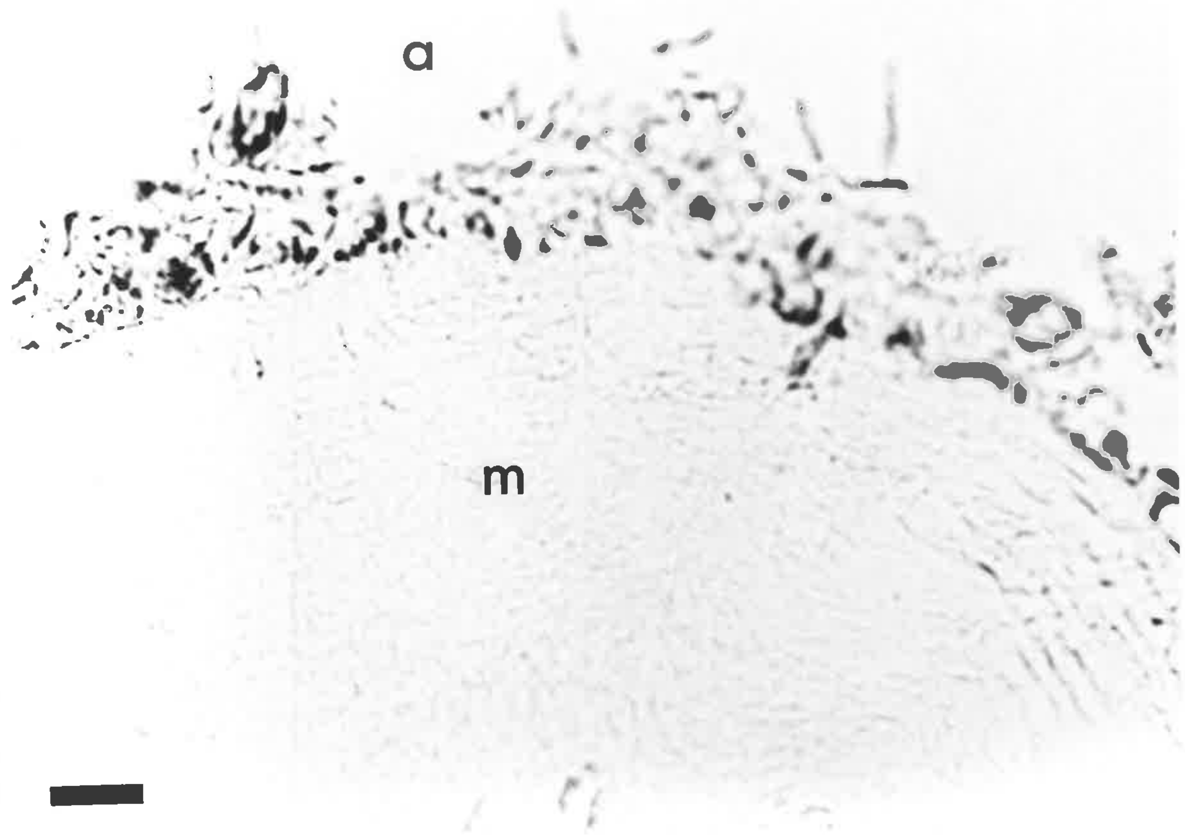
## DISCUSSION

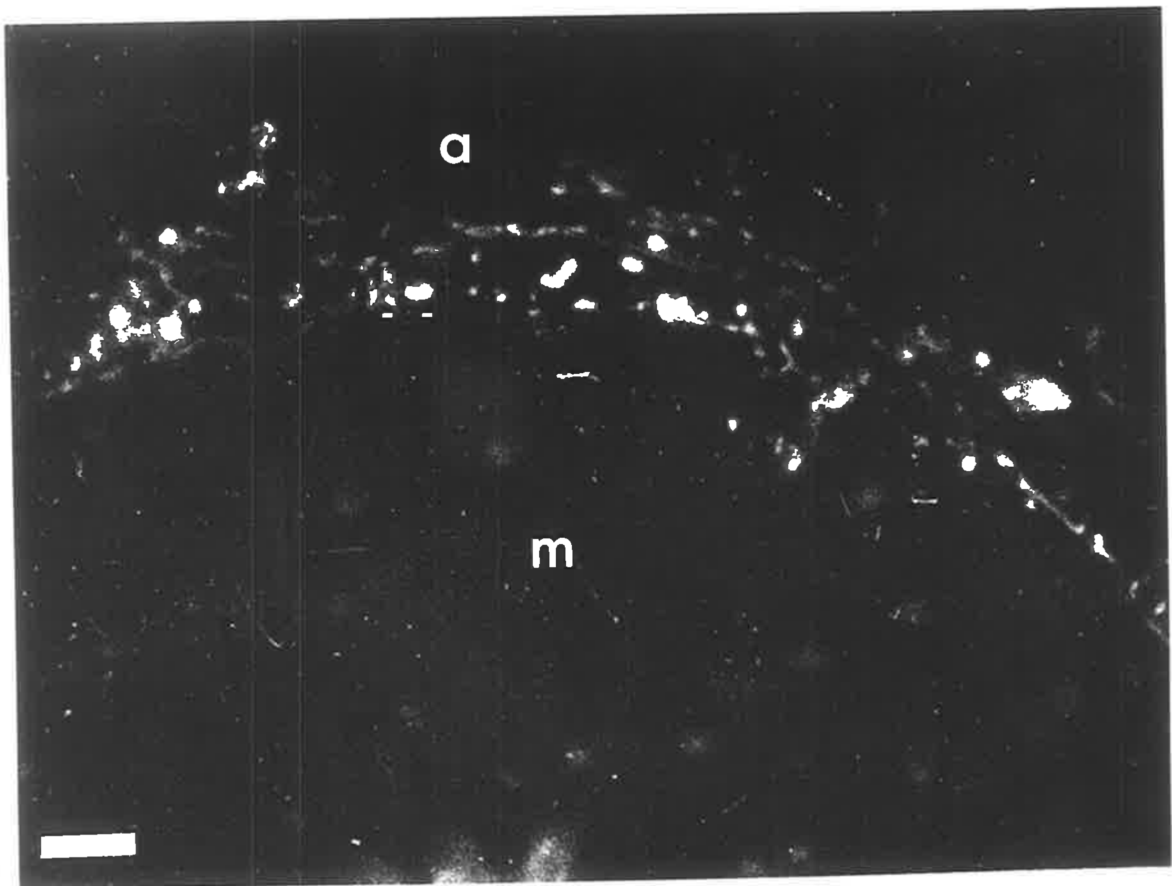
This approach, while enabling ChE and catecholamine to be visualised in the same section, cannot be considered satisfactory in that regard for purposes of comparison, since the stain and fluorescence were very weak when both were present. The fluorescence, in particular, was seen in fewer areas than with other techniques when a visible stain was developed. No result was obtained for the simultaneous demonstration of AChE and catecholamine.

Figure 3.4

Two photomicrographs of one section of rabbit ear artery, in the region of the medial-adventitial border, illuminated with (upper panel) light from a tungsten lamp and (lower panel) light from a mercury vapour lamp. Scale, 10μ.
a, adventitia; m, media.

Treated for the simultaneous demonstration of ChE and catecholamine, as described in the text. Butyrylthiocholine incubation, 90 minutes. No inhibitors.


a
m


a
m

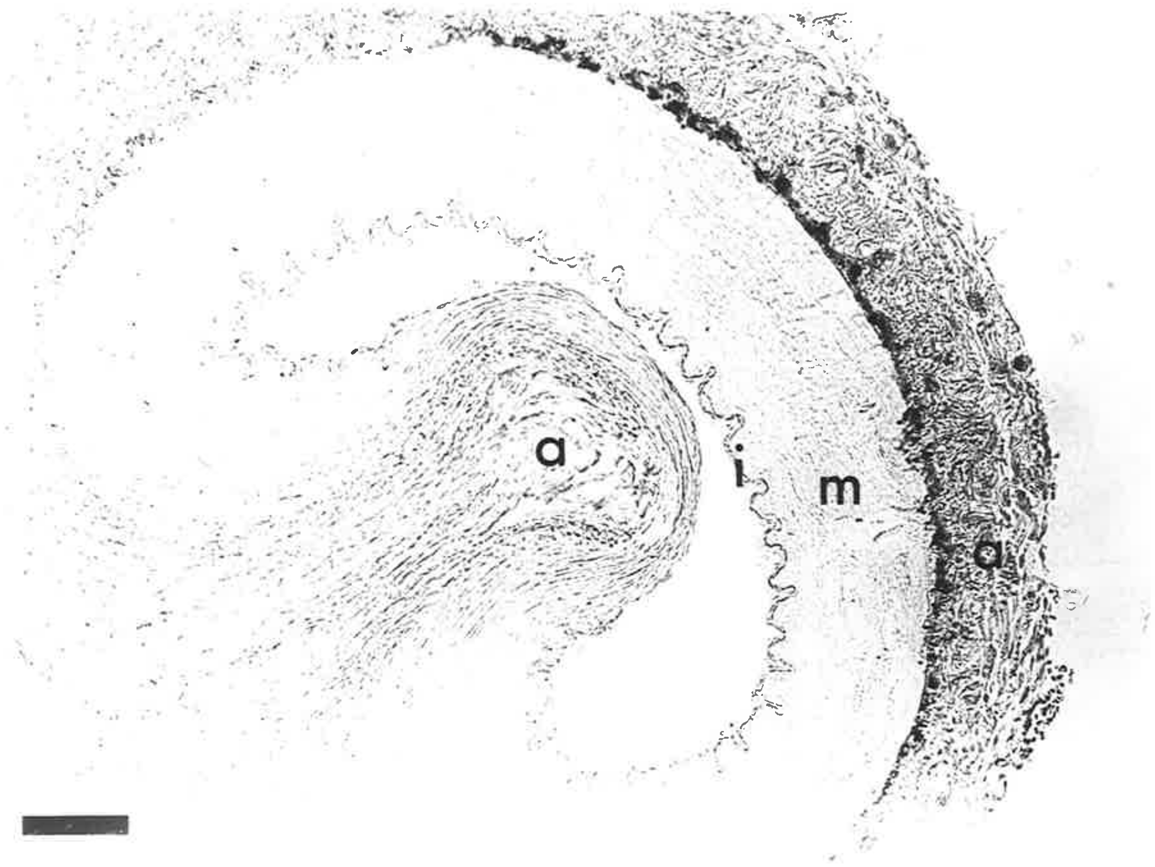
But the technique was of value in allowing an indirect demonstration that fluorophore forms at the same sites as ChE and AChE stain. Incubation in a cholinesterase staining medium prevented fluorescence only if the enzyme was active. This result is open to only one interpretation, namely that the deposition of stain prevented the fluorescence, perhaps by chemical inhibition of fluorophore production, or perhaps by optical interference during microscopy. Since heavy staining prevented all catecholamine fluorescence, it may be concluded that all potentially fluorescent structures, that is, all adrenergic nerves (Eranko, 1967), had enzyme nearby. In the light of this conclusion it would be unwise to speculate on any comparison between the two panels of Figure 3.4, which can be considered to represent the 'threshold' for stain development on one hand and fluorophore inhibition on the other.

The technique described above is not likely to have a wide application in other tissues, since the penetration of stain using the Karnovsky and Roots (1964) method is limited. Teravainen (1969) noted that the penetration of stain in muscle using the similar Karnovsky (1964) method was at least 10 microns, but the adventitia in close-trimmed arteries in the present study was at least 20 and up to 40 microns thick. It is more than likely that the poor localisation of stain observed in the present study was due to the depth of the staining structures, and that this depth approached the limit of penetration of the reagents. Figure 3.5 illustrates the effect of the depth of the staining structures on the development of ChE stain and catecholamine fluorescence.

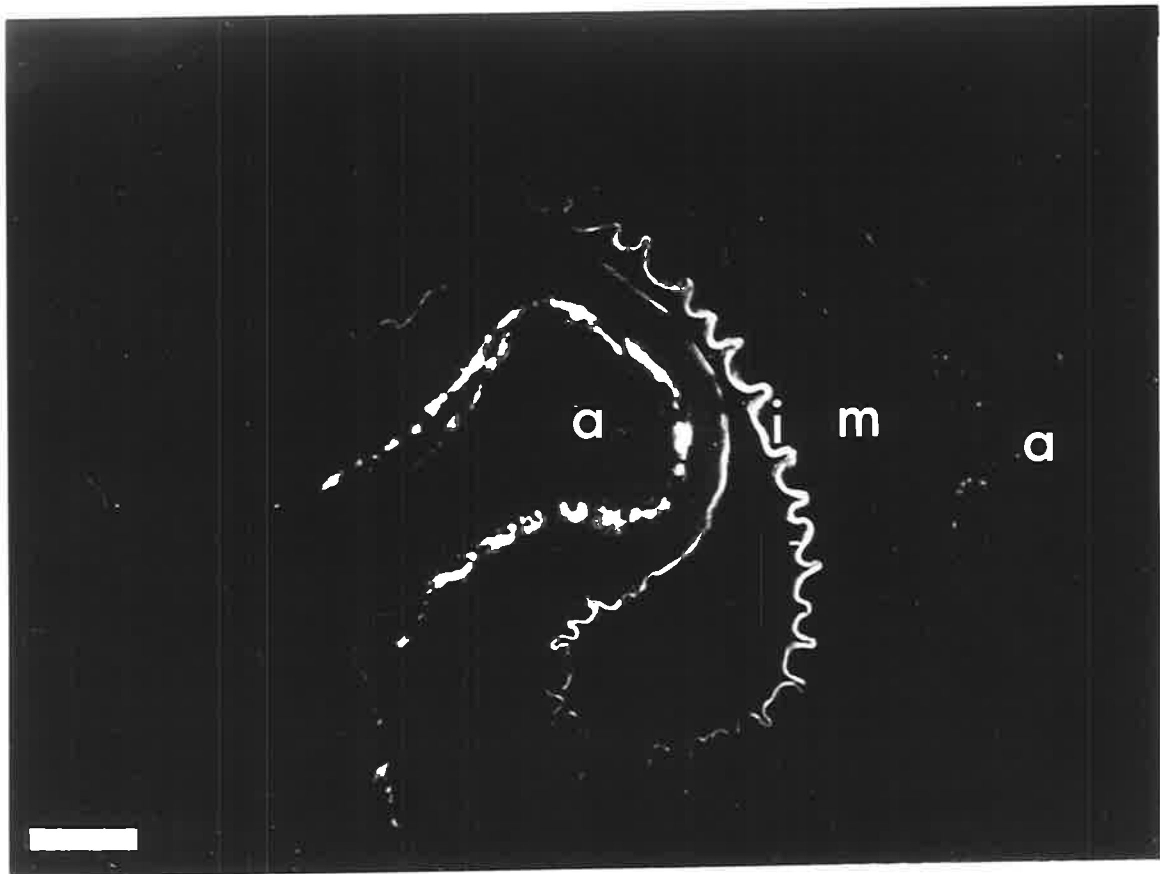
Figure 3.5

Two photomicrographs of one section of rabbit ear artery, illuminated with (upper panel) light from a tungsten lamp and (lower panel) light from a mercury vapour lamp. Scale, 100µ. a, adventitia; m, media; i, intima.

Treated for the simultaneous demonstration of ChE and catecholamine, as described in the text. Butyrylthiocholine incubation, 4 hours. No inhibitors.



a
i
m
a

a
i
m
a

## GENERAL DISCUSSION

The results described in this Chapter showed that there was an extremely close morphological association between cholinesterases and the sympathetic transmitter in the rabbit ear artery. No evidence for discrete, separate adrenergic and cholinergic innervations of the type described by Bell (1968) in the uterine artery of the guinea-pig or by Schenk and El Badawi (1968) in dog and cat vessels was demonstrated. But it could not be concluded on the basis of the present light-microscopic investigations whether the enzymes were associated with single adrenergic nerve fibres, since in sympathetically innervated tissues axons lie in groups or bundles (Grillo, 1966). Fibres with enzyme activity could lie alongside others without such activity, a possibility emphasised by Eranko (1967) in a review of the histo-chemistry of nervous tissue. It is not unreasonable to suggest also that Schwann cells, which are associated with axons, might contain one or both of the enzymes.

The resolution allowed by light microscopy was not sufficient to examine these latter possibilities. An electron microscopic study of the rabbit ear artery was made, and is described in Chapter 4.

## SUMMARY

1. Cholinesterases and catecholamines showed a similar pattern of distribution at the medial-adventitial border of the rabbit ear

artery, when demonstrated in adjacent transverse sections.

2. Using a histochemical technique it was shown indirectly that all adrenergic nerve fibres had ChE and AChE nearby.

3. The results indicated that there was an extremely close morphological relation between cholinesterases and adrenergic nerve fibres in the rabbit ear artery.

# CHAPTER FOUR

## ELECTRON MICROSCOPE STUDIES

The light microscopic investigations of Chapters 2 and 3 indicated that there was a very close morphological relationship between cholinesterases and adrenergic nerves in the wall of the rabbit ear artery. Two main anatomical possibilities were consistent with this relationship, first that the enzymes were associated with a single population of adrenergic axons, and second that the enzymes indicated the presence of separate cholinergic nerves which were part of the sympathetic system, and which were distributed in the same nerve bundles as the adrenergic fibres.

This Chapter describes an electron-microscopic investigation of the rabbit ear artery. Although the study was directed mainly towards the nerves in the artery wall, and the relation of cholinesterases to the nerves, the general ultrastructural morphology of the vessel is also briefly outlined.

## MATERIALS AND METHODS

Segments of rabbit ear artery were obtained as described on p. 2.5. Eighteen rabbits were used in this study, and the general scheme of treatment was as follows.

Arteries were fixed at room temperature in either 5% glutaraldehyde or formaldehyde-glutaraldehyde-acrolein (Appendix, page 2) in phosphate, S-collidine or cacodylate buffer, pH 7.4. Initial contact with fixative was made in one of three ways:

(i) fixative was dripped onto the exposed vessel *in situ*;

(ii) the vessel was removed from the ear and pinned out to its measured *in vivo* length on a wax sheet, and fixative applied;

(iii) the vessel was removed, quickly cannulated at the proximal end with a polythene cannula (heat drawn No. 3 Sterivac tubing), and gently perfused with fixative at approximately 5 ml/min by hand syringe while immersing the vessel in fixative.

Within minutes of the first contact with fixative each artery was cut transversely with razor blades into approximately 1 mm thicknesses while wet to give 10-20 segments. The small rings of tissue were in contact with the fixative solution for a total of 20-30 minutes, and were then held overnight at 4°C in the corresponding buffer saturated with sucrose.

The tissue was post-fixed in phosphate-buffered 1% osmium tetroxide pH 7.4 for 25-30 minutes at room temperature, and rapidly rinsed in several changes of distilled water. Some specimens were blockstained with 2% aqueous uranyl acetate for 30 minutes. The specimens were dehydrated with ethanol and embedded in Araldite (Fluka) according to the schedule shown in the Appendix, page 3. The rings of tissue were carefully orientated during the embedding procedure, and again orientated

and trimmed during mounting so that a small area including the junction of the media and adventitia could be cut in transverse or longitudinal section. Some sections of other areas were also made. Thin sections (silver to pale gold) were cut with glass knives on a Huxley Cambridge ultramicrotome, and stained with 2% uranyl acetate in 50% methanol for 5-20 minutes, and then with basic lead citrate (Reynolds, 1963) for 3-5 minutes. The sections, which were on uncoated copper grids, were examined with a Hitachi Hu 11B electron microscope.

Hydroxydopamine treatment.

Freshly removed ear artery segments from four rabbits were treated *in vitro* for 30-60 minutes in 5-hydroxydopamine (5OHDA, A B Biotec, Stockholm), 10 ug/ml or 100 ug/ml in Krebs solution or Hanks balanced salt solution (Grand Island Biological Co., New York) at 37$^{\circ}$C. Control segments were held in the corresponding solution without 5OHDA. The segments were then fixed as described above.

Cholinesterase stain.

Segments of artery from three rabbits were stained for either ChE or AChE, using the thiocholine-based staining medium of Karnovsky and Roots (1964). After 5 minutes initial fixation in formaldehyde-glutaraldehyde-acrolein, the tissue rings were rinsed in two changes of malate buffer pH 6.0, and then treated with inhibitors and staining

media as described in Chapter 2, with combinations of substrates and inhibitors as shown in Table 2.1 (page 2.11). Incubation times of 1-5 hours for ChE and controls, and 1-24 hours for AChE and controls, were used. At the end of incubation the specimens were briefly rinsed in malate buffer, and the treatment schedule for unstained tissue re-commenced at the fixation stage.

## RESULTS

Representative electron micrographs are grouped together at the end of this section.

### Tunica intima.

The intimal surface of the artery consisted of endothelial cells on the luminal side of a loose collagen network, which was about 10,000 Å thick. Medial to this was the internal elastic lamina, a broad (7,000-15,000 Å) relatively homogeneous band with few discontinuities. Between the lamina and the smooth muscle was a second zone containing collagen fibres. Some cell profiles characteristic of fibroblasts were seen in this zone.

### Tunica media.

The medial layer was composed of smooth muscle cells, of similar form to that described by Somlyo and Somlyo (1968), and intercellular elements. The orientation of the myofilaments, as seen in transverse and longitudinal sections of the artery, suggested that most cells throughout the media were circularly arranged. Although medial cells were seen as close as 200 Å to each other, no tight junctions, nexuses or desmosomal contacts were seen between medial cells in the present study. The media was about 15-20 smooth muscle cells thick and no bodies of cells other than smooth muscle were seen within this layer. The intercellular spaces contained collagen fibres but apparently no elastic bundles, and no axon profiles were seen within the media.

Some fibroblastic processes were seen between the outermost smooth muscle cells.

Tunica adventitia.

The adventitia consisted primarily of dense parallel arrays of collagen fibres, with elastin bundles, fibroblasts, and neural tissue. The elastin bundles were apparently randomly arranged throughout the adventitia, and were also seen close to the outer cells of the media in the area of the external elasic lamina seen using light microscopy, and could be visualised as forming a very open meshwork in that region. Fibroblasts were characterised by their long and tenuous processes, and their lack of basement membrane. Some fibroblast-like cells contained an extensive rough endoplasmic reticulum. The neural components of the adventitia are described in detail below.

Neural elements.

Groups of axons were found throughout the adventitia, particularly near the medial-adventitial border. The axons within each group or bundle were associated with Schwann cell processes, and the number of axons in each group varied. Single axons with Schwann cells were sometimes seen, and less commonly single 'naked' axons, without Schwann cells. The majority were in bundles of 3 or 4 near the media, and in larger bundles of up to 30 in the inner and middle adventitia. Whole bundles or single axons were surrounded by a basement membrane about 500 Å in thickness, and the axons and Schwann cells showed

trilaminar 80 Å thick plasma membranes with lead and uranium staining. The nuclear regions of Schwann cells were seen in some sections, in which case the cells were basically similar in appearance to fibroblasts, being distinguished by their basement membrane and the presence of axons partially enfolded within their cytoplasm.

The shape and content of the individual axons in section suggested that they were varicose, as is sometimes observed using light microscopic techniques. The intervaricosity (internodal) regions were about 1500 Å in diameter, and contained neurotubules and neurofilaments. The varicosity regions (nodes), which were about 3000 Å in diameter, contained in addition spherical or ovoid vesicles, and often one or more mitichondria. Each vesicle was bounded by a trilaminar membrane similar in appearance to the axon membrane, and the vesicular contents were of variable electron density when compared with the remainder of the axoplasm. The majority of the vesicles were in the range 350-550 Å in diameter, although some of up to 900 Å were present. On the basis of vesicle size and a subjective assessment of the presence of granularity or a dense core within the vesicle, vesicles were classified as either small granular (less than 600 Å diameter), as small clear, or as large (greater than 600 Å diameter). Some of the large vesicles showed evidence of granularity, others did not. Data from fifteen axons selected at random is presented in Table 4.1. In all of several hundred axon profiles examined, when vesicles were present a majority showed either granularity or a dense core, and were less than 600 Å in diameter.

TABLE 4.1 Numbers of different types of vesicle in 15 axon profiles in nerve bundles near the medial-adventitial borders of three arteries.

| Axon no. | Small granular | Small agranular | Large | Total |
|---|---|---|---|---|
| 1 | 10 | 3 | 2 | 15 |
| 2 | 6 | 1 | 0 | 7 |
| 3 | 4 | 1 | 0 | 5 |
| 4 | 55 | 3 | 3 | 61 |
| 5 | 4 | 0 | 1 | 5 |
| 6 | 23 | 3 | 2 | 28 |
| 7 | 17 | 5 | 0 | 22 |
| 8 | 8 | 3 | 2 | 13 |
| 9 | 27 | 2 | 7 | 36 |
| 10 | 3 | 0 | 0 | 3 |
| 11 | 5 | 2 | 2 | 9 |
| 12 | 80 | 16 | 11 | 107 |
| 13 | 4 | 1 | 0 | 5 |
| 14 | 7 | 2 | 1 | 10 |
| 15 | 6 | 1 | 0 | 7 |
| Total | 259 | 43 | 31 | 333 |
| Percentage | 78 | 13 | 9 | 100 |

The closest approach of an axon to the outermost cells of the media seen in the present study was 1800 Å. Large nerve to muscle distances are common in medium-sized blood vessels (Grillo, 1966; Somlyo and Somlyo, 1968). Table 4.2 lists the minimum nerve to muscle distance, and the number of axons in each bundle, for ten nerve bundles selected on the basis of their relative closeness to the media.

5-hydroxydopamine treatment.

In all artery segments treated with 5OHDA *in vitro* the axons showed differences when compared with control segments. In 5OHDA-treated segments the axon membrane was often incomplete, and the axon contents were sometimes displaced to one side of a clear space. The electron-density of the vesicles was in all cases greater than in control segments, when viewed as the average of each axon's contents. The form of the vesicles varied, with a number of large, dense-cored vesicles and some smaller, apparently flattened, granular vesicles (Figure 4.4).

AChE and ChE staining.

Prolonged incubation (16-20 hours) was required for the demonstration of AChE stain. The degree of stain deposition varied with the depth in each block of tissue at which the section was made, but in each section all axons near the medial-adventitial border showed

TABLE 4.2 Minimum nerve to muscle distance, and the number of axons in each nerve bundle, for groups of axons from three arteries selected on the basis of their relative proximity to the smooth muscle.

| Bundle no. | Nerve-muscle distance | Number of axons |
|---|---|---|
| 1 | 450 mu | 2 |
| 2 | 180 mu | 2 |
| 3 | 500 mu | 8 |
| 4 | 640 mu | 1 |
| 5 | 300 mu | 2 |
| 6 | 250 mu | 1 |
| 7 | 900 mu | 3 |
| 8 | 240 mu | 6 |
| 9 | 650 mu | 3 |
| 10 | 850 mu | 5 |

a similar degree of staining, whether cut through a varicosity or intervaricosity region (Figure 4.5). Difficulties encountered were deposition of electron-dense crystals throughout the tissue, and tissue appearance suggestive of poor fixation. However, discrete areas of apparently non-crystalline electron-dense material were seen between the Schwann cell and axon membranes. No such stain was evident in tissue treated with BW284C51 throughout the normal stain procedure.

Heavy ChE stain was observed after 30 minutes incubation in the appropriate medium. Although stain localisation was generally not as good as with AChE treatment, non-crystalline deposits were seen in the same areas, that is, between the axons and Schwann cell membranes (Figure 4.6). Crystalline deposits were seen associated with other parts of the Schwann cell, in low-powered views.

Note on fixation.

The tissue with fewest signs of poor fixation was from arteries bathed in and gently perfused with formaldehyde-glutaraldehyde-acrolein in 0.1M phosphate buffer pH 7.4, as the initial fixation stage. This solution appeared to give better results than 5% glutaraldehyde in buffer, although the general tissue appearance was similar in both cases. A systematic examination of fixation procedures was not made.

Figure 4.1

Electron micrograph of a thin section of rabbit ear artery, in the region of the medial-adventitial border.

Longitudinal section.

| | | | |
|---|---|---|---|
| SM | smooth muscle cells | A | axon |
| N | nucleus | S | Schwann cell |
| Ct | collagen in transverse section | B | basement membrane |
| Cl | collagen in longitudinal section | | |

Inset: close approach between two smooth muscle cells deeper in the media of the same artery.

Formaldehyde-glutaraldehyde-acrolein/osmium fixation.

Lead citrate - uranyl acetate stain.

Scale, 1μ (approximate). Inset scale, 0.5μ(approximate).

Approximate magnification, x 25,000. Inset, x 50,000.

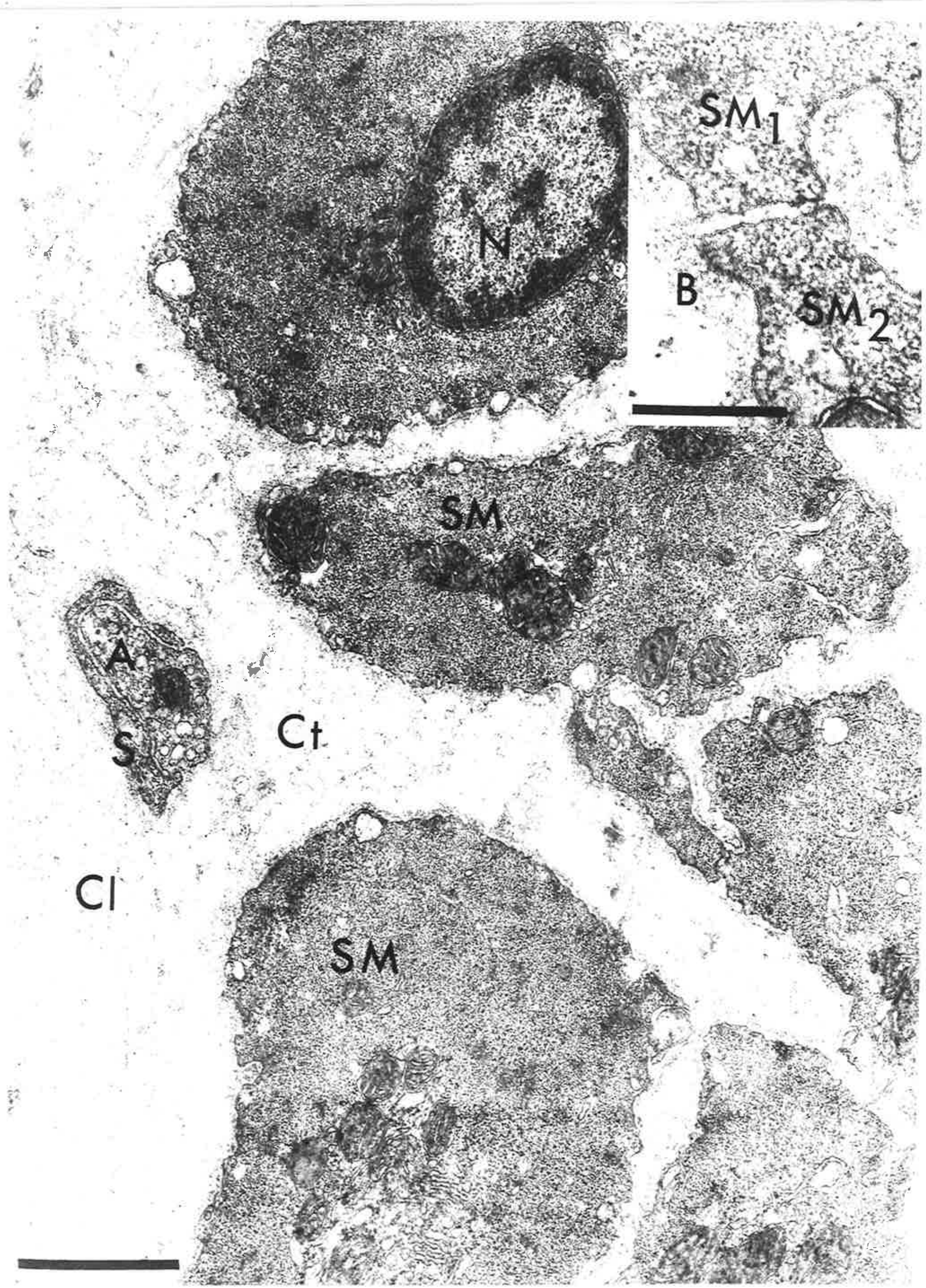

N
SM1
B
SM2
SM
A
S
Ct
Cl
SM

Figure 4.2

Electron micrograph of a thin section of rabbit ear artery, in the region of the medial-adventitial border.

F fibroblastic process

C collagen

S Schwann cell

A axon

E elastin

SM smooth muscle

arrows indicate small granular vesicles

Three axons are shown, A1 is cut through a varicosity region, A3 is cut through an intervaricosity region.

Formaldehyde-glutaraldehyde-acrolein/osmium fixation.

Lead citrate - uranyl acetate stain.

Scale, 0.5μ (approximate).

Approximate magnification, x 60,000.

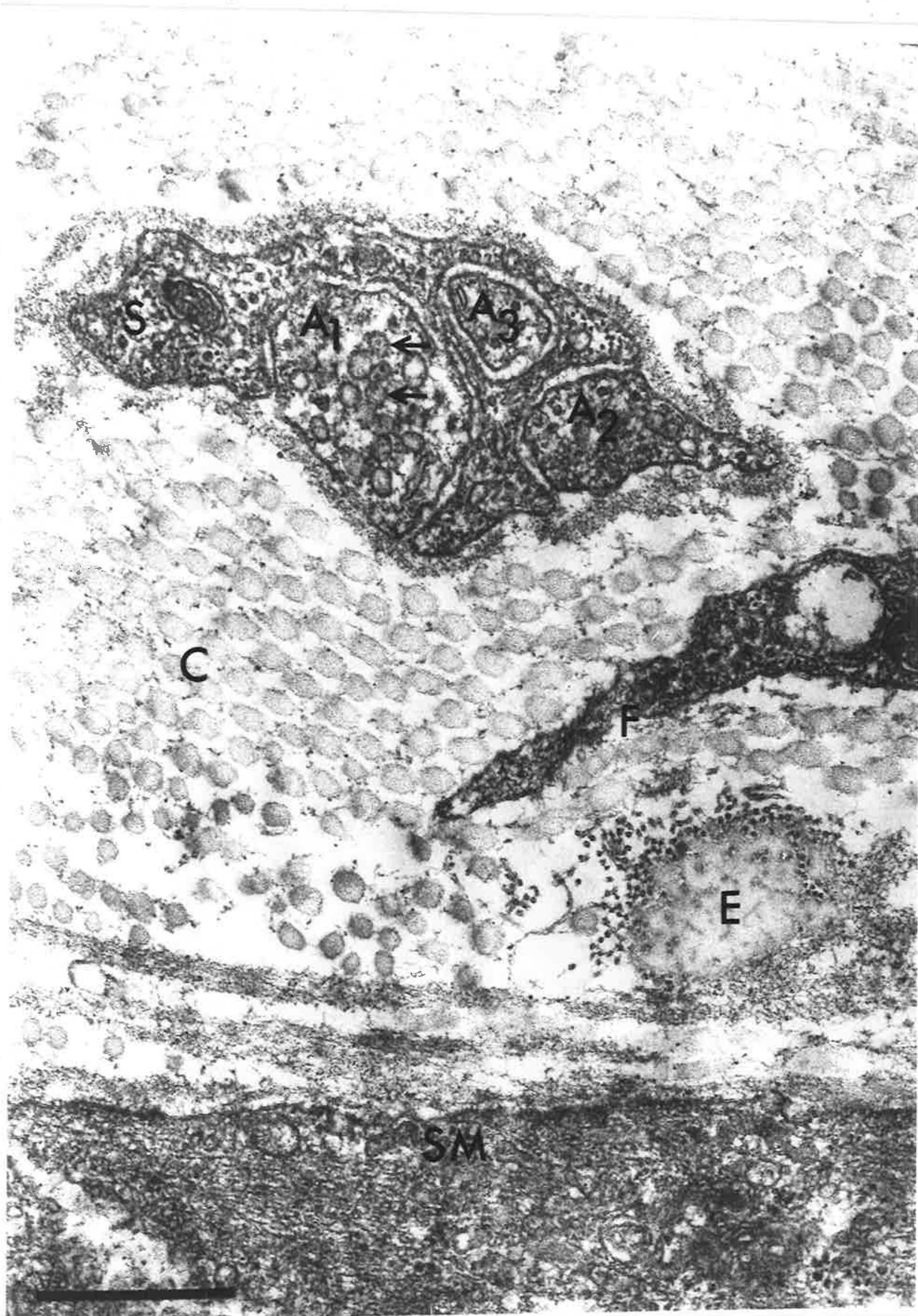

S
A1
A3
A2
C
F
E
SM

Figure 4.3

Electron micrograph of a thin section of rabbit ear artery, in the inner adventitia. Showing a single axon and Schwann cell, and a nearby smooth muscle cell.

| | |
|---|---|
| A | axon |
| S | Schwann cell |
| SM | smooth muscle |
| C | collagen |
| M | mitochondrion |

arrows show small granular vesicles

Inset 'naked axon', without Schwann cell, in a different region of the same section, at a similar magnification.

Formaldehyde-glutaraldehyde acrolein/osmium fixation.

Lead citrate - uranyl acetate stain.

Scale, 0.5μ (approximate).

Approximate magnification, x 80,000.

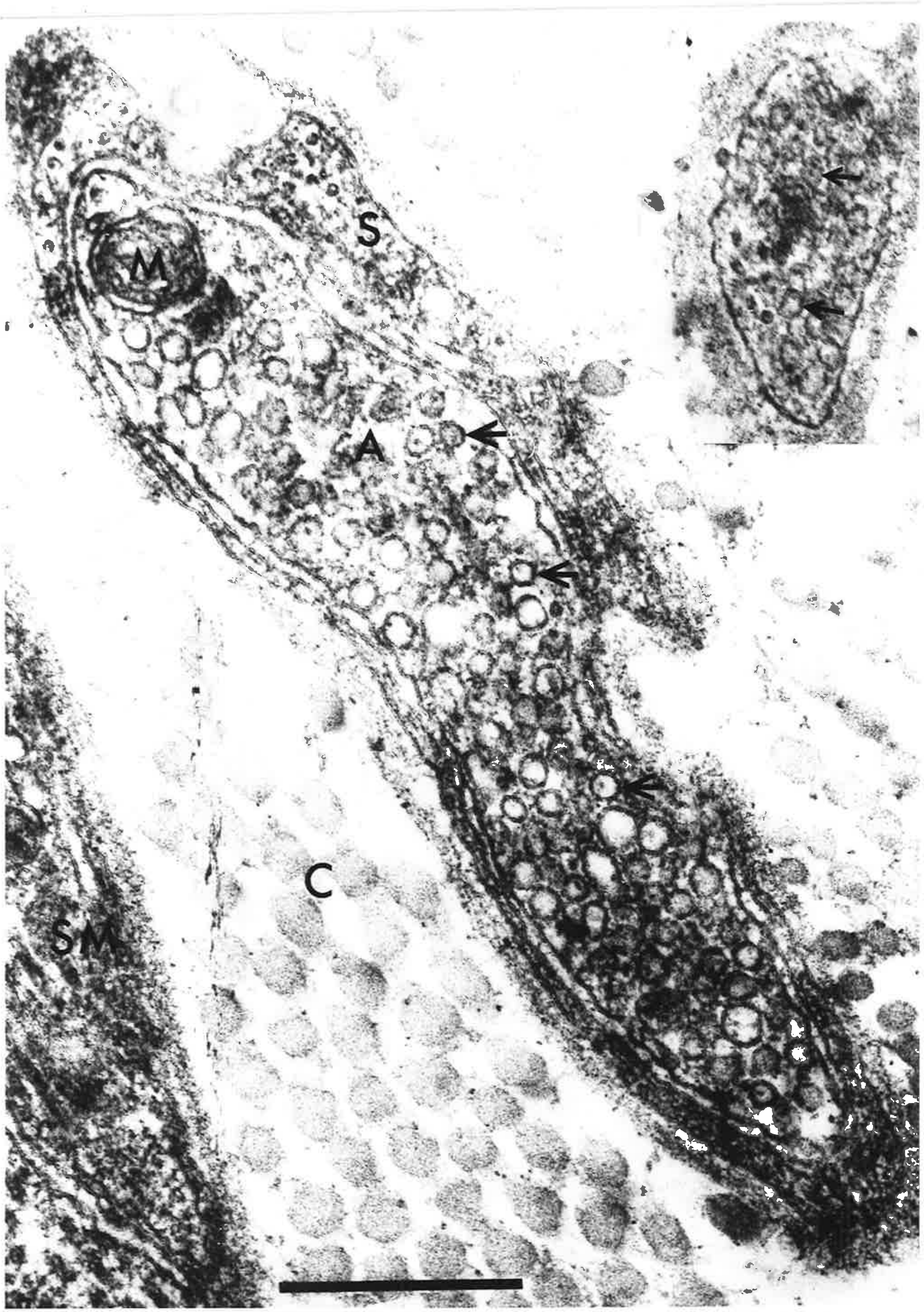

S
M
A
C
SM

Figure 4.4

Electron micrograph of a thin section of rabbit ear artery, in the inner adventitia.

E elastin

A axon

C collagen

arrows indicate dense-cored vesicles

Treated *in vitro* with 5-hydroxydopamine 10 μg/ml in Krebs bicarbonate containing ascorbic acid 1:30,000 for 30 minutes before fixation.

Glutaraldehyde/osmium fixation

Lead citrate - uranyl acetate stain.

Scale, 0.5μ (approximate)

Approximate magnification, x 60,000

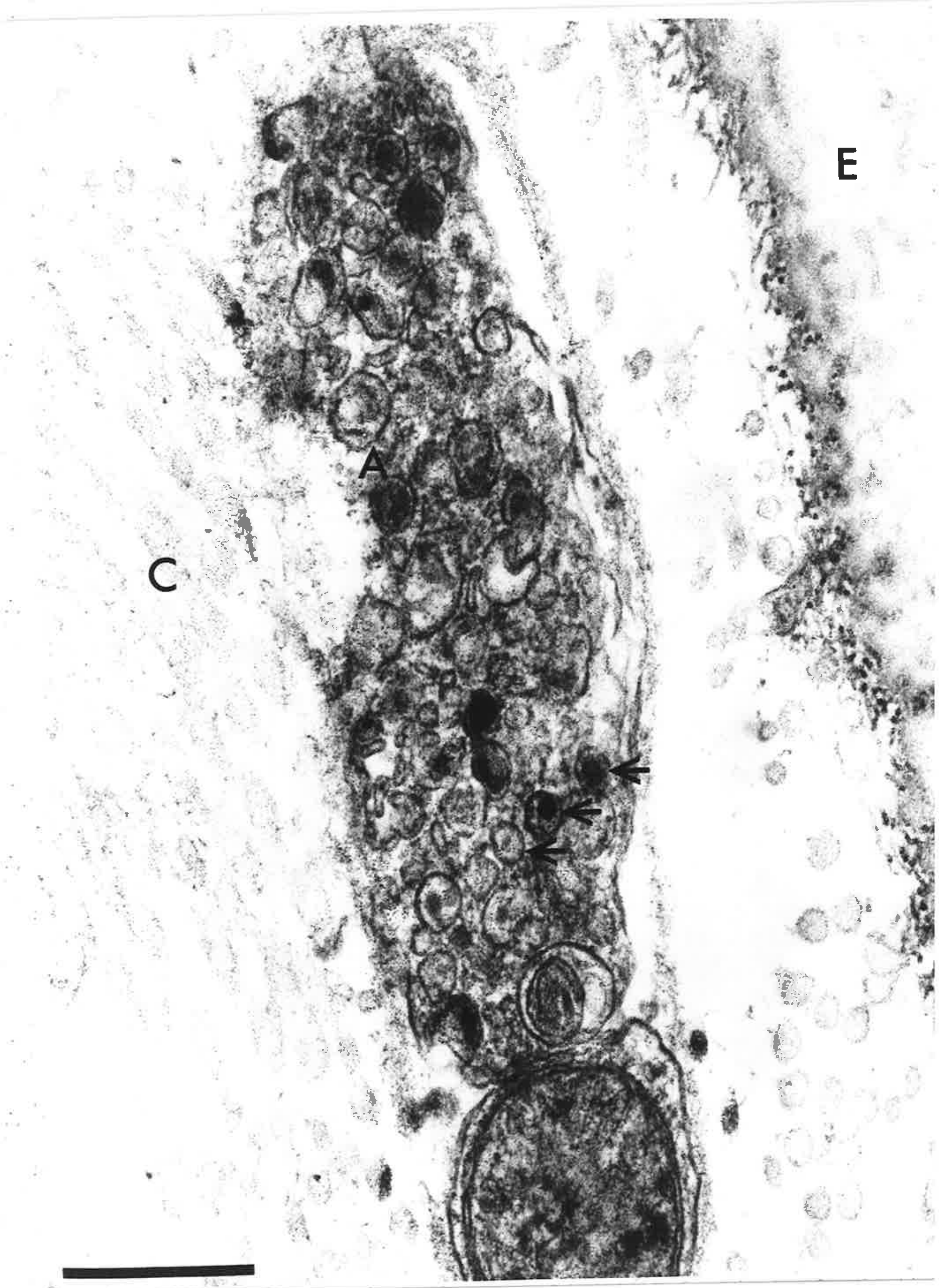

E
A
C

Figure 4.5

Electron micrograph of a thin section of rabbit ear artery treated for AChE, showing a bundle of axons near the medial-adventitial border.

| | | | |
|---|---|---|---|
| F | fibroblastic process | A | axon |
| S | Schwann cell | M | mitochondrion |

Arrows show areas of stain deposition.

Fixed for five minutes in formaldehyde-glutaraldehyde-acrolein.

Pre-incubated for one hour in $10^{-5}$M *iso*OMPA.

Incubated for 24 hours in acetylthiocholine incubation medium at 37°C.

Fixed in formaldehyde-glutaraldehyde-acrolein/osmium.

Lead citrate - uranyl acetate stain.

Scale, 1μ (approximate)

Approximate magnification, x 40,000

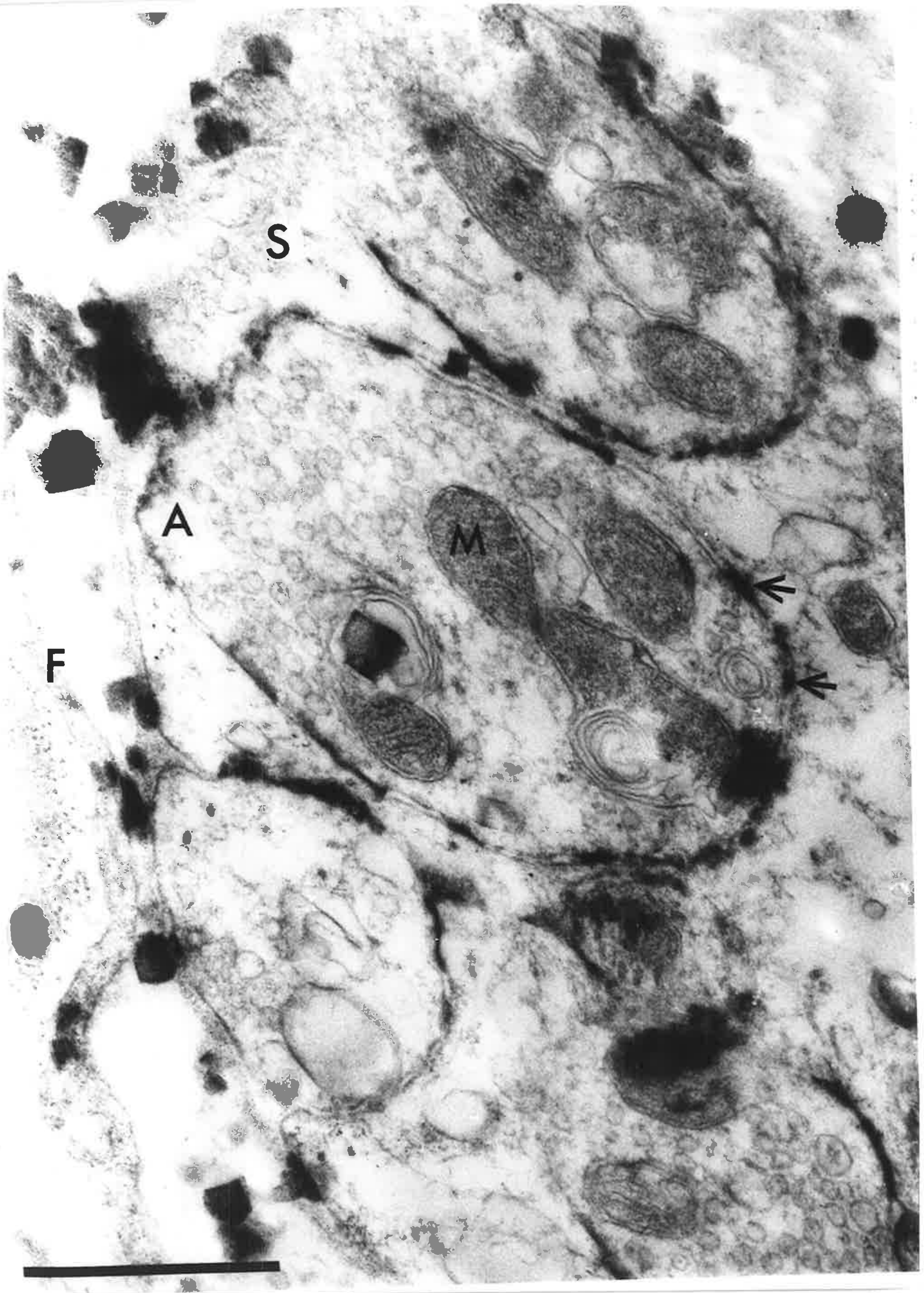

S
A
M
F

Figure 4.6

Two electron micrographs of thin sections of two rabbit ear arteries, both treated for the demonstration of ChE, in the inner adventitia region.

| | |
|---|---|
| F | fibroblastic process |
| C | collagen |
| S | Schwann cell |
| A | axon |

Both arteries were fixed in formaldehyde-glutaraldehyde-acrolein for five minutes, then incubated in butyrylthiocholine incubation medium for 30 minutes at 22°C.

Fixed in formaldehyde-glutaraldehyde-acrolein/osmium.

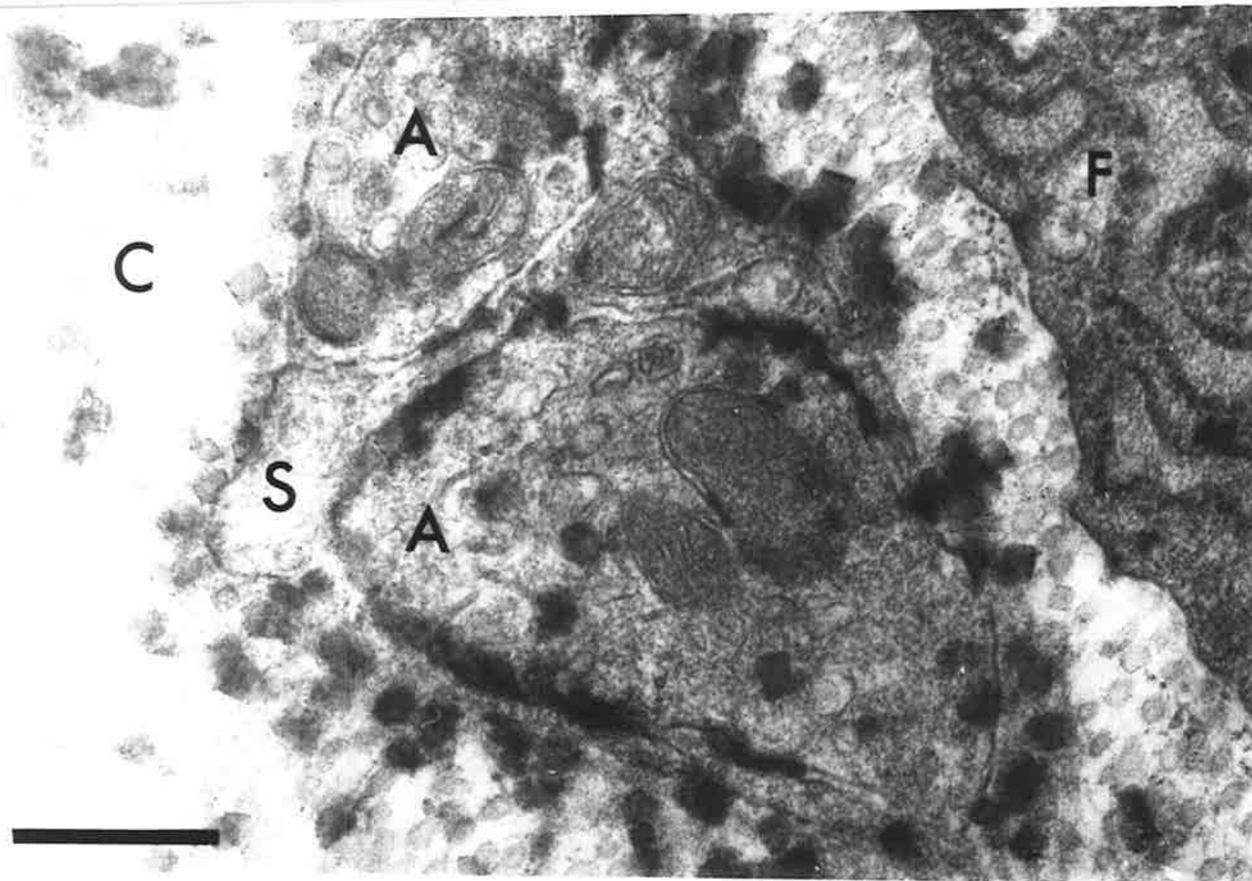
Lead citrate - uranyl acetate stain.

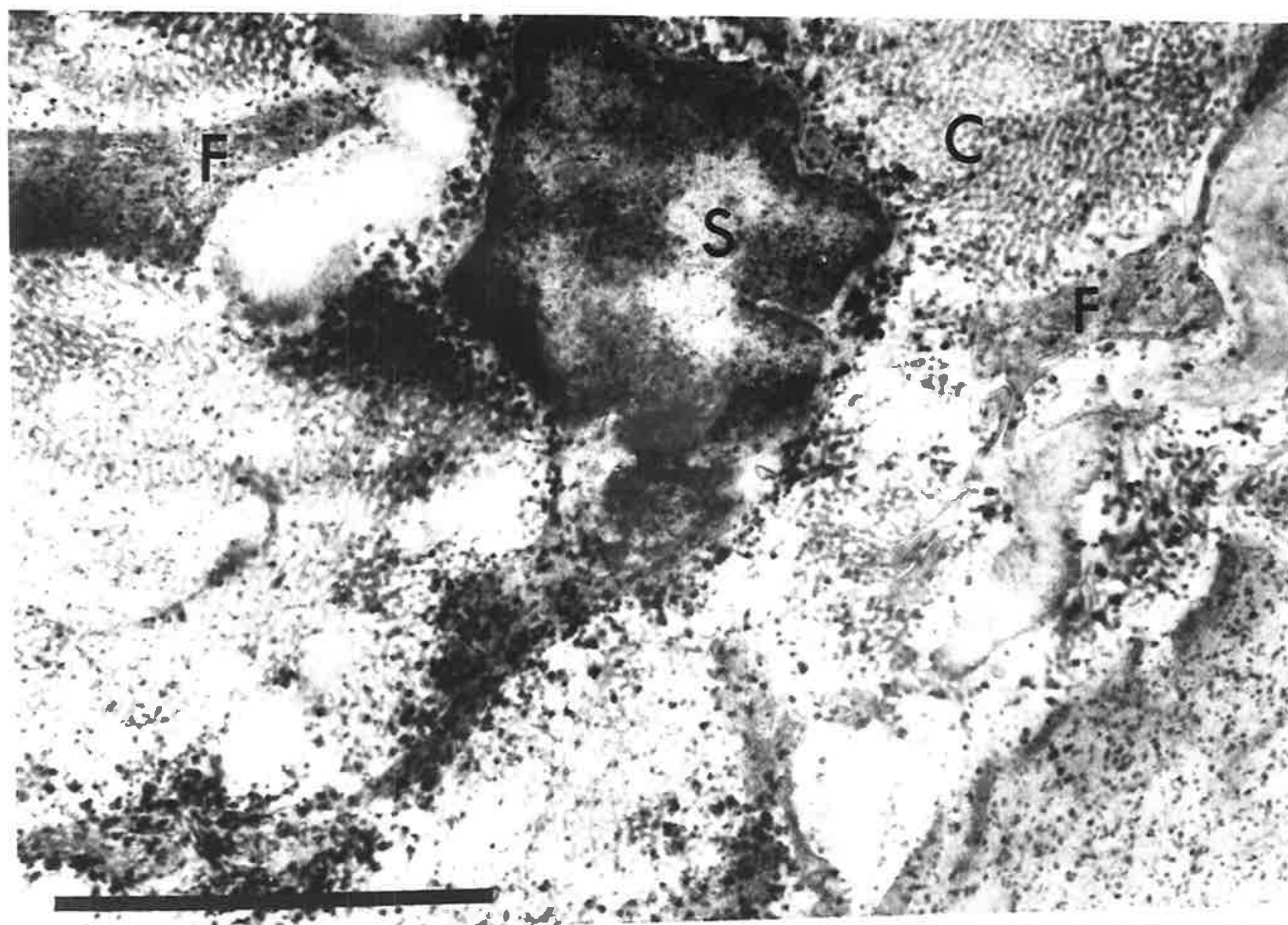
Upper panel; scale, 0.5μ (approximate)

Approximate magnification, x 50,000.

Lower panel; scale 5μ (approximate).

Approximate magnification, x, 10,000.


A
C
F
S
A


F
C
S
F

DISCUSSION

The main ultrastructural finding of importance to the present work was the demonstration of what appeared to be a single population of axons in the rabbit ear artery. The axons contained mostly small granular vesicles, and exhibited an AChE stain reaction in association with the axon membrane.

Hokfelt (1968) showed that small granular vesicles were characteristic of monoamine-containing neurons. The importance of the method of tissue preparation in the appearance of vesicles was noted by Grillo (1966), and Machado(1967) indicated that primary fixation with glutaraldehyde gave a higher proportion of granular to agranular vesicles than primary osmium tetroxide fixation. The size and granularity of the vesicles in all axons in the present work suggested that the axons were adrenergic. Iwayama, Furness and Burnstock (1970) showed that the adrenergic and cholinergic axons in rat cerebral vessels could be further distinguished by the increase in granularity of the former group after *in vivo* hydroxydopamine treatment. *In vivo* treatment was not possible in the rabbit because of the large quantity of the drug required, but *in vitro* application of 5OHDA increased the granularity of the vesicles in all axons, which substantiated the adrenergic nature of the nerves. The damage to the axon structure observed with such application was probably due to osmotic effects related to the drug, since it was not seen after control (drug-free) treatment. The use of a balanced salt solution did not prevent the

damage, but further experiments may overcome this difficulty.

AChE was found in association with all axons in sections of appropriately treated tissue. But long incubation times (20 hours or more) were required to demonstrate the enzyme. Robinson and Bell (1967) demonstrated AChE activity in axons of toad bladder using 20 or 40 minute incubation times, and Silva, Farrell and Smith (1968) saw AChE stain in axons in mouse colon after 20 minutes incubation . These axons were considered to be cholinergic. Shute and Lewis (1965) regarded intense AChE activity of the axon membrane as a reliable criterion for the classification of a neuron as cholinergic, while Eranko (1967) considered such activity as only suggestive of cholinergic nature. The AChE activity seen in this study could in no way be regarded as intense. Thompson and Tickner (1953) determined the AChE activity of the rabbit ear artery by manometric assay, and found that the level of activity was low. Therefore, it is reasonable to propose that the axons observed in the present study were not cholinergic, and that there was no evidence for a cholinergic innervation in the vessel. It may be concluded that AChE was associated with the axon membranes of the artery's sympathetic nerves.

The possibility must be considered that ChE and AChE together represented one enzyme complex, with a single distribution. The two groups ChE and AChE cannot be considered absolute, as was pointed

out by Chessick (1954), and may simply be representative of a range of properties in relation to substrates and inhibitors. At the ultrastructural level both ChE and AChE were seen to be primarily associated with the cleft between axons and Schwann cells. ChE was observed in relation to non-axons regions of Schwann cells as well, but this may simply reflect a factor related to enzyme activity, since when staining was present in these regions it was extremely heavy in the axonal areas. On the basis of the present results it cannot be concluded whether AChE was related primarily to the axon or the Schwann cell, and in this regard it is unfortunate that single 'naked' axons were so uncommon in the artery. Further studies may reveal more exactly the relation between cholinesterases and sympathetic axons, in the rabbit ear artery.

While this work was in progress Eranko, Reichardt, Eranko and Cunningham (1970) demonstrated AChE activity on the axon membrane of sympathetic nerve terminals in the rat pineal gland. Burn(1971) has interpreted this result as evidence in favour of the 'cholinergic link' hypothesis that acetylcholine takes part in noradrenaline release from sympathetic nerves, and a similar interpretation of the present findings could be made. However, although the degree of correlation between AChE and acetylcholine in nerves has been described as 'fairly good' by Jacobowitz and Koelle (1965), both their observations and those of Koelle (1963) indicated that the demonstration of AChE within a nerve did not prove that acetylcholine was present. So it can be stated that, in particular because of the low AChE activity suggested by the

present results, the monoamine-containing neurons in the rabbit ear artery need not also contain acetylcholine. AChE at such a level may not be functional, and could simply indicate a property common to the nature of all neural tissue but which is not active in this case, as was discussed by Koelle (1963).

The presence of ChE, and its disappearance after superior cervical ganglionectomy (Chapter 2) is not readily explained in functional terms. The relation of the enzyme to Schwann cells and their processes observed in this work was consistent with the association of the enzyme with Schwann cells in other tissues (Koelle, 1961) and with other glial cells (Hebb, 1957; Koelle, 1963; Eranko, 1967). It is possible that some metabolic interchange takes place between axons and Schwann cells, and ChE may be associated in some way with such an action. Alternatively, ChE may protect axons from choline esters which might inhibit their function. If ChE was primarily associated with the Schwann cells, as seemed the case, it must be postulated that some changes occurred in these cells when the axons degenerated since the enzyme was then not apparent in light-microscope sections (Chapter 2). Ultrastructural investigations of arteries at different times after superior cervical ganglionectomy may indicate the nature of such changes. Two arteries were examined 32 and 54 days after ganglionectomy respectively, but were not stained for cholinesterases. No axon or Schwann cell profiles were recognised, suggesting that the Schwann cells had either

degenerated or assumed another form, possibly fibroblastic. An interesting observation regarding the structure of these arteries, which was not of direct application to the present work, is described in the Appendix, page 8.

SUMMARY

1. At the ultrastructural level the rabbit ear artery was shown to contain a single and uniform population of axons near the medial-adventitial border.
2. On the basis of the morphology of neuronal vesicles and their ability to take up 5-hydroxydopamine the axons were classified as adrenergic.
3. The axons exhibited weak AChE activity in association with their axon membrane, as shown by a histochemical technique.

# CHAPTER FIVE

## CHOLINE ACETYLASE

It is an essential prerequisite of the 'cholinergic link' hypothesis that acetylcholine should be present in the terminal region of sympathetic nerves. In the rabbit ear artery, the only indication of the presence of acetylcholine revealed by a search of the literature was that provided by the data of Armin, Grant, Thompson and Tickner (1953), which was based on indirect pharmacological evidence open to other interpretations, particularly in the light of more recent concepts such as neuronal uptake of catecholamine. If acetylcholine is present in the vessel, in the terminals of sympathetic fibres, it would be necessary that it should either be synthesised *in situ*, or be transferred from a synthetic site in the cell body to the terminal by axoplasmic flow. In view of the ability of nerves in isolated preparations, such as the rabbit ear artery (see the following Chapters), to continue releasing noradrenaline for many hours, and even days, after removal from the animal and therefore from the cell body, the latter possibility would seem very unlikely. If a cholinergic element did take part in the function of sympathetic nerves in the rabbit ear artery, the acetylcholine synthesising enzyme choline acetylase (ChAc) should therefore be present in the vessel.

Samples of untreated and sympathetically denervated rabbit ear arteries were assayed for ChAc, using the technique of McCaman and Hunt (1965). Sections of similar vessels were treated histochemically for the demonstration of ChAc, using the technique of Burt (1970).

## MATERIALS AND METHODS

### Assay procedure.

Choline acetylase activity was determined using the technique of McCaman and Hunt (1965), who measured the synthesis of $(C_{14})$-acetyl-choline from $(C_{14})$ acetyl-coenzyme A and choline. The two labelled compounds were separated by precipitation of the labelled acetylcholine with ammonium reineckate (Reinecke's salt). The labelled acetylcholine-reineckate was washed and quantified using a scintillation counter.

Eight semi-lop eared rabbits were used in this study. Three rabbits were prepared by unilateral excision of a superior cervical ganglion, as described on page 2.7, 24-32 days before use. The animals were sacrificed by stunning and exsanguination. Segments of the central ear arteries were removed as described on page 2.5, rinsed in distilled water, weighed, and homogenised in ice-cold double-distilled water using a pre-chilled pestle and mortar. When the activities of experimental and control side artery homogenates were to be compared, 0.1 ml of each homogenate was oven-dried overnight and weighed to check that the homogenates were of similar concentration. Activities were, however, expressed in terms of wet weight of tissue. All subsequent steps except incubation and scintillation counting were carried out in a cold room (temperature 5-6$^{o}$C), and the assay tubes were held in an ice-water bath as much as was possible. Centrifugation was carried out using an MSE refrigerated centrifuge at 0-3$^{o}$C.

Four to ten samples of each homogenate were assayed separately as described by McCaman and Hunt (1965). The assay procedure is outlined in Figure 5.1. The composition of the incubation mixture is shown in the Appendix, page 4. The incubation time was 30, 60 or 90 minutes at 37-39$^{o}$C, and each centrifugation step was of 15 minutes duration at 3,000 revolutions per minute. Samples of tissue homogenates which had been boiled in sealed vials for 30 minutes were assayed, and enzyme-free assays (blanks) were made by omitting tissue homogenate from the incubation mixture. The sensitivity of the technique was monitored using homogenates of whole mouse brain (Swiss Albino mice, bred at the Central Animal House, University of Adelaide), or of rabbit anterior cerebral cortex, removed from the stunned and bled animals. To test the loss of labelled acetylcholine during the procedure, known amounts of acetyl-($C_{14}$)choline (Radiochemical Centre, Amersham) were treated identically with assay samples. Results indicated that at least 95% of the labelled acetylcholine was recovered. The reaction product in the assay procedure, and other labelled compounds, were dissolved in 15 ml of a scintillation solution (Appendix, page 4) and their activity estimated using a Packard TriCarb liquid scintillation spectrometer, model 3310, at a cabinet temperature of 12$^{o}$C.

Histochemistry.

Cryostat sections of 6 arteries from 4 rabbits were treated for the demonstration of ChAc using the technique of Burt (1970), which is

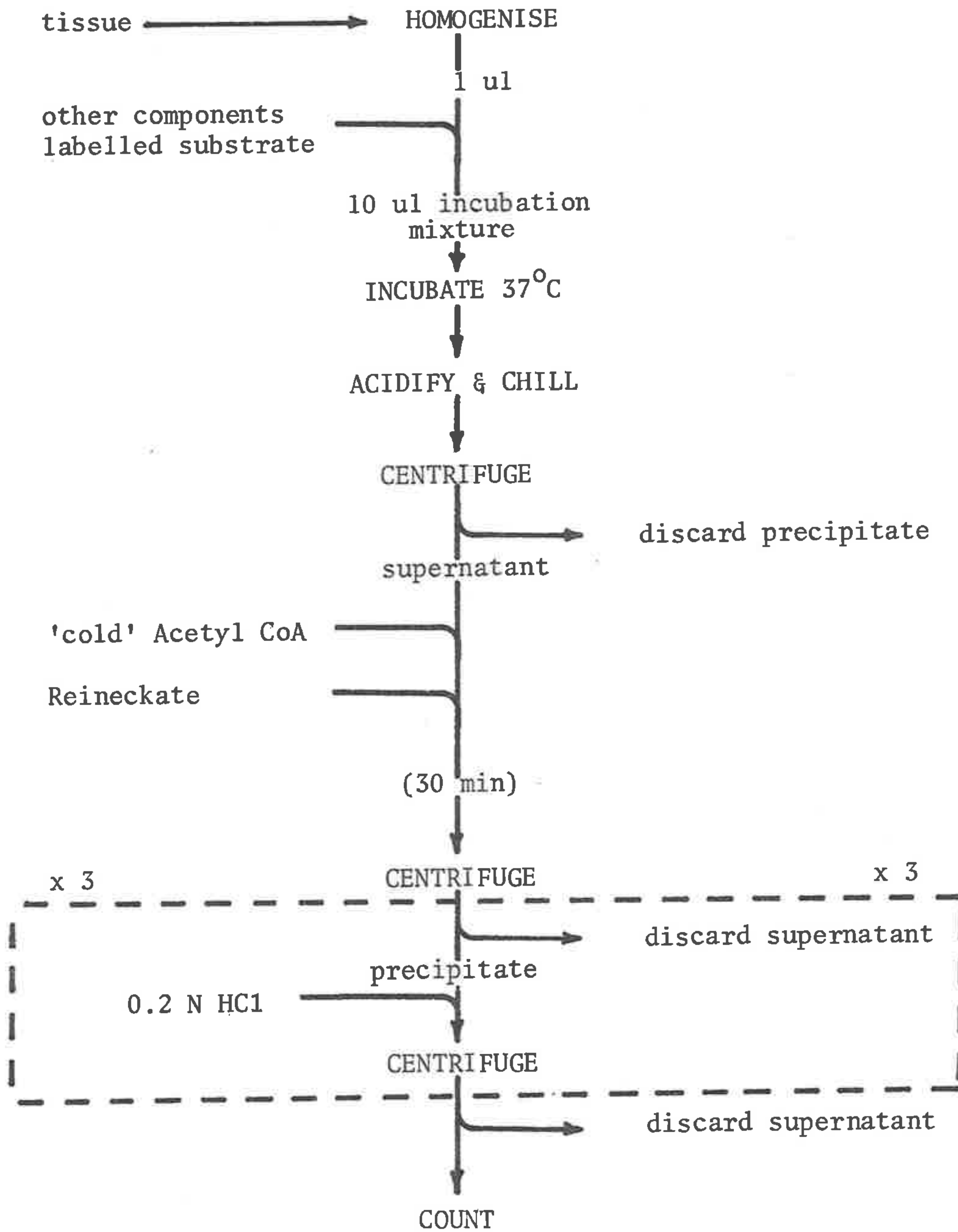



Figure 5.1

Showing the steps of the choline acetylase assay procedure described in the text.

based on the formation of an insoluble lead mercaptide from Coenzyme A, a product liberated during acetylcholine synthesis. Two of the rabbits had undergone unilateral superior cervical ganglionectomy 21 and 24 days respectively before sacrifice. Rabbit cervical spinal cord and superior cervical ganglia, taken from the animals at sacrifice, were treated as control tissues. The arteries and other tissues were frozen in liquid nitrogen, and sections 4 or 8 microns in thickness cut on a cryostat (American Optical Co.). The sections were dried onto glass slides, treated for ChAc as described by Burt (1970), and examined on a Zeiss S.V. microscope using light field illumination. Photomicrographs were taken using Ilford Pan F film and a 35 mm Zeiss Ikon camera with an automatic exposure device.

## RESULTS

### Enzyme assay.

Table 5.1 shows the results of one experiment in which the activity of samples of tissue was compared with the activity of samples from the same homogenates which had been boiled for 30 minutes before assay.

TABLE 5.1 Scintillations per minute in assay groups of rabbit tissue

| | Unboiled | Boiled |
|---|---|---|
| Anterior cerebral cortex | 3,720 ± 103 | 67.3 ± 8.2 |
| Ear artery | 58.9 ± 4.5 | 59.2 ± 7.1 |
| Blank (no tissue) | 45.7 ± 2.7 | |

Note: each group represents the mean ± the standard error of the mean for six samples of the homogenate.

Brain homogenates were markedly more active than boiled brain samples, while no difference of this kind was observed with artery samples. In seven experiments no significant differences (that is, less than the 5% confidence level using Student's 't' test) were observed between artery and boiled artery homogenates. Similarly in three experiments

no significant differences were observed between arteries taken from the ears on the experimental and control sides of rabbits which had undergone unilateral superior cervical ganglionectomy (Table 5.2). Blanks gave counts which were usually lower than those for boiled tissue, particularly when the tissue concentration in the assay mixture was high.

TABLE 5.2 Scintillations per minute in assay groups of experimental and control side rabbit ear arteries from animals which had undergone unilateral superior cervical ganglionectomy.

| Rabbit no. | Days[a] | Left artery | Right artery |
|---|---|---|---|
| 1 | 32 | 51.2 ± 13.2 | 48.3 ± 6.7 |
| 2 | 26 | 81.8 ± 6.6 | 85.4 ± 8.6 |
| 3 | 24 | 58.9 ± 4.5 | 62.4 ± 5.3 |

[a]Days = number of days between ganglionectomy and sacrifice

Note: each group represents the mean ± the standard error of the mean for six samples of the homogenate.

Histochemistry

In sections of superior cervical ganglion and spinal cord treated for the demonstration of ChAc, stained areas, similar to those described by Burt (1970) in rat brain, were evident. The areas of stain possibly indicated terminal button ChAc activity, and were not evident in control incubated sections (without acetyl-Coenzyme A). Panels A and B of Figure 5.2 show an active and a control incubated section of superior cervical ganglion.

No areas of stain were evident in sections of rabbit ear artery treated identically with sections of cord or ganglion which did show stain. With prolonged incubation times (3 hours or more) apparently nonspecific stain was seen in both active and control incubated sections. No distinction could be made between active and control incubated sections, or between sympathectomised and control arteries when treated for ChAc. Panel C of Figure 5.2 shows a section of a control side artery, treated for the demonstration of ChAc.

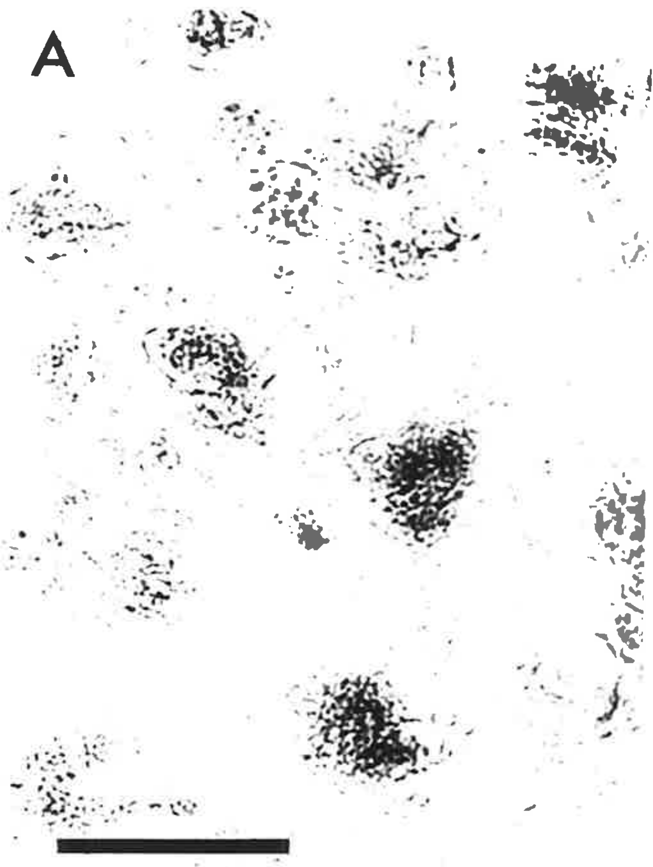
Figure 5.2

Panels A and B.

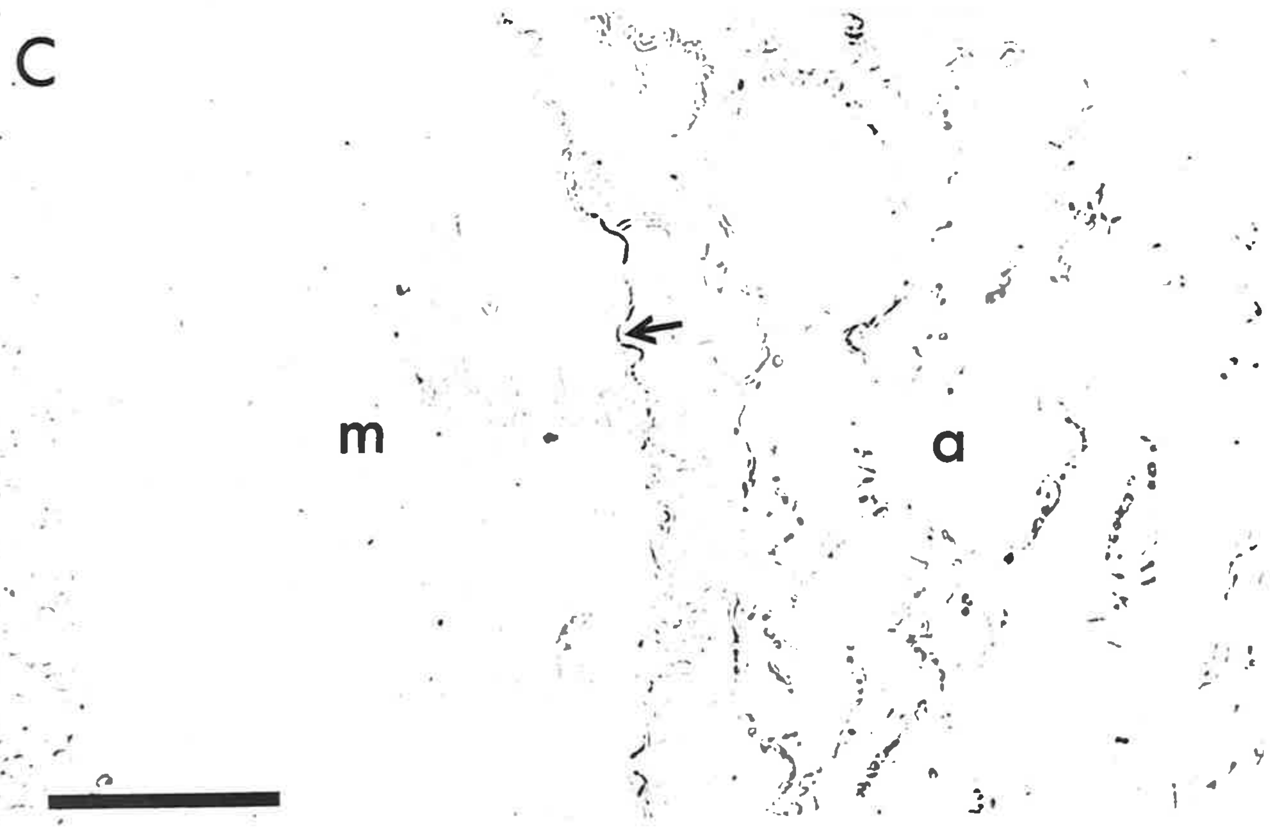
Photomicrographs of two sections of a rabbit superior cervical ganglion treated (a) for the demonstration of ChAc and (b) as a control, both as described in the text. Incubation time 180 minutes. Scale, 50μ.

Panel C.

Photomicrograph of a transverse section of rabbit ear artery, treated for the demonstration of ChAc as described in the text. Incubation time 180 minutes. m, media; a, adventitia; the medial-adventitial border is arrowed. Scale, 50μ. The dark regions in the adventitia, which were also apparent in control and untreated sections, changed with focus and were presumably a result of optical effects. No stain attributable to ChAc activity was evident.


A

B


C
m
a

DISCUSSION

In the assay procedure the activity of cerebral cortex homogenates was markedly reduced by boiling, but artery homogenates gave counts which were in no case higher than those for the corresponding boiled tissue. If it is assumed that boiling inactivates ChAc, this result indicated that the artery tissue contained no detectable enzyme. Counts for boiled samples of either artery or brain were usually greater than the blank, which might suggest that either an increase of reineckate trapping of ($C_{14}$)acetyl-Coenzyme A occurred in the presence of tissue homogenate, or that the tissue had a slight, heat stable acetylcholine synthetic ability. However, it was evident that the rabbit ear artery contained no detectable heat labile ChAc activity corresponding to the activity observed in brain homogenates.

Superior cervical ganglionectomy is followed, in the rabbit, by degeneration of sympathetic nerves in the ear artery on the experimental side (de la Lande, Frewin and Waterson, 1967). In the present study no difference in ChAc activity was observed between sympathectomised and control arteries. Similarly, in the histochemical study no ChAc stain was observed in sections of rabbit ear artery.

The compound 4-(1-naphthylvinyl)-pyridine (NVP) has been reported to exhibit evidence of ChAc inhibition at the neuromuscular junction (Hemsworth and Foldes, 1970). In a preliminary study using the assay

technique described in this Chapter, and tissue from two rabbits, the presence of NVP $10^{-4}$M during the incubation stage was found to depress the activity of brain homogenates to less than 10% of the control level, but such treatment did not affect the results of artery sample assays. This provided supportive evidence for a lack of detectable ChAc activity in the vessel.

Although no evidence of ChAc activity in the artery was observed, criticism of the present study could be made on the grounds of poor sensitivity in both of the techniques. The histochemical procedure in particular did not show a strong stain reaction with tissues known to have high ChAc activities, and so any activity in the artery could have been below the threshold for detection. In the assay experiments, samples of rabbit anterior cerebral cortex yielded 0.9-1.5 micromoles of $(C_{14})$acetylcholine per gram wet weight of tissue per hour, an activity comparable with that reported by McCaman and Hunt (1965), and the limit of sensitivity of the technique could be calculated as in the range of $10^{-9}$ moles per gram wet weight per hour. But the very small proportion by weight of neural tissue in the artery, as suggested by morphological studies, may not have been sufficient to influence the ChAc activity of the total artery homogenate. In attempts to overcome this difficulty, both the substrate and homogenate concentrations were increased in the hope that any differences between artery samples, boiled and control or sympathetically denervated and control, might become evident. This caused an increase in the absolute counts with

cerebral homogenates, although the calculated activity decreased, and also an increase in the blank counts. But no evidence of arterial ChAc activity was seen.

It can be stated only that the present study failed to demonstrate ChAc activity in the rabbit ear artery. A similar result was obtained by Ehinger et al (1970), who bio-assayed acetylcholine from the cat iris and found no detectable acetylcholine related to the sympathetic innervation. The development of more sensitive techniques for demonstrating acetylcholine or ChAc will undoubtedly be followed by a more definite statement concerning acetylcholine in sympathetic nerves.

## SUMMARY

Using both an enzyme assay and a histochemical technique, the present study failed to demonstrate choline acetylase activity in the rabbit ear artery.

# CHAPTER SIX

## ACETYLCHOLINE IN THE ISOLATED PERFUSED RABBIT EAR

This Chapter is brief, and describes a relatively simple phenomenon. But it deserves inclusion in the thesis as the starting point for the investigations described in the Chapters that follow. An important observation was made, namely that if the tone of isolated perfused vessels was raised, the vessels became sensitive to dilator stimuli. The demonstration of dilator responses to nanogram quantities of acetylcholine (ACh) in the isolated perfused rabbit ear was also of importance in its own right.

The study began as an investigation of antidromic vasodilatation, a phenomenon which remains basically unexplained. In early experiments it was noted that the isolated perfused rabbit ear, as well as showing responses related to the above phenomenon, dilated in response to low dose levels of ACh. The investigation into antidromic vasodilatation was not carried further, and is described in the Appendix, pages 9-14 (Appendix Six), as it is of interest but is not directly related to the subject of the thesis. After initial characterisation of the ACh phenomenon in the whole ear, investigations into its action were continued in the isolated perfused central ear artery.

## MATERIALS AND METHODS

Isolated whole rabbit ears were perfused using the method of de la Lande, Paton and Waud (1964).

Semi-lop eared rabbits of either sex were bred at the Central Animal House, University of Adelaide. The weights of the animals varied from 1.5-3.0 Kg. Rabbits were anaesthetised with 10 ml/Kg or more of a 25% solution of ethyl carbamate (urethane) injected intraperitoneally. Heparin, 1,000 units total dose irrespective of weight, was injected into an ear vein of the anaesthetised animal.

After wetting the fur of the dorsal surface of the ear close to the base with water, the proximal parts of the central artery and the great auricular nerve were exposed and cleaned of their surrounding tissue by incision and blunt dissection. The relative positions of the central artery and vein and the great auricular nerve in the rabbit ear are shown in Figure 6.1. The central artery was tied off with a cotton ligature at the proximal end of the ear, and a polythene cannula (heat drawn No. 3 Sterivac tubing) was inserted into the lumen of the artery through an oblique cut just distal to this tie. The artery was then cut through between the ligature and the cannula, and the whole ear was severed from the animal at the same level. The second ear was sometimes removed from the anaesthetised animal in the same way, either some minutes or several hours later.

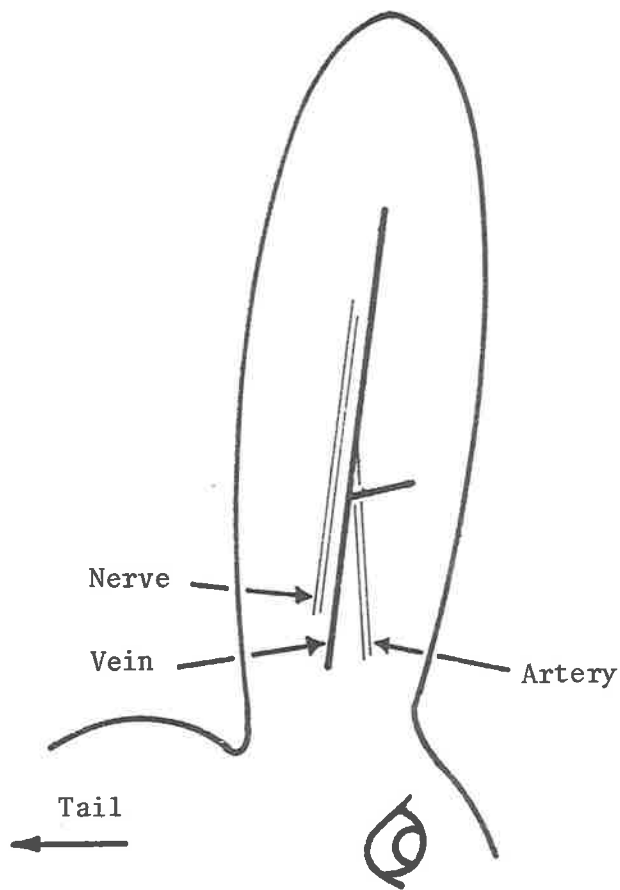



Figure 6.1

A diagram of a rabbit ear, showing the relative positions of the great auricular nerve, the central vein and the central artery. The diagram represents the left ear, held erect and viewed from the right side of the animal.

The cannulated ear was placed in a 37°C air warming chamber and perfused with a physiological saline solution at 5.5-6.0 ml/min. The saline solution, which is referred to throughout this thesis as Krebs or Krebs bicarbonate, was bubbled with 95% oxygen and 5% carbon dioxide and maintained at 37°C. Perfusion pressure was recorded with a pressure transducer (Ether Engineering) and a potentiometric recorder (Rikadenki Kogyo Co.). Details of the warming chamber, the perfusion pump and the recording apparatus and the composition of the Krebs solution are found in the Appendix. When two ears from the same animal were perfused the second was usually not cannulated until the experiment with the first was complete, although in some experiments both ears were perfused at the same time using two pumps and a two-channel recording system. Figure 6.2 is a diagram of the perfusion system for one ear.

Drugs were applied to the ear via the perfusion stream. They were either added to the reservoir of perfusion fluid (infusion) or were injected in a volume of 0.05-0.1 ml into the perfusion stream just proximal to the cannula through a rubber cuff (injection). Details of the source and dilution of all drugs used in this study are found in the Appendix, page 7. Concentrations of drugs are expressed as weight over volume, and injected doses simply as weight of the drug. The general sequence of each experiment was to apply test infusions of ornithine vasopressin (POR8) to determine the concentration which would raise the perfusion pressure to 100 mm Hg or more, and then to

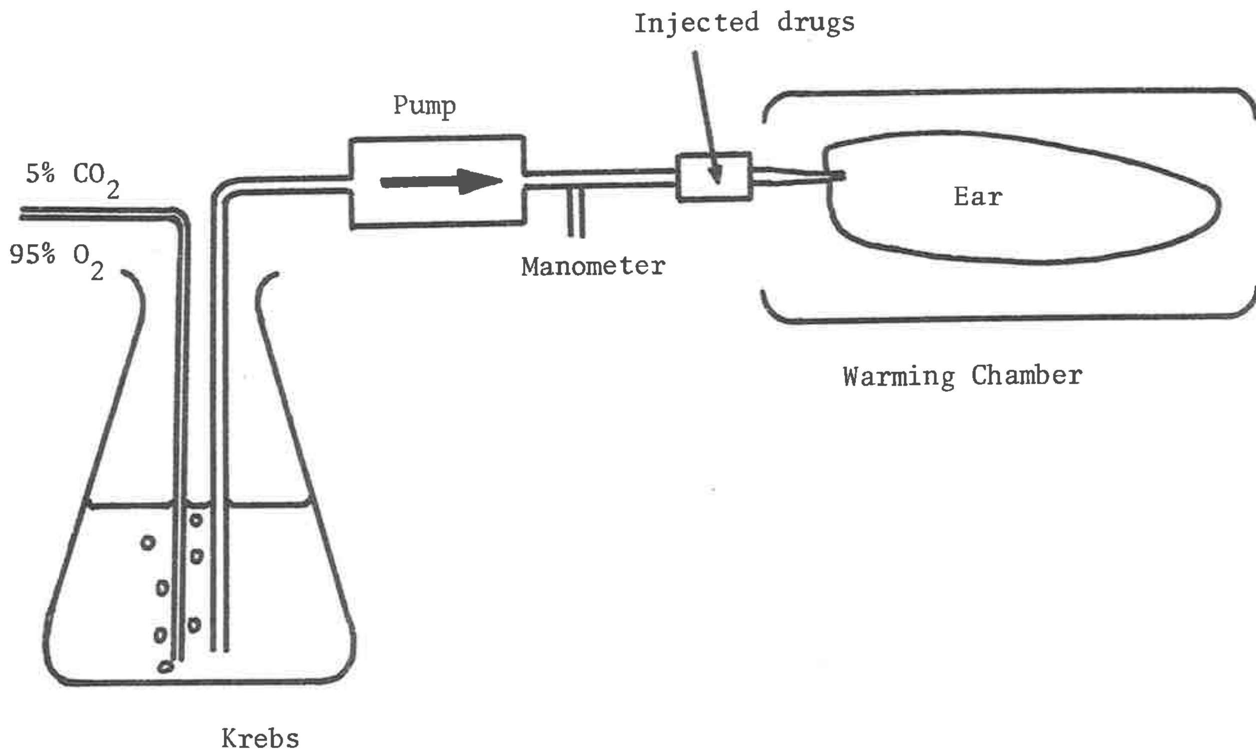



Figure 6.2

Diagram of the apparatus used to perfuse the isolated whole rabbit ear.

infuse POR8 at this concentration for several hours. During this time vasodilator stimuli, either nerve impulses (see Appendix Six) or the drugs acetylcholine or isoprenaline, were applied. Only one long term infusion of POR8 was made in each ear.

## RESULTS

In this work the terms *constriction* and *dilatation* will be used to describe the factors causing rise and fall of perfusion pressure. In vessels perfused at a constant rate perfusion pressure varies with flow resistance. It is recognised that in the whole ear changes in factors other than vessel diameter, notably the action of arteriovenous anastomoses in changing the total length of the vessels, may influence perfusion pressure. The above terms therefore involve a broad generalisation, but they have the advantage of common usage.

The perfusion pressure of the experimental system without drugs was in the range 25-40 mm Hg for different ears. Injections of ACh up to 10 μg did not cause constriction or dilatation of the ear vessels, only the transient increase in pressure corresponding to the injection of the drug being evident on the pressure record. This increase was seen with injections of all drugs, and with injections of saline, in both the ear and its isolated central artery.

### Infusion of POR8.

Long term infusion of POR8 caused a rapid increase in perfusion pressure which reached a peak and then declined to a steady raised level, where it remained for several hours (Figure 6.3). The concentration of POR8, the range of the perfusion pressure and the duration of the infusion are shown for each of ten experiments in Table 6.1. POR8

TABLE 6.1 The concentration of POR8, the range of the perfusion pressure during the infusion and the duration of the infusion for each of a series of 10 experiments using the isolated perfused whole rabbit ear.

| Experiment no. | POR8 concentration | Perfusion pressure | Duration |
|---|---|---|---|
| 1 | $2 \times 10^{-3}$ IU/ml | 110-80 mm Hg | 4.5 hours |
| 2 | $3 \times 10^{-3}$ IU/ml | 75-60 mm Hg | 3.8 hours |
| 3 | $5 \times 10^{-3}$ IU/ml | 90-110 mm Hg | 4.0 hours |
| 4 | $3 \times 10^{-3}$ IU/ml | 70-50 mm Hg | 3.7 hours |
| 5 | $3 \times 10^{-3}$ IU/ml | 90-110 mm Hg | 2.5 hours |
| 6 | $2 \times 10^{-3}$ IU/ml | 100-130 mm Hg | 4.0 hours |
| 7 | $3 \times 10^{-3}$ IU/ml | 60-80 mm Hg | 3.8 hours |
| 8 | $5 \times 10^{-3}$ IU/ml | 120-140 mm Hg | 3.5 hours |
| 9 | $5 \times 10^{-3}$ IU/ml | 110-150 mm Hg | 4.0 hours |
| 10 | $2 \times 10^{-3}$ IU/ml | 110-160 mm Hg | 2.8 hours |

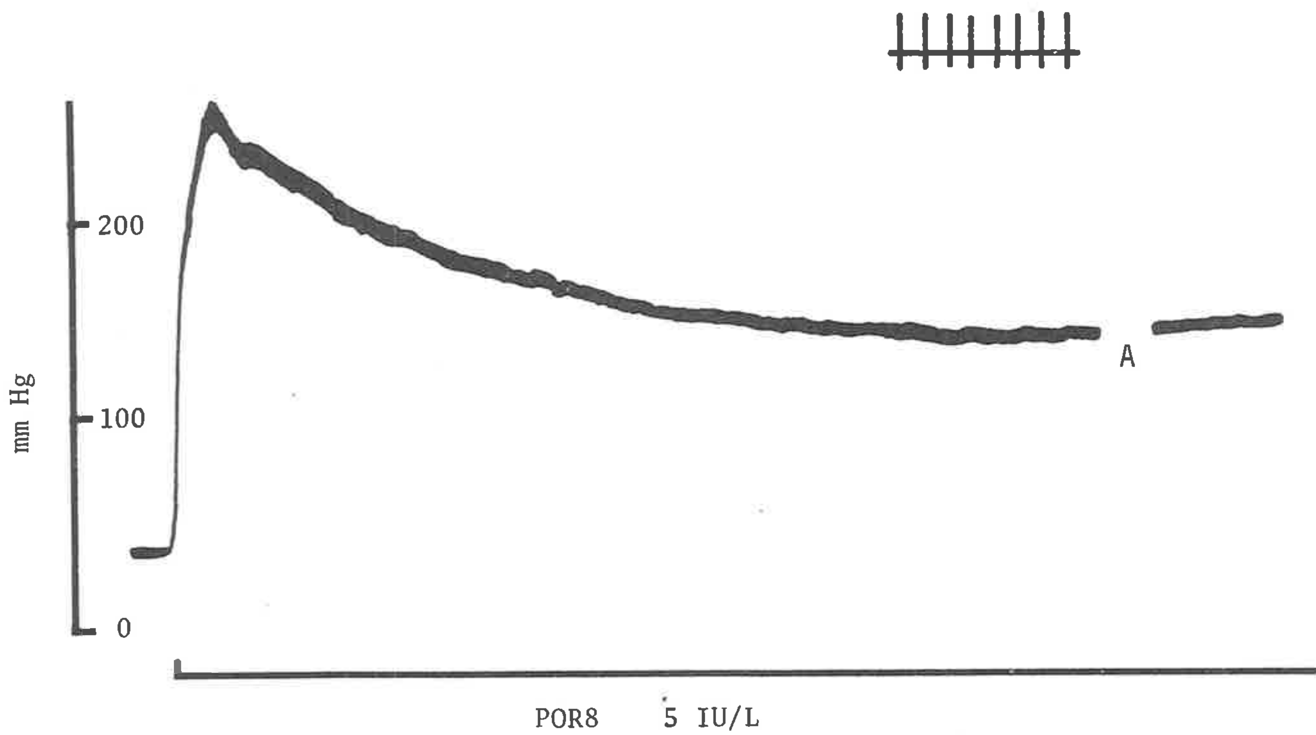



Figure 6.3

Showing the response to an infusion of POR8 5 IU/L in an isolated whole rabbit ear perfused with Krebs solution. The break at A omits three hours of the trace. Pressure scale in mm Hg, time course in minutes.

was effective in raising the perfusion pressure for at least two hours in each of twenty-seven experiments. All ears became oedematous after perfusion for several hours, and experiments were terminated when the sensitivity to dilator stimuli was lost.

Acetylcholine during POR8 infusion.

Injections of ACh caused dilator responses in each of 27 ears. The threshold for ACh's action varied between 2 ng and 10 ng in different ears, and the action was dose-dependent to approximately 100 ng (0.1 μg). The dilator response was rapid in onset, reaching a maximum within ten seconds of the injection. The pressure returned to the pre-injection level within one minute. Figure 6.4 shows dilator responses to ACh. In the presence of atropine 0.1 μg/ml injections of ACh of less than about 1 μg had no effect, and injections of ACh above this level caused vasodilatation.

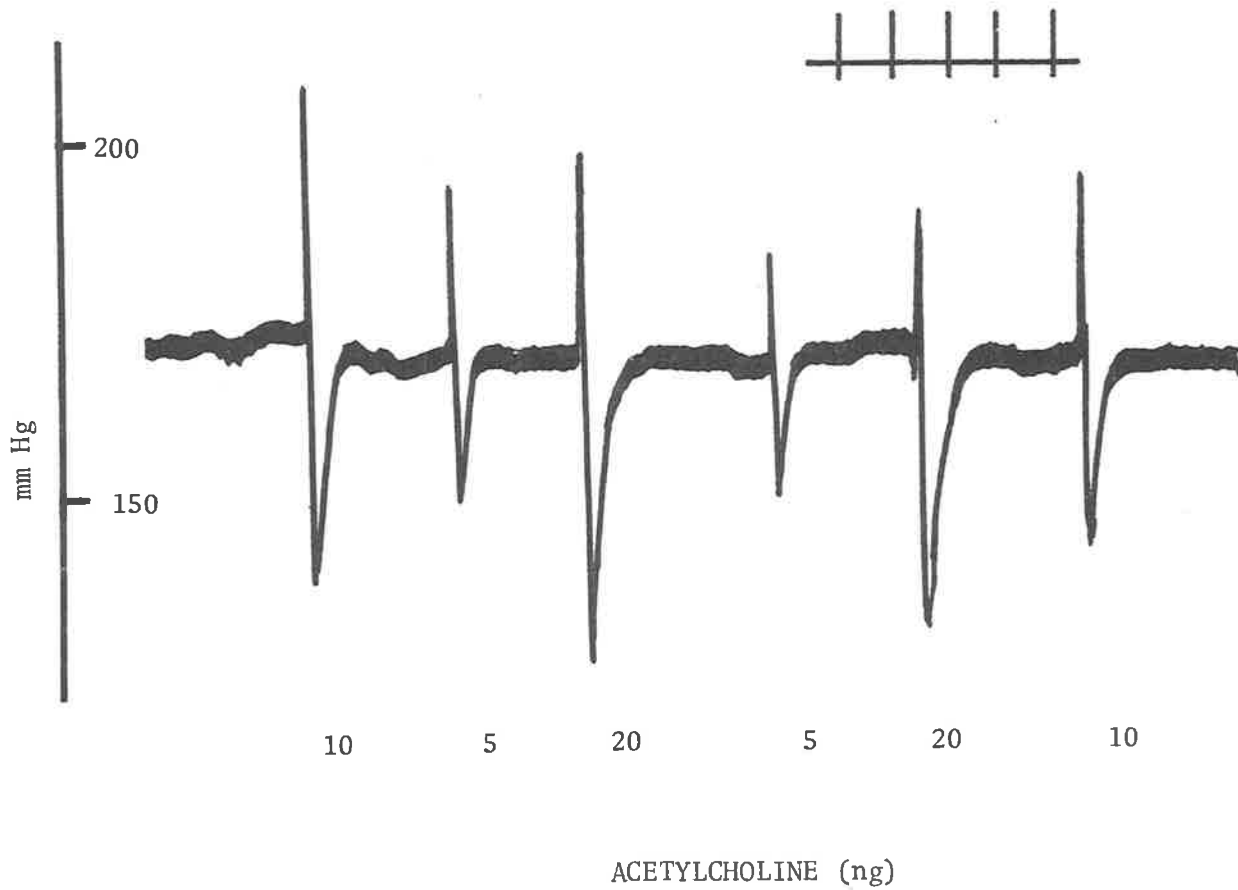



Figure 6.4

Showing responses to injections of acetylcholine in an ear perfused with Krebs solution containing POR8 5 IU/L. Numerals indicate dose of acetylcholine in nanograms (ng). Pressure scale in mm Hg, time trace in minutes. The upward deflection corresponds to the injection of the drug.

## DISCUSSION

Acetylcholine in nanogram quantities dilated the vessels of the isolated rabbit ear when the tone of the vessels had been raised with POR8. Similar sensitivity to ACh was reported in the blood-perfused ear of the anaesthetised rabbit (Holton and Perry, 1951) but not in the isolated perfused ear (Holton, 1956). In the experiments related to antidromic vasodilatation (see Appendix Six) the dilator response was also not seen unless the tone of the ear vessels was raised with POR8. This suggests that in isolated vascular preparations it may be necessary to raise the tone of the vessels to see the true sensitivity to dilator stimuli. Jeliffe (1962) noted that concentrations of ACh in the nanogram range relaxed rabbit aortic chains if they had been pre-constricted.

Experiments were undertaken in a relatively simple vascular system, the isolated perfused central artery of the rabbit ear, to further examine the effects of ACh. An important observation in the present Chapter, that ACh caused dilatation in the isolated whole ear when there was presumably no sympathetic nerve activity, is discussed in Chapter 9.

## SUMMARY

1. In the isolated whole rabbit ear, perfused with Krebs bicarbonate containing POR8, ACh in nanogram quantities caused vasodilatation.
2. The vasodilator action of ACh was prevented by atropine.

# CHAPTER SEVEN

## ACETYLCHOLINE AND SYMPATHETIC VASOCONSTRICTION

In morphological studies in preceding Chapters of this thesis a close association between cholinesterases and sympathetic nerve terminals was demonstrated in the rabbit ear artery. This Chapter describes experiments designed to test the physiological significance of this association, and also to examine further the dilator action of acetylcholine (ACh) observed in the perfused whole ear (Chapter 6).

As a starting point, the dilator effect of ACh seen when the tone of the artery was raised by sympathetic nerve stimulation, which was noted briefly by de la Lande and Rand (1965), was analysed in greater detail. The ability of anticholinesterase and anticholinergic agents to modify this effect was then examined in the hope that this might point to a role of endogenous ACh in the effects of stimulation.

At the time this study was commenced Malik and Ling (1969) reported that ACh both facilitated and depressed the response to sympathetic nerve stimulation in isolated rat mesenteric vessels, and considered that these results supported the 'cholinergic link' hypothesis of Burn and Rand (1960). Their results and interpretation were supported by a later study, contemporary with the present, of Rand and Varma (1970) in the isolated rabbit ear artery. However, as indicated by the studies in this and the following Chapter (the initial results

of which were reported at the same time as those of Rand and Varma - Hume, Waterson and de la Lande, 1970), the interaction between ACh and nerve stimulation is probably much more complex than that envisaged in the simple cholinergic link hypothesis proposed by the above workers.

Of necessity a division has been made in the presentation and discussion of the results of experiments in the isolated perfused artery. This Chapter deals only with results related to the interaction between ACh and responses to periarterial stimulation, and the effects of other agents on this interaction, since consideration of this facet alone is quite complex. It should be pointed out that in most of the experiments to be described constrictor responses to noradrenaline were used for purposes of comparison, and that the effect of ACh on these responses was notably different from ACh's effects on the responses to periarterial stimulation. This aspect, and studies which developed from it, are described in the next Chapter.

## MATERIALS AND METHODS

### Perfusion of the isolated rabbit ear artery.

Rabbits were prepared for cannulation of the rabbit ear artery as is described on page 6.2 for the whole ear. After exposure, the central artery was dissected free of surrounding tissue to the level of the artery's first major branch (see Figure 6.1). A cannula (heat drawn No. 3 Sterivac tubing) was inserted into the proximal part of the artery as described for the whole ear, and a finer cannula (No. 2 Sterivac) was inserted just proximal to the branch, using a similar technique but with the tip of the cannula pointing centrally. This left a segment of artery some 15-20 mm in length between the cannulae. The double cannulated segment was removed from the animal and placed in a dish of warm Krebs solution and gently flushed through with the same fluid. As soon as was practical the artery was set up in a vertical double jacketed organ bath, and bathed extraluminally and perfused intraluminally with Krebs solution bubbled with 5% carbon dioxide 95% oxygen. The perfusion system was maintained at 37$^{o}$C unless specificied otherwise in the text. The distal (upper) cannula was held in a beam device adjusted to apply 1 gram tension to the artery. Figure 7.1 illustrates the perfusion set-up, which is essentially that described by de la Lande, Cannell and Waterson (1966). The perfusion flow rate was in the range 5.5-6.5 ml/min, but constant during each experiment. Changes in perfusion pressure were measured

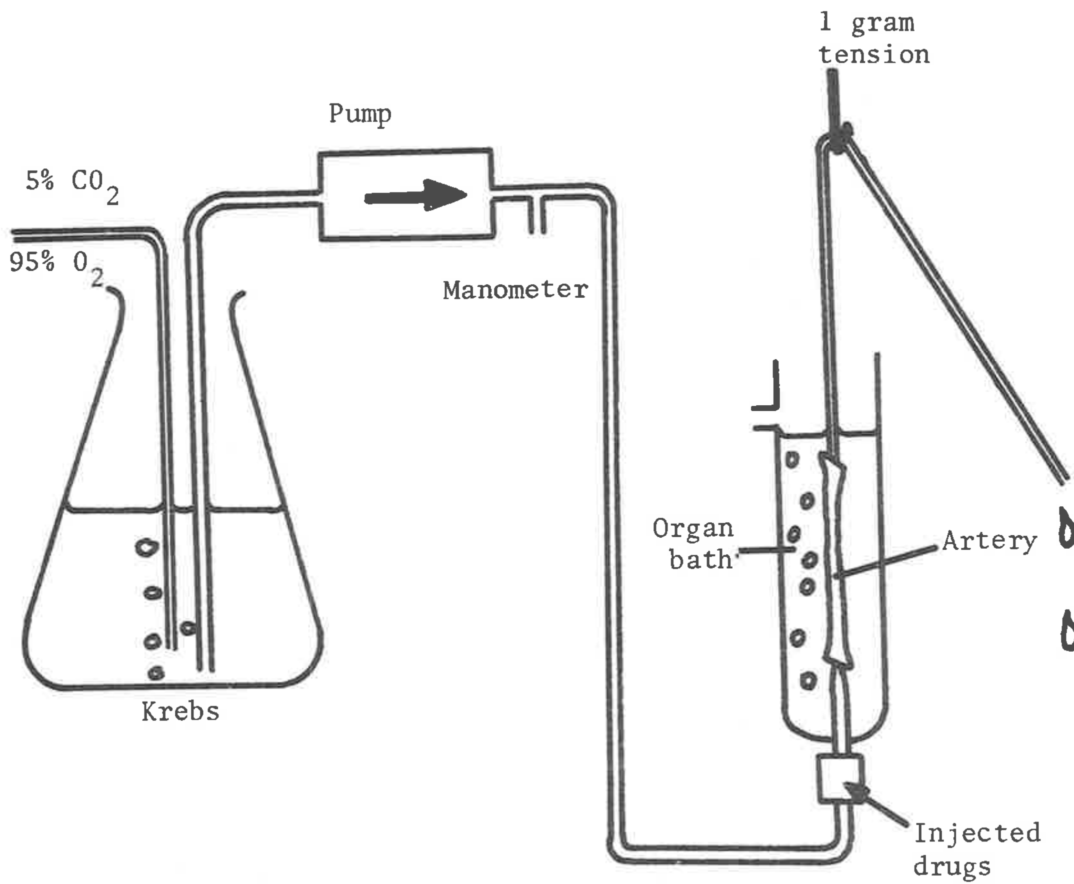



Figure 7.1

Diagram of the apparatus used to perfuse the isolated, double cannulated, rabbit ear artery.

with a mercury manometer and recorded on a Kymograph or with a pressure transducer (Ether Engineering) and a potentiometric recorder (Rikadenki Kogyo Co.) (see Appendix, page 6). In most experiments two arteries from the same animal were perfused at the same time, using two recording systems.

In some experiments arteries were cannulated at the proximal end only, as described by de la Lande and Rand (1965) and perfused with McEwen's solution (McEwen, 1956). The procedure for removal and perfusion of the artery was similar to that described above, but a distal cannula was not used, and the artery was not under tension in the bath.

Electrical stimulation.

Bipolar platinum electrodes (see Appendix, page 6) were placed around the artery in the region where the lumen contained the tip of the proximal cannula, but distal to the cotton ligature around the vessel, as illustrated in Figure 7.2. Square wave pulses of 0.3 or 1 msec duration (pulse length) were applied with either a Grass S4 or an Eilco 6418 stimulator, at a voltage adjusted to be maximal for each experiment. Pulses were applied at different frequencies either in ten second trains with intervals of three or six minutes between trains, or continuously for up to one hour.

Drugs.

In double cannulated arteries, drugs could be applied separately

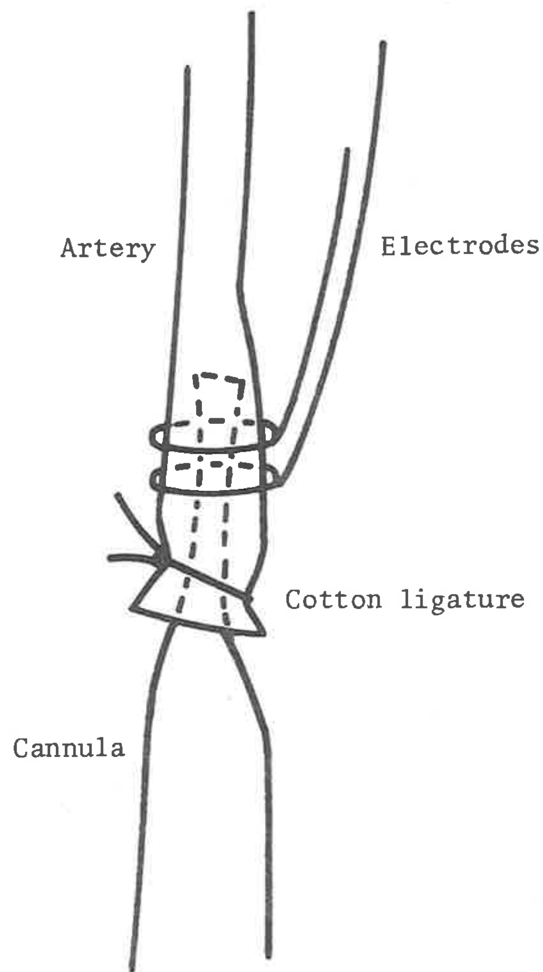



Figure 7.2

Diagram showing the position of the ring electrodes in relation to the proximal cannula and the isolated artery.

to the intraluminal or extraluminal surfaces. The effluent from the distal cannula did not mix with the bathing fluid.

Drugs were applied in one or more of three ways:

(i) *injection* - small volumes of solutions of drugs (0.05 - 0.1 ml) were injected into the perfusion stream just proximal to the artery;

(ii) *infusion* - drugs were added to the reservoir of perfusion fluid;

(iii) *extraluminal administration* - drugs in a volume of 0.05 - 0.5 ml were added to the 10 ml organ bath and were washed out by flushing the bath twice with 20 ml lots of fresh Krebs solution.

The duration of the types of application described in (ii) and (iii) above varied from less than one minute up to several hours. In single cannulated arteries drugs were added by injection or infusion only. Details of the source and dilution of all drugs used in this study are found in Appendix, page 7.

Neither electrical pulses nor drugs were applied to arteries during the first hour of perfusion.

## RESULTS

### Effect of ACh on perfusion pressure.

Acetylcholine had no effect on the resting perfusion pressure of 4 arteries when applied intraluminally (by infusion) or extraluminally in concentrations of $10^{-6}$ μg/ml (1 pg/ml) to 10 μg/ml. Injections of ACh in a similar range of doses (1 pg-100 μg) also had no effect on the resting perfusion pressure, in both normal arteries and in the presence of atropine 0.1 μg/ml or 1 μg/ml (6 arteries).

### Response to periarterial stimulation.

The patterns of response to periarterial electrical stimulation in the isolated perfused rabbit ear artery have been described by de la Lande and Rand (1965), and are evident in the figures which follow in the present work. Stimulation caused vasoconstriction in the arterial segment, with a consequent rise in the pressure in the perfusion system. Arteries differed in their responses, but biphasic responses as noted by de la Lande, Frewin and Waterson (1967) and later investigated by Bevan and Waterson (1971) were commonly observed. In experiments where arteries were stimulated with intermittent ten second trains of periarterial pulses, over the first hour the responses at first decreased and then rose to a steady level (Figure 7.3). In most arteries the responses tended to increase in magnitude over several hours, but if this tendency was marked (Figure 7.4) the experiment was discontinued.

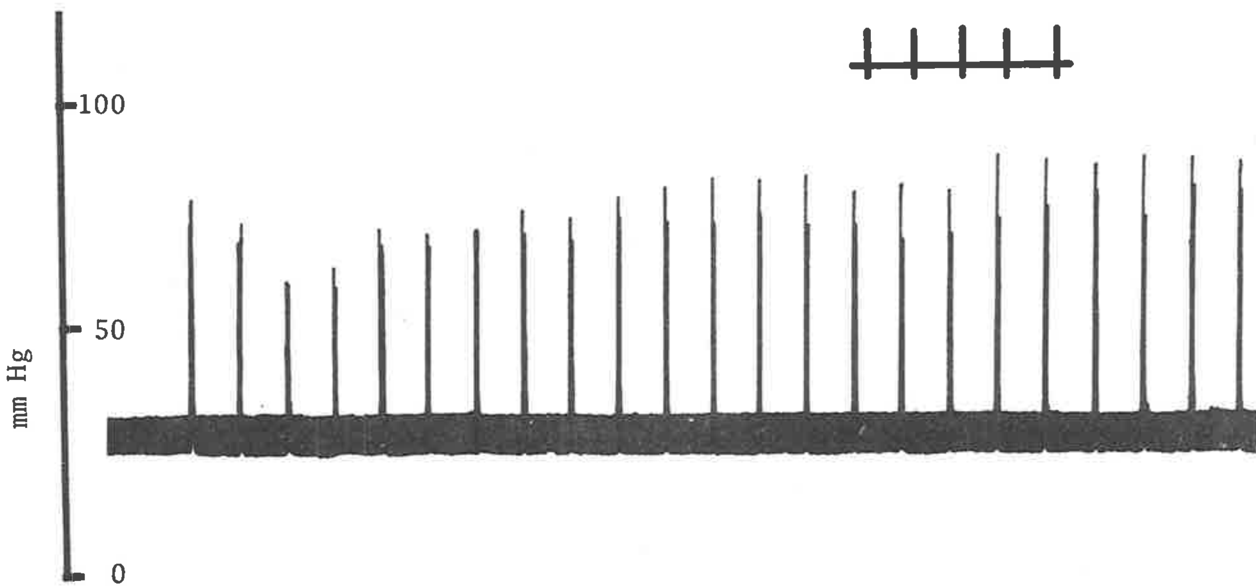



Figure 7.3

Showing the responses to intermittent trains of periarterial electrical pulses (60V, 1 msec duration, 3 pulses/sec, 30 pulses) at four minute intervals during the first 50 minutes of stimulation. No drugs were applied. Time scale in four minute intervals, pressure scale in mm Hg.

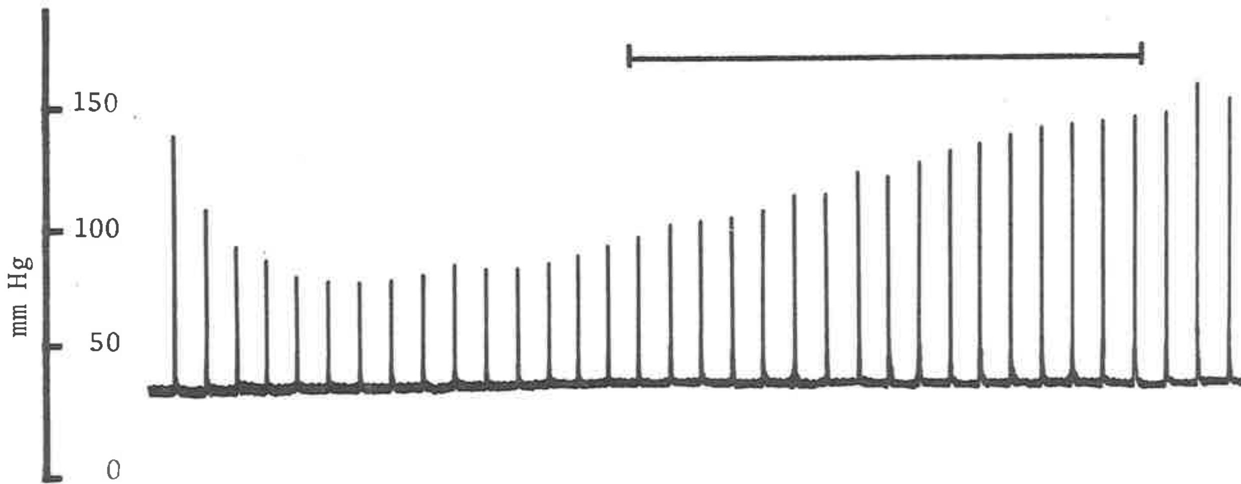



Figure 7.4

Showing the responses to intermittent trains of periarterial electrical pulses (50v, 0.3 msec duration, 30 pulses) at 3 minute intervals during the first two hours of stimulation. No drugs were applied. Time scale shows one hour. Pressure scale in mm Hg.

Depression of responses by ACh.

ACh, applied intraluminally or extraluminally, depressed the response to periarterial stimulation. The effect was demonstrated either by addition of ACh during a sequence of intermittent ten second trains of pulses (Figure 7.5), or by adding ACh during sustained stimulation, when the pressure was raised (Figure 7.6). The first method was used in most experiments, and was suitable for comparing the effects of ACh on different stimulus parameters, and on responses to alternate applications of electrical pulses and drugs. The second method was used for determining the time course of the inhibition, and also for assessing the effects of injected ACh. With either method, the inhibitory effect of ACh was shown to be dose dependent (Figure 7.7), the threshold varying from 2 ng/ml-20 ng/ml in different arteries. The inhibitory effect was rapid in onset on application of ACh, and the responses or the perfusion pressure returned rapidly to the pre-ACh level on washout of the drug. The intraluminal and extraluminal potencies of ACh were of the same order, and ACh was more effective in depressing responses to low frequency periarterial stimulation than to high, as shown in Figure 7.8. This latter effect was noted in each of 4 arteries in which stimulus frequency was varied in the range 1-32 or 1-64 pulses/sec.

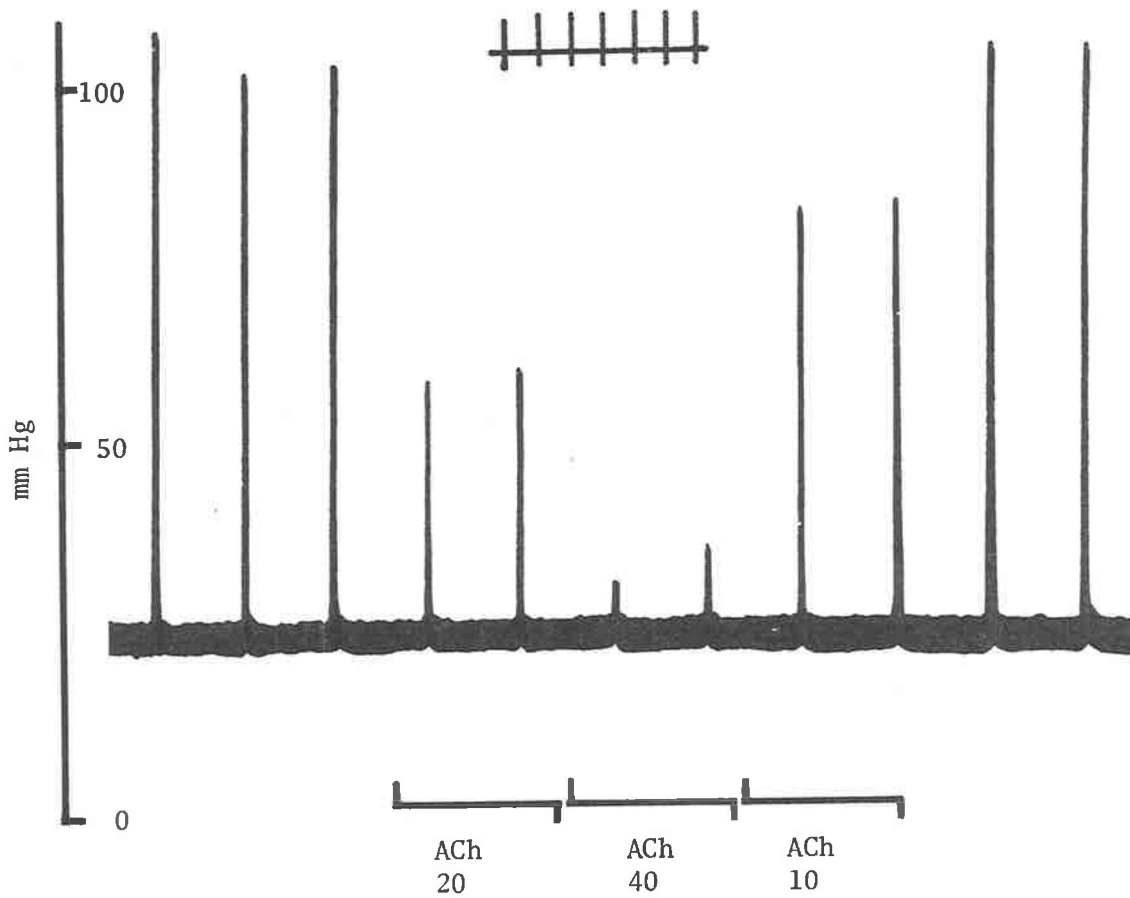
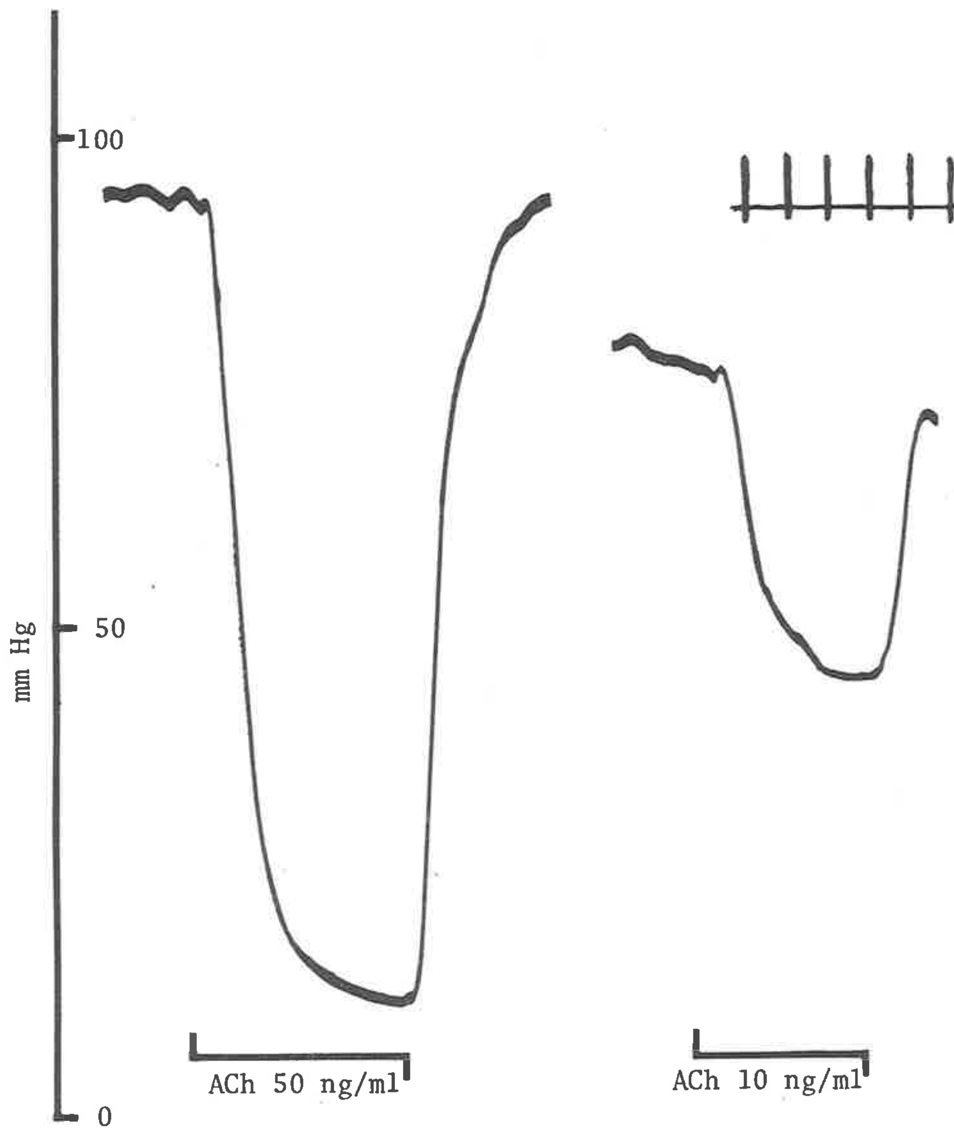



Figure 7.5

Effect of extraluminal ACh 20, 40 and 10 ng/ml on the response to periarterial electrical stimulation (40 V, 1 msec duration, 3 pulses/sec, 30 pulses). Time scale in minutes, pressure scale in mm Hg.




<u>Figure 7.6</u>

Effect of extraluminal ACh 50 ng/ml and 10 ng/ml applied during continuous periarterial electrical stimulation (40 V, 1 msec duration, 2 pulses/sec). Time scale in minutes, pressure scale in mm Hg.

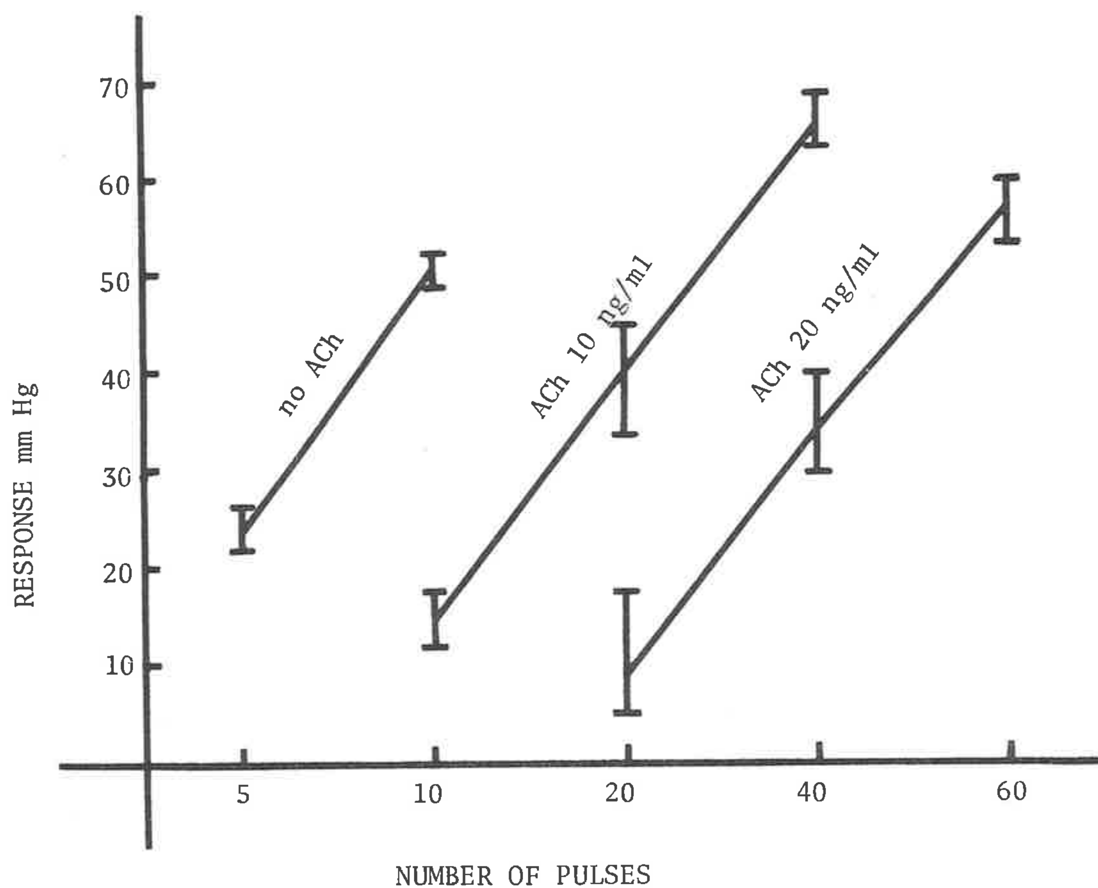



Figure 7.7

An experiment showing the effects of ACh on responses to periarterial electrical stimulation. Ordinate: response (mm Hg). Abscissa: number of pulses at 30V, 1 msec duration, 10 pulses/sec. The magnitude of responses in one artery in the presence of ACh 10 and 30 ng/ml applied extraluminally are shown. The mean and range of three responses are shown for different numbers of pulses.

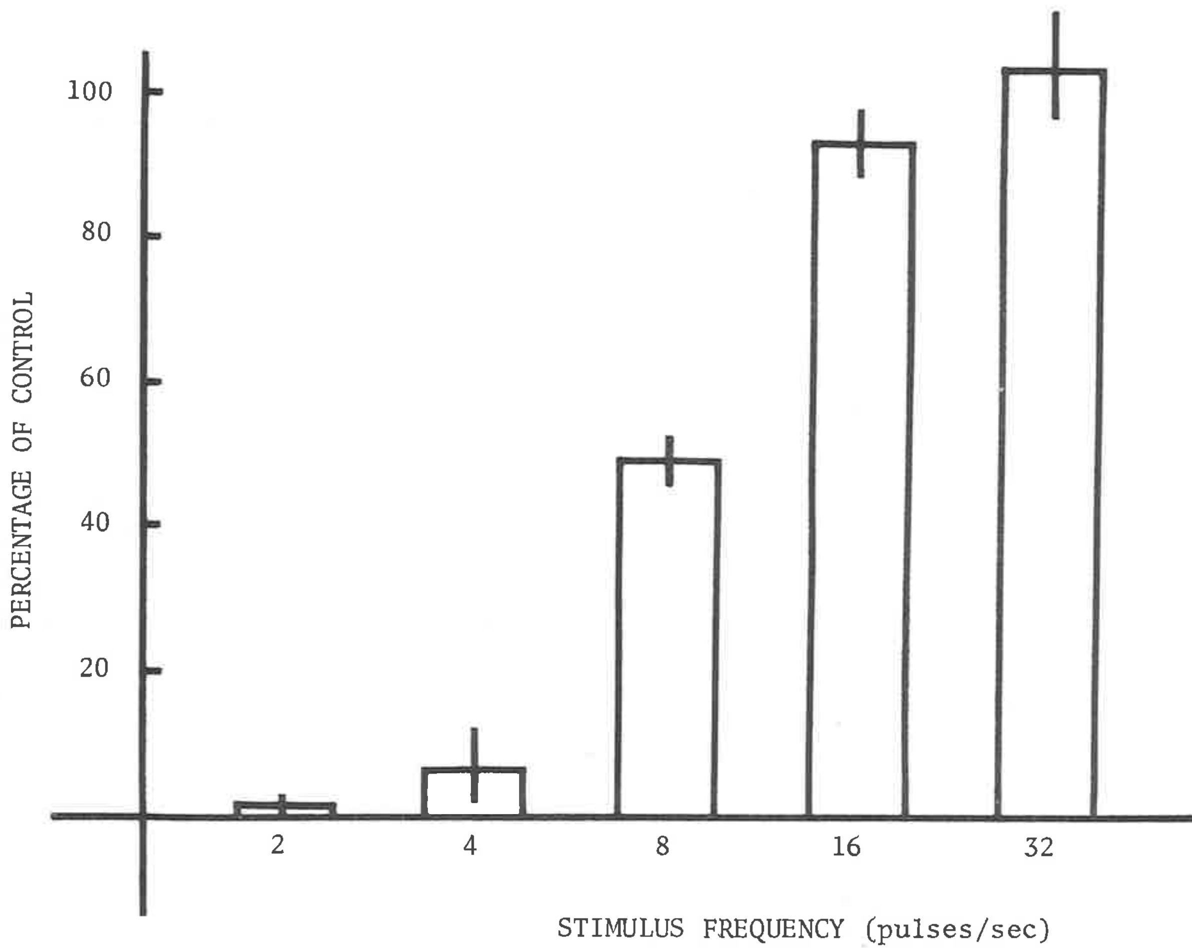



Figure 7.8

Showing the effect of intraluminal ACh 0.1 μg/ml on the maximum response to continual periarterial stimulation (50V, 0.3 msec duration) at different frequencies in one artery. The height of each column represents the mean of two responses in the presence of ACh expressed as a percentage of the control at the same frequency, and the vertical bar the range.

Possible potentiation of responses by ACh.

In concentrations below those which caused inhibition, ACh did not cause potentiation of responses. Attempts to demonstrate the phenomenon described by Malik and Ling (1969) were made by applying ACh in low concentrations (10 pg/ml-500 pg/ml) for 30-120 minutes at stimulus frequencies of 1-20 pulses/sec. No change in responses related to the ACh application was observed in 5 arteries perfused at 37°C. In 2 of 4 arteries perfused at 24°C the response to stimulation (7 pulses/sec) increased during the period of application of ACh 50 pg/ml, but in both cases the increase was explicable in terms of spontaneous increase of sensitivity. In one of the arteries the response to injected noradrenaline also increased during ACh, and neither increase was reversible on ACh washout. In the second case the responses in a control artery, perfused without ACh, also increased over the same time course.

The responses to periarterial stimulation sometimes exceeded the pre-ACh levels in the 10 minutes after washout of a dose of ACh which had caused depression (Figure 7.9). The increases were of the order of 10% of the response height, and were observed in only 15 of 46 arteries in which such an effect was sought. The slight and variable nature of the increases made analysis difficult, but the following observations suggested that the effect may not have been due to a direct action of ACh.

(i) When, *in the absence of ACh*, the stimulus was omitted for a period corresponding to the normal period of application of ACh (approximately 12 minutes) a similar slight increase in the height of the first one or two responses after resuming the stimulus sequence was observed in 7 out of 13 arteries (Figure 7.10).

(ii) An increase was not observed (14 arteries) on addition or washout of ACh when the depressant effect of ACh was prevented by the simultaneous application of atropine (see atropine, below).

(iii) Since it might be argued that the lack of increase described in (ii) above indicated that the potentiation was a muscarinic action of ACh, atropine was added either prior to or simultaneously with ACh washout. In 8 of 15 arteries, the responses *were* restored transiently to a level slightly above that of the pre-ACh responses (Figure 7.11).

As it was possible that the use of Krebs solution and double cannulated arteries may have contributed to the failure to demonstrate direct potentiation by ACh, 4 arteries were perfused under the conditions of Rand and Varma (1970), that is, using single cannulated arteries and McEwen's solution. The effects of ACh on the responses of the latter arteries were qualitatively identical with those on the double cannulated arteries described above.

Atropine-like compounds.

Neither atropine 0.1 μg/ml, propantheline 6 μg/ml nor hyoscine n-butylbromide 2 μg/ml altered the response to periarterial stimulation, but these drugs at the stated concentrations raised the threshold for the action of ACh on the responses to periarterial stimulation to approximately 1 μg/ml and reduced ACh's inhibitory action (Figure 7.12). These effects were observed in each of 12 arteries.

Cholinesterase inhibitors.

Physostigmine 0.3 μg/ml, di*iso*propylfluorophosphate (DFP) 5 μg/ml or tacrine 10 ng/ml did not alter the responses to periarterial stimulation at 1-20 pulses/sec when applied for up to two hours in a total of 8 arteries. These drugs at the stated concentrations consistently potentiated the depressant effect of ACh on the responses, both with respect to magnitude and threshold (Figure 7.13). The effect of ACh was not tested until after more than 30 minutes exposure to the anticholinesterase drug. ACh below the level for depression of responses had no effect.

The effects of higher concentrations of cholinesterase inhibitors were examined. Physostigmine 2 μg/ml and above potentiated the response of the artery to periarterial stimulation, the potentiation increasing with increase of physostigmine concentration (Figure 7.14). The effect was observed in each of 8 arteries, using stimulus frequencies of 2-10 pulses/sec. The potentiation was rapid in onset on

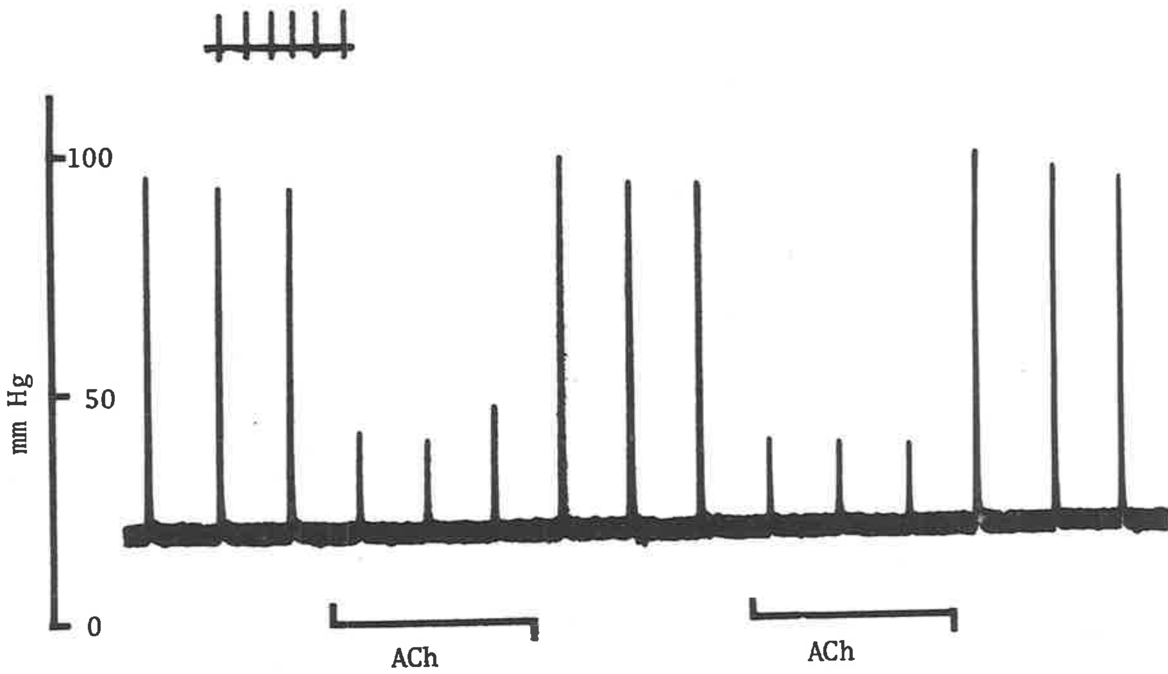



Figure 7.9

Effect of extraluminal ACh 0.1 μg/ml on the responses to periarterial electrical stimulation (40 V, 0.3 msec duration, 3 pulses/sec, 30 pulses). Time scale in minutes, pressure scale in mm Hg.

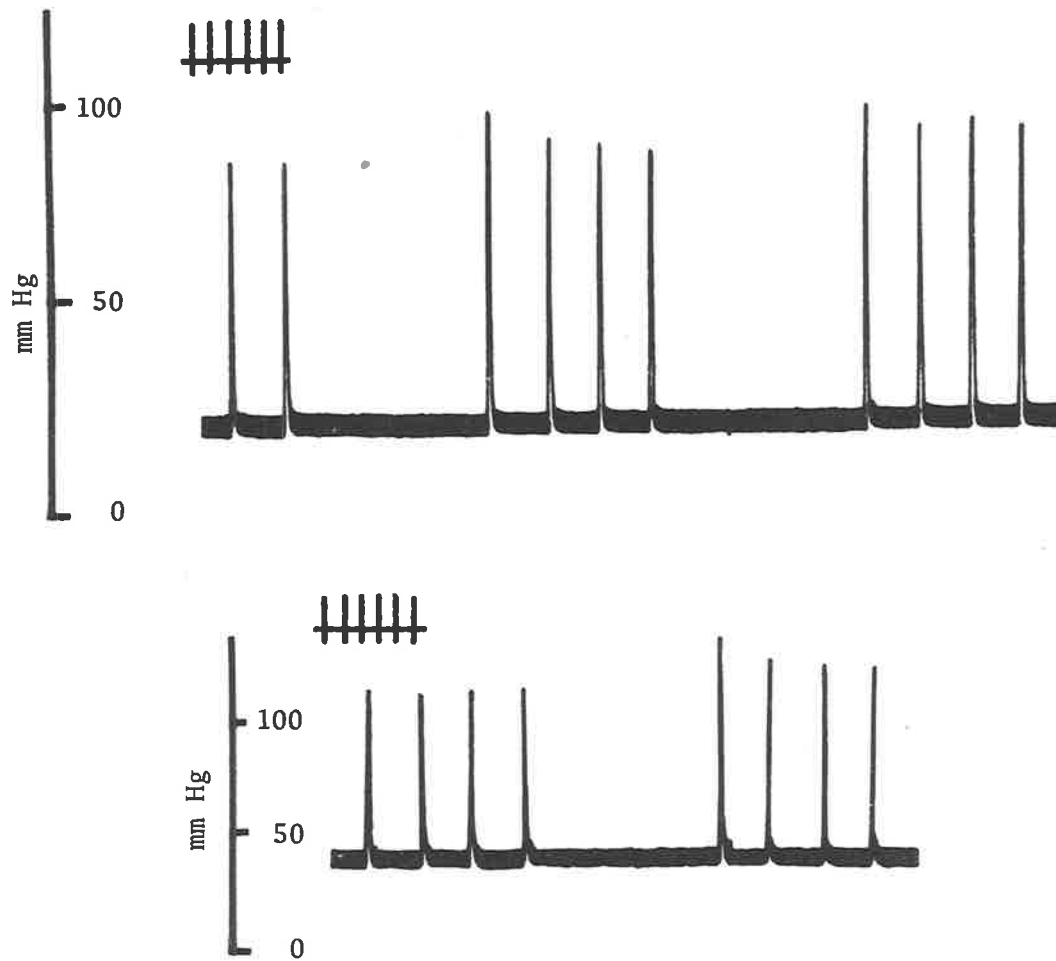



Figure 7.10

Showing responses to periarterial electrical stimulation in two arteries from different animals. Upper trace; 40 V, 1 msec duration, 3 pulses/sec, 30 pulses. Lower trace: 40 V, 0.3 msec duration, 2 pulses/sec, 20 pulses. No drugs were applied. Time scale in minutes, pressure scale in mm Hg.

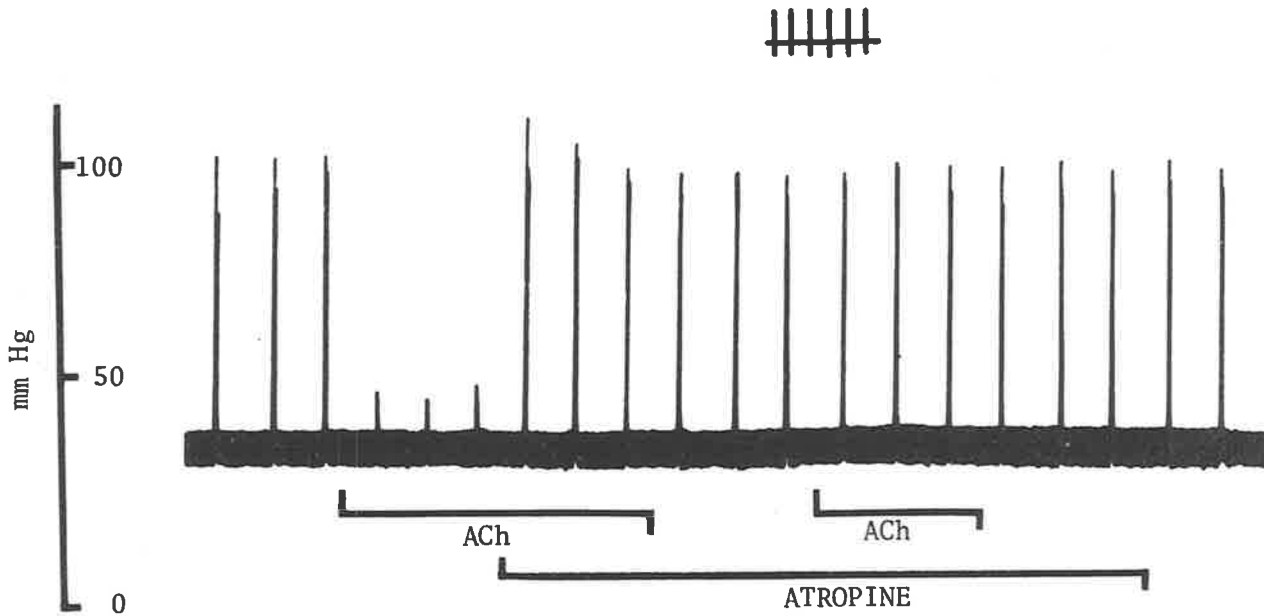



Figure 7.11

Showing the effect of extraluminal ACh 0.1 μg/ml and extraluminal atropine 0.1 μg/ml on the responses to periarterial electrical stimulation (40 V, 1 msec duration, 2 pulses/sec, 20 pulses). Time scale in minutes, pressure scale in mm Hg.

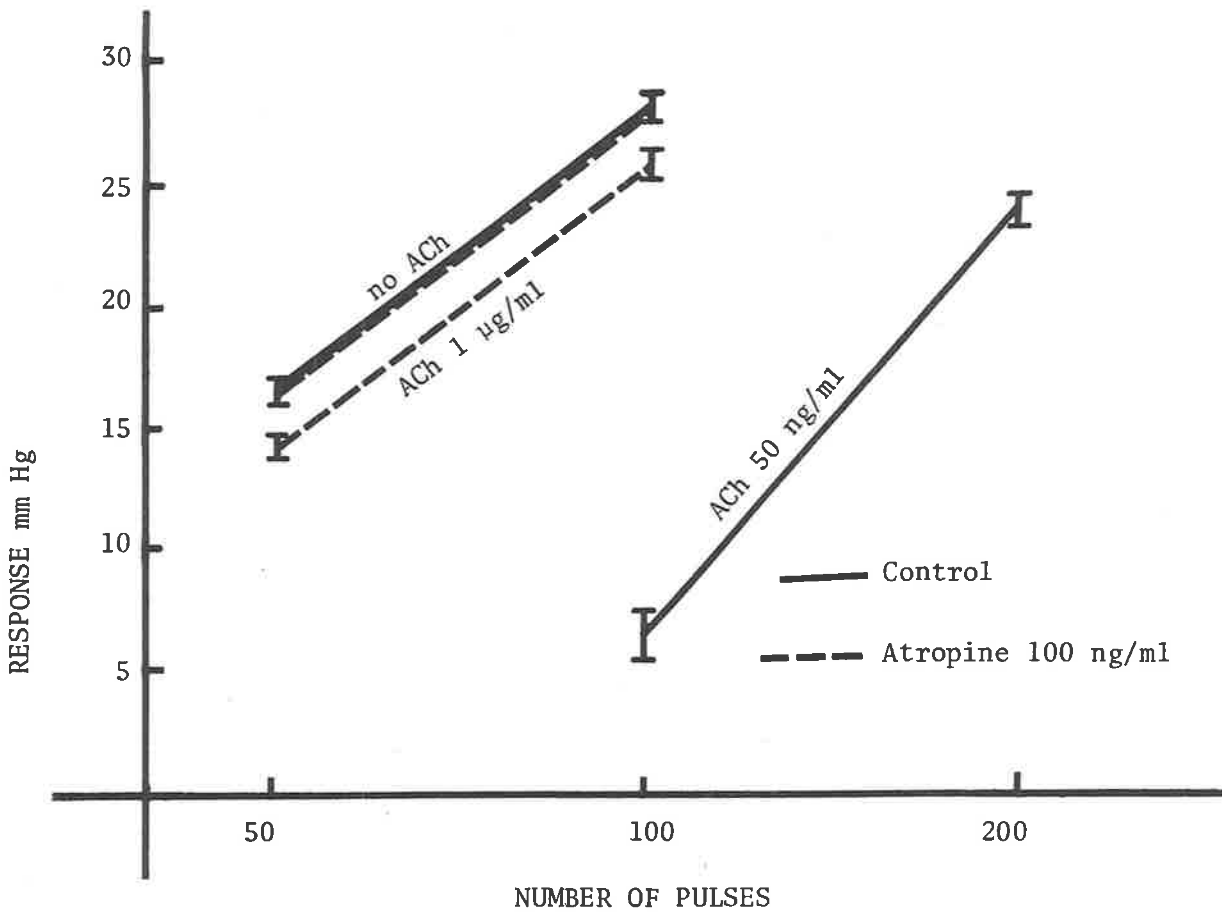



Figure 7.12

An experiment showing the interaction between ACh, atropine and the responses to periarterial electrical stimulation. Ordinate: response (mm Hg). Abscissa: number of pulses at 30V, 1 msec duration, 10/sec. The magnitude of the responses in one artery is shown before (no ACh) and during extraluminal acetylcholine (either ACh 50 ng/ml or ACh 1 μg/ml) in the absence of atropine (unbroken lines) and in the presence of intraluminal and extraluminal atropine 100 ng/ml (broken lines). The mean and range of three responses are shown for different numbers of pulses.

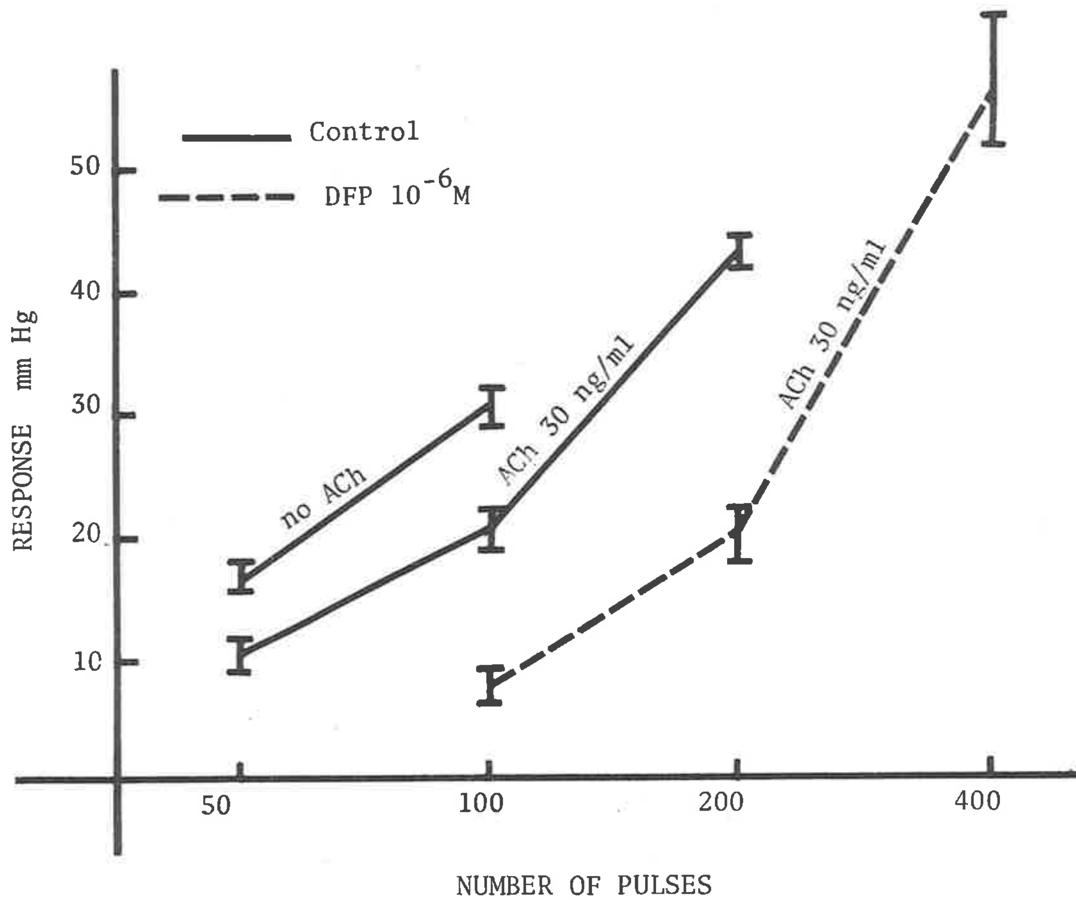



Figure 7.13

An experiment showing the effect of ACh and DFP on the responses to periarterial electrical stimulation. Ordinate: response (mm Hg). Abscissa: number of pulses at 35V, 1 msec duration, 10/sec. The magnitude of the responses in one artery is shown before (no ACh) and during extraluminal acetylcholine (ACh 30 ng/ml) in the absence of DFP (unbroken lines) and in the presence of DFP $10^{-6}$M applied intraluminally and extraluminally for at least 30 min. (broken lines). The mean and range for responses are shown for different numbers of pulses.

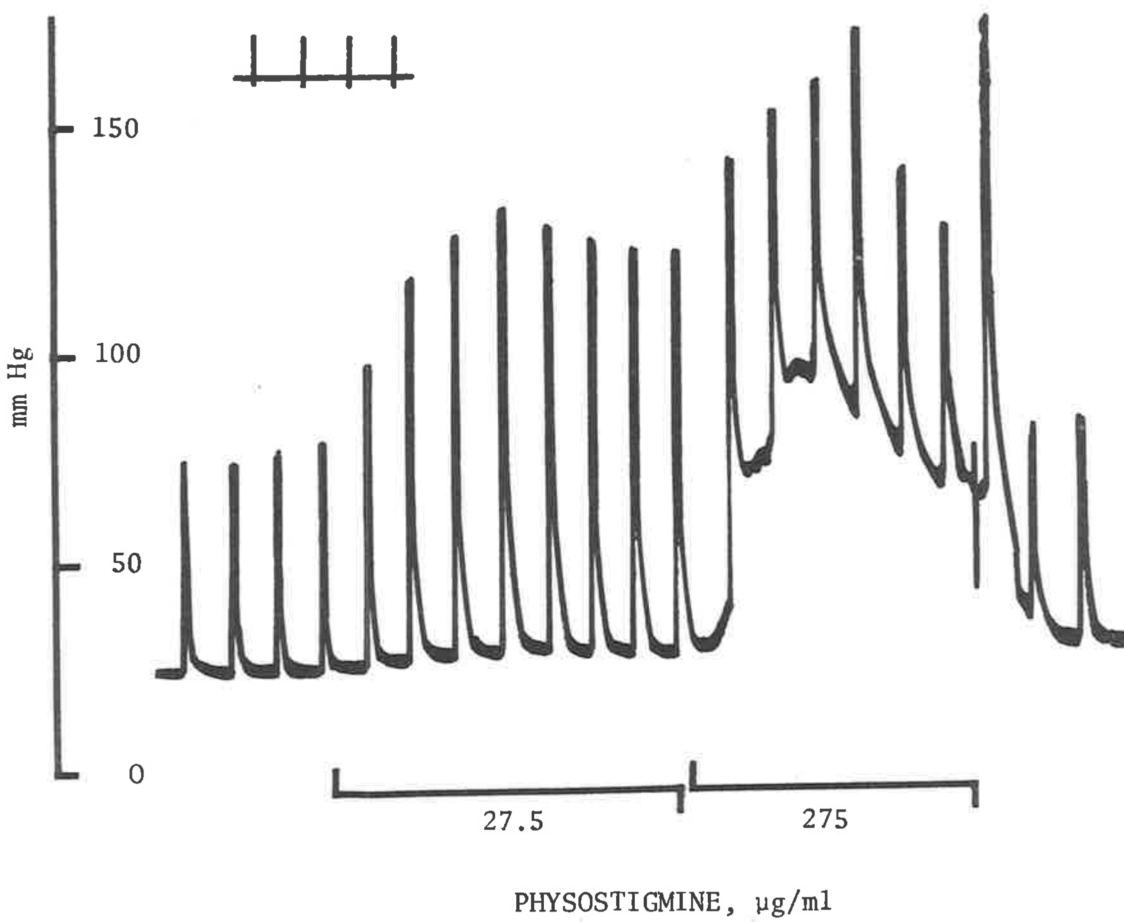



Figure 7.14

Effect of extraluminal physostigmine 27.5 and 275 μg/ml on the responses to periarterial electrical stimulation (50 V, 1 msec duration, 2 pulses/sec, 20 pulses). Numerals indicate the concentration of physostigmine in μg/ml. Time scale shows four minute intervals, pressure scale in mm Hg.

application of the drug, and the responses decreased in magnitude after washout. But the effect of ACh in depressing the responses to periarterial stimulation, which was enhanced after the application of physostigmine, remained enhanced for more than one hour (Figure 7.15). Neither the AChE inhibitor BW 284C51 nor the ChE inhibitor *iso*OMPA increased the responses to periarterial stimulation in concentrations up to 100 μg/ml in 4 arteries. In 3 arteries extraluminal neostigmine 10 μg/ml and 100 μg/ml potentiated the response to periarterial stimulation (2 pulses/sec). The responses returned to the pre-neostigmine levels within 1-2 minutes after washout of the drug, and the phenomenon was repeated several times in the course of one hour in each of the vessels. In 2 arteries DFP180 μg/ml, extraluminally applied, potentiated the response to periarterial stimulation (2 pulses/sec). The potentiation was rapidly reversible on washout of the drug, and was repeated on a second application less than an hour later.

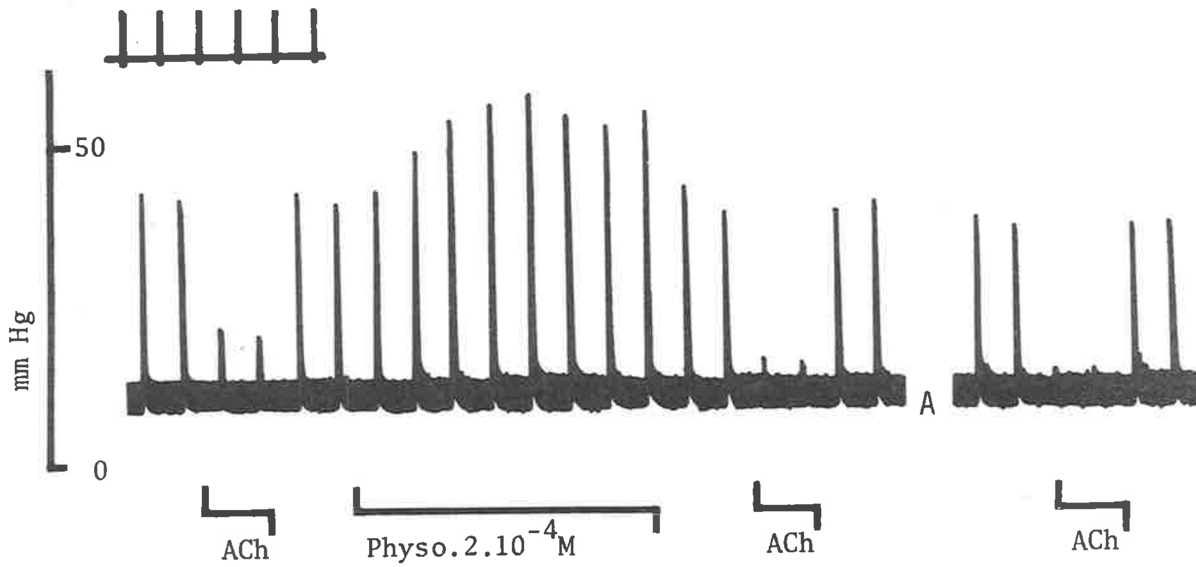



Figure 7.15

Effect of extraluminal ACh 20 ng/ml and extraluminal physostigmine 8 μg/ml on the responses to periarterial electrical stimulation (40 V, 1 msec duration, 3 pulses/sec, 30 pulses). The break at A omits 36 minutes of the record. Time scale shows four minute intervals, pressure scale in mm Hg.

## DISCUSSION

It has been established both by de la Lande and Rand (1965) and by de la Lande, Frewin and Waterson (1967) that the isolated perfused rabbit ear artery constricts in response to transmural electrical stimulation via the action of the artery's perivascular sympathetic nerves. In the present work, where periarterial electrodes were employed (method 2 of de la Lande and Rand, 1965), the obligatory role of the sympathetic nerves in the response was checked by perfusing five sympathetically denervated arteries (Chapter 8). These vessels did not respond to stimulation using parameters which caused extreme constriction in control arteries.

### Depression of responses by ACh.

The present results showed that low levels of ACh depressed the response of the isolated rabbit ear artery to periarterial stimulation, and that this action was muscarinic, since it was prevented by atropine and similar compounds. The same result was reported independently by Rand and Varma (1970). A muscarinic vasodilator effect of ACh and other choline derivatives was noted by Dale (1914), and it is possible that the depression of responses in the rabbit ear artery was due to such an action. But Rand and Varma (1970) interpreted the phenomenon as evidence that ACh may act to inhibit the release of noradrenaline from sympathetic nerves. This interpretation is discussed, and further experiments related to it are described in Chapter 8.

Possible potentiation of responses by ACh.

In this work no conclusive evidence for a facilitatory role of exogenous ACh on noradrenaline release was seen. Other workers have described four types of action by ACh on the responses to sympathetic nerve stimulation which might suggest such an action, and these are discussed in turn in relation to the present results below.

(i) In arteries perfused at 37°C, ACh 10 pg/ml-500 pg/ml had no effect on the response to stimulation when applied for up to two hours, using a wide range of stimulus frequencies. Malik and Ling (1969) observed potentiation of responses to sympathetic nerve stimulation in rat mesenteric vessels perfused at 22°C in the presence of ACh 50 pg/ml. They did not report on this effect at 37°C. In the present study such increases were seen in two arteries perfused at 24°C, but these increases were explicable in terms of spontaneous increase in sensitivity. It should be noted that the perfusion system described by Malik and Ling (1969) differs from that described in this work. Their perfusion rate was 25 ml/min compared with 5.5-6.5 ml/min, and their perfusion pressure was 85 mm Hg compared with 10-20 mm Hg. These factors may be responsible for differences in results under otherwise similar conditions.

(ii) Rand and Varma (1970) noted facilitation of responses to sympathetic nerve stimulation in the rabbit ear artery after brief infusions or single injections of ACh. They observed that this action

was not altered by atropine 0.1 μg/ml. In the present study no such effect of ACh was observed in the presence or absence of atropine, or in double or single cannulated arteries, unless ACh first caused a depression of responses (see below). The failure to observe this type of facilitation in this work cannot be explained.

(iii) After washout of ACh which had caused a depression of responses to periarterial stimulation, the responses sometimes exceeded their pre-ACh height. A similar phenomenon was observed both by Malik and Ling (1969) in rat mesenteric vessels and by Rand and Varma (1970) in the rabbit ear artery. But analysis of the present results, using a large number of arteries, indicated that the increase was a function of the prior depression, rather than a direct action of ACh (see page 7.9). It is possible that the increase was related to rest in a system which had stabilised under conditions of repeated regular stimuli, since similar increases were also seen in some vessels after a pause in repeated applications of exogenous noradrenaline.

(iv) There is a body of favourable evidence concerning the release of noradrenaline from sympathetic nerve terminals by high concentrations of ACh, which has been reviewed by Furchgott (1955) and more recently by Somlyo and Somlyo (1970). In the rabbit ear artery Furchgott, Steinsland and Kirpekar (1971) have demonstrated such release by ACh, measuring the output of isotopically-labelled noradrenaline. In the present work, where a constrictor response would have indicated

such an action of ACh, no evidence of noradrenaline release was seen. It is suggested that the response to any noradrenaline released may have been masked by the action of ACh on the arterial smooth muscle, which is discussed in the next Chapter.

Cholinesterase inhibitors

Cholinesterase inhibitors in concentrations which were effective in other systems (Long, 1963) enhanced the depressant effect of exogenous ACh on the response to periarterial stimulation, indicating both that the artery contained cholinesterases which could influence ACh and that the inhibitors were effective in the vessel. Yet such concentrations of inhibitors alone did not alter the response of the artery to stimulation. Therefore, if there was a role for ACh in the noradrenaline release mechanism, as suggested in the 'cholinergic link' hypothesis, this would seem not to be influenced by anticholinesterase drugs at the levels used, and not influenced by the functionally demonstrable cholinesterase.

Higher concentrations of physostigmine did potentiate the response to periarterial stimulation. A similar phenomenon was observed in rat mesenteric vessels perfused at 30°C by Malik (1970). Malik interpreted the phenomenon in terms of the 'cholinergic link' concept, but two main aspects of the present results were not consistent with such a suggestion.

(i) The doses of the drug needed to cause the potentiation were extremely high. Data on effective dose levels of physostigmine in the rabbit was provided by the work of Shelley (1955), who observed that in the isolated rabbit duodenum physostigmine's effect on muscle tone was first noted at $2.7 \times 10^{-8}$M, and reached a maximum at $2.7 \times 10^{-6}$M, the full effect being noted in less than 10 minutes. When estimating inhibition of ChE and AChE in rabbit gut homogenates the same author found that $10^{-6}$M inhibited 65% and $10^{-5}$M nearly 100%, of both enzymes' activity. de la Lande and Porter (1963) also noted greater sensitivity to anticholinesterases in the isolated guinea-pig ileum preparation than in an enzyme assay system using the same tissue. But in the present work physostigmine $10^{-5}$M had no effect on the magnitude of responses to periarterial stimulation, and higher concentrations still ($10^{-4}$M and $10^{-3}$M) were required to elicit potentiation of responses.

(ii) The time course of the effect also seemed inconsistent with anti-cholinesterase action. In the present work recovery from the potentiation was relatively rapid after washout of the drug, while enhancement of ACh's depressant effect persisted in the same vessels; Watson (1970) showed that $2 \times 10^{-6}$M physostigmine, applied for 15 minutes to the isolated rat superior cervical ganglion, potentiated responses for at least two hours after removal of the drug.

These observations suggest that physostigmine may have acted, at least in part, by some effect other than inhibition of cholinesterases,

possibly by directly influencing the sensitivity of the arterial smooth muscle. Indeed Ehrenpreis, Bigo-Gullino and Avery (1965) observed that physostigmine in high concentrations contracted rabbit aortic strips, an action which they also considered was probably unrelated to cholinesterase inhibition. Malik (1970) noted that neostigmine and DFP in high concentrations had similar effects to physostigmine in isolated rat mesenteric vessels. The present work supports his results, with the additional observation of the reversibility of the effects, but it is suggested that these drugs shared a common action not related to cholinesterase inhibition. Neither *iso*OMPA nor BW284C51, which are selective inhibitors of ChE and AChE respectively (Bayliss and Todrick, 1956) potentiated the responses to periarterial stimulation at high or low concentrations.

<u>SUMMARY</u>

1. Exogenous ACh exerted a depressant action on the responses to periarterial electrical stimulation, which was prevented by atropine and enhanced by inhibitors of cholinesterase. This effect is examined further in the next Chapter.
2. No evidence was seen for a facilitatory role of exogenous ACh, directly attributable to a primary action of the drug, on the response to periarterial stimulation.

3. A facilitatory role for endogenous ACh was possibly indicated by the effects of high concentrations of physostigmine, but the doses of the drug required and other factors strongly suggested that these effects could be explained in other terms.

# CHAPTER EIGHT

## ACETYLCHOLINE AND DRUG INDUCED VASOCONSTRICTION

In the present experiments, the effects of acetylcholine (ACh) on the constrictor responses to two drugs, noradrenaline and histamine, were studied in order to shed further light on the mechanism of ACh's inhibitory effect on the response to periarterial electrical stimulation.

Noradrenaline (NA) was selected for comparison because it is the sympathetic neurotransmitter, and hence its interaction with ACh might be expected to reveal whether the latter substance exerted its effect on periarterial (nerve) stimulation by a neuronal or extraneuronal mechanism. At the time this study was commenced, Malik and Ling (1969) reported a potent and highly selective inhibitory effect of ACh on the response of the perfused mesenteric artery bed of the rat to periarterial nerve stimulation, compared with the effect of ACh on the response to intraluminal exogenous NA. In the case of the isolated rabbit ear artery the logical worth of such a simple comparison may be questioned, since there is indirect evidence that the response to intraluminal NA may be mediated by smooth muscle cells in a different region of the artery wall to those mediating the response to endogenous NA. This possibility arises from the consi-

deration that endogenous NA is released into the vicinity of the medial-adventitial border, whereas there is evidence that very little intraluminally applied NA reaches this region of the artery wall (de la Lande et al., 1970; de la Lande and Jellett, 1972). In contrast, extraluminally applied NA rapidly gains access to this region. Kalsner (1972) has shown differential activation of the inner and outer muscle cell layers of the rabbit ear artery. In the present study, therefore, particular attention was paid to the routes of administration of both ACh and NA.

The role of neuronal uptake of NA poses a further complication to the interpretation of comparisons between endogenous and exogenous NA. The evidence of de la Lande, Frewin and Waterson (1967) suggests that neuronal uptake of NA plays a much more important role in the response to extraluminal NA than in the response to intraluminal NA. Their evidence was based on the magnitude of the potentiation of these responses by cocaine and by chronic sympathetic denervation. In addition their data suggests that extraluminally applied NA is influenced by neuronal uptake to a far greater extent than is the response to endogenous NA released by nerve stimulation. For these reasons, a number of comparisons in the present study were made in the presence of cocaine to ascertain whether neuronal uptake of NA also influences its interaction with ACh. Comparisons were also made in chronically denervated arteries.

Finally, histamine was used as a test constrictor agent on the assumption that its constrictor effect is not mediated by adrenergic mechanisms. This assumption is supported by evidence from these laboratories that in the rabbit ear artery the response to histamine is not decreased by (a) cocaine (de la Lande, Frewin and Waterson, 1967), (b) chronic sympathetic denervation (de la Lande and Waterson, unpublished), and (c) phentolamine, in concentrations which caused a 10- to 100-fold antagonism of the constrictor effects of NA (de la Lande and Campbell, unpublished).

## MATERIALS AND METHODS

1. General.

The preparation of arteries, and the methods of perfusion and drug application were as described in Chapter 7.

2. Chronic sympathetic denervation.

Five rabbits were treated by unilateral excision of a superior cervical ganglion 23 to 56 days before sacrifice as described in Chapter 2. That the arteries on the operated side were denervated was demonstrated by their failure to display monoamine fluorescence when treated by the histochemical method of Falck and Owman (1965), and by the failure of the perfused arteries to respond to periarterial electrical stimulation.

3. Constrictor responses.

The nature of the constrictor response of the isolated perfused rabbit ear artery to NA has been extensively documented elsewhere (de la Lande and Rand, 1965; de la Lande, Cannell and Waterson, 1966; Bevan and Waterson, 1971) and comprises,

(i) to intraluminal injection; a transient increase in perfusion pressure,

(ii) to sustained application of either intraluminal or extraluminal NA; a rapid initial increase in perfusion pressure (spike phase) followed by a sustained increase (steady state response).

The nature of the response to histamine is similar. The dose or concentration of constrictor agent applied was chosen to give a perfusion pressure increase of between 50 and 100 mm Hg. The requirement varied between arteries, and the following were applied throughout the study;

(i) NA by intraluminal injection, 0.005 μg to 0.025 μg (in a volume of 0.05 to 0.1 ml).

(ii) sustained extraluminal NA, 0.05 μg/ml to 1.2 μg/ml.

(iii) sustained extraluminal NA in the presence of cocaine and in denervated arteries, 0.002 μg/ml to 0.2 μg/ml.

(iv) sustained intraluminal NA, in untreated, cocaine treated and denervated arteries, 0.01 μg/ml to 0.3 μg/ml.

(v) histamine, whether by sustained intraluminal or extraluminal application, in the range 0.2 μg/ml to 1.0 μg/ml.

4. Interaction between constrictor agents and ACh.

The degree of depression of constrictor responses by ACh was expressed in one of two ways, namely,

(i) on a linear scale ranging from 1.0 (no depression) to 0 (total depression, i.e. no response), the mean of the first 3 responses in the presence of ACh being compared with the mean of the preceding 3 control responses as unity, and,

(ii) as a dose ratio, i.e. as the dose of constrictor in the

absence of ACh divided by the dose giving the same magnitude response in the presence of ACh, as calculated from comparison of dose-response curves.

The latter method of expression necessitated determinations at at least two dose or concentration levels, which in some experiments was not possible. With either method a value of 1.0 represents no depression, and 0 total depression. In all tables experiments where depression is expressed as a dose ratio, i.e.(ii) above, are marked with an asterisk. Experiments were numbered in sequence for each of the four groups of interactions studied,

(i) between ACh and NA,

(ii) between ACh and NA in the presence of cocaine 1 μg/ml,

(iii) between ACh and NA in sympathetically denervated arteries,

(iv) between ACh and histamine.

## RESULTS

### 1. ACh and intraluminally injected NA.

ACh 0.01 µg/ml, which depressed the mean response to periarterial electrical stimulation to 0.51 of the control level (n = 10), was without effect on the response to injected NA in each of 4 arteries. However, in higher concentrations (0.1 µg/ml and 1.0 µg/ml), ACh depressed the responses to NA in 4 out of 9, and in 4 out of 4 arteries, respectively (Table 8.1).

TABLE 8.1 Effect of ACh on responses to injected NA.

| | ACh (µg/ml) | | | |
|---|---|---|---|---|
| Expt. No. | 0.01 | 0.1 | 1.0 | 10 |
| 5. | 1.0 | 1.0 | | |
| 13. | 1.0 | 1.0 | 0.25 | |
| 14. | 1.0 | 1.0 | 0.4 | |
| 17. | | 0.53 | 0.77 | |
| 18. | | 0.73 | | |
| 19. | | 0.75 | 0.5 | 0.35 |
| 34. | | 1.0 | | |
| 38. | 1.0 | 0.15 | | |
| 39. | | 0.75 | | |
| Mean | 1.0 | 0.77 | 0.48 | |
| ± S.E. of mean | ±0 | ±0.09 | ±0.11 | |

By comparison, ACh had the following effects on the responses to periarterial electrical stimulation (supramaximal voltage, less than 5 pulses per second);

ACh 0.01 μg/ml depressed responses to 0.51 ± 0.06 (n = 10) of the control,

ACh 0.1 μg/ml depressed responses to 0.10 ± 0.05 (n = 16) of the control,

ACh 1.0 μg/ml completely abolished responses in each of 12 experiments.

Figure 8.1 illustrates the much greater effect of ACh on the response to periarterial stimulation, compared with the effect on injections of NA.

2. ACh and sustained intraluminal NA.

ACh in concentrations of 0.01 μg/ml and 0.1 μg/ml was without effect on the responses to sustained intraluminal NA (3 and 8 arteries respectively). However, the response was depressed in 2 of 10 arteries when ACh was applied in the relatively high concentration of 1.0 μg/ml (Table 8.2).

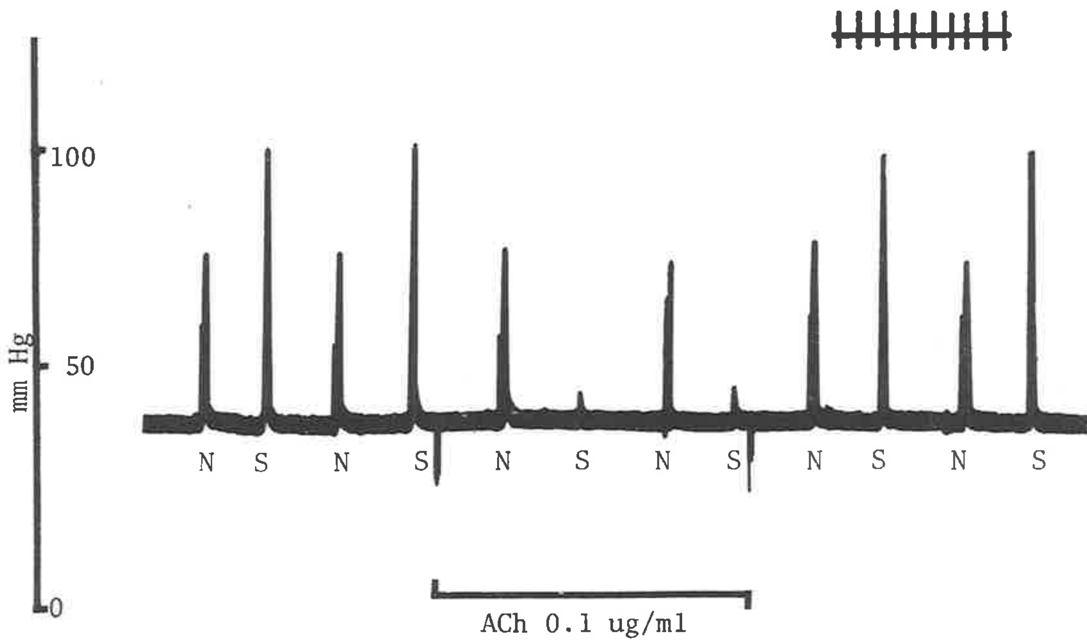



Figure 8.1 Effect of intraluminal ACh 0.1 ug/ml on the responses to injections of NA, 0.01 ug in 0.1 ml(N) and to trains of periarterial electrical pulses at 60 Volts, 1 msec duration, 3 pulses/sec, 5 sec trains (S). Time scale in minutes, pressure scale in mm Hg.

TABLE 8.2 Effect of ACh on sustained application of intraluminal NA.

| | ACh (μg/ml) | | | |
|---|---|---|---|---|
| | 0.01 | 0.1 | 1.0 | 10 |
| Expt. No. | | | | |
| 1, 2, 3 | 1.0 | 1.0 | 1.0 | 1.0 |
| 20 | | | 0.85 | |
| 21 | | | 0.7 | |
| 22, 23, 28, 29, 35 | | 1.0 | | |
| Mean | 1.0 | 1.0 | 0.91 | 1.0 |
| ± S.E. of the mean | | | ±0.06 | |

3. ACh and sustained extraluminal NA.

Again ACh was without effect in the concentration 0.01 μg/ml, but in the concentration 0.1 μg/ml did cause depression in 10 of 29 arteries. ACh was without effect in the remaining 19 arteries when applied at 0.1 μg/ml, and a noteworthy feature was that in 4 of these latter arteries increasing the ACh concentration to 10 μg/ml still failed to cause depression of the responses to NA (Table 8.3, see next page).

4. Effect of route of administration of ACh.

In the preceding experiments ACh was applied either intra-luminally or extraluminally, and maintained in the medium while

TABLE 8.3 Effect of ACh on sustained application of extraluminal NA.

| | ACh (μg/ml) | | | |
|---|---|---|---|---|
| | 0.01 | 0.1 | 1.0 | 10 |
| Expt. No. | | | | |
| 4, 7, 10 | 1.0 | 1.0 | 1.0 | |
| 6, 8, 9 | 1.0 | 1.0 | 1.0 | 1.0 |
| 11 | | | 1.0 | |
| 12 | | 1.0 | 1.0 | 1.0 |
| 22, 23, 35, 28, 29, 32, 35, 39, 40 | | 1.0 | | |
| 15 | 1.0 | 0.8 | 0.45 | |
| 20 | | 1.0 | 0.65 | |
| 21 | | 1.0 | 0.6 | |
| 24 | | 0.84 | | |
| 26 | | 0.68 | | |
| 27 | | 0.83 | | |
| 30 | 1.0 | 0.12 | | |
| 31 | | 0.4 | | |
| 36 | | 0.57 | | |
| 37 | | 0.45 | | |
| 38 | | 0.2 | | |
| Mean | 1.0 | 0.85 | 0.88 | 1.0 |
| ± S.E. of mean | | ±0.05 | ±0.06 | |

determinations of NA sensitivity were made. The effects of the two routes of ACh administration are shown in Table 8.4 (page 8.12), for ACh 0.1 μg/ml. Inspection of the data does not point to a major influence of the route of application of ACh. The effect of the two routes was compared in the same artery in 4 instances; again there was no obvious difference between the effects (or lack of effects) of ACh in each case. Statistical comparison (Student's t test) of all data for ACh-NA interaction for intraluminal versus extraluminal ACh showed no significant difference in degree of depression ($p > 0.2$).

5. <u>Effect of route of administration of NA.</u>

Table 8.4 is a summary of data in which the mean depression is defined in terms of the routes of application of ACh and NA. For ease of presentation the data refers to only one concentration of ACh, 0.1 μg/ml.

TABLE 8.4 Mean effect ± standard error of the mean of ACh 0.1 μg/ml on responses to NA, classified by route of administration of the drugs.

| | Extraluminal ACh | Intraluminal ACh |
|---|---|---|
| Extraluminal NA | 0.89 ± 0.05 (n = 21) | 0.78 ± 0.11 (n = 8) |
| Intraluminal NA[a] | 0.97 ± 0.03 (n = 8) | 0.85 ± 0.08 (n = 12) |

[a]includes data for injected intraluminal NA plus sustained intraluminal NA.

There was no significant difference (Student's t test) between the effects of intraluminal and extraluminal NA, or between any of the four groups shown in the above Table.

The noteworthy findings related to simple ACh-NA interactions were that

(i) ACh did exert an effect on NA, but not in all arteries, and

(ii) irrespective of the route of application of the drugs, the magnitude of inhibition of responses to periarterial electrical stimulation was far greater than the effects on NA.

6. Effect of cocaine.

Tables 8.5 and 8.6 summarise the interaction between ACh and NA in the presence and absence of cocaine 1 μg/ml. In all experiments, the pre- and during-cocaine effects were examined in the same artery. Again there was considerable variability in effects between arteries; the results were as follows:

(a) extraluminal ACh, extraluminal NA. In some experiments inhibition of the response to NA appeared slightly more marked in the presence of cocaine (Table 8.5), but statistical analysis of all experiments (dose ratio data and other data were treated separately) showed no significant difference with cocaine present or absent (Student's t test). In different experiments the effects of ACh ranged from complete inhibition of the response to periarterial electrical stimulation without depressing responses to NA (Figure 8.2), to comparable inhibition of both responses (Figure 8.3).

(b) other combinations. Although the remaining combinations of ACh and NA application were examined in less detail, the results (Table 8.6) did not point to any major differences between these interactions and those described above. There was considerable variation between arteries, but in the majority the inhibition of responses to electrical stimulation was much more profound than the responses to NA, both alone and in the presence of cocaine. The route of application of NA did not appear to exert an important

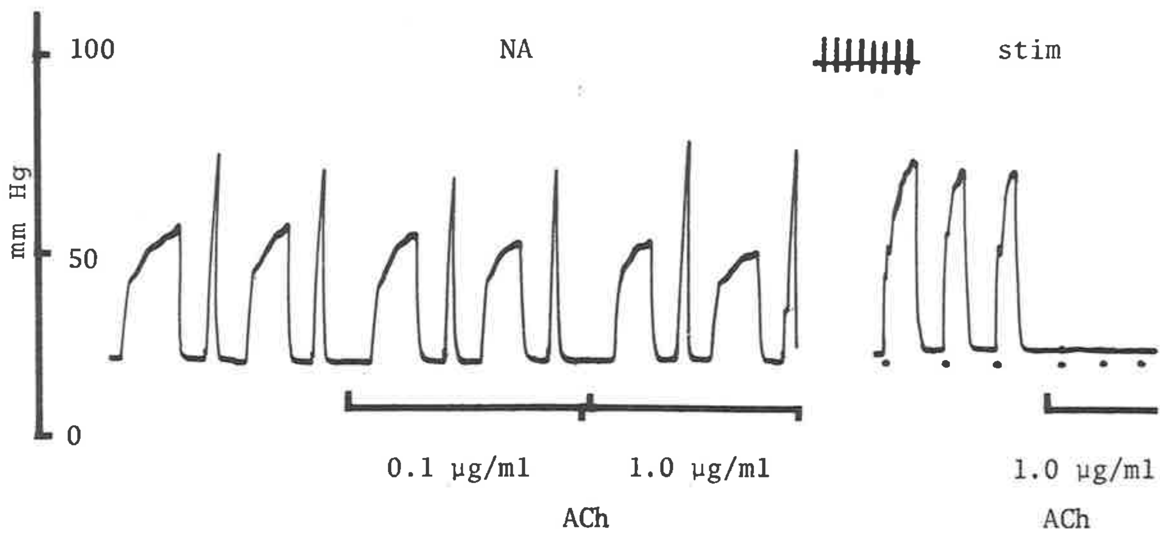



Figure 8.2. Effect of intraluminal ACh 0.1 μg/ml and 1.0 μg/ml on the responses to (left) NA applied alternately by injection, 0.04 μg in 0.1 ml, and extraluminally, 0.05 μg/ml, and (right) to periarterial electrical stimulation 60 Volts, 1.5 pulses/sec, continuous for the duration of the bar below. Cocaine 1 μg/ml present in the intraluminal medium. Pressure scale in mm Hg, time scale in minutes.

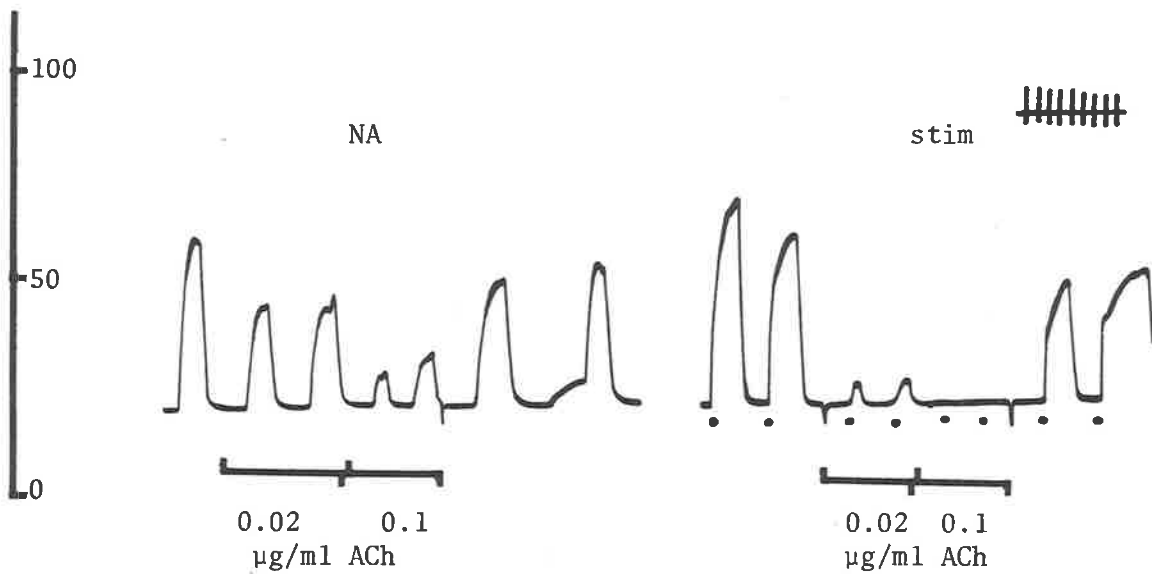



Figure 8.3. Effect of intraluminal ACh 0.02 μg/ml and 0.1 μg/ml on the response to (left) extraluminal NA 0.02 μg/ml and (right) to periarterial electrical stimulation 60 Volts, 0.5 pulses/sec. Cocaine 1 μg/ml present in the intraluminal medium. Pressure scale in mm Hg, time scale in minutes.

influence; those experiments in which comparisons between routes of application of NA were studied in the same artery are summarised separately in Table 8.7.

TABLE 8.5 Effect of extraluminal ACh on responses to extraluminal NA in the presence and absence of cocaine, and effect on responses to periarterial electrical stimulation.

| Expt. No. | ACh μg/ml | No cocaine | Cocaine | Elect.[1] |
|---|---|---|---|---|
| 5 | 0.1 | 1.0 | 1.0 | 0 |
| 6 | 0.1 | 1.0 | 1.0 | 0 |
| 14 | 0.1 | 0.12 | 0.15 | 0 |
| 15 | 0.1 | 0.4 | 0.15 | 0.7 |
| 18* | 0.1 | 1.0 | 1.0 | 0.15 |
| 9* | 0.1 | 0.7 | 0.6 | |
| 10* | 0.1 | 1.0 | 0.6 | |
| 11* | 0.1 | 0.7 | 0.6 | |
| 12* | 0.1 | 0.9 | 0.8 | |
| 2* | 0.1 | | 0.9 | 0.5 |
| 3* | 0.1 | | 0.65 | 0.8 |
| 4* | 0.1 | | 0.75 | 0.55 |
| 1* | 0.05 | 0.9 | 0.5 | 0.05 |
| 13* | 0.2 | 1.0 | 0.9 | |
| 14 | 0.025 | 1.0 | 0.5 | 0.3 |
| 14 | 0.05 | 0.44 | 0.5 | 0.5 |

[1] periarterial electrical stimulation at supramaximal voltage and frequency < 5/sec.

Experiments marked with asterisk, depression as dose ratio (see text); remainder, responses compared with control as unity.

TABLE 8.6 Effect of ACh 0.1 μg/ml to NA in the absence and presence of cocaine, and effect on responses to electrical stimulation.

Ext ACh, ext NA - see Table 8.5

Ext ACh, int NA

| Expt. No. | No Cocaine | Cocaine | Elect. |
|---|---|---|---|
| 19 | 1.0 | 1.0 | 0 |
| 7* | 1.0 | 1.0 | 0.5 |
| 8* | 1.0 | 1.0 | 0.5 |
| 18* | 0.8 | 1.0 | 0.15 |

Int ACh, ext NA

| Expt. No. | No Cocaine | Cocaine | Elect. |
|---|---|---|---|
| 19 | 1.0 | 1.0 | 0 |
| 7* | 1.0 | 1.0 | 0.5 |
| 8* | 1.0 | 1.0 | 0.5 |
| 21* | 0.8 | 0.45 | 0.55 |
| 20* | 0.6 | 0.35 | 0 |

Int ACh, int NA

| Expt. No. | No Cocaine | Cocaine | Elect. |
|---|---|---|---|
| 19 | 1.0 | 1.0 | 0 |
| 7* | 1.0 | 1.0 | 0.5 |
| 8* | 1.0 | 1.0 | 0.5 |

TABLE 8.7 Comparisons of effects of ACh on intraluminal and extraluminal NA in the same artery, in the absence and presence of cocaine.

| | | No Cocaine | | Cocaine | | |
|---|---|---|---|---|---|---|
| Expt. No. | ACh | Int NA | Ext NA | Int NA | Ext NA | Elect. |
| 7* | int | 1.0 | 1.0 | 1.0 | 1.0 | 0.5 |
| 8* | int | 1.0 | 1.0 | 1.0 | 1.0 | 0.5 |
| 18* | ext | 0.8 | 1.0 | 1.0 | 1.0 | 0.15 |
| 19 | int | 1.0 | 1.0 | 1.0 | 1.0 | 0 |

The presence of cocaine did not alter the pattern of interaction between ACh and NA observed in experiments where cocaine was not applied. ACh did depress the response to NA in some arteries but not in others, and in nearly all cases ACh depressed the responses to periarterial electrical stimulation to a much greater degree.

7. Effect of chronic sympathetic denervation.

TABLE 8.8 Effect of ACh* on responses to extraluminal NA in experimental and control side arteries from rabbits which had undergone unilateral superior cervical ganglionectomy.

| Expt. No. | Days[a] | Experimental (denervated) side | Control side |
|---|---|---|---|
| 1. | 27 | 0.8 | 1.0 |
| 2. | 26 | 0.7 | 0.85 |
| 3. | 23 | 1.0 | 1.0 |
| 4. | 56 | 0.65 | 0.7 |
| 5. | 47 | 0.55 | 0.6 |

[a]days = number of days between ganglionectomy and sacrifice.

*ACh 0.1 ug/ml intraluminal

Although in 4 of the 5 experiments there appeared to be a slight trend towards a greater effect of ACh on the denervated artery, statistical analysis failed to show a significant difference between the groups ($p > 0.5$, Student's t test).

## 8. Interaction between ACh and histamine.

In a majority of arteries tested, ACh 0.1 μg/ml inhibited the responses to histamine (Table 8.9). Figure 8.4 shows two examples of the response inhibition. The effect was more marked with intraluminal ACh application, irrespective of the route of histamine administration; statistical analysis showed that there was a significant difference at the 10% confidence level between intraluminal and extraluminal ACh. Table 8.10 shows separately those experiments in which both intraluminal and extraluminal ACh were applied; in each case the greater sensitivity of the responses to intraluminal ACh was apparent.

In 5 arteries the effects of ACh on the responses to peri-arterial electrical stimulation were also examined. The results are presented separately in Table 8.11 and show that, with one exception (artery 5), the effects on electrical stimulation were more marked.

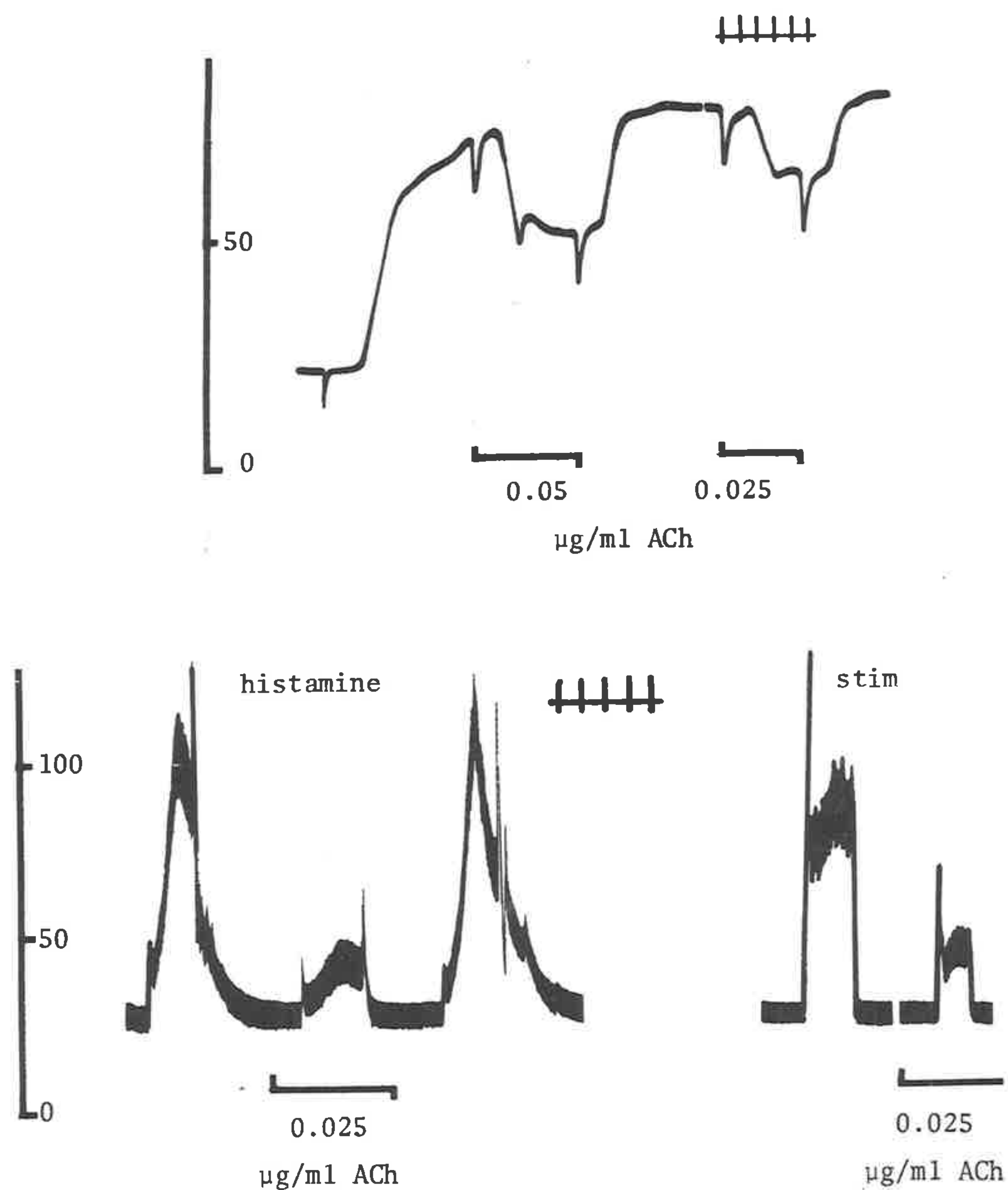



Figure 8.4.

Upper panel: Effect of intraluminal ACh 0.05 and 0.025 μg/ml on the response to continuous application of intraluminal histamine 0.5 μg/ml.

Lower panel: Effect of extraluminal ACh 0.025 μg/ml on the responses to (left) extraluminal histamine 0.3 μg/ml, and to (right) periarterial electrical stimulation 50 Volts, 1 pulse/sec.

Pressure scales in mm Hg, time scales in minutes.

TABLE 8.9 Effect of ACh on constrictor response to histamine.

Extraluminal ACh

| Expt. No. | Route of application of histamine | | ACh concn.(μg/ml) | | |
|---|---|---|---|---|---|
| | | | 0.01 | 0.1 | 1.0 |
| 1 | ext | | 1.0 | 0.75 | 0.45 |
| 2 | ext | | 0.87 | 0.45 | 0 |
| 4 | int | | | 1.0 | 0.95 |
| 5 | int | | | 1.0 | 0.58 |
| 9[a] | int | | | | 1.0 |
| | | mean | 0.93 | 0.80 | 0.60 |
| | | ± S.E. | ±0.06 | ±0.13 | ±0.18 |

Intraluminal ACh

| Expt. No. | Route of application of histamine | | 0.01 | 0.1 | 1.0 |
|---|---|---|---|---|---|
| 1 | ext | | 0.7 | 0.45 | |
| 2 | ext | | 0.47 | 0.2 | |
| 3 | ext | | 0.85 | 0.6 | 0.53 |
| 4 | ext | | | 0.1 | |
| 7 | ext | | | 0.4 | |
| 4 | int | | 0.95 | 0.65 | 0.65 |
| 5 | int | | 0.54 | 0 | |
| 8 | int | | 0.4 | 0 | |
| 10[a] | int | | | | 1.0 |
| | | mean | 0.65 | 0.30 | 0.59 |
| | | ± S.E. | ±0.09 | ±0.09 | ±0.06 |

[a]histamine injected as a bolus

TABLE 8.10 Comparisons of effect of intraluminal and extraluminal ACh on the constrictor response to histamine.

| Expt. No. and route of application of histamine | ACh route | ACh concn. (μg/ml) | | |
|---|---|---|---|---|
| | | 0.01 | 0.1 | 1.0 |
| 1. (ext) | int | 0.7 | 0.45 | |
| | ext | 1.0 | 0.75 | 0.45 |
| 2. (ext) | int | 0.47 | 0.2 | |
| | ext | 0.87 | 0.45 | 0 |
| 4. (int) | int | 0.95 | 0.65 | 0.65 |
| | ext | | 1.0 | 0.95 |
| 5. (int) | int | 0.54 | 0 | |
| | ext | | 1.0 | 0.58 |

TABLE 8.11 Effect of intraluminally applied ACh on constrictor responses to histamine and to periarterial electrical stimulation.

| Expt. No. | Stimulus | ACh concn. (μg/ml) | | | | |
|---|---|---|---|---|---|---|
| | | 0.01 | 0.025 | 0.05 | 0.1 | 1.0 |
| 4 | histamine (int) | 0.95 | | | 0.65 | 0.65 |
| | electrical | | | | 0 | 0 |
| 5 | histamine (int) | 0.54 | | | 0 | |
| | electrical | 0.58 | | 0.32 | 0 | |
| 6 | histamine (ext) | | 0.17 | | 0.1 | |
| | electrical | | 0.2 | | 0 | |
| 7 | histamine (ext) | | | | 0.4 | |
| | electrical | | | | 0.2 | |

## DISCUSSION

By demonstrating that in most arteries ACh exerts a more potent effect on the responses to stimulation via the nerves than on the responses to exogenous NA, irrespective of the route of application of the latter or its uptake into nerve terminals, the present results provide strong evidence in favour of the concept that ACh has a neuronal site of action in the rabbit ear artery. This evidence must however be termed indirect, and cannot on its own be considered conclusive. The concept of a neuronal site of action has been subsequently validated in the same artery by the direct demonstration of inhibition by ACh of the release of tritiated NA during sympathetic nerve stimulation (Steinsland, Kirpekar and Furchgott, 1971).

ACh also apparently exerts an extraneuronal action, since it did depress the responses to exogenous NA in some untreated, cocaine-treated and sympathetically denervated arteries. It is also likely that depression of the response to histamine was due to an extra-neuronal action since, as was outlined in the Introduction, there is evidence that the response to histamine is not mediated by adrenergic receptors in this tissue. The extraneuronal effect was clearly more variable than the neuronal effect, and in most arteries was too weak to be regarded as a serious factor contributing to the depression

of the response to periarterial electrical stimulation. The cause of the variability in this effect between arteries was not clear. There was no obvious relationship between the ACh depressor potency and the constrictor potency of NA on the arteries, when these factors were compared for all experiments; the cocaine-treated arteries were in general 10 to 50 times more sensitive to extraluminal NA than untreated arteries, yet the considerable variation of ACh effect was still apparent. In the concluding stages of this study the interaction was re-examined in a different laboratory and building, with separate personnel, perfusion equipment and drugs, but variability between arteries remained a notable factor. It can only be concluded that the concentration of receptors mediating the extraneuronal effect varied between arteries, or that the receptors were extremely sensitive to the conditions of perfusion.

The results described in this Chapter therefore provide strong indirect evidence in favour of a potent neuronal action of ACh in the isolated rabbit ear artery, and also provide direct evidence for a less potent, and variable, extraneuronal action. While the latter action of ACh was not strong, the evidence in its favour appears indisputable.

SUMMARY

1. ACh, in concentrations which depressed the response of the isolated rabbit ear artery to periarterial electrical stimulation, had a much less potent effect on the responses to exogenous NA, irrespective of the route of application of the drugs.

2. The depressor potency of ACh on exogenous NA was not increased by cocaine treatment or by chronic sympathetic denervation.

3. ACh depressed the constrictor response to histamine, but in general the effect of ACh on the response to periarterial electrical stimulation was more marked.

4. It is concluded that the isolated rabbit ear artery has cholinergic inhibitory receptors related to the sympathetic nerve terminals, and also inhibitory receptors related to the arterial smooth muscle, the activity of the latter varying widely between arteries.

# CHAPTER NINE

## GENERAL DISCUSSION

The trend of early data in both the morphological and functional arms of this study indicated that there might be an intimate relationship between sympathetic and cholinergic systems in the rabbit ear artery, and that therefore intrinsic cholinergic factors might be involved in the normal adrenergic function of the vessel. The design and sequence of subsequent experiments was influenced by this possibility, and in particular by a specific concept of cholinergic involvement in sympathetic function, the 'cholinergic link' hypothesis of Burn and Rand (1965).

In histochemical studies at the light-microscope level it was shown that the distribution of the enzymes ChE and AChE was similar to that of the perivascular sympathetic nerves, and that both enzymes disappeared following removal of the superior cervical ganglion. A variety of histochemical techniques was used to demonstrate the similarity of distribution of the enzymes and the catecholamine-containing nerves. Electron-microscopic studies indicated that the vessel received a homogeneous, adrenergic innervation in the medial-adventitial border region, with no separate cholinergic nerve supply. Specific localisation of AChE at the electron-microscope level showed that the enzyme was associated with

the axon membrane of the adrenergic fibres, although the activity of the enzyme was extremely low. The more active ChE appeared to be related to the Schwann cells which accompany the nerve axons.

These morphological studies pointed to a functional connection between cholinergic and adrenergic structures in the artery wall, and the results of pharmacological studies appeared at first to support such a possibility. In the isolated perfused whole rabbit ear, ACh was observed to have a potent dilator action when the tone of the vessels was raised with the synthetic polypeptide POR8. The effect of ACh on the responses of the isolated perfused central artery of the ear was then examined; ACh was found to exert a potent inhibitory effect on the response to periarterial electrical stimulation, that is, to stimulation via the sympathetic nerves. This inhibitory effect was prevented by anti-muscarinic agents, and enhanced by cholinesterase inhibitors. On the other hand, no facilitatory action of exogenous ACh on the response was observed. Responses were seen to exceed the pre-ACh level after depression in some cases, but this effect was readily explicable in terms other than direct facilitation by ACh. Facilitation of responses by very high concentrations of anticholinesterase drugs was also seen, but again this could be explained in terms other than enhancement of the action of an endogenous ACh mechanism. Neither anticholinergics nor anticholinesterases, when applied in concentrations which modified the inhibitory effects of exogenous ACh, altered in any way the response

of the artery to stimulation via the sympathetics. Hence it was concluded that the interaction between ACh and sympathetic nerve stimulation in this artery was pharmacological rather than physiological, that is, that although exogenous ACh altered the response of the artery to sympathetic nerve stimulation endogenous ACh was not involved in the mechanism of the response. This conclusion was supported by the failure to demonstrate the ACh-synthesising enzyme choline acetylase in the artery, either histochemically or by enzyme assay.

Further experiments were designed to determine the site of action of ACh in depressing the response to periarterial electrical stimulation. It was shown that in the great majority of arteries ACh had a much less potent depressor effect on the responses to exogenous NA than it did on responses to stimulation via the nerves; this relation was not altered by changing the routes of administration of the drugs, by treatment of the arteries with cocaine, or by chronic sympathetic denervation. This constituted strong indirect evidence for a neuronal site of action of ACh in the artery, evidence basically in agreement with that of a contemporary and similar study in the same artery (Rand and Varma, 1970). In addition, in the present study ACh was observed to depress the responses to exogenous NA in some arteries, and also to depress the response to histamine, thus indicating an extraneuronal, smooth muscle site of action of ACh at low concentrations. An

extraneuronal site of action of ACh had been indicated in the isolated perfused whole ear in the present study, and also by Graham, Suhaila and Tai (1971) in the same experimental system. It was therefore concluded that the artery had both cholinergic inhibitory receptors related to the sympathetic nerve terminals and inhibitory receptors related to the arterial smooth muscle, but that the former were chiefly responsible for the depression of responses to periarterial electrical stimulation by ACh.

The 'cholinergic link' hypothesis of Burn and Rand (1965) was a major factor in the design of the present investigation. The histochemical demonstration of acetylcholinesterase in close association with the sympathetic nerve terminals in the artery wall was consistent with the provisions of the hypothesis, but no further evidence in its favour was observed. There was no demonstration of a direct facilitatory action of endogenous or exogenous ACh in the response of the artery to stimulation via the sympathetic nerves in the present work, and such a demonstration would be essential to substantiate the hypothesis. Rand and Varma (1970) argued that depression of responses to stimulation via the sympathetics by ACh, which they observed and which was also seen in the present study, constituted evidence in favour of a facilitatory 'cholinergic link' in the nerve terminals, suggesting that excess ACh would block obligatory 'link' receptors. However, in the absence of the demonstration of a facilitatory action of ACh at lower concentrations

such argument is unreasonable. It must therefore be concluded that the results of the present study do not support the hypothesis.

Shortly before this thesis was submitted, Steinsland, Furchgott and Kirpekar (1973) reported in detail pertinent observations which had earlier been presented only in brief (Steinsland, Kirpekar and Furchgott, 1971). The results of their study confirmed many of the observations reported in Chapter 7 of this thesis, and which had been published previously (Hume, de la Lande and Waterson, 1972). Of particular interest was the additional demonstration by Steinsland et al. that, in ear arteries which had been exposed to tritiated NA, ACh decreased the release of radioactivity during subsequent nerve stimulation, thus providing direct evidence for a neuronal site of action of the ester. In addition to pharmacologically characterising the ACh receptors as muscarinic, Steinsland et al. concluded on the basis of the effects of atropine in particular that, *"the inhibition of NE release by exogenous ACh in no way gives support to the cholinergic link hypothesis."*

The implications of the present findings to the question of the mechanism of vasodilatation by ACh *in vivo* are of interest. It is evident from the present results that ACh might exert a potent dilator action when the tone was raised by local sympathetic activity, but that it might not do so when the tone was raised by circulating catecholamines. In this regard it is noteworthy that the type of

interaction seen in the rabbit ear artery appears to apply to at least one other isolated vascular system, the isolated perfused rat mesenteric vessels (Malik and Ling, 1969). It would be of interest to examine the interaction in isolated arteries from heart, brain and muscle to determine whether the sensitivity of the sympathetic response to ACh might be related in any way to the characteristic regional responses to generalised sympathetic activity. The recent findings that chronic treatment with high doses of guanethidine causes complete and permanent degeneration of peripheral adrenergic fibres in rats (Burnstock et al., 1972) promises an interesting line of investigation in the present context, as it would allow comparison of the effects of ACh on the circulation of totally sympathectomised and control whole animals. The selective action of ACh which has been observed in an isolated artery in this study could therefore be examined on the whole body level. The possibility of lowering the blood pressure with drugs which mimic the effects of ACh on the peripheral vascular neuroeffector system as a therapeutic measure would logically follow such investigations. Acetylcholine has been used in such a therapeutic role, but it has the major disadvantage of brevity of action. A suitable therapeutic analogue would need to be relatively slowly hydrolysed, and predominantly muscarinic, although the potency demonstrated in the present study at low concentrations might allow avoidance of both nicotinic and muscarinic effects elsewhere if the drug could be

delivered to the desired effector site without the use of high doses.

No evidence was seen in either the morphological or functional parts of this work to indicate the presence or action of a system of cholinergic nerves in the rabbit ear artery, but as was discussed elsewhere in this thesis the artery is very probably not typical in this regard. It is possible that in the physiological control of peripheral circulation in other systems endogenous ACh from cholinergic nerves might selectively depress sympathetically induced tone, while being relatively ineffective against circulating catecholamines. It is also possible that in such a system the cholinergic-adrenergic interaction might take place at the nerve endings, entailing economy of transmitter, rather than at the smooth muscle. To propose such systems of control on the basis of the present results requires, however, considerable extrapolation, and it may be that the type of interaction observed in the rabbit ear artery does not obtain in vessels with dual innervation. Such possibilities remain to be explored.

Some data not directly applicable to the present study, but which may be of interest in related fields, was obtained during the course of the investigation. An examination of the ultrastructure of the rabbit ear artery confirmed the presence of apparently uniform adrenergic innervation just outside the medial layer. The synaptic cleft was relatively wide, and no nexuses or close contacts between

adjacent smooth muscle cells were observed. Also of interest was the observation of possible changes in the density of the adventitial elastin following degeneration of sympathetic nerves in the vessel, which may be related to the aetiology of essential hypertension (Appendix Five). Investigations using the isolated perfused whole rabbit ear, as well as providing results applicable to the present work, re-opened possible investigations into the nature of antidromic vasodilatation by providing a suitable system for study of the phenomenon (Appendix Six). The principle involved in that system, namely raising the tone of the perfused vessels before applying dilator stimuli, may also be of importance in the investigation of other dilator phenomena in isolated vascular models.

Much remains to be discovered about the role of cholinergic influences in vascular tone control. The application of the techniques used and developed in this study to other blood vessels may provide useful information, particularly if those vessels prove to have an active cholinergic innervation, whether sympathetic or parasympathetic in origin. Much of the present knowledge of peripheral dilator mechanisms depends on the study of whole vascular beds, in some cases in intact animals. While the use of such experimental systems has advantages, experiments often rely heavily on the use of antiadrenergic, anticholinergic and anticholinesterase drugs for identification of adrenergic and cholinergic effects. The specificity of action of some of these drugs in

this application has been challenged by Honig and Myers (1968). The continued development and use of isolated vascular models in this field of study, as an adjunct to other methods of investigation, may prove as fruitful as the use of isolated adrenergic systems is doing in the study of vasoconstrictor mechanisms.

# APPENDIX ONE - HISTOCHEMISTRY

## Cholinesterase staining.

The incubation medium for ChE or AChE contained the following:

| | |
|---|---|
| sodium citrate | 5mM |
| copper sulphate | 3mM or 6mM |
| potassium ferricyanide | 0.5mM |
| AThI or BuThI | 2mM |

In the study described in Chapter 2 the compounds were added to 0.1M sodium hydrogen malate adjusted to pH 5.95-6.0 with NaOH. In the study described in Chapter 3 the compounds were added to Krebs bicarbonate (see Appendix Four) adjusted to pH 5.95-6.0 with HCl. In either case, the thiocholine salt (acetylthiocholine iodide or butyrylthiocholine iodide, Koch Light) was added as the solid. The other compounds were held in aqueous stock solutions for up to one month. Inhibitors were either diluted from the solid (*iso*OMPA and BW284C51) or from a 0.1M stock solution in propylene glycol (DFP) immediately before use. DFP and *iso*OMPA were from Koch Light, BW284C51 from Burroughs Wellcome.

## Catecholamine fluorescence.

Paraformaldehyde powder (Merck) was prepared for use in the development of fluorophore by storage in 5 gram lots over sulphuric acid in 10 inch dessicator jars. The acid was in different concentrations in different

jars to provide relative humidities from 50% to 94%. For most experiments the concentration of acid was 34% (for subsequent whole tissue treatment, see below) and 15% (for cryostat section treatment, see Chapter 2, part A) providing relative humidities of 70 and 94 respectively (Handbook of Chemistry and Physics, Chemical Rubber Publishing Co., Cleveland, Ohio, 44th Edition, 1962-3 pp.2092-2093, 2596). The acid was changed weekly.

Artery segments were frozen in a mixture of acetone and dry-ice (solid $CO_2$), freeze-dried at 20-40 microns Hg air pressure at $-35^{o}C$ to $-30^{o}C$ for 16-20 hours, and exposed to formalin vapour at $80^{o}C$ for 60-75 minutes in a sealed 1 litre jar containing a beaker of paraformaldehyde (see above). The specimens were vacuum embedded in paraffin wax, and transverse sections 7 microns in thickness were cut and mounted on glass slides with Entellan (Merck) diluted with an equal volume of xylol. Between the freezing and embedding stages the segments were within numbered recesses in an aluminium container, designed to allow transfer of gases and liquids without loss of the tissue.

## APPENDIX TWO - ELECTRON MICROSCOPY

### Formaldehyde-glutaraldehyde-acrolein.

The fixative was prepared as follows:

2 grams of fresh paraformaldehyde powder (Merck) was dissolved in 25 ml

water at 60-70°C, and N NaOH was added until the mixture cleared. 10 ml 25% aqueous glutaraldehyde (Fluka), 63 ml 0.1M phosphate buffer pH 7.4 and, while stirring vigorously, 3 ml 100% acrolein (Analar) were added. The fixative was used within ten minutes, and the excess discarded.

Dehydration and embedding.

Araldite (Fluka) was made up in the following proportions and mixed for at least 30 minutes before use:

| | |
|---|---|
| 'A' | 10 ml |
| 'B' | 10 ml |
| 'C' | 0.4 ml |
| 'D' | 0.4 ml |

The embedding procedure was as follows:

1. 2-3 minute changes of 50% ethanol<br>75% ethanol<br>95% ethanol<br>100% ethanol (twice)<br>propylene oxide (Analar) (twice)
2. Overnight in a mix of equal proportions of propylene oxide and Araldite at room temperature.
3. One hour in Araldite at 60°C in vacuo.
4. Re-orientate specimens in Araldite.
5. Cure for 2-3 days at 60°C.

## APPENDIX THREE - CHOLINE ACETYLASE ASSAY

### Incubation medium

The incubation medium (assay mixture) contained the following in a total volume of 11 ul for each sample:

| | |
|---|---|
| potassium phosphate | 0.08M |
| sodium chloride | 0.3M |
| magnesium sulphate | $2 \times 10^{-2}$M |
| choline chloride (BDH) | $5 \times 10^{-3}$M |
| physostigmine sulphate[a] | $2 \times 10^{-4}$M |
| bovine serum albumen (CSL) | 0.05% |
| ($C_{14}$) acetyl Coenzyme A[b] | $10^{-5}$M - $4 \times 10^{-5}$M |
| tissue (wet weight) | 20-330 ug |

### Scintillant.

The scintillant contained the following, in dioxane (Analar):

| | |
|---|---|
| napthalene | 150 grams/litre |
| PPO (Packard) | 10 grams/litre |
| POPOP (Packard) | 0.3 grams/litre |

---

[a]Nutritional Biochem.

[b]Radiochemical, Amersham

## APPENDIX FOUR - PERFUSION EXPERIMENTS

### Krebs bicarbonate solution.

The perfusion fluid was a Krebs bicarbonate solution of the following composition:

| | grams/litre | millimolar |
|---|---|---|
| NaCl | 6.90 | 120.0 |
| KCl | 0.35 | 4.7 |
| $CaCl_2$ | 0.28 | 2.5 |
| $MgCl_2$ | 0.10 | 1.1 |
| $NaHCO_3$ | 2.10 | 25.0 |
| $KH_2PO_4$ | 0.16 | 1.0 |
| Glucose | 1.00 | 5.5 |

All compounds except $CaCl_2$ and $MgCl_2$ were dissolved in the required volume of distilled water, then $CaCl_2$ and $MgCl_2$ were added from standardised 10% stock solutions. The total solution was filtered before use, and gassed with 5% carbon dioxide and 95% oxygen (Carbogen). The pH of the gassed solution was 7.4.

### Perfusion pump.

The perfusion pump was designed by O.Saxby, Department of Pharmacology, University of Oxford, and made at the Medical School workshop, University of Adelaide. Flow was induced in a silicone rubber tube by the pressure of moving rollers along the tube. The pump was driven by an adjustable speed electric motor.

Warming chamber.

The ear warming chamber was designed by M. Tyler, of the Department of Human Physiology and Pharmacology, University of Adelaide. It consisted of a 75 mm diameter perspex tube, 130 mm in length, with partially closed ends. The external surface of the tube was lined with a coiled 10 mm diameter polythene tube through which flowed water at 37$^{o}$C.

Periarterial electrodes.

Periarterial electrodes were made of 20 gauge platinum wire rings, of 3 mm internal diameter and placed 2 mm apart, encased in a short formed tube of Araldite (Selleys, Aust.) so that the inner surfaces of the rings were uncoated and on the luminal surface of the Araldite tube.

Pressure recorders.

Pressure changes were measured and recorded using one of two systems:

1. A Palmer mercury manometer (Condon model) and a Palmer kymograph (Model Super-Ten) using a 12 inch diameter drum.
2. A pressure transducer (Ether Engineering, England, type 2BP15MK5) and a twin channel potentiometric recorder (Rikadenki Kogyo Co., Japan, Model D-24), using 20 mV or 50 mV attenuator settings.

Drugs.

The following drugs were used in perfusion experiments in this study:

acetylcholine perchlorate (B.D.H.)

atropine sulphate (Farmer, Hill)

cocaine hydrochloride (Fauldings)

di*iso*propyl fluorophosphate (DFP) (Koch Light)

histamine diphosphate (Koch Light)

hyoscine-n-butylbromide (Boehringer)

*iso*prenaline hydrochloride (Winthrop)

neostigmine methyl sulphate (Roche)

1-noradrenaline bitartrate (Sigma)

ornithine$^{8}$ vasopressin (POR8) (Sandoz)

propranolol hydrochloride (I.C.I.)

propantheline (Searle)

physostigmine sulphate (Nutritional Biochem.)

tacrine (Woods)

tetra*iso*propylpyrophosphoramide (*iso*OMPA) (Koch Light)

BW284C51 (Burroughs Wellcome)

All drugs were diluted in 0.9% NaCl. ACh was kept in a stock solution of 1 mg/ml at 4$^{o}$C. DFP was kept in a stock solution of $10^{-1}$M in propylene glycol at 4$^{o}$C. Solutions of noradrenaline contained 0.1 mg/ml ascorbic acid.

## APPENDIX FIVE - STRUCTURAL CHANGE IN DENERVATED VESSELS.

In arteries taken from 2 animals several weeks after unilateral superior cervical ganglionectomy, electron-microscopic examination suggested that the vessels from the operated side showed a greater number of elastin bundles in the adventitia than those from the control side.

Experimental and control side arteries were taken from 12 rabbits which had undergone unilateral superior cervical ganglionectomy 4-53 days before sacrifice, and were treated with conventional elastic stains (Gomori's aldehyde fuchsin, orcein). Sections were examined un the light microscope by two observers, using a 'blind' technique, to compare the density of elastic staining in experimental and control side arteries. In each of 9 animals (12-53 days) the artery assessed as having denser elastic staining was from the operated (sympathectomised) side, and in the remaining 3 animals (4, 7 and 36 days) no difference was detected by either observer. This result may be of importance in demonstrating the ability of vessels to make rapid structural adjustments to changes in the state of innervation, and may be related to concepts of structural vascular changes as factors in essential hypertension (Sivertson, 1970) and to the demonstration of a decline in demonstrable catecholamine in human blood vessels with advancing age (Frewin, Hume, Waterson and Whelan, 1971).

The light-microscopic investigation described above was carried out in association with W.J. Sampson, a Vacation Scholar in the Department of Oral Biology, University of Adelaide, in January 1971.

## APPENDIX SIX - ANTIDROMIC VASODILATATION

### Historical introduction

In 1876 Stricker observed that stimulation of the peripheral side of a dog's cut posterior spinal nerve root caused flushing and a temperature rise in the area of sensory distribution of that root. Stricker had therefore indicated that vasodilator fibres left the spinal cord through the posterior roots, which seemed contrary to accepted nerve distribution theory. Sherrington (1897) found that dorsal roots contained only afferent fibres, but Bayliss (1901) confirmed Stricker's results, showing that sympathetic denervation did not alter the response, and that extirpation of the dorsal root ganglion abolished it. Bayliss stated, "...the vasodilator action in question is conveyed along what are called afferent fibres, but in an efferent direction. This....I shall speak of as antidromic conduction."

Two suggestions made an object of interest of what otherwise may simply have been a minor curiosity. Lewis (1927) noted similarities between the flare response to injury in skin and the response to stimulation of the dorsal root, and proposed that both might act by releasing a dilator substance from sensory endings, the former by means of an 'axon reflex' in the branching nerve. Since it would be reasonable to expect a neuron to liberate the same substance at either end, Dale (1935) pointed out that the identification of a peripheral dilator

substance in sensory nerves could indicate the nature of the central sensory transmitter.

Feldberg (1926) described a dilator response in the blood vessels of the rabbit ear after stimulation of the great auricular nerve, the ear's main sensory trunk. This phenomenon was studied in depth by Holton and others (Holton and Perry, 1951; Holton and Holton, 1952, 1954; Hilton and Holton, 1954; Holton, 1953, 1956, 1959). They used either the ear of the intact rabbit, or the isolated ear perfused at constant pressure with blood substitutes. Dilatation and constriction were monitored by measuring changes in light transmission through a peripheral part of the ear. Dorsal root and other nervous system extracts were found to cause dilatation similar to that following sensory nerve stimulation. Acetylcholine and histamine each caused dilatation in the ear, and their actions were blocked by atropine and mepyramine respectively; these latter compounds did not affect antidromic vasodilatation. Inhibitors of cholinesterase, while potentiating acetylcholine's dilator action, reduced the response to sensory nerve stimulation. Attention was given to the possibility that adenosine triphosphate (ATP) might mediate the dilation since the compound had a potent dilator action. But no conclusion was reached regarding the identity of the transmitter (Holton, 1959).

While perfusing ears with Ringer-Locke solution Hilton and Holton (1954) were unable to obtain dilator responses to sensory nerve stimulation.

The phenomenon was seen when ears were perfused with a mixture containing salts, erythrocytes, dextran and adrenaline (Holton, 1956), but the experiments proceeded with difficulty and often failed. In the present study it was decided to perfuse the isolated ear with Krebs bicarbonate solution, and to raise the tone of the perfused vessels with a directly-acting compound which would be unlikely to interfere with dilator responses. The vasopressin analogue ornithine$^{8}$ vasopressin (POR8, Sandoz) was chosen in this role.

## Materials and Methods.

Isolated whole rabbit ears were perfused using the method of de la Lande, Paton and Waud (1964) as is described in Chapter 6, and long term infusions of POR8 were applied. The great auricular nerve was placed over unshielded bipolar platinum electrodes in a bowl of paraffin oil. Square wave pulses of electrical stimulation were applied with an Eilco 6418 stimulator. Various stimulus parameters were used, and are described in Results, below. In addition to infusions of POR8, injections of acetylcholine (see Chapter 6) and of isoprenaline, and infusions of atropine and propranolol, were applied.

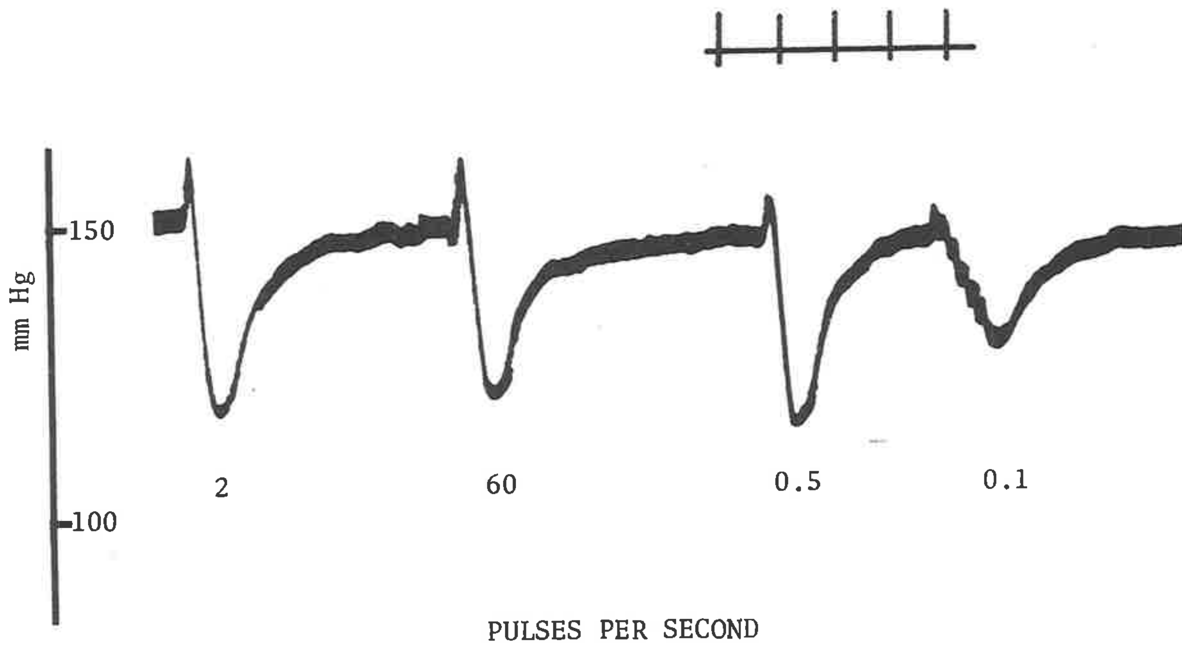
## Results.

Electrical stimulation of the great auricular nerve caused a slow dilatation in 19 of 21 ears perfused with Krebs solution containing POR8 2-5 IU per litre. When ears were perfused with Krebs solution

alone dilatation was not observed, and in some ears a slight, transient constriction was seen. The parameters of electrical stimulation were varied to find the optimal values for the dilator effect. The threshold voltage was 10-15 volts, and the response increased with greater voltages up to, but not beyond, 30-40 volts. Similarly, the magnitude of the response increased with increasing pulse duration up to, but not beyond 1 msec. In the frequency range 1-60 pulses per second the response to a set number of pulses did not alter greatly with frequency (Appendix, figure 1), while at a given frequency of stimulation the magnitude of the responses varied directly with the number of pulses (Appendix, Figure 2). In some arteries the dilatation was preceded by a transient vasoconstriction. The dilatation phase increased over 15-30 seconds, often reaching a maximum 10-20 seconds after stimulation had ceased. The perfusion pressure returned to the pre-stimulus level slowly, the whole response taking 2-3 minutes.

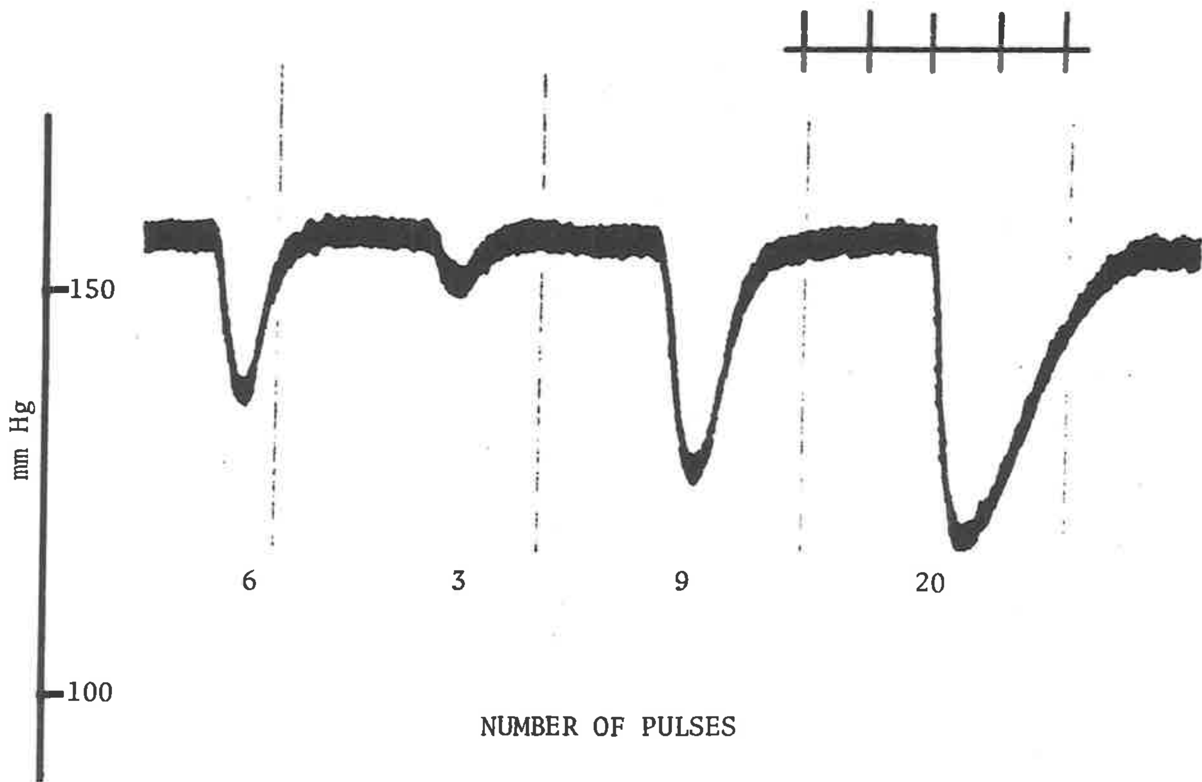
As was described in Chapter 6, injections of acetylcholine caused dilator responses, and these responses were not seen in the presence of atropine 0.1 μg/ml. Atropine 0.1 μg/ml did not affect the responses to sensory nerve stimulation. Injections of isoprenaline, 2 ng or more, were followed by dilatation in each of 13 ears (Appendix figure 3). The dilatation was rapid in onset, but the return to the pre-injection perfusion pressure was slower than with acetylcholine, taking up to 2 minutes. Propranolol hydrochloride

Appendix figure 1

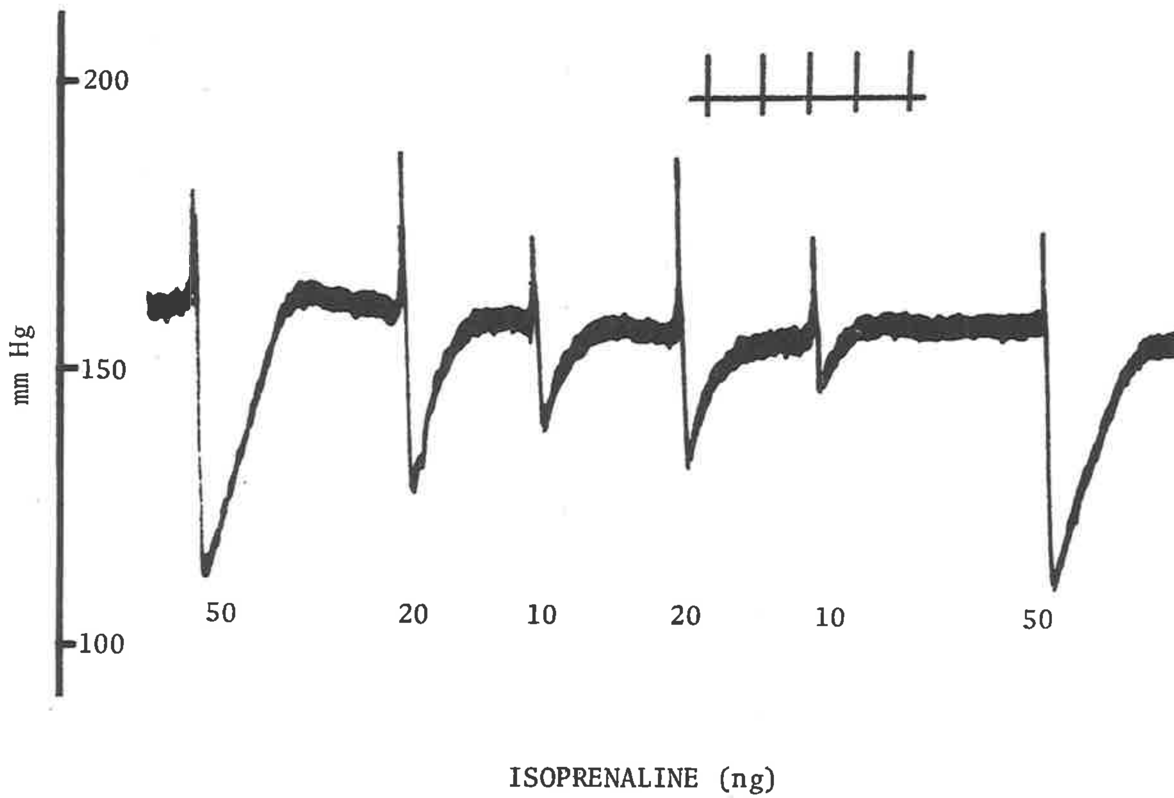



Showing responses to stimulation of the great auricular nerve in an ear perfused with Krebs solution containing POR8 5 IU/L. Numerals indicate the frequency of stimulation, in pulses per second, at 40 V, 1 msec. In each case a total of six pulses were applied. Pressure scale in mm Hg, time scale in minutes.

Appendix figure 2




Showing responses to stimulation of the great auricular nerve in an ear perfused with Krebs solution containing POR8 3 IU/L. Numerals indicate number of pulses at 40 V, 1 msec and 1 pulse/sec. Pressure scale in mm Hg, time trace in minutes.




ISOPRENALINE (ng)

Showing responses to injected doses of isoprenaline in an ear perfused with Krebs solution containing POR8 4 IU/L. Numerals indicate dose of isoprenaline in ng. Pressure scale in mm Hg, time scale in minutes. The upward deflection corresponds to the injection of the drug.

1 μg/ml prevented the dilator effect of injections of isoprenaline below 0.5 μg, while the responses to stimulation of the great auricular nerve were only slightly depressed by propranolol 1 μg/ml in 10 of 13 experiments and this depression was not reversible on propranolol washout.

## Discussion

The results described above indicated that the vessels of the isolated perfused rabbit ear had the capacity to dilate in response to stimulation of the great auricular nerve when the tone of the vessels had been raised with POR8. It is interesting to note that the successful synthetic perfusion media described by Holton (1956) contained adrenaline 5 x $10^{-9}$ (5 ng/ml), although the reason for its addition was not stated; it is suggested that this compound raised the tone of the vessels, and allowed dilatation to occur. In the experiments of Hilton and Holton (1954) where Ringer-Locke alone was used the tone of the vessels may have been low, and dilatation therefore difficult to demonstrate.

The dilatation which followed stimulation of the great auricular nerve in the present study was very probably the same phenomenon as that described by Holton and Perry (1951), since both the mode of stimulation and the pattern of response were similar. While this work has provided a new, simple model in which to study the phenomenon,

investigations beyond the application of atropine and propranolol have not at present been made. The lack of effect of these agents suggested that the phenomenon was neither muscarinic nor β-adrenergic. The recent evidence of non-adrenergic, non-cholinergic nerves presented by Burnstock, Campbell, Satchell and Smythe (1970) suggests new lines of enquiry. It was proposed that ATP might mediate the action of these nerves. Holton (1959) demonstrated ATP liberation in the ear after stimulation of the sensory trunk, and suggested that ATP might be the transmitter released from the sensory endings as a result of antidromic impulses. The possibility that non-adrenergic, non-cholinergic motor nerves may travel in the great auricular would be of interest to those who consider, like Grigor'eva (1962), that the concept of antidromic conduction and vasodilatation is 'naive and unphysiological'.

Summary.

1. A dilator response to stimulation of the great auricular nerve was observed in isolated rabbit ears, perfused with Krebs bicarbonate containing POR8.
2. The phenomenon was very probably that described elsewhere as antidromic vasodilatation.
3. The magnitude of the response varied with the number of electrical pulses applied, but not the frequency of stimulation within a wide range.

4. The phenomenon was very probably neither muscarinic nor β-adrenergic.

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