ACTIONS AND INTERACTIONS OF VASOACTIVE DRUGS AND SYMPATHETIC NERVES IN THE RABBIT EAR

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

by

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SUMMARY

The actions of sympathomimetic drugs on blood vessels of the the rabbit ear have been well documented. Many of the results were derived from experiments using constant pressure perfusion techniques, but in the present study the constant volume perfusion method of de la Lande, Paton and Waud (1964) was used, in which the temperature of the perfused vessels was adjusted to approximate conditions found *in vivo*.

A further modification of previous methods was the perfusion of an isolated artery so arranged that drugs could be applied either to the intraluminal or the extraluminal surface of the artery, and the responses of drugs applied to either surface of the vessel separately recorded (de la Lande, Cannell and Waterson, 1966). Application of this method to study vasoconstriction produced by noradrenaline showed the amine to be much more active intraluminally than extraluminally. Because of the known ability of sympathetic nerve terminals to take up noradrenaline, the possibility was investigated that the low sensitivity of the artery to extraluminal noradrenaline was due to uptake by sympathetic nerves in the wall of the artery.

An established histochemical method designed to outline sympathetic nerve terminals by causing fluorescence of the neurotransmitter substance (Falck, 1962) was applied to the rabbit ear

artery and it was found that (in common with many other blood vessels) the rabbit ear artery possessed a dense network of noradrenergic fluorescent structures at the medial-adventitial border, and outside the smooth muscle layer of the artery. In the fluorescence studies autofluorescence of certain tissues was marked, and a method was devised to use Evans blue dye to modify the autofluorescence.

With confirmation of the position of the sympathetic nerves in the artery, the role of the nerves in the uptake and release of noradrenaline was investigated, using cocaine to block uptake by the nerves, chronic denervation of the artery to destroy the nerves, and reserpine to cause depletion of noradrenaline in the artery. One finding was that cocaine caused much greater potentiation of extraluminal noradrenaline than of intraluminal noradrenaline, and the results of the experiments led to the formulation of an hypothesis which is presented in this thesis. A diagrammatic model of an artery was used to explain differences in sensitivity of the artery to intraluminal and extraluminal noradrenaline.

Experiments were designed to examine the effects on the artery of endogenous noradrenaline (released by nerve stimulation). It was found that cocaine caused potentiation of the responses to nerve

stimulation but that the effects resembled potentiation of intraluminal rather than of extraluminal noradrenaline, a result not readily explained in terms of the hypothesis.

Tyramine, which has been shown to have both direct and indirect (noradrenaline releasing) actions in the rabbit ear artery (Farmer, 1966), was found in the present study to be much more active extraluminally than intraluminally when applied to the ear artery. A series of experiments was designed to examine this action of tyramine and an explanation for the difference in sensitivities is presented using the model hypothesis.

Comparisons were also made of the responses of the isolated perfused ear and the isolated perfused artery to several sympathomimetic drugs, in an endeavour to locate the site of action of the drugs in the rabbit ear vessels.

Included in the study was an investigation of the vasoconstrictor effects of thiopentone and methohexitone in the rabbit ear. The barbiturate experiments were conducted to further test the possibility that the constrictor action of thiopentone is due to release of noradrenaline from artery walls (Burn, 1960).

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.

The results have been presented in part to meetings of the Australian Physiological Society (1966) and the International Association for Dental Research (Australian Section) 1964, 1965, 1966 and 1967. Some of the material presented has been published in the following journals.

Br. J. Pharmac. Chemother. 28, 255-272 (1966)

Br. J. Pharmac. Chemother. 31, 82-93 (1967)

Nature (Lond.) 214, 313-314 (1967)

J. Oral Therap. Pharmacol. 3, 462-467 (1967)

Aust. J. exp. Biol. med. Sci. 45, 301-308 (1967)

Aust. J. exp. Biol. med. Sci. 45, 309-311 (1967)

Circ. Res. 21, Supp. III, 177-181 (1967).

Ann. A.C.D.S. 1, 60-69 (1967)

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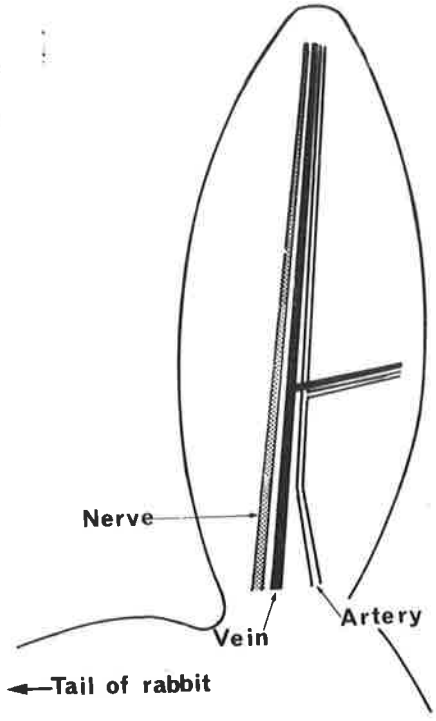
CHAPTER 1

GENERAL INTRODUCTION

The rabbit external ear is perfused with blood through a dense network of blood vessels which anastomose freely. These vessels are readily accessible for experimental operative procedures and for direct observation of their size. The ear contains some cartilage and voluntary muscle, but most of its vascular system allows perfusion of skin and other superficial structures, so the blood vessels of the ear are in close contact with the rabbit's environment. It has been shown (Grant, Bland and Camp, 1931) that the rabbit ear is highly reactive to changes in body temperature, and it is likely that the ear has an important role in maintaining homeostasis in the rabbit.

The suitability of the rabbit ear as a model to provide information on the neural control of blood vessels was recognised by a number of early workers. Fletcher (1898) discussed the work of Schiff, who in 1867 obtained constriction of rabbit ear blood vessels by electrical stimulation of the great auricular nerve in the normal ear (figure 1.1), and following extirpation of the superior cervical ganglion and some post-ganglionic nerves to the ear. He concluded that the vasoconstrictor fibres of the great auricular nerve arose from the spinal cord, and passed in the roots of the cervical nerves, which send branches to the

Figure 1.1



A diagram of the erect left ear of a rabbit showing the convex surface of the ear as seen from the midline of the skull, and showing the relative positions of the ventral (great) auricular nerve, the central vein, and the central (main) artery of the ear.

great auricular nerve. Fletcher confirmed some of Schiff's results and made further observations. Firstly, that vasoconstriction was more evident in the distal part of the artery after stimulation of the great auricular nerve. Secondly, that the proximal one-third of the artery showed a moderate constriction after stimulation of the great auricular nerve, and thirdly, that the middle one-third of the artery was little affected by stimulation of this nerve. However, stimulation of the cervical sympathetic chain caused constriction of the whole artery, and stimulation of the pre-ganglionic fibres leading to the stellate ganglion caused effects which were identical with stimulation of the great auricular nerve. Fletcher observed that the terminal bifurcation of the blood vessels in the ear was least affected by cervical sympathetic stimulation. He made the interesting observation that the application of nicotine to the stellate ganglion in the rabbit abolished the response to stimulation of the cervical sympathetic chain central to the stellate ganglion. Prior to this, Langley (1893) had reported the results of nerve stimulation of vasoconstrictor fibres to the rabbit ear. Langley found that the majority of the vasoconstrictor fibres ran in the external carotid branch from the superior cervical ganglion. Stimulation of this branch caused constriction of the whole artery, but the effect was slight at the base of the ear. However, stimulation of the third cervical nerve caused vascular constriction near the tip of the ear. Often in Langley's experiments two-thirds of the artery

constricted but the one-third near the base did not. The work of Schiff, Fletcher and Langley suggested the following possibilities:

1. That the vasoconstrictor fibres in the great auricular nerve reach the ear artery via the stellate ganglion of the rabbit.
2. That cervical nerves, possibly the third cervical nerve, supply constrictor fibres to vessels in the tip of the ear, and that these fibres are contained in the great auricular nerve.
3. Most of the vasoconstrictor fibres pass in the external carotid branch of the superior cervical ganglion.

Gaskell (1880) published a paper on effects of changes in acidity or alkalinity on isolated frog hearts, and the blood vessels in the perfused leg of the frog. By measuring the rate of flow of effluent from the perfused frog leg, Gaskell found that lactic acid increased the calibre of blood vessels, and that alkaline solutions diminished the size of the vessels, as judged by the decrease in flow of effluent from the perfused limb. Discussing the possibility that the alkalinity or acidity of the tissues affected vascular tone, Gaskell wrote the following passage, aptly describing observations which in many respects are consistent with modern concepts:

"Is there any likelihood that the muscle of the smaller arteries

should be affected by the nature of the lymph fluid of the tissue they supply? From what is known of the structure of the arterioles it seems highly probable that such must be the case. We find in them an inner elastic membrane intervening between the blood stream and the well-developed muscular layer, while the outer elastic layer is absent, the adventitia lying immediately against the muscular coat. This adventitia is composed of loosely compacted fibres containing spaces within their meshes which are in all probability continuous with the spaces of the tissue itself. With such an arrangement as this it seems highly probable that the naked muscular fibres of the smaller arteries must be continually bathed by the lymph fluid of the tissue they supply, and that it is the constitution of this fluid which must influence them rather than that of the blood circulating in their interior."

Gaskell's assumption was that the tonicity of the blood vessels was in fact due to the alkalinity of the tissue fluids.

Further interest in the perfusion of blood vessels was taken by Rischbieter (1913) who reported experiments in which the isolated rabbit ear was perfused with a physiological saline solution. Rischbieter credits Bissemski as the originator of the method. The Bissemski method (as reported by Rischbieter) was to bleed a rabbit and then remove the ears. A cut was made in the gap between the two protruding cartilages on the dorso-medial aspect of the ear and the posterior auricular artery

located medial to the great auricular nerve. One centimetre of the artery was isolated, and a cannula inserted into the lumen of the artery, which was then ligated at the base of the ear. A physiological saline solution was syringed through the artery until fluid emerging from the veins became clear. The ear was fixed under a glass plate, and the cannula connected to a burette containing physiological saline solution maintained at a pressure of 30-40 centimetres of the fluid. The saline was allowed to flow through the ear and the fluid emerging from the vein was collected and the drops counted. The Bissemski method was used by other workers with little variation in principle (Burn and Dutta, 1948; Burn and Hutcheon, 1949; Miyake, 1952). Rischbieter examined the vasoconstrictor effects of adrenaline and hypophysin.

Feldberg (1926) stated that there was doubt about the precise innervation of the vessels of the rabbit ear. However, experiments showing changes in the diameter of the vessels had provided evidence of vasoconstrictor fibres. Feldberg pointed out that the external ear of the rabbit is supplied by the fifth and seventh cranial nerves, while the facial nerve, the auriculo-temporal nerve, and the nerves from C1 and C2 also innervate the ear. Feldberg said that the sensory nerves of the ear came from the auriculo-temporal nerve and from C1 and C2. After extirpation of the superior cervical ganglion the blood vessels were at first dilated, but only for a few days. Dilatation persisted longer in

the central artery than in the other ear vessels. Feldberg found that for complete denervation it was essential to remove the stellate ganglion, and described a method for its removal. After removal of both the superior cervical ganglion and the stellate ganglion the vessels remained dilated. In two out of three rabbits where only the stellate ganglion was removed the dilatation persisted. A further observation by Feldberg was that the vasodilator distribution was the same as the sensory nerve distribution, and nearly the same as the distribution of vasoconstrictor fibres. He concluded that:

1. The vasoconstrictor fibres for the external ear run in the posterior facial nerve, the ventral (great) auricular nerve and the dorsal auricular nerve. Constrictor fibres of the posterior facial nerve pass in the superior cervical ganglion and those of the ventral and dorsal auricular nerves in both the superior cervical ganglion and the stellate ganglion (although the dorsal auricular nerve obtained very few fibres from the stellate ganglion).

2. Vasodilator fibres run in the ventral and dorsal auricular nerves. They could not be found in the auriculo-temporal nerve. Each nerve supplies a special region similar to the sensory distribution of the auriculo-temporal nerve. Feldberg suggested a standard terminology for these nerves, the anterior auricular nerve should be called the ventral auricular and

that the posterior auricular nerve should be called the dorsal auricular nerve.

Feldberg stimulated the separate nerves and mapped out the regions where the blood vessels were constricted following nerve stimulation. He found that the ventral auricular nerve supplied an area at the distal and medial border of the ear and that the dorsal auricular nerve supplied constrictor fibres to the lateral and distal portion of the ear. The proximal half of the ear constricted on stimulation of the peripheral end of the cut sympathetic nerve.

Grant, Bland and Camp (1931) employed direct observation of ear vessels exposed to cold. With only the distal part of one ear immersed in cold water, heat was lost and body temperature was quickly lowered, although the ear temperature fluctuated. During and after exposure to cold the capillaries remained constricted but the resistance vessels (arterioles and pre-capillary sphinters) dilated and considerable anastomosis occurred. Grant, Bland and Camp reported that the mechanism of the vasodilatation was obscure but was probably not mediated by histamine. They described the effects of denervation caused by cutting the ventral and dorsal auricular nerves and by removing the superior cervical ganglion. This denervation produced dilated vessels no longer responding to changes in body temperature and they confirmed that the auricular branch of the trigeminal nerve still supplied sensory fibres

to the basal part of the lateral surface of the ear. However, the denervated ear still gave the usual responses to distal one-third cooling. Even after trigeminal section some of the nerves remained (as shown by histological section) and these nerves ran from the auricular branch of the vagus (that is Arnold's nerve as described in the rabbit by Loven in 1870) but which was known by Schiff in 1867 (according to Grant, Bland and Camp). Arnold's nerve supplied the proximal half to two-thirds of the ear chiefly on the lateral, but also on the dorsal surface, and did not ramify in the distal one-third of the ear. In the intact animal it contained both sensory and vasomotor fibres. The vasoconstriction of the areas on the lateral and dorsal surfaces, and including the central artery, produced by electrical stimulation of Arnold's nerve, did not occur after removal of the superior cervical ganglion. Thus the vasomotor fibres of this nerve must come via the superior cervical ganglion. By combining their observations with those of Feldberg (1926) Grant, Bland and Camp summarised the nerve supply to the rabbit ear as follows:

Sensory nerves

1. The ventral (great, anterior) auricular nerve.
2. The posterior, (dorsal), auricular nerve.
3. The auriculo-temporal branch of the trigeminal nerve.
4. The auricular branch of the vagus nerve (Arnold's nerve).

Sympathetic nerves

Post-ganglionic sympathetic fibres emanate from the superior cervical and stellate ganglia. Fibres from the superior cervical ganglion pass to the ear through the branches of the motor facial nerve, the ventral and dorsal auricular nerves, and through Arnold's nerve. The fibres from the stellate ganglion pass by way of its vertebral ramus to the ventral and dorsal auricular nerves. The auricular branch of the trigeminal nerve carries vasomotor fibres for some of the ear vessels.

Further work by Grant (1935) described direct observation of the vessels and nerves of the rabbit ear before and after denervation. After denervation dilatation occurred, but gradually vessel tone returned to normal. The two ears then gave equal constrictor responses to various stimuli. However, Grant noted that body temperatures had less effect on the state of the blood vessels of the denervated ear. An interesting observation was that the rectal temperature was usually $2^{\circ}C$ higher than the temperature of the ear, the normal rectal temperature being $39^{\circ}C$, and the ear temperature 37°. Grant found evidence of only constrictor fibres to the rabbit ear vessels. There were greatly increased responses to almost all stimuli after denervation, and there was no difference in the effect of total denervation compared with extirpation of the superior cervical ganglion. Grant suggested that an adrenaline-like substance released during muscular or nervous activity caused

constriction in the sensitised vessels of the denervated ear. He said that this did not come from the pituitary or from the adrenal glands. Grant also found that histamine dilated the small ear vessels and constricted the larger arteries, confirming the work of Feldberg (1927).

Grant and Thompson (1963) reported on the nerve supply to blood vessels in the rabbit external ear and the association of cholinesterase with this nerve supply. Grant and Thompson found cholinesterase to be distributed throughout the course of the nerves. They also reported that some sympathetic nerve fibres survived ganglionectomy and some even survived "total denervation". It was found that cholinesterase activity was halved by ganglionectomy and virtually abolished by superimposing sensory nerve section.

Perfusion of the rabbit ear via the carotid artery after decapitation was employed by Gaddum and Kwiatowski (1938). Outflow through a venous cannula was measured using an outflow recorder. Gaddum and Kwiatowski examined the constrictor actions of ephedrine on the blood vessels of the rabbit ear.

Burn and Dutta (1948) perfused the rabbit ear in two ways, using the perfusion method of Gaddum and Kwiatowski (1938), and the method described by Rischbieter, employing the outflow recorder of Stephenson (1948). They examined the effects of atropine, benadryl, pethidine,

procaine and quinidine in the ear, which all inhibited the constrictor action of adrenaline. Gowdey (1948) perfused the rabbit ear by the method of Gaddum and Kwiatowski and demonstrated a reversal by tolazoline of the constrictor action of adrenaline on the rabbit ear blood vessels. Burn and Hutcheon (1949) reported the constrictor action of noradrenaline in rabbit ear vessels and found this to be reversed by tolazoline, as was that of adrenaline. Luduena, Ananenko, Siegmund and Miller (1949) studied the vasoconstrictor effects of laevo and dextro adrenaline and noradrenaline on the perfused rabbit ear. They found that l-noradrenaline was one-and-a-half to two-and-a-half times as potent as l-adrenaline, and that l-noradrenaline was twelve to eighteen times more potent than d-noradrenaline, as vasoconstrictors in the rabbit ear.

Miyake (1952) investigated the potentiating effects of cocaine and procaine on the pressor response to adrenaline in rabbit ear blood vessels. Miyake perfused by the method of "Pissemski" but probably meaning Bissemski quoted by Rischbieter, (1913). Miyake found that cocaine and procaine potentiated vasoconstriction by adrenaline, and made the important observation that enzyme inhibition alone was not responsible for the potentiation of adrenaline by cocaine, and by procaine, in the rabbit ear.

The rabbit ear was now established for use as a vascular model.

However, the perfusion methods used did not always provide stable experimental conditions. The perfused ear and the perfusion fluid were normally at room temperature, and the perfusion system allowed prolonged constriction of the ear vessels, with consequently long exposures to perfused drugs. A major improvement in the technique of recording from the perfused rabbit ear was that employed by de la Lande, Paton and Waud (1964) in which a constant volume pump delivered physiological saline solution heated to 37°C, and bubbled with a mixture of 5% carbon dioxide in oxygen. Flow rates and temperatures approaching those found with in vivo conditions allowed more accurate observations of changes in vessel tone, measuring changes in pressure in the perfusion system as changes in diameter of the perfused vessels.

de la Lande, Paton and Waud also perfused the isolated central ear artery, and the sympathetic innervation of this artery was subsequently examined by de la Lande and Rand (1965). The perfusion system was further modified by de la Lande, Cannell and Waterson (1966) to provide separate observations of responses to the intraluminal and extraluminal application of drugs. This enabled comparisons to be made of the sensitivity of the intraluminal and extraluminal surfaces, with an opportunity to observe the behaviour of drugs diffusing into the blood vessel wall. A system was now available to obtain information on the behaviour of a variety of drugs acting on the blood vessels of the rabbit

ear and particularly on the central artery of the rabbit ear.

The development by Falck (1962) of the fluorescent histochemical identification of catecholamines in nerve tissue, made possible the combined morphological and perfusion studies reported in this thesis. Examination of the anatomical structure of the central artery of the rabbit ear and the arrangement of its sympathetic nerves, aided the design of experiments which were to test the role of the nerves in the function of the artery. A particular nerve function examined was that of conservation of sympathetic transmitter substance by its re-absorption into the nerves. Until recently the sympathetic nerve transmitter substance in the rabbit ear was thought to be adrenaline, as Gaddum and Kwiatowski (1938) reported the presence of significant amounts of adrenaline released in the ear by ephedrine. Outschoorn (1952) found that noradrenaline was the predominant amine in the rabbit ear. de la Lande and Head (1967) took segments of rabbit ear artery and estimated catecholamine content by fluorimetric assay. They found noradrenaline to be the predominant amine present in this vessel. This finding is supported by fluorescence histochemical studies of the central artery which suggest that noradrenaline is present in discrete areas in the wall of the vessel (de la Lande and Waterson, 1967a; Waterson and Smale, 1967).

Scope of project

The present project was commenced originally to compare the vasoconstrictor activity of the barbiturate anaesthetic drugs thiopentone and methohexitone, and to further test the hypothesis presented by Burn and Hobbs (1959) that thiopentone caused constriction of the ear vessels, and that this was induced by the release of noradrenaline. Comparisons were made with other sympathomimetic substances and changing sensitivity to noradrenaline became a problem in the assessment of results. Therefore the nature of the vascular sensitivity changes was studied in greater detail. This involved histological as well as pharmacological studies on the ear artery. As a result, an experimental hypothesis has been developed which has proved useful in interpreting the role of the sympathetic nerves in responses of the artery to certain drugs. Reports of the histological and pharmacological studies on the ear artery form a major part of this thesis, and are presented accordingly in the earlier chapters. In later chapters the results of comparisons of the activity of the rabbit ear and its isolated central artery are presented, and are followed by the results of the barbiturate experiments.

CHAPTER 2

MORPHOLOGICAL STUDIES

When the present study was commenced, the main information on the relationship between nerves and blood vessels of the rabbit ear was that derived from the classical studies of Grant and his coworkers. (Grant, Bland and Camp, 1931; Armin and Grant, 1953; Armin, Grant, Thompson and Tickner, 1953; Grant and Thompson, 1963). Their findings have been described in the introductory chapter of this thesis, and in summary comprised evidence that the main artery of the rabbit ear was associated with a dense network of nerve fibres, some of which showed strong staining for cholinesterase. This is consistent with the findings of Holton and Rand (1962) who found physiological evidence for the presence of cholinergic nerves in the rabbit ear.

Histological evidence for a sympathetic innervation was found by Grant and Thompson, (1963), who noted that many of the nerves accompanying the blood vessels of the perichondrium of the ear degenerated after removal of the homolateral superior cervical ganglion. The bioassay data of Burn and Rand (1958) indicated that the skin of the rabbit ear contained noradrenaline and this was presumably associated with sympathetic nerves in the walls of the blood vessels. Physiolo-

gical evidence of Gaddum and Kwiatowski, (1938), and Stinson (1961), that the vessels of the rabbit ear constricted following sympathetic post ganglionic and central artery peri-arterial stimulation constituted further evidence that noradrenergic or adrenergic fibres were present and were closely associated with the artery wall. Later de la Lande, Paton and Waud, (1964) and de la Lande and Rand, (1965) showed that small segments of the isolated major ear artery were highly sensitive to the constrictor effects of peri-arterial stimulation, and the effects were characteristically those elicited by excitation of sympathetic nerves. These properties of the isolated artery were abolished by removal of the homolateral superior cervical ganglion in the rabbit one to three weeks previously. Hence the combined physiological and histological evidence pointed strongly to extensive sympathetic innervation of the central artery of the rabbit ear.

A major limitation of the classical techniques used by Grant and other workers was that they failed to reveal the true nature of the nerves surrounding the ear arteries, although the dense ground plexus was shown in some detail by Grant and Thompson. It seemed appropriate therefore to apply the fluorescent histochemical method developed by Falck (1962), which shows the location of catecholamines in tissues, to the problem of establishing the precise distribution of the sympathetic innervation in the central artery of the rabbit ear.

The advantage of the Falck method is that it detects localised concentrations of catecholamines and hence serves to highlight those nerves which are rich in sympathetic neurotransmitter, that is the fine nerve terminals which may escape resolution by classical light microscopy. It had already been shown in sections of brain and other tissues that blood vessels showed fluorescence characteristic of noradrenaline only in the outer portion, or adventitia of the vessel. (Falck, 1962; Norberg and Hamberger, 1964; Fuxe and Sedvall, 1965)

In preliminary experiments, attempts were made to apply the Falck method to stretched preparations of the ear artery, employing modifications described for the iris by Malmfors (1965). In a few preparations a dense network of fluorescent structures was seen, but the attempts were abandoned as the artery wall was too thick. It was evident that only application of the technique to thin sections of the artery would reveal useful information.

A number of problems were encountered in applying the Falck method to artery sections. The technique as used by Falck involved freeze drying of the tissue, exposure to formaldehyde gas to convert the amines (noradrenaline will be assumed unless otherwise indicated), to isoquinolines. The tissues were blocked in paraffin wax and sectioned. One problem in applying the method to the rabbit ear

was difficulty in handling small segments of isolated artery, and another was that the comparison of segments of perfused artery and unperfused controls required prolonged storage of some arteries. Little fluorescence due to formaldehyde treatment was detected until the paraformaldehyde powder used was standardised for water content (Hamberger, Malmfors and Sachs, 1965).

In the present study the Falck method was used successfully in more than 200 arteries. The specificity of the method for noradrenaline was tested in several ways.

1. Reserpine pretreatment, and chronic sympathectomy, procedures which are known to cause disappearance of noradrenaline from tissues.

2. Replenishment of the tissues with exogenous noradrenaline after depletion caused by reserpine.

3. The tests of specificity for catecholamine fluorescence described by Falck and Owman, (1965) and the borohydride specificity test of Corrodi, Hillarp and Jonsson, (1964).

4. Development and application of a method using Evans blue dye to eliminate the interference caused by

autofluorescence.

5. In an associated study, de la Lande and Head, (1967), analysed the artery directly by the fluorimetric method of von Euler and Lishajko, (1959), for the presence of noradrenaline and adrenaline.

The relevant experimental procedures and results are described in detail. Reference is also made to results obtained in other tissues. The latter tissues were those which were included for comparative purposes, such as rat and guinea pig iris and rat fat pad, and tissue such as dental pulp where ready availability offered a useful opportunity for exploring the innervation of blood vessels in the human. However, the bulk of the studies in this chapter are devoted to the morphology of the rabbit ear artery. It is emphasised that these studies were in most instances carried out concurrently with physiological studies on the artery, and are described separately only to facilitate presentation.

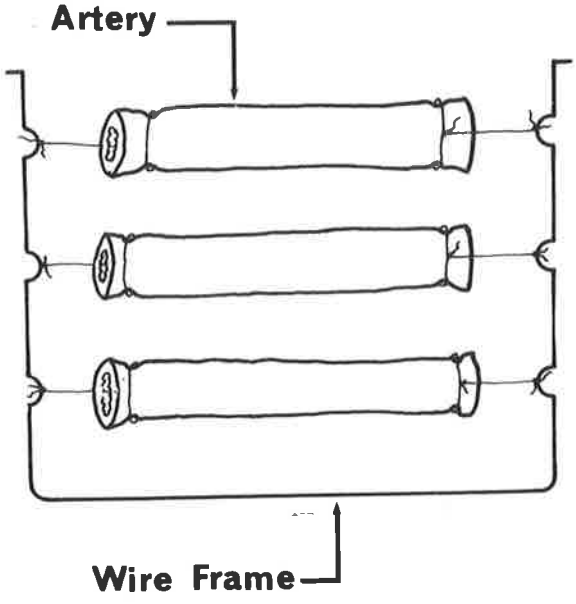
MATERIALS AND METHODS

A. Histology

1. Preparation of sections of the ear artery

Male and female lop-eared rabbits were anaesthetised with urethane, 2 grams/Kg intraperitoneally. The central ear artery was exposed by blunt dissection and a segment at least 1 cm long excised from the proximal part of the ear. The artery was tied with cotton thread to a wire frame (Figure 2.1) stretching being avoided, and frozen in a mixture of acetone and dry ice. (Fujiwara, Tanaka, Honjo and Okegawa, 1965). The frame and artery were rapidly transferred to a freeze drying apparatus (Thermovac, Model FD/3) which had been previously cooled to minimise the risk of thawing of the tissue. Freeze drying was continued for from 12 hours (for thin specimens) to 72 hours (for the thickest specimens) at temperatures of -50°C to -35°C, and pressures of 20-50 microns of mercury. The tissue was removed from the freeze dryer and placed in a one litre glass jar with 5 grams of paraformaldehyde powder (Merck) which had been stored over sulphuric acid for at least one week at a relative humidity of 70% (See appendix, page 5). The jar was covered with a metal lid, sealed with a rubber ring, and placed in an oven preheated to 80°C for either one hour or for three hours. The selection of one or three hours depended on the

Figure 2.1



Showing method of holding segments of artery during freeze drying and formaldehyde treatment.

particular amine under investigation, namely noradrenaline or adrenaline respectively. Falck and Owman (1965) showed that heating for one hour was necessary for the development of the noradrenaline fluophore, and for three hours for development of the adrenaline fluophore.

The formaldehyde treated tissue was vacuum infiltrated with paraffin wax at 60°C and blocked in paraffin wax. Transverse sections were cut at 5 to 7 microns thickness and further prepared as follows:

(i) Tissue treated with formaldehyde vapour for one hour was mounted in Entellan (Merck) and xylol mixture.

(ii) Tissue treated for three hours was mounted in liquid paraffin, since organic solvents such as xylol greatly reduce adrenaline, but not noradrenaline fluorescence (Falck and Owman, 1965).

The sections were examined with a Leitz Ortholux microscope. Fluorescence was produced with an HBO 200 mercury vapour lamp using a 3 mm Schott BG 12 excitation filter, and 490 to 530 millimicron barrier filters. A Leitz Orthomat camera and Kodak Photoflure film were used for photography. Either an oil immersion dark field

condenser or a phase contrast system and dry dark field condenser were used . Photographic exposures varied from 90 seconds to more than 5 minutes.

The above procedure differs from that of Falck in the following points (see diagram in figure 2.2);

1. The method of rapid freezing was the acetone/ dry ice method described for fluorescence studies by Fujiwara, Tanaka, Honjo and Okegawa (1965), whereas Falck used isopentane cooled in liquid nitrogen.

2. After completion of freeze drying, the preparations were transferred directly to the jar containing paraformaldehyde, whereas Falck used a perspex box containing phosphorus pentoxide as a dessicant to protect the specimens from moist air during transfer to numbered receptacles. In the present study specimens were identified by coloured cotton (for tying to frames) and by numbered containers.

Figure 2.2

MODIFICATION OF THE FLUORESCENT HISTOCHEMICAL METHODS

PROCEDURE	FALCK & OWMAN	PRESENT STUDY
RAPID FREEZING	PROPANE-PROPYLENE MIXTURE COOLED IN LIQUID NITROGEN.	ACETONE AND DRY ICE MIXTURE.
FREEZE DRYING	PRESSURE 10^{-6} TORR.	PRESSURE 2×10^{-3} to 5×10^{-3} TORR.
	TEMPERATURE $-20^{\circ}C$.	TEMPERATURE $-50^{\circ}C$ to $-35^{\circ}C$
PARAFFIN INFILTRATION	SPECIMENS NOT EXPOSED TO ATMOSPHERE BEFORE INFILTRATION.	SPECIMENS EXPOSED TO ROOM AIR BEFORE INFILTRATION.
MICROSCOPY	OIL IMMERSION CONDENSER USED.	DRY CONDENSER SYSTEM USED.

Comparison of the histochemical method described by Falck and Owman (1965) with the method described in the present study.

2. Preparation of stretched tissues

(i) Iris muscle

Irises from albino guinea pigs, rats and rabbits were frequently used as comparative or control preparations to test the validity of the technique, particularly when occasional arteries unexpectedly failed to show fluorescence. (Usually such failures were found to be due to imperfect freeze drying through power failure or leaks in the system, or to unsuitable water content of the paraformaldehyde).

The irises were stretched on glass slides and air dried over phosphorus pentoxide for at least one hour (Malmfors, 1965). After drying the preparations were treated with formaldehyde vapour in the same way as the freeze dried tissue. They were examined immediately after formaldehyde treatment, usually unmounted, but some were mounted in the media used for the artery.

(ii) Epididymal rat fat pad

The tissue was used since it contains serotonin (in mast cells) as well as sympathetic nerves in small blood vessels. The rats were stunned and bled, and the epididymal fat stretched on a glass slide and then treated in an identical fashion to the iris (see above).

(iii) Auricles

The auricle of the toad "Bufo marinus" was used as an example of a sympathetically innervated tissue rich in adrenaline rather than noradrenaline (quoted by Cooper, de la Lande and Tyler, 1966). Auricles from the Sleepy Lizard "Tiliqua rugosa" which contain mainly noradrenaline (Cooper, de la Lande and Tyler, 1966) were used for comparison. The auricles were stretched on glass slides and subsequently prepared in an identical fashion to the iris muscle (see above).

(iv) Rabbit and human dental pulp

Rabbit dental pulp was selected as a further example of vascular tissue from the rabbit which allowed comparison with the intramural innervation observed in the ear blood vessels. However, in view of the ready availability of human dental pulp, and the paucity of histological information on sympathetic innervation of human blood vessels, the opportunity was taken to examine blood vessels in human pulp. Rabbit dental pulps were taken from teeth extracted from rabbits anaesthetised with urethane, and the human pulps were taken from teeth extracted using local anaesthesia. (See appendix page 5). The pulp tissue was treated as follows:

Pulps were extirpated after extraction of the teeth, frozen in acetone and dry ice mixture, and treated and sectioned

by the same procedures as used for the rabbit ear artery.

3. Other histological procedures

Standard methods of sectioning and staining arteries for ordinary light microscopy were as follows:

Segments of artery were fixed in formal saline, dehydrated and cleared before blocking in paraffin wax, melting point 60°C. Sections were cut at 5 to 7 microns thickness and were stained with Van Gieson stain, although some were stained with Mallory and Orcein stains to demonstrate elastic fibres.

In many experiments serial sections of freeze-dried formaldehyde treated artery were stained with Van Gieson stain after dewaxing and clearing. This was done to ensure that no gross morphological changes occurred in the frozen and formaldehyde treated arteries.

4. Tests of specificity

The following tests were applied to show that the methods used were specific for catecholamines:

(i) Some sections of formaldehyde treated tissue were floated on water before mounting, as

water eliminates catecholamine fluorescence (Falck and Owman, 1965).

(ii) Some tissue was treated by the usual methods, with the exception that no paraformaldehyde was present in the jar during the heat treatment.

(iii) The borohydride test for specificity of catecholamine fluorescence (Corrodi, Hillarp and Jonsson, 1964) was applied. Sections of formaldehyde treated arteries were washed in 0.1% sodium borohydride dissolved in 90% isopropanol. They were later re-exposed to formaldehyde vapour. Great difficulty was experienced in retaining the very small sections of artery on slides during these procedures.

5. Elimination of autofluorescence

The following procedure was developed to eliminate autofluorescence in the rabbit ear artery. Evans blue dye 50 µg/ml in Krebs bicarbonate solution was perfused for 10 minutes, with both intraluminal and extraluminal surfaces of the artery exposed to the perfusate. The dye was applied before formaldehyde treatment and

examination of the artery using fluorescence microscopy.

To test the effect of the dye on catecholamine fluorescence in another tissue irises from two albino guinea pigs were treated by the method of Malmfors (1965). The iris from the left eye was placed in a solution of Evans blue, 50 µg/ml in Krebs bicarbonate solution for 10 minutes while the contralateral iris was placed in Krebs bicarbonate solution. After air-drying and formaldehyde vapour treatment the stretched irises were examined by fluorescence microscopy.

Further sections of artery were prepared by standard histological methods following fixation in formal saline. Some sections were stained for 10 minutes with Evans blue 50µg/ml, and compared with unstained sections using fluorescence microscopy.

B. Treatment of animals

1. Denervation of arteries

This was carried out by removal of the homo lateral superior cervical ganglion one to four weeks previously. Details are presented on page **3.16.**

2. Reserpine pretreatment

Reserpine was dissolved in 20% ascorbic acid and either injected intraperitoneally 3 to 72 hours previously or injected intravenously ½ to 4 hours before removal of the artery. Details of dosage are presented on page 3.18.

3. Replenishment of noradrenaline

The above procedures 1 and 2 were intended to cause the disappearance of noradrenaline from the artery wall. In order to replace noradrenaline in the artery wall, the following procedure, based on that of Malmfors (1965), was evolved.

(i) In vivo experiments

Three rabbits anaesthetised with urethane received a dose of reserpine 2.5 mg/kg intravenously, followed two hours later by nialamide 100 mg/kg intraperitoneally. Two hours later one artery was removed and placed in Krebs bicarbonate solution for 10 minutes. Immediately following excision of the artery the rabbit was given 0.5 mg/kg of noradrenaline intravenously over a period of 5 minutes, and the artery from the opposite ear removed. The two arteries were then treated histochemically (described on page 2.6) for noradrenaline.

(ii) In vitro experiments

In 5 experiments, noradrenaline was depleted with reserpine, and replenished in the isolated perfused artery. The central arteries from both ears were perfused by the single cannula method (page 3.11) for 30 minutes. Reserpine, 50 µg/ml was recirculated from a volume of 100 ml for 15 minutes, while Krebs bicarbonate solution was recirculated in the opposite ear artery from a similar container. Fresh pieces of artery were taken from each ear before commencement of perfusion, and prepared for freeze drying. Two hours after the reserpine perfusion, nialamide 2 mg/ml was recirculated in both arteries from a volume of 100 ml for 15 minutes. After a further two hours, a piece of the distal end of each artery was removed and prepared for freeze drying. A perfusion of noradrenaline 20 ng/ml was perfused through the reserpinised and control arteries for 10 minutes, and noradrenaline 10 µg/ml was added to the extraluminal fluid in the organ bath. After 5 minutes the noradrenaline was washed out, and the arteries perfused with Krebs solution for 5 minutes. Both remaining segments of the arteries were prepared for freeze drying. The six pieces of artery were then freeze dried, formaldehyde treated, and examined by fluorescence microscopy as described on pages 2.6 to 2.8.

In 4 experiments similar depletion of noradrenaline was followed by attempted repletion in the presence of cocaine 10 µg/ml perfused through the artery. In each experiment the cocaine treated artery was compared with a control (not treated with cocaine) artery. The experimental procedure is shown diagrammatically in figure 2.3.

Figure 2.3

STAGE	EXPERIMENTAL TIME IN MINUTES	PERFUSION	
		CONTROL ARTERY	EXPERIMENTAL ARTERY
1	0-30	KREBS	KREBS
2	30-45	KREBS	RESERPINE 50 µg/ml
3	45-165	KREBS	KREBS
4	165-180	NIALAMIDE 2 mg/ml	NIALAMIDE 2 mg/ml
5	180-300	KREBS	KREBS
6	300-310	NORADRENALINE 20 ng/ml I.L.	NORADRENALINE" 20 ng/ml I.L.
7	310-315	10 µg/ml E.L.	10 µg/ml E.L.
8	315-320	KREBS	KREBS

I.L. = intraluminally

E.L. = extraluminally

Experimental plan for the in vitro depletion and repletion of noradrenaline. In 5 experiments segments of each artery were taken for histochemical study prior to perfusion, at the conclusion of stage 4 and at the conclusion of stage 8.

In 4 experiments in which cocaine was used to block repletion, the above procedure was varied so that both the control and experimental arteries were perfused with reserpine, and the experimental artery was perfused with cocaine (10 µg/ml I.L. and E.L.) for 10 minutes prior to noradrenaline perfusion.

RESULTS

1. Sections of rabbit ear artery

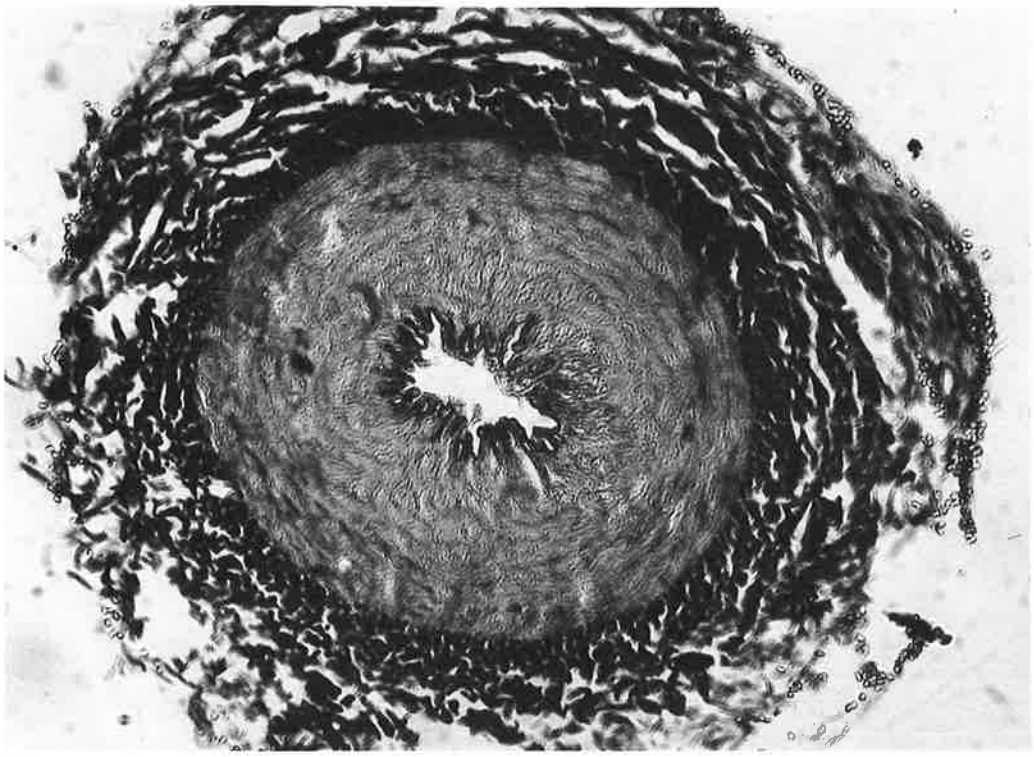
(i) Classical staining

The appearance of the artery after staining by the Van Gieson method is shown in figure 2.4. The media of the artery stains yellow in this method, while the adventitia stains pink to red. The medial-adventitial border is clearly defined, and the intima is shown as a folded membranous structure. The smooth muscle layer is relatively thick, and in most arteries in transverse section there were about 10 smooth muscle cells between the intima and the adventitia. The total diameter of the artery was of the order of 500 microns.

(ii) Formaldehyde treated tissue

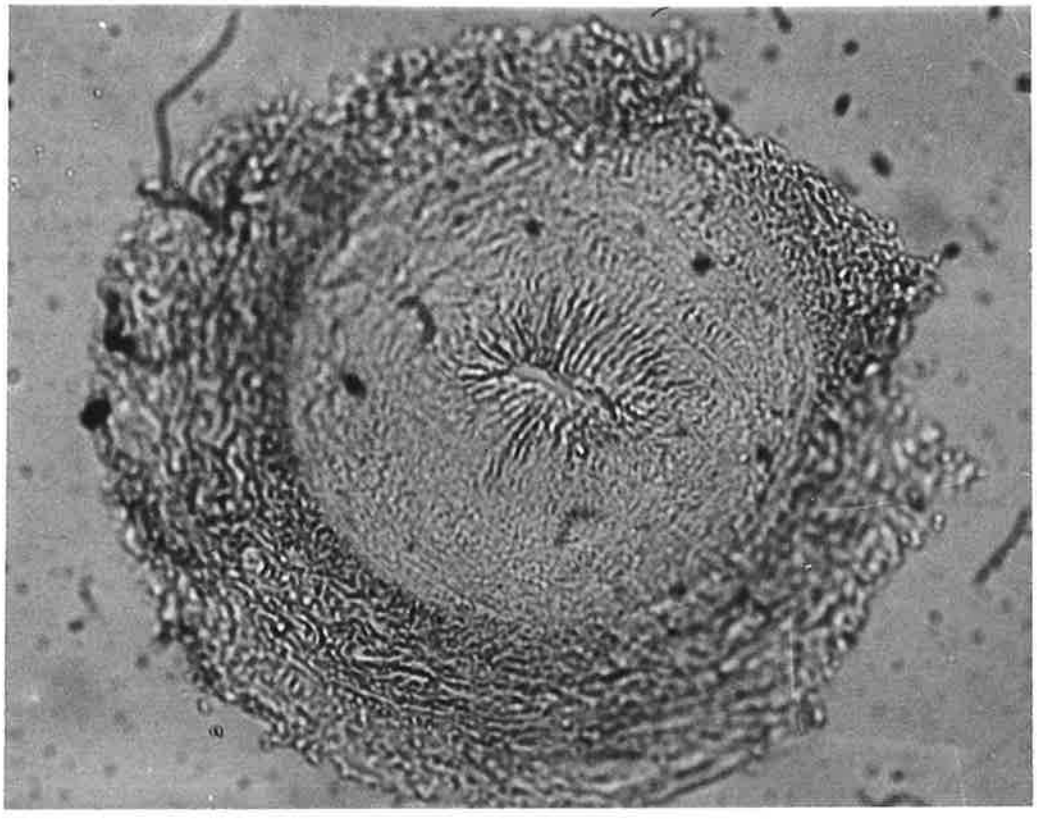
An unstained section of artery treated with formaldehyde vapour, and illuminated with light from a tungsten lamp is shown in figure 2.5. The medial-adventitial border is clearly shown. The identical field is shown in figure 2.6, but illuminated with light from a mercury vapour lamp with suitable filters to view catecholamine fluorescence. Two areas of fluorescence are seen, one at the intima, and one at the medial-adventitial border. The intimal fluorescence appeared as a greenish yellow line, and is the auto-

Figure 2.4



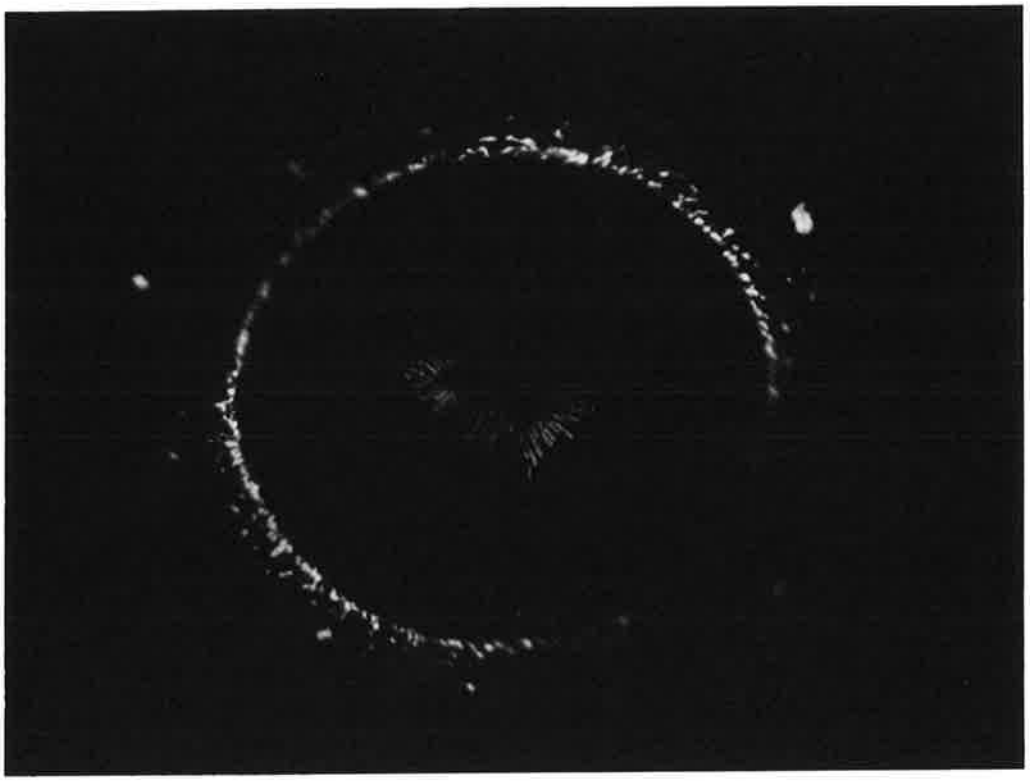
Transverse section of rabbit ear artery showing the clearly defined adventitia (dark stained outer layer) and the lighter media. The medial-adventitial border is clearly defined. The folded intima is at the centre of the section.
Van Gieson stain.
Scale 100 microns.

Figure 2.5



Transverse section (unstained) of rabbit ear artery, illuminated with light from a tungsten filament.
The border of the media and the adventitia is shown (compare with figure 2.4).
The identical field illuminated by an ultra-violet lamp is shown in figure 2.6.
Scale 100 microns.

Figure 2.6



Transverse section of rabbit ear artery. Shows noradrenergic fluorescence at the medial-adventitial border, and auto-fluorescence of the intima. Identical field to that shown in figure 2.5.
Formaldehyde treatment 1 hour.
Scale 100 microns.

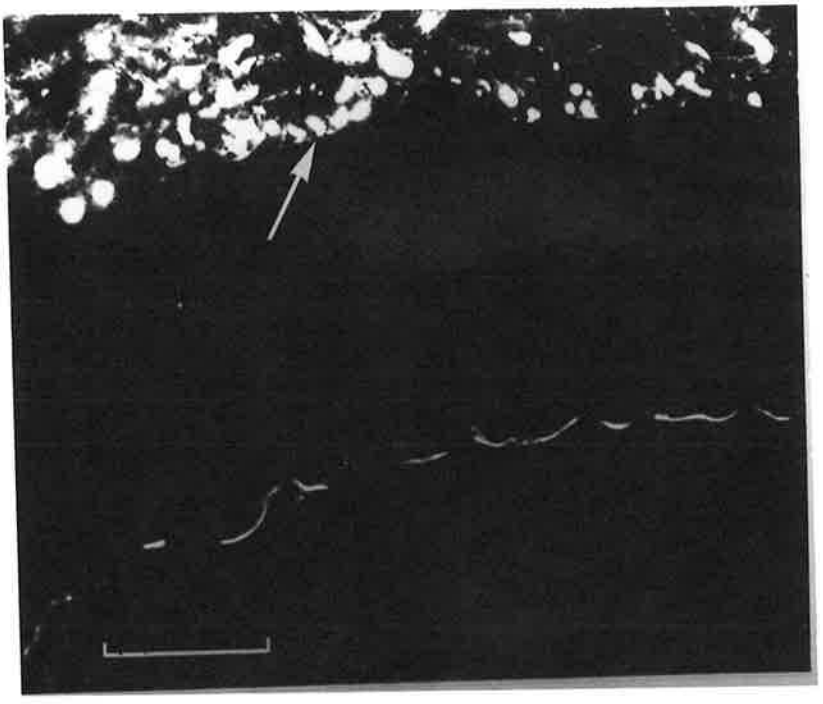
fluorescence reported in many tissues, and particularly near the intimal surface of blood vessels (Falck, 1962; Fuxe and Sedvall, 1965). The outer fluorescence appeared as a ring of bright green to greenish-yellow fluorescent structures, which were located at the medial-adventitial border. The position of the fluorescence was confirmed by phase contrast microscopy of the same field.

The bright ring of specific fluorescence was restricted to the outer border of the smooth muscle layer. Careful examination of many arteries under high power, (up to X400) and using oil immersion objectives and condensers, failed to show fluorescence in any part of the smooth muscle layer. (figure 2.7).

(iii) Identity of fluorescent structures as noradrenergic

a. Sections exposed to water before microscopy did not show a layer of fluorescent structures at the medial-adventitial border. Autofluorescence at the intima appeared unchanged when the sections were compared with serial sections not exposed to water. (Falck and Owman, 1965).

Figure 2.7



Transverse section of wall of rabbit ear artery. The arrow shows noradrenergic fluorescence at the medial-adventitial border. The irregular single line is intimal autofluorescence. There is no fluorescence shown in the media. Formaldehyde treatment 1 hour. Scale 50 microns.

b. Tissue heated in the absence of formaldehyde vapour did not show fluorescence at the medial-adventitial border although intimal autofluorescence was evident.

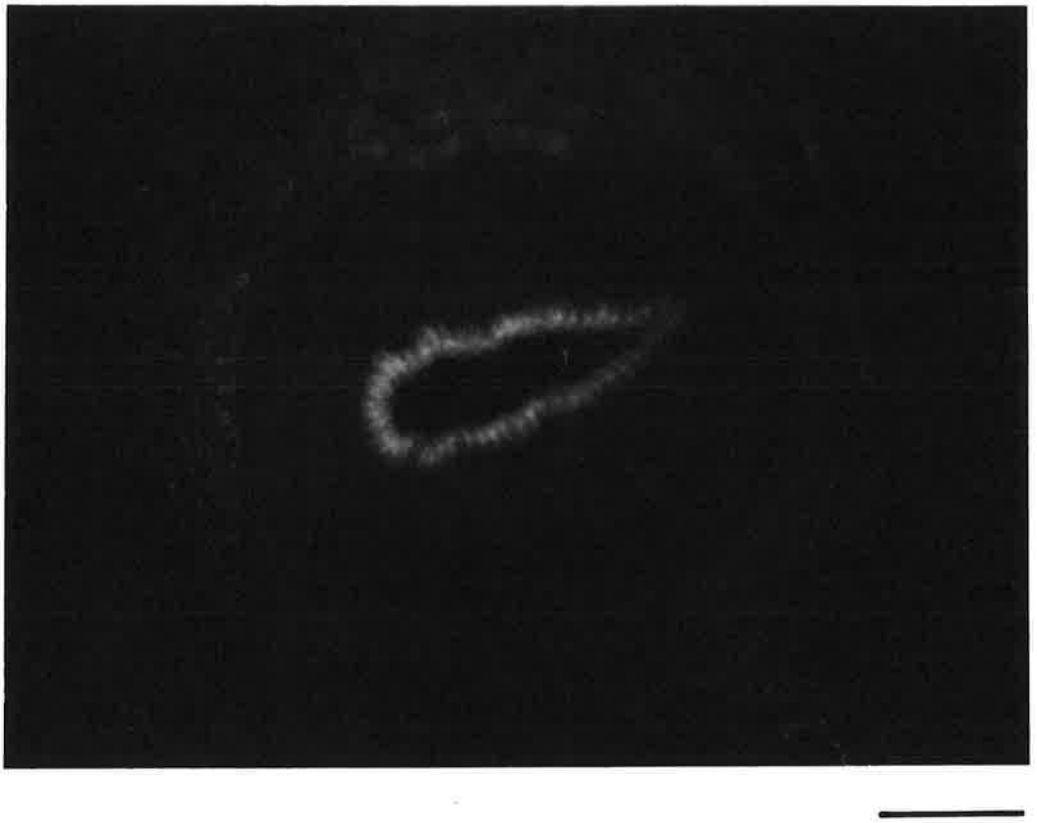
c. The borohydride test for specificity of monoamines was applied to sections of arteries in three experiments. Borohydride solution eliminated the fluorescence, which was later restored by further formaldehyde treatment. (Corrodi, Hillarp and Jonsson, 1964).

d. Arteries taken from rabbits treated with reserpine at least three hours before removal of the arteries (see page 3.18) did not show fluorescence at the medial-adventitial border after formaldehyde treatment, although the usual autofluorescence was visible near the intima. In those rabbits given reserpine intravenously less than two hours before taking the artery specific fluorescence was occasionally observed. The intravenous dose of reserpine was 2.5 mg/kg.

e. Rabbit ear arteries were denervated by removal of the homolateral superior cervical ganglion 2 to 25 days before use. Segments of the denervated arteries were treated by the Falck method, together with segments of untreated control arteries taken from the contralateral ear. The denervation procedures are described in detail on page 3.16. Other segments of the same arteries were perfused with Krebs bicarbonate solution as described on page 3.10. The effectiveness of denervation was judged by the lack of response of the denervated artery to electrical stimulation (page 3.15) compared with the control artery from the opposite ear. In no case was the bright outer layer of fluorescence observed in a formaldehyde treated artery taken from the denervated ear. A denervated and a control artery from the same rabbit are shown in figures 2.8 and 2.9.

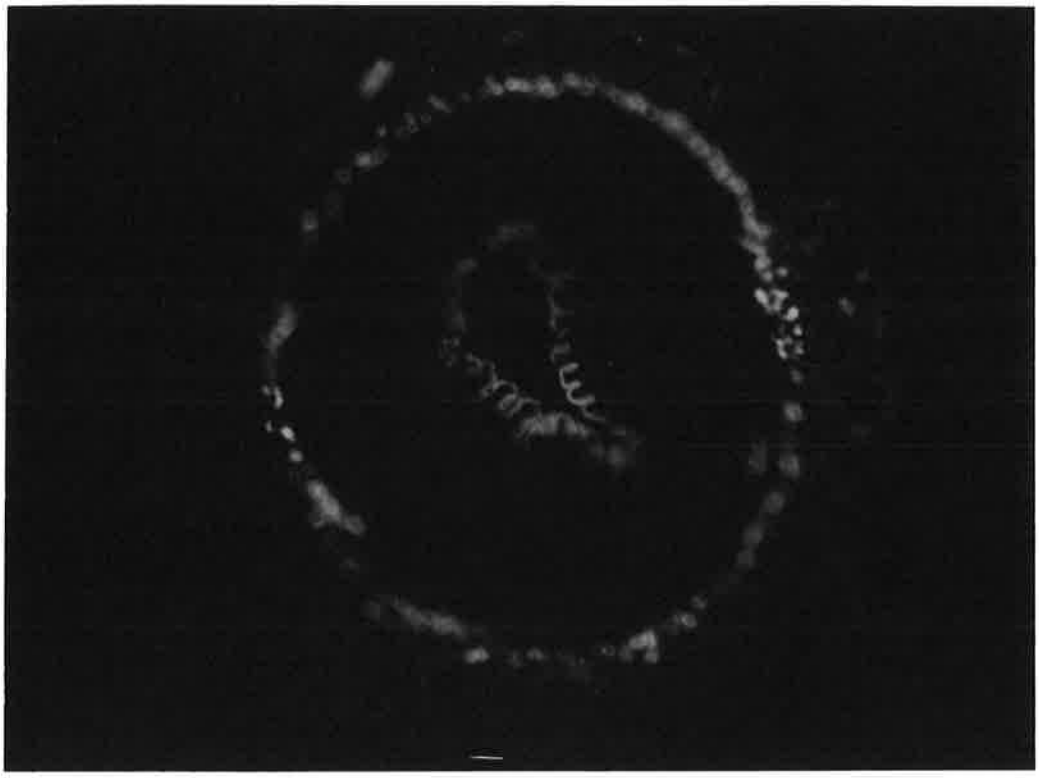
f. Perfusion of the artery with Evans blue resulted in the marked modification of the autofluores-

Figure 2.8



Transverse section of chronically denervated rabbit ear artery.
No noradrenergic fluorescence is shown.
The usual intimal autofluorescence is visible.
Compare with control artery figure 2.9.
Formaldehyde treatment 1 hour.
Scale 100 microns.

Figure 2.9



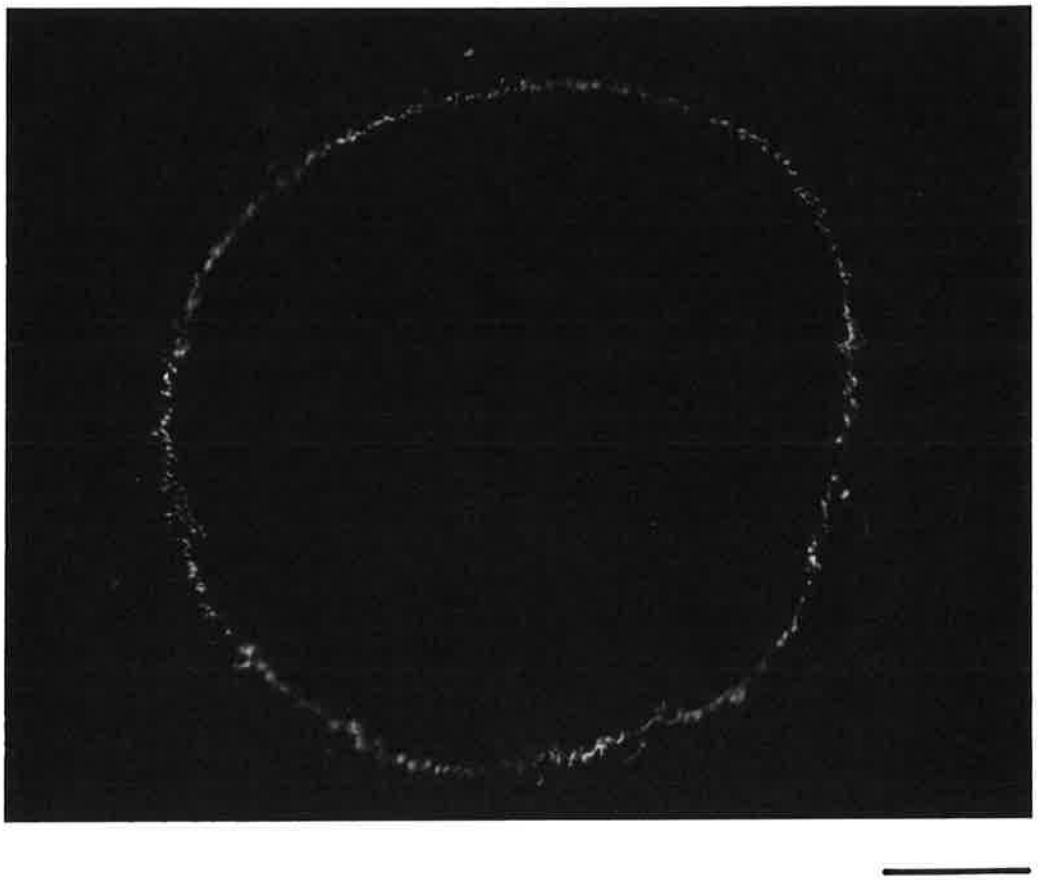
Transverse section of rabbit ear artery.
Noradrenergic fluorescence at the medial-adventitial border and autofluorescence at the intima.
Control for denervated artery (compare with figure 2.8).
Formaldehyde treatment 1 hour.
Scale 100 microns.

cence of the intima, the lumen now giving a dull red appearance. The fluorescence at the outer border of the smooth muscle was unchanged. An artery typical of 10 treated with Evans blue is shown in figure 2.10. Under the conditions of photography used the red colour of the intima is not reproduced.

The guinea pig irises treated with Evans blue showed specific fluorescence which was identical to that in the control (untreated with Evans blue) irises. Sections of arteries prepared by standard histological methods, and stained with Evans blue, showed no green autofluorescence, while unstained sections showed the usual bright green autofluorescence at the intima.

The preceding observations provide strong evidence that the bright green fluorescence at the medial-adventitial border is due to noradrenaline contained in or closely associated with sympathetic nerve endings.

Figure 2.10



Transverse section of rabbit ear artery after treatment with Evans blue dye.
Noradrenergic fluorescence is shown at the medial-adventitial border, but there is no autofluorescence at the intima.
Formaldehyde treatment 1 hour.
Scale 100 microns.

(iv) Ear artery after replenishment with noradrenaline

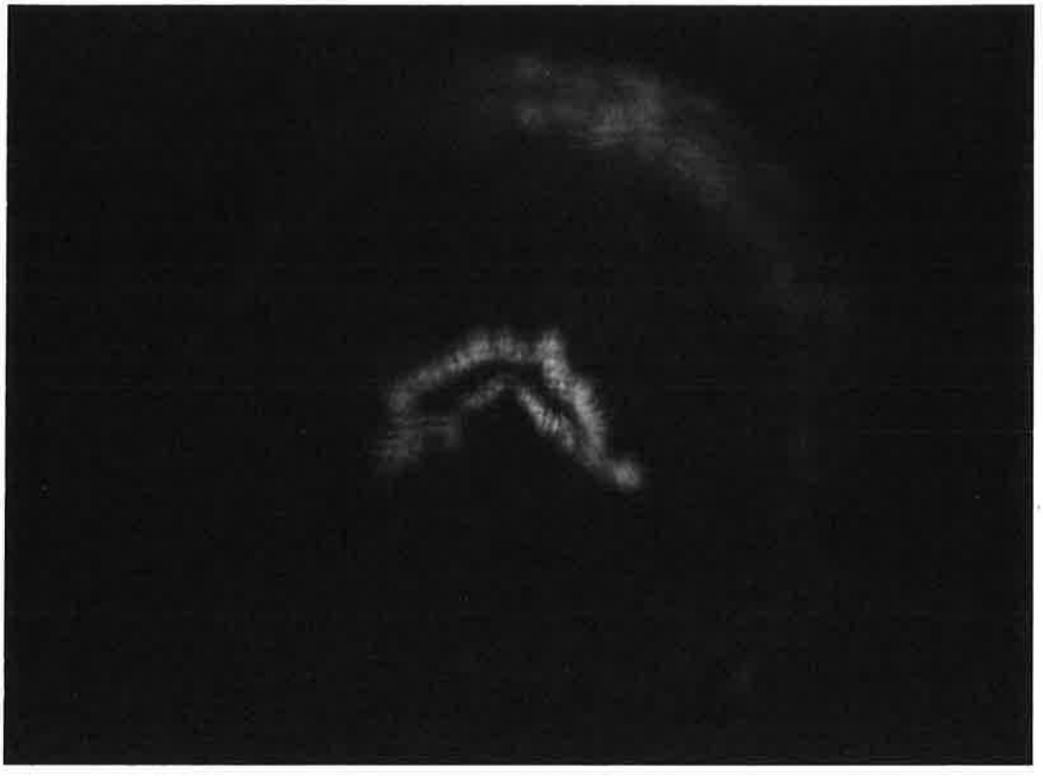
a. In vivo experiments

Arteries from rabbits treated with reserpine and nialamide (see page 2.14) showed no outer layer of fluorescence (figure 2.11) while arteries from the opposite ears of the same rabbits showed bright fluoreseence at the medial-adventitial border after the infusion of noradrenaline. A typical comparison is shown in figures 2.11 and 2.12.

b. In vitro experiments

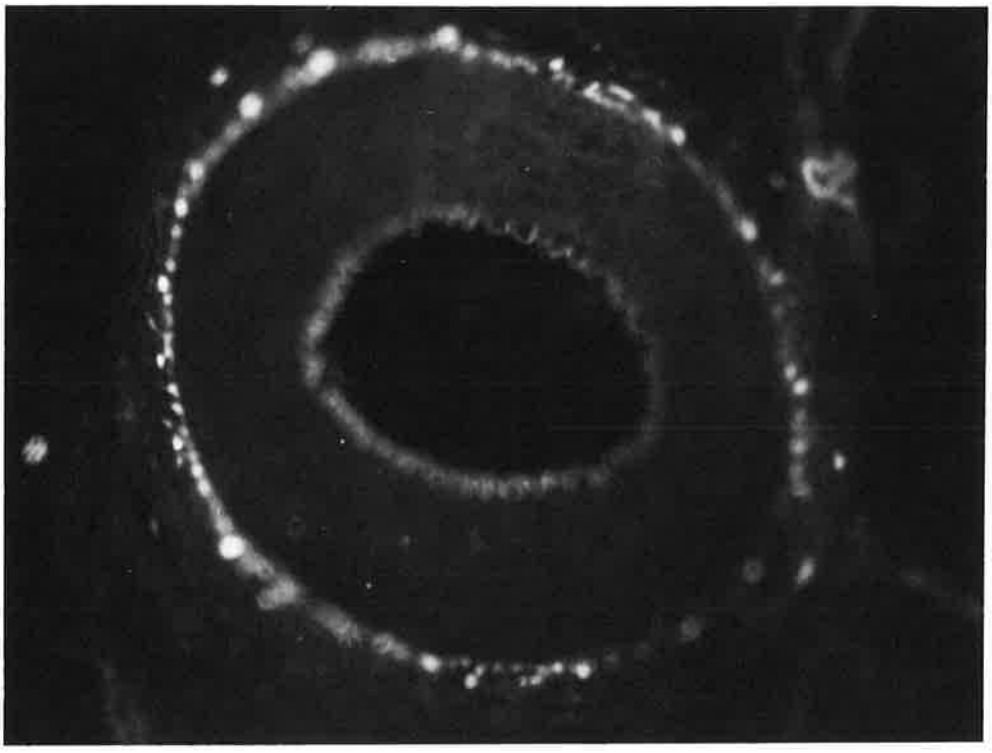
The arteries perfused with reserpine and nialamide did not show fluorescence at the medial-adventitial border, while the arteries perfused with nialamide only did show this fluorescence. The noradrenaline perfused arteries all showed fluorescence at the medial-adventitial border. It was therefore concluded that the perfused noradrenaline was taken up into the neural storage sites. A

Figure 2.11



Transverse section of rabbit ear artery after depletion of noradrenaline *in vivo*. There is no noradrenergic fluorescence. Compare with repleted artery in figure 2.12.
Formaldehyde treatment 1 hour.
Scale 100 microns.

Figure 2.12



Transverse section of rabbit ear artery after depletion of noradrenaline, and subsequent repletion of noradrenaline *in vivo*.
Noradrenergic fluorescence at the medial-adventitial border.
Compare with figure 2.11.
Formaldehyde treatment 1 hour.
Scale 100 microns.

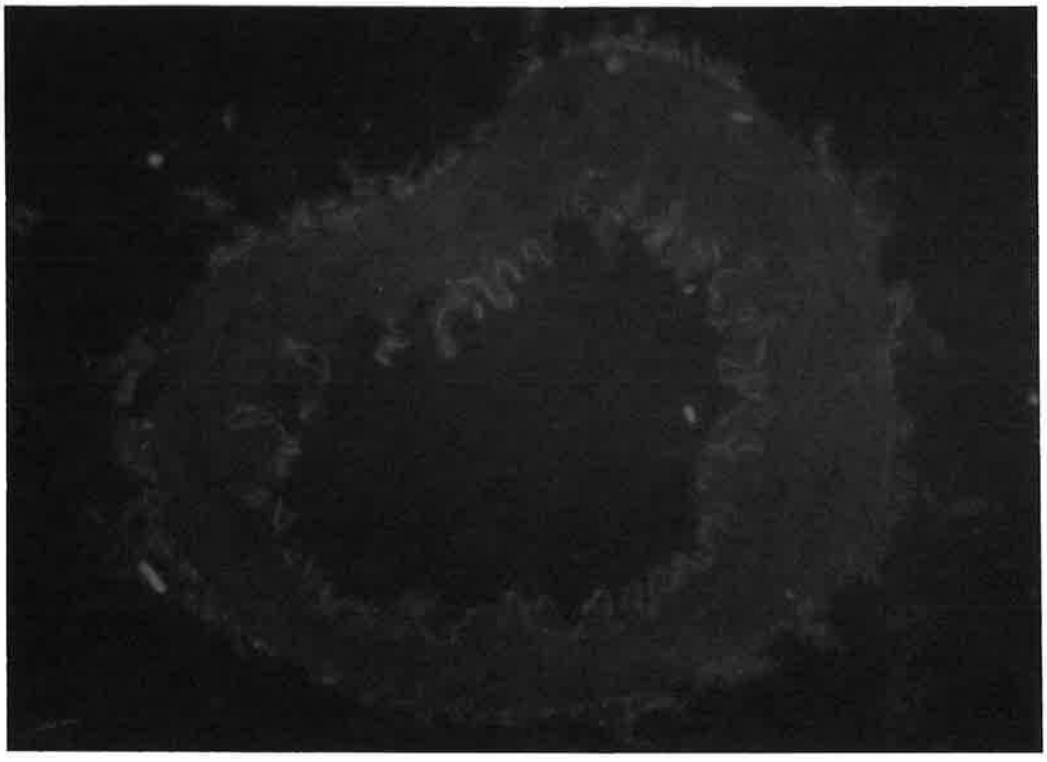
typical comparison is shown in figures 2.13 and 2.14. In 4 arteries in which cocaine was used to block uptake of noradrenaline, the fluorescence at the medial-adventitial border was very greatly modified. The four control arteries in this series of experiments all showed bright outer fluorescence after exposure to noradrenaline. A typical comparison of a cocaine treated artery and a control (not treated with cocaine) artery is shown in figures 2.15 and 2.16). In all arteries the autofluorescence at the intima was clearly visible.

(v) Other tissues

Rat fat pad

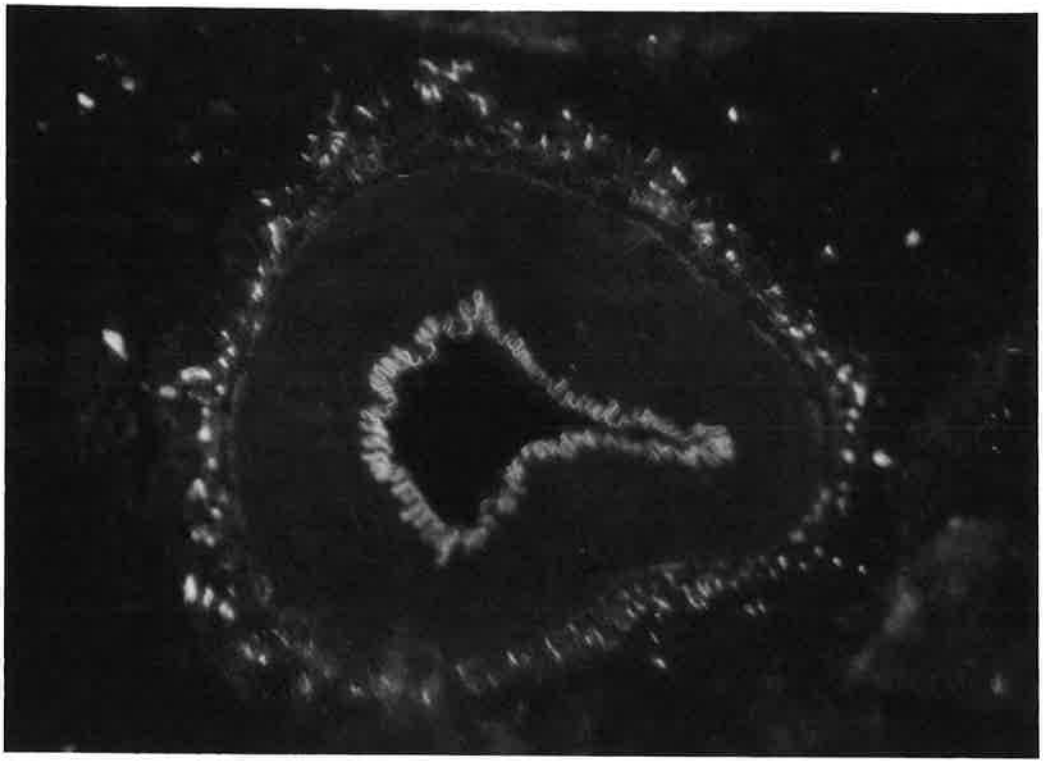
The stretched tissue preparations of rat fat pad showed green specific fluorescence associated with blood vessels, and probably due to noradrenaline, and discrete cells with bright yellow fluorescence, similar to that described by Falck and Owman (1965) for serotonin. The green fluorescence disappeared and the yellow fluorescence was greatly reduced 24 hours after reserpine pretreatment.

Figure 2.13



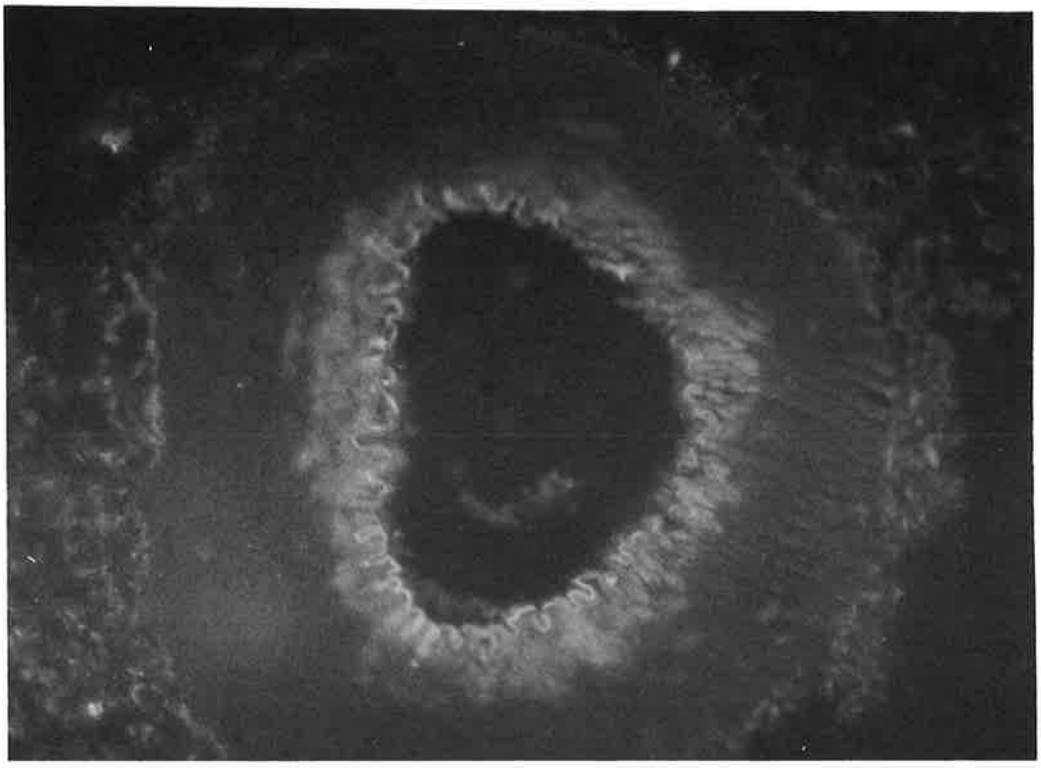
Transverse section of rabbit ear artery after depletion of noradrenaline *in vitro*.
There is no noradrenergic fluorescence.
Compare with figure 2.14.
Formaldehyde treatment 1 hour.
Scale 100 microns.

Figure 2.14



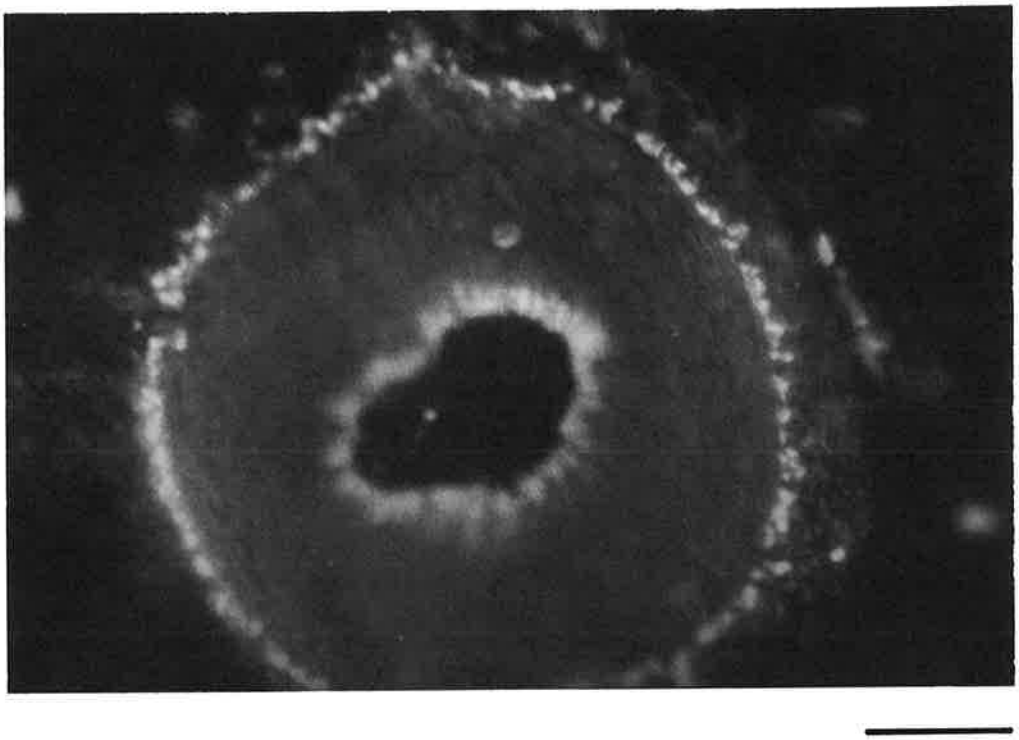
Transverse section of rabbit ear artery after depletion of noradrenaline, and subsequent re-exposure to noradrenaline 10 μg/ml *in vitro*.
Noradrenergic fluorescence is shown at the medial-adventitial border, and autofluorescence at the intima.
Formaldehyde treatment 1 hour.
Scale 100 microns.
Compare with figure 2.13

Figure 2.15



Transverse section of rabbit ear artery after depletion of noradrenaline *in vitro* and exposure to noradrenaline in the presence of cocaine 10 μg/ml.
Shows autofluorescence at the intima but no noradrenergic fluorescence at the medial-adventitial border.
(Compare with control artery figure 2.16).
Formaldehyde treatment 1 hour.
Scale 100 microns.

Figure 2.16



Transverse section of rabbit ear artery after depletion of noradrenaline and subsequent repletion in vitro.
Shows noradrenergic fluorescence at medial-adventitial border.
Control for artery in figure 2.15.
Formaldehyde treatment 1 hour.
Scale 100 microns.

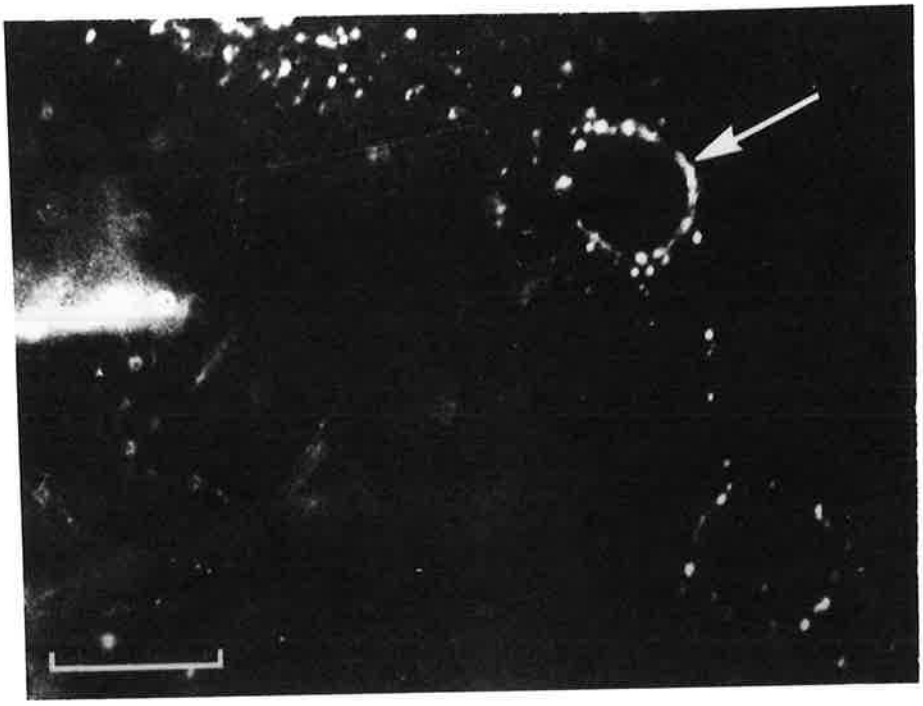
Iris muscle

The stretched iris muscle preparations showed bright green fluorescence similar to that described by Malmfors (1965).

Sections of dental pulp and other tissue

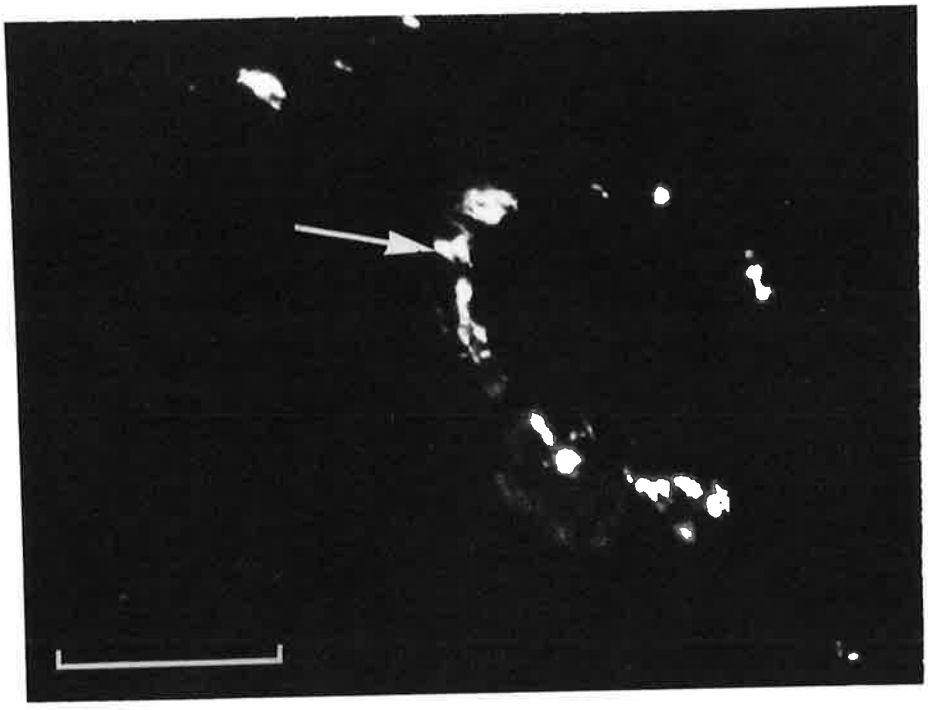
Sections of rabbit and human dental pulp showed bright green fluorescence external to the smooth muscle layer of the blood vessels. Typical examples of these and other tissues examined are shown in figures 2.17 to 2.22.

Figure 2.17



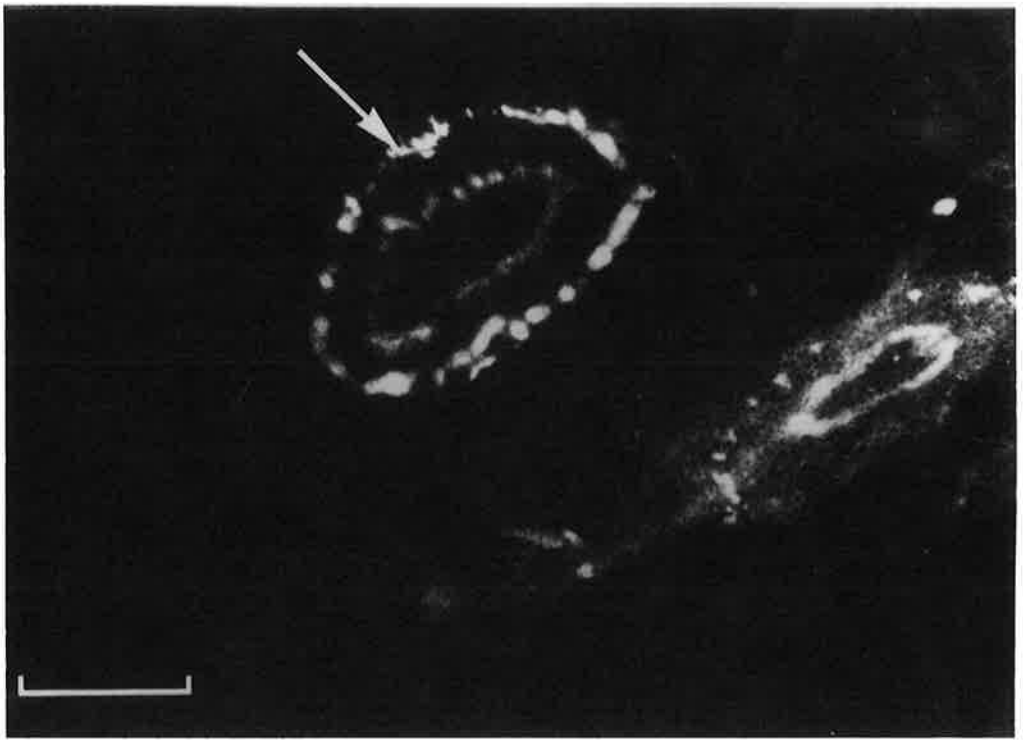
Transverse section of rabbit dental pulp.
The arrow shows fluorescent structures at the outer border of a blood vessel.
Formaldehyde treatment 1 hour.
Scale 100 microns.

Figure 2.18



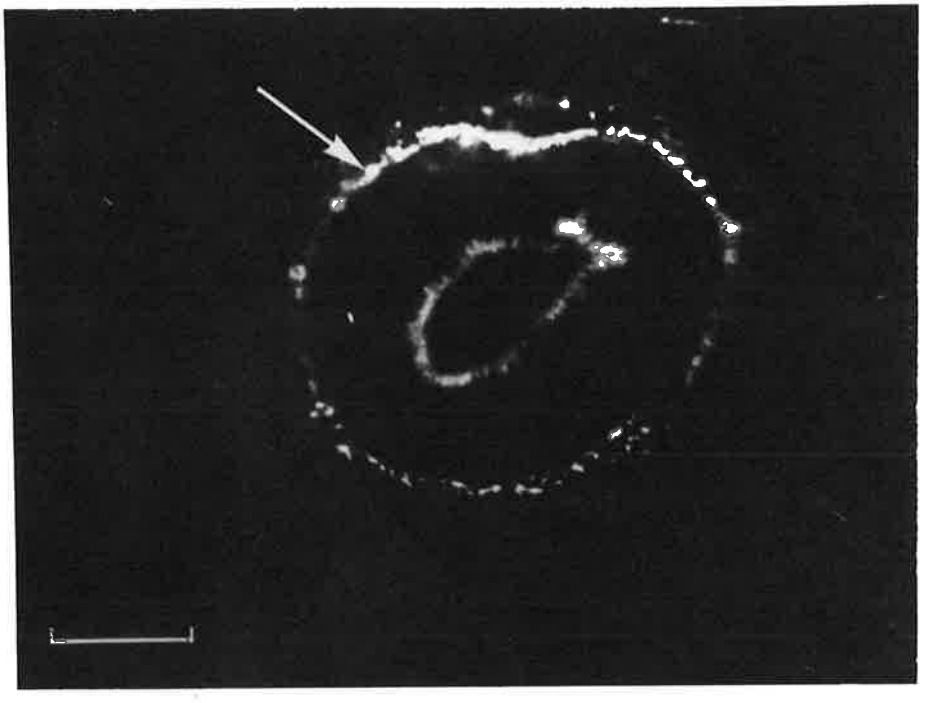
Transverse section of human dental pulp.
The arrow shows fluorescence at the outer border of a blood vessel.
Formaldehyde treatment 1 hour.
Scale 50 microns.

Figure 2.19



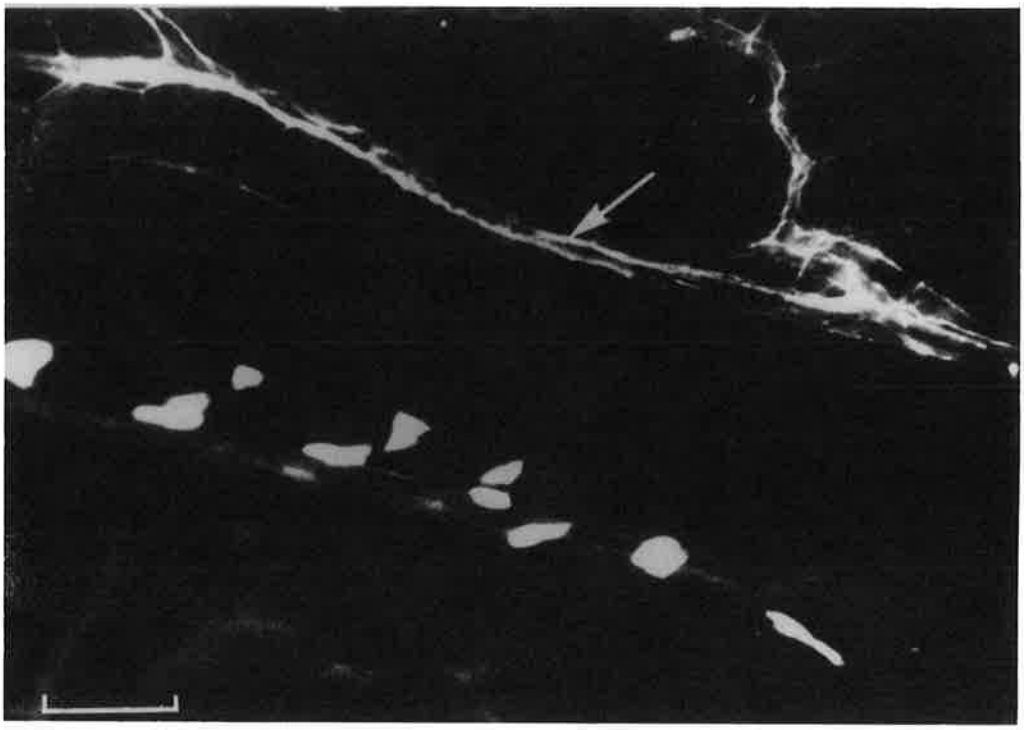
Transverse section of rabbit inferior dental artery.
The arrow shows fluorescence at the medial-adventitial border.
Formaldehyde treatment 1 hour.
Scale 100 microns.

Figure 2.20



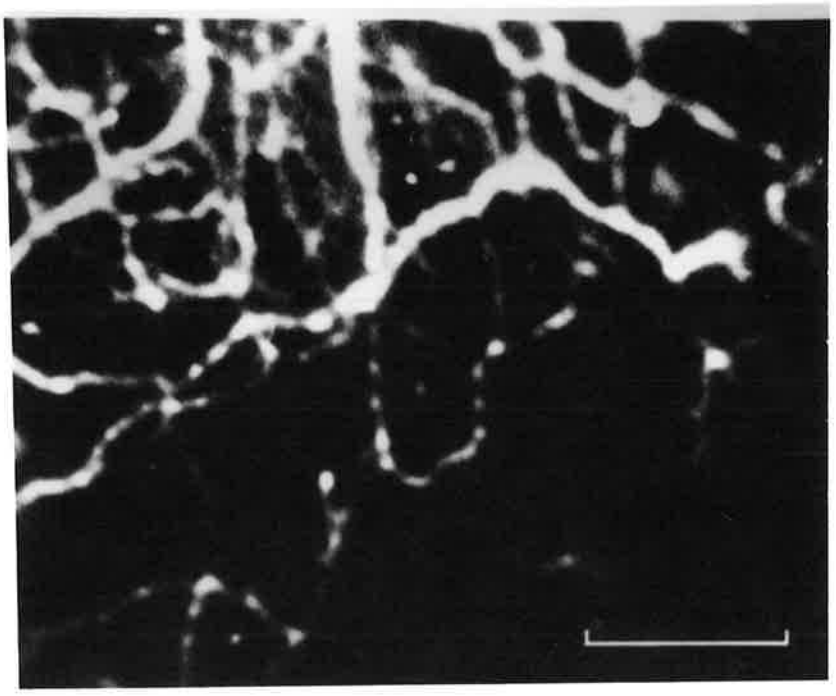
Transverse section of artery from the paw of a young rhesus monkey.
The arrow shows typical noradrenergic fluorescence at the medial-adventitial border.
Autofluorescence is shown at the intima.
Formaldehyde treatment 1 hour.
Scale 100 microns.

Figure 2.21



Stretched rat epididymal fat pad.
Formaldehyde treatment 2 hours.
The arrow shows a blood vessel (green fluorescence).
The discrete cells in the lower part of the picture show bright yellow fluorescence typical of serotonin.
Scale 100 microns.

Figure 2.22



Stretched whole mount of lizard auricle.
1 hour formaldehyde treatment.
Scale 100 microns.

DISCUSSION

The formaldehyde treatment of tissues by the Falck method results in fluorescence which is highly specific for catecholamines and for serotonin (Corrodi and Jonsson, 1967). Positive identification of the substance causing fluorescence at the medial-adventitial border of the rabbit ear artery has not been made in the present study, but indirect evidence suggests that the substance is noradrenaline. Evidence to support this assumption is as follows. (The fluorescence occurring at the medial-adventitial border of the artery will now be referred to as "specific fluorescence".)

1. The specific fluorescence was generated by treatment with formaldehyde vapour, and did not appear in the absence of formaldehyde treatment.

2. Specific fluorescence was easily quenched by exposure of tissue sections to water. Monoamine fluorescence is similarly quenched by water. (Falck and Owman, 1965).

3. Exposure to sodium borohydride abolished the specific fluorescence, which was regenerated by further exposure to formaldehyde. This is a test for the

presence of biogenic monoamines. (Corrodi, Hillarp and Jonsson, 1964).

These findings indicate that the specific fluorescence was due to the presence of monoamines. The following results aid in the identification of the monoamines responsible for the fluorescence.

1. The colour of the specific fluorescence was bright green to greenish-yellow under the conditions of microscopy used. The green fluorescence could be due to catecholamines, but is unlikely to be caused by serotonin, which gives a strong yellow fluorescence (Falck and Owman, 1965).

2. In the present study, extended time for formaldehyde treatment did not alter the appearance of the specific fluorescence. Bright specific fluorescence was obtained after treatment with formaldehyde for one hour at 80°C. Primary catecholamines such as dopamine and noradrenaline may be responsible for fluorescence after this period, but secondary amines (such as adrenaline) and serotonin may require exposure to formaldehyde for up to three hours at 80°C (Falck and Owman, 1965).

2. Mounting sections in organic solvents may greatly reduce adrenaline fluorescence (Falck and Owman, 1965) but mounting in Entellan and xylol had no apparent effect on the specific fluorescence in the rabbit ear artery.

3. Fluorimetric assay of the rabbit ear artery by de la Lande and Head (1967) proved that the artery contains much more noradrenaline than adrenaline.

Although these findings suggest that the monoamine responsible for the specific fluorescence is noradrenaline, the colour of the observed fluorescence indicating the presence of noradrenaline, adrenaline, or dopamine, is not always a reliable indication, as very high concentrations of catecholamines may give a decidedly yellow fluorescence (Norberg, 1967). However, in the present study, the specific fluorescence was predominantly green, and where a greenish-yellow colour was seen the autofluorescence of the intima was also a greenish-yellow, suggesting that a factor other than the formaldehyde treatment was contributing to the yellowish tinge. The fact that strong specific fluorescence appeared after one hour suggests that this fluorescence is not due to adrenaline. Prolonged formaldehyde treatment (three hours)

failed to alter the appearance of the specific fluorescence, although determination of the characteristics of such fluorescence by eye is not always reliable (Corrodi and Jonsson, 1967). However, as the green colour of the specific fluorescence and the conditions of treatment probably exclude serotonin, and as the conditions of treatment and mounting of sections probably exclude adrenaline, the amine responsible for the specific fluorescence may be either dopamine or noradrenaline. The assay results of de la Lande and Head (1967) support the belief that the amine is not adrenaline. The discovery by von Euler (1946) that noradrenaline is the sympathetic neurotransmitter substance in most mammalian systems lends support to the identification of the amine as noradrenaline.

That the specific fluorescence is related to sympathetic nerves is indicated by the effects of sympathectomy, which prevented the appearance of specific fluorescence in all of the arteries so treated. Furthermore, pretreatment with reserpine which is known to deplete sympathetic nerves of noradrenaline prevented the appearance of specific fluorescence.

The results of experiments in which arteries depleted by reserpine were exposed to noradrenaline indicate that a noradrenaline

uptake mechanism is active under these conditions. Cocaine is known to block the uptake of noradrenaline, and in this study cocaine prevented re-establishment of specific fluorescence by noradrenaline after depletion by reserpine. Evidence that noradrenaline is taken up by sympathetic nerves is discussed later in the thesis (page 3.2). The appearance of the specific fluorescence observed after repletion with noradrenaline was identical in colour and in distribution with that seen in normal control tissue. This finding further supports the assumption that noradrenaline in sympathetic nerves is responsible for the green fluorescence in the rabbit ear artery.

Perfusion of arteries with Evans blue dye altered the characteristics of the autofluorescence noted in this study in the rabbit ear artery. Autofluorescence may obscure specific fluorescence in some tissues (Norberg, 1967) and its modification by Evans blue is a further test of the specificity of the formaldehyde induced fluorescence, which was unchanged by Evans blue.

The evidence discussed above may be summarised as follows:

1. The specific fluorescence is generated by formaldehyde treatment. It is not autofluorescence of the tissues.

2. The specific fluorescence is due to the presence of a biogenic monoamine.

3. The specific fluorescence is caused by either dopamine or noradrenaline.

4. The specific fluorescence is related to the' presence in the artery of sympathetic nerves, and is likely to be noradrenaline contained in these nerves.

The results of the fluorescence studies indicate that the sympathetic innervation of the rabbit ear artery is dense. In the three stretched artery preparations in which specific fluorescence was noted, its appearance was similar to that of the iris described by Malmfors (1965) in which a dense sympathetic ground plexus was evident. Grant and Thompson (1963) have shown a dense nerve plexus associated with the rabbit ear artery, which degenerated after sympathectomy. Therefore the results of this study support the earlier view that the innervation of the rabbit ear artery is predominantly sympathetic in origin.

This observation is consistent with the high sensitivity of the artery to periarterial stimulation observed by Stinson

(1961) and de la Lande and Rand (1965).

No evidence was found in any rabbit ear artery of sympathetic innervation within the smooth muscle layer. This is consistent with observations of small arteries in other tissues (see page 2.3).

Rhodin (1967) and Verity and Bevan (1966) in electron microscope studies on small arteries in rabbit fascia found little evidence of innervation in the smooth muscle layer. Although the findings in fluorescence studies support this view, the resolution obtainable in fluorescence microcopy is very much less than that obtained with the electron microscope. However, interpretation of electron micrographs is sometimes contentious, and artefacts produced in preparing tissues may alter the morphological picture. Therefore there is only indirect evidence that sympathetic nerves rarely if ever penetrate the smooth muscle layer of small arteries. Hence it cannot be assumed that there is no sympathetic innervation in the media of the rabbit ear artery. This point is important in consideration of the way in which released sympathetic transmitter substance causes constriction of the smooth muscle fibres in the media.

The distribution of sympathetic nerves in the wall of the

rabbit ear artery was an important consideration in the design of experiments described in subsequent chapters in this thesis.

CHAPTER 3

PREFACE

This and later chapters describe studies on the physiological properties of the ear artery. In particular, the sensitivity of the artery to exogenous noradrenaline, and to endogenous noradrenaline released by electrical stimulation and by drugs, is considered in relation to the position of the nerves in the artery wall.

CHAPTER 3

INFLUENCE OF SYMPATHETIC INNERVATION ON VASCULAR SENSITIVITY TO NORADRENALINE.

INTRODUCTION

There is now a mass of evidence that the sympathetic neurotransmitter substance accumulates in nerve fibres in peripheral tissues. Evidence for the site of this accumulation was largely indirect before the development by Falck (1962) of a histochemical method for demonstration of biogenic monoamines (described in Chapter 2). This fluorescence method allowed the demonstration of the neurotransmitter in the autonomic ground plexus formed by terminal nerve fibres.

Previously the possibility that exogenous catecholamines were taken up by peripheral storage areas had been suggested by Burn (1932). Later other workers demonstrated an increase in catecholamine content in dog, cat and rat hearts after the administration of large doses of adrenaline and noradrenaline *in vivo*, (Nickerson, Berghout and Hammerstrom, 1950; Raab and Humphreys, 1947). The demonstration by Axelrod, Weil-Malherbe and Tomchick

(1959) that after giving small doses of H^3 adrenaline to mice, some of the amine could be detected many hours later suggested that although degradation by enzymes could account for some loss of the amine, it was likely that storage in some tissues also occurred.

Using sensitive fluorimetric assay techniques, Stromblad and Nickerson (1961) showed an accumulation of noradrenaline and adrenaline in rat salivary glands and heart. They suggested that tissue uptake could be an important factor in the non-metabolic inactivation of catecholamines. Evidence that the peripheral tissue uptake sites of catecholamines were sympathetic nerves was given by Whitby, Axelrod, Weil-Malherbe (1961), who found that noradrenaline uptake was greatest in those tissues with rich sympathetic innervation. This hypothesis has been supported by the results of denervation studies, autoradiographic studies, and by histochemical methods. The accumulated evidence has been reviewed in detail by Iversen (1967).

In the present study, it was found that the central artery of the rabbit ear was richly supplied with sympathetic nerves. Because of the suggestion that uptake into sympathetic nerves is largely responsible for terminating the physiological activity of noradrenaline and adrenaline, the question was raised as to how

the uptake mechanisms could modify the responses of the artery to exogenous noradrenaline.

To determine the effectiveness of the uptake mechanisms the drug cocaine was used, since cocaine has long been known to potentiate some effects of catecholamines (Frohlich and Loewi, 1910; Tainter and Chang, 1927; Burn and Tainter, 1931). Miyake (1952) demonstrated potentiation by cocaine of adrenaline induced constriction in the perfused rabbit ear. Furchgott (1955) reviewed the theories relating to cocaine's potentiation of catecholamines. It was proposed by Macmillan (1959) that the cocaine potentiation of catecholamines was due to block of uptake of the amines by neural storage sites. The action of cocaine on blood vessels was studied by Furchgott, Kirpekar, Rieker and Schwab (1963), using strips of rabbit aorta.

The perfused rabbit ear artery was used in the present study to examine the influence of the sympathetic innervation on responses to noradrenaline. The rabbit ear artery preparation has been shown to be highly sensitive to noradrenaline and to other vasoconstrictors. de la Lande, Paton and Waud (1964) and de la Lande and Rand (1965) perfused the isolated rabbit ear and the isolated rabbit ear artery with Krebs bicarbonate solution at constant volume, and recorded

changes in the diameter of the perfused vessels as pressure changes. They found that noradrenaline, histamine, angiotensin, serotonin and electrical periarterial stimulation all caused constriction. Cocaine potentiated the constrictor effect of injected noradrenaline, and of nerve stimulation.

Consideration of the morphological picture led to the design of experiments which tested the role of the storage and uptake noradrenergic structures in responses of the artery to exogenous noradrenaline. This point became important when the studies on single cannulated arteries confirmed that cocaine had a relatively small effect on noradrenaline induced vasoconstriction. It was observed also in experiments in which arteries were perfused so that the intraluminal and extraluminal noradrenaline did not mix (described on page 3.9), that extraluminal noradrenaline was far less active than intraluminal noradrenaline. These observations are documented in the first part of the results section, and drew attention to the possibility that the distribution of the sympathetic nerves could be an important factor in modifying responses to noradrenaline applied to the artery by different routes. Therefore most of the studies in this chapter are devoted to an analysis of this relationship. They are subdivided as follows:

1. The effect of the route of application of noradrenaline on vascular sensitivity.

2. The interactions of noradrenaline and cocaine.

3. The effects on noradrenaline sensitivity of denervation and depletion of neurotransmitter substance.

4. Additional studies on cocaine.

MATERIALS AND METHODS

Experimental methods described in this chapter are:

Perfusion of the isolated ear

Perfusion of the isolated artery

Stimulation of periarterial sympathetic nerves in the central artery

Pretreatment of rabbits

The most commonly employed materials and methods for each of the above procedures aredescribed. Modifications of the methods in later chapters are referred to separately under the heading "Materials and Methods" for those chapters. A list of drugs used in the study, and their origin, appears in the Appendix together with their manner of preparation for use in the study.

Preparation of rabbits

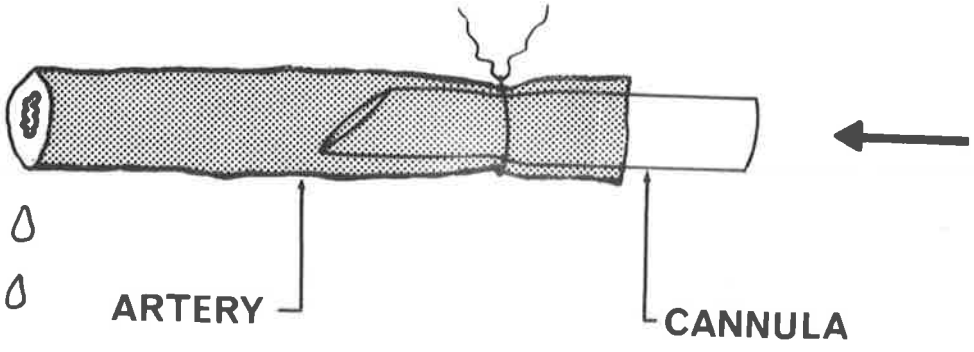
Male and female semi-lop eared rabbits were bred at the Central Animal House of the University of Adelaide. The weights of the animals varied from 1.5 to 3.0 Kg, although most of the rabbits used in the study weighed from 1.5 to 2.0 Kg. The rabbits were not starved before experimental use, except where anaesthesia was required for a recovery operation such as sym-

pathectomy. Anaesthesia was induced with urethane 8 ml/Kg of a 25% solution being injected intraperitoneally, with increments given as required. Before cannulation of the ear artery heparin 1000 units/Kg was injected intravenously into an ear vein. Polythene cannulas were made by heat drawing No. 3 Sterivac tubing in such a way that a slight bulge near the tip of the cannula facilitated tying in the artery. (figure 3.1).

Perfusion of the isolated ear

Rabbits were usually anaesthetised with urethane but some were stunned and bled and cannulation performed after death. The selected ear was placed so as to make prominent the cartilage near the base of the ear, where pulsation of the central artery could usually be seen or felt. An incision was made in the skin after wetting the fur with Krebs bicarbonate solution, and by blunt dissection a portion of the central artery exposed as near as possible to the base of the ear. A short segment of the artery, usually about 1 cm in length, was gently cleaned of adherent tissue by blunt dissection. A cotton thread was placed under the artery which was then tied off as near to the proximal end of the ear as possible. A polythene cannula was inserted into the lumen of the central artery and firmly tied in position. The whole ear including the

Figure 3.1



Showing how the bulge near the tip of the cannula aided its retention in the artery.

cannula was severed from the animal distal to the ligated central artery. The cannulated ear was quickly transfered to a plastic warming chamber designed by M.J. Tyler of this department and connected to the perfusion apparatus (figure 3.2) . The perfusion chamber was warmed to 37°C and the perfusion fluid was a Krebs bicarbonate solution (Appendix page 3) bubbled with 95% oxygen and 5% carbon dioxide. This solution, maintained at 37°C, was pumped through the ear at rates varying from 6 to 12 mls/min by means of a roller pump (designed by O. Saxby, Department of Pharmacology, University of Oxford) delivering constant volume. Perfusion pressure was measured with a mercury manometer, constriction being recorded as an increase in perfusion pressure. The ear perfused in this manner was very sensitive to temperature changes and care was taken to exclude air currents.

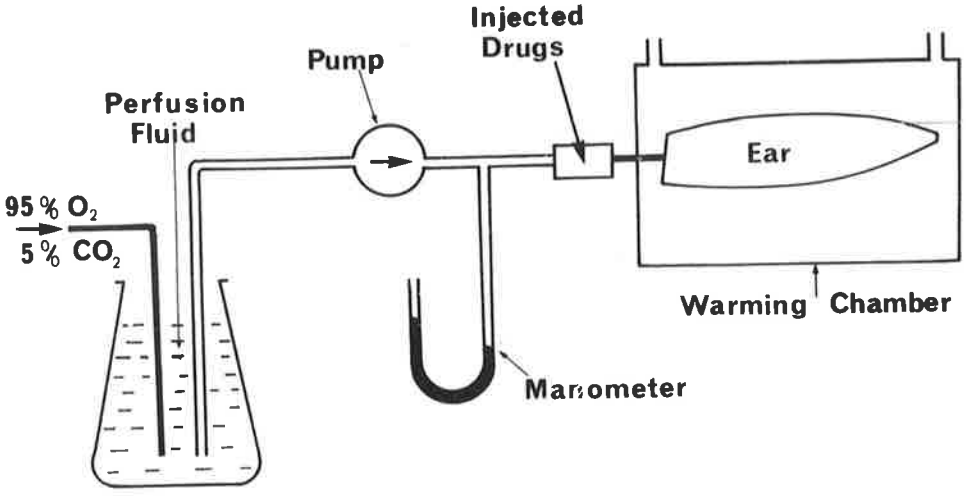
Drugs were dissolved in a 0.9% saline solution and injected either through rubber tubing into the perfusion stream proximal to the ear, or were added to the perfusion reservoir. A possible limitation to this perfusion method was the great increase in water content of the perfused ear which on some occasions was measured at more than a 100% increase in weight of the whole ear (see table 3.1). In addition spasm of the perfused vessels occurred very easily after slight movements of the perfusion apparatus or of the

TABLE 3.1

Expt. No.	Weight of ear in grams		% gain in weight
	Before perfusion	After perfusion	
1	17	51	200
2	15	69	360
3	10	21	110
4	16	34	112
5	18	52	188

The weight of the ear before and after perfusion is shown for each of 5 experiments and in the last column the percentage increase in weight during the perfusion is indicated.

Figure 3.2



EAR PERFUSION APPARATUS

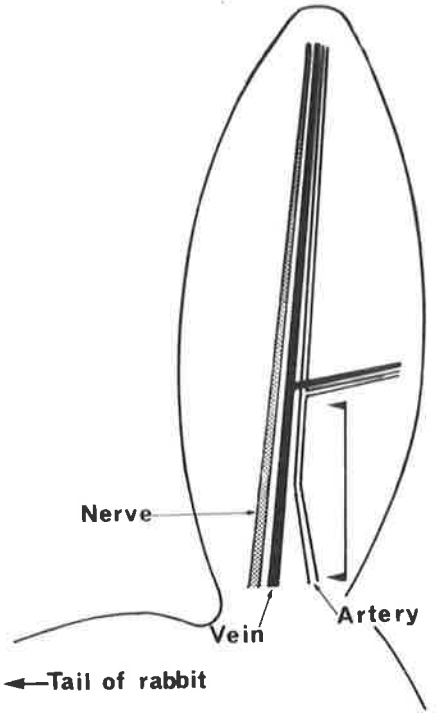
Diagram of the apparatus used to perfuse the isolated ear of the rabbit.

warming chamber. The method described for perfusion of the ear is that of de la Lande, Paton and Waud (1964).

Perfusion of the isolated artery (double cannulation)

The preparation of the animal for cannulation and subsequent perfusion of the isolated central artery was similar to that for perfusion of the whole ear. After exposure of the central artery near the base of the ear the vessel was freed from surrounding tissue from the point where the artery emerges from deeper structures near the base of the ear to the first major branch of this artery. (figure 3.3). A cannula was inserted into the proximal portion of the artery as for the whole ear, and a finer cannula drawn from No. 2 Sterivac tubing was inserted into the distal part of the selected arterial segment, but proximal to the first major branch. This usually left for perfusion a free segment of artery approximately 15 mm in length, but varying from 5 mm to 20 mm or more. The double cannulated artery was immediately transferred to a dish containing warm gassed Krebs bicarbonate solution and as soon as possible set up in a double jacketed organ bath and perfusion fluid pumped through the proximal cannula, the effluent emerging through the finer distal cannula. In most experiments slight tension was applied to the distal or superior cannula to avoid the complication.

Figure 3.3



A diagram of the erect left ear of a rabbit showing the convex surface of the ear as seen from the midline of the skull, and showing the relative positions of the ventral (great) auricular nerve, the central vein, and the central (main) artery of the ear. The double arrows show the segment of the artery used in the perfusion experiment.

of kinking of the artery, which occasionally occurred with the considerable elongation often seen during constriction. The apparatus used is shown in figure 3.4 . The method of artery perfusion using double cannulation was that described by de la Lande, Cannell and Waterson, (1966);

Perfusion of the isolated artery (single cannulation)

An alternative method of arterial perfusion was that described by de la Lande and Rand (1965) and de la Lande and Harvey (1965) in which only a proximal cannula was used and the effluent allowed to escape into the perfusion fluid accumulating in the organ bath (figure 3.5). The essential difference between the two methods was that in the method of double cannulation, drugs could be applied to either the intraluminal or the extraluminal surfaces of the artery separately, and in such a way that no admixture of the extraluminal and intraluminal fluids occurred. With the method of single cannulation of the artery drugs which were perfused through the lumen of the vessel escaped from the cut end of the artery and then bathed the extraluminal surface as they were contained in the fluid in the organ bath. No tension was applied to the distal end of the artery. This method was subsequently employed by Farmer (1966) and Gillespie (1966).

Figure 3.4

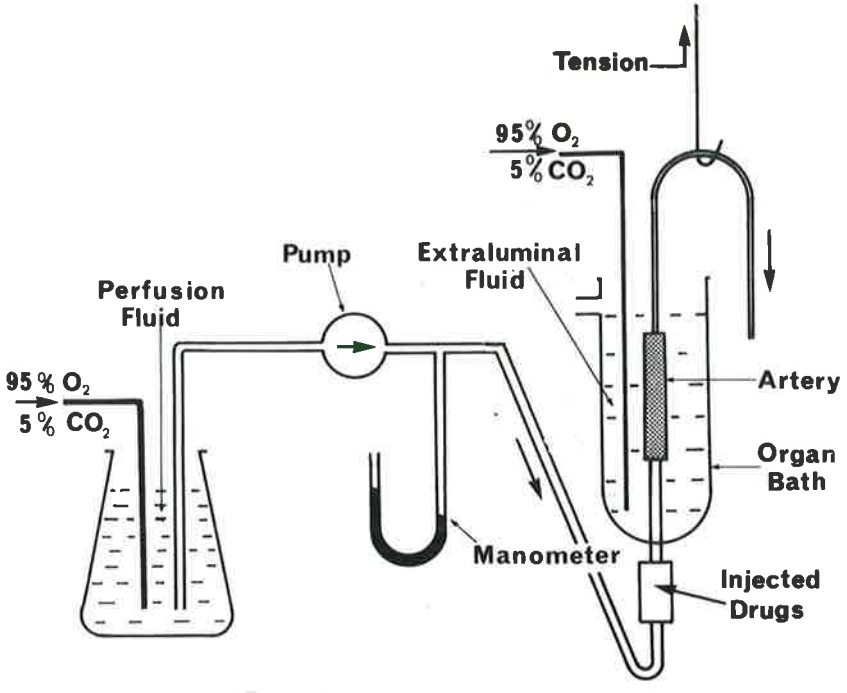


Diagram of the apparatus used to perfuse the isolated central artery of the rabbit ear.
Double cannula method.

Figure 3.5

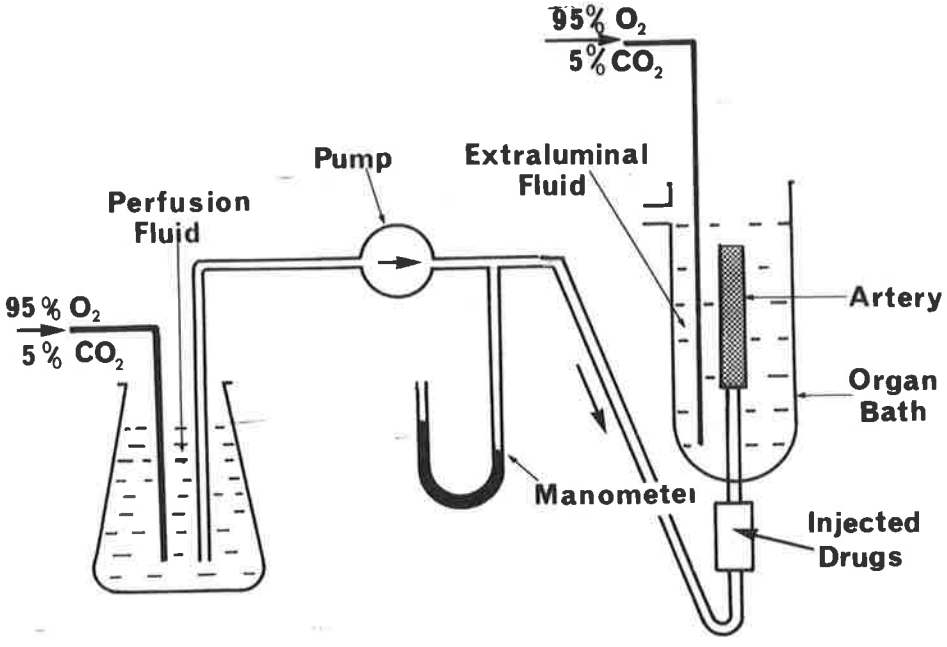


Diagram of the apparatus used to perfuse the isolated central artery of the rabbit ear.

Single cannula method.

Comments on perfusion methods

The temperature of the organ bath and ear warming chamber was maintained at 37°C (the rabbit ear temperature in vivo , Grant, 1935) by suitable adjustment of the heating element in the circulating pump. Usually a bath temperature of 38°C gave a temperature of 37°C in the organ bath and warming chamber under the prevailing laboratory conditions. A mixture of 5% carbon dioxide in oxygen was bubbled through the intraluminal perfusion fluid in the heating bath before the fluid passed through the pump, and a separate gas tube was placed in the solution surrounding the artery in the organ bath. (See figure 3.4). The organ bath used was of 10 ml capacity. Concentrations of drugs were recorded as wgt/ml, both in the perfusion fluid and where applicable in the extraluminal fluid.

Although the pump delivered perfusion fluid at constant volume, flow rate through the artery varied with diversion of perfusion fluid into the mercury manometer as pressure within the system increased. However, these changes in flow rate were slight, and in experiments using a pressure transducer to show changes in perfusion pressure, responses to various drugs appeared to be identical in character with those in which a mercury manometer was used to measure changes. The "dead space" in the apparatus, as measured

by the volume of fluid contained in the tubing leading from the perfusion reservoir through the pump to the artery, was between 3.5 and 4.0 ml. The time taken for fluid to reach the artery from the perfusion reservoir is shown for different flow rates in table 3.2. The times were those taken for perfusion of dye to reach the artery from the perfusion reservoir. The dye solutions did not advance through the tubing as a square front, but rather as an elongated cone, with its apex pointing well in front of the main stream of dye. Therefore it is likely that the first contact of drug and artery would not strictly relate to the concentration of drugs in the perfusion reservoir, Estimation of time of onset for intraluminal drug application was less accurate than that for extraluminal application.

Tests for leakage of the artery perfused by the double cannula method were made as follows: by observations of the level of extraluminal fluid in the organ bath during perfusion of the artery, and by the perfusion of Evans blue dye through the artery, with photometric comparison of intraluminal and extraluminal solutions taken during the dye perfusion. A typical result of a dye test is shown in figure 3.6.

TABLE 3.2

	Time taken for dye to reach artery from perfusion reservoir		
Expt. No.	1	2	3
Flow Rate ml/min			
4	54	55	54
6	39	37	36
8	26	26	26
10	20	20	20

The figures show time in seconds for Evans blue dye to travel from the perfusion reservoir to the artery.
In the great majority of artery experiments, the flow rate was between 6 and 8 ml/minute.

Figure 3.6

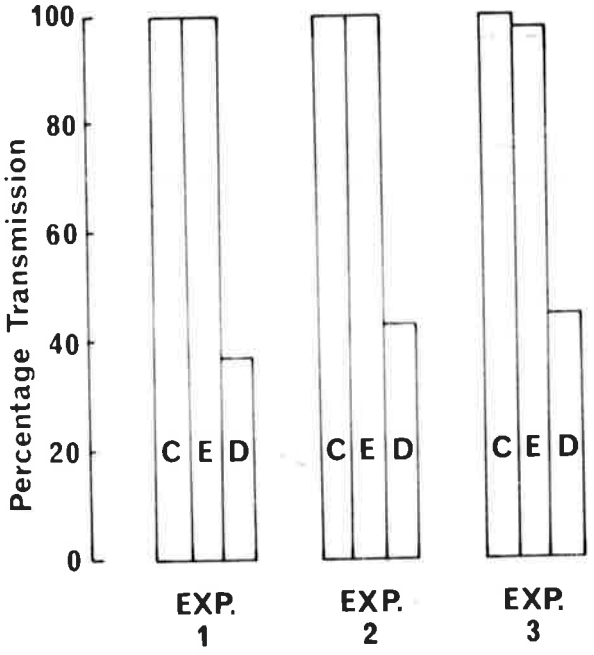


Diagram of the results of three dye tests for the presence of leaks in a perfused artery (double cannulated). The height of the columns represents transmission of light through the fluid sample.

C = control perfusion solution.

E = extraluminal fluid after 10 minutes intraluminal dye perfusion.

D = intraluminal dye perfusion fluid.

The results of experiment 3 suggest that the artery developed a slight leak, while no leak was apparent in experiments 1 and 2.

Kinking of the artery

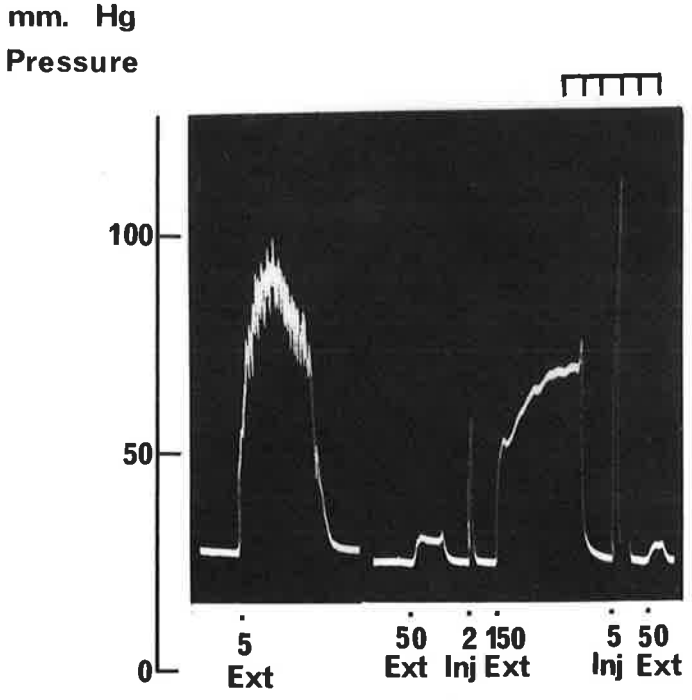
In arteries perfused by the double cannula method, and when slight tension was not applied to the distal end of the artery, elongation of the artery during vasoconstriction was sufficient to allow kinking of the artery and subsequemt blocking of the lumen, recognisable by a sharp rise in perfusion pressure until the obstruction was overcome. This led to a fluctuating pattern of the tracing typically shown in figure 3.7. Many arteries displayed localised areas of constriction, giving an "hour glass" appearance to the artery. These constrictions were commonly, although by no means always, adjacent to the tip of a cannula, and in their presence a fluctuating tracing similar to that in figure 3.7 resulted.

Typically, the arteries increased in sensitivity during perfusion experiments, which usually lasted from 4 to 8 hours. However, the greatest increase in sensitivity occurred during the first hour of perfusion. In most experiments, therefore, the artery was perfused for at least one hour before administration of drugs.

Method of measuring sensitivity changes

Constrictor responses to drugs were measured by the

Figure 3.7



Showing a fluctuating constrictor response to noradrenaline 5 ng/ml (extraluminally and in the presence of cocaine) and the sustained response (in the absence of cocaine) to extra-luminal noradrenaline, 150 ng/ml. The numerals indicate ng/ml.
Time trace in minutes.

maximum rise in perfusion pressure occurring during (or with intraluminal injections immediately following) the application of the drug. Concentration-response curves were derived from responses recorded in duplicate or in triplicate at two or three concentration levels. Changes in sensitivity to noradrenaline produced by drugs, or relative sensitivity to intraluminal and extraluminal noradrenaline were measured in terms of concentrations producing equivalent maximum responses. This difference in sensitivity was expressed as the sensitivity ratio. Where the two dose-response curves under consideration were similar in shape and slope, the mean distance apart of the curves was used to estimate the sensitivity ratio, otherwise minimum and maximum values were calculated and the ratio expressed as a range.

Stimulation of periarterial sympathetic nerves in the central artery

A Grass stimulator, model S4, was used to deliver short pulses through platinum electrodes. The electrodes were usually placed periarterially in the whole ear, but in several experiments needle electrodes were placed in the tip of the ear, and in the perfusion stream proximal to the ear. During perfusion of the isolated artery, electrodes were placed in the organ bath, or occasionally one electrode was placed in the organ bath and one in

the proximal perfusion stream. Pulses were usually of 1 millisecond duration, from 2 to 10 per second, the train of impulses being applied for 10 seconds for single responses. Voltage was supramaximal, and ranged from 20 to 100 volts.

Pretreatment of rabbits

Rabbits were pretreated to modify the function of neurotransmitter storage sites in the blood vessels of the ear by sympathetic denervation and by pretreatment with reserpine.

A Sympathetic denervation

The ear blood vessels of 20 rabbits were denervated by extirpation of the left superior cervical ganglion; in some cases the operation was extended to include sectioning of the ventral and dorsal auricular nerves. The method for sympathetic denervation was that followed by de la Lande and Rand, (1965). Sterile technique was used and anaesthesia was usually induced and maintained with ether by the drop method, after premedication with 2.5 mg/kg of atropine sulphate. In some rabbits anaesthesia was induced with methohexitone, 10 mgm/kg intravenously. After preparation of the skin of the neck with a mixture of cetrimide and chlorhexidine, and before incision, 1 ml of 2% lignocaine containing

adrenaline 12.5 micrograms/ml was infiltrated into the incision area.

A midline incision was made in the neck of the rabbit, the trachea exposed by blunt dissection, the carotid artery isolated, and the cervical sympathetic nerve identified. The superior cervical ganglion in the rabbit lies at the level of the angle of the mandible and this was usually the mid-point of the neck incision. The ganglion was held in mosquito forceps and the pre-ganglionic and post-ganglionic nerve fibres cleared as far as possible. After removal of the ganglion penicillin-sulfanilamide powder mixture was insufflated into the wound which was closed in layers with interrupted sutures. In some rabbits after closure of the neck wound separate incisions were made in the ear to remove at least 1 cm from the dorsal and ventral auricular nerves near the proximal portion of the ear. After skin closure a plastic film was sprayed over the suture line. Some animals were given intramuscular procaine penicillin 100,000 units/kg. In some of the later operations both the penicillin-sulfanilamide powder and the intramuscular injection of procaine penicillin were omitted.

The effectiveness of superior cervical ganglionectomy was aparent by constriction of the pupil and by early vasodilata-

tion of the ear on the operated side. Several animals were used 48 hours after denervation but most were used from 14 to 23 days after denervation.

Effectiveness of the denervation procedure was tested by:

1. Examination by fluorescence microscopy after formaldehyde treatment. In no case after denervation was specific fluorescence observed at the outer border of the smooth muscle of the rabbit ear artery.

2. Electrical stimulation of the artery. Occasionally a slight response to stimulation occurred but this was always of very much less magnitude than the response in the control artery taken from the opposite (untreated) ear.

B Reserpine pretreatment

Reserpine was used to deplete tissue stores of catecholamines. Pretreatment was given in a variety of ways and varied from an intraperitoneal dose of 0.1 mg/kg of reserpine daily for 10 days to an intravenous dose of 5 mg/kg intravenously 2 hours before use (Muscholl and Vogt, 1958; Burn and Rand, 1958; Fleming and Trendelenburg, 1961; Reinert, 1963; Levy and Richards, 1965.

A convenient method which was used for a majority of the rabbits was a single dose of 2.5 mg/kg intraperitoneally 24 hours before use. Usually longer term treatments with reserpine resulted in lethargy and restricted food intake. The effectiveness of reserpine pre-treatment was tested by the methods used after denervation, that is by fluorescence studies and by the effects of electrical stimulation. In three rabbits reserpine was given intravenously in a dose of 2.5 mg/kg not less than 3 hours before removal of the reserpine-treated artery. Prior to administration of reserpine a control artery had been removed from the opposite ear. In this way it was possible to perfuse a control artery and then perform an identical experiment in the reserpine-treated artery. Pre-treatment with reserpine effectively eliminated specific catecholamine fluorescence, and the response to electrical stimulation in reserpine-treated rabbits was either eliminated or was very much smaller than that seen in the untreated arteries.

RESULTS

1. Types of response to noradrenaline

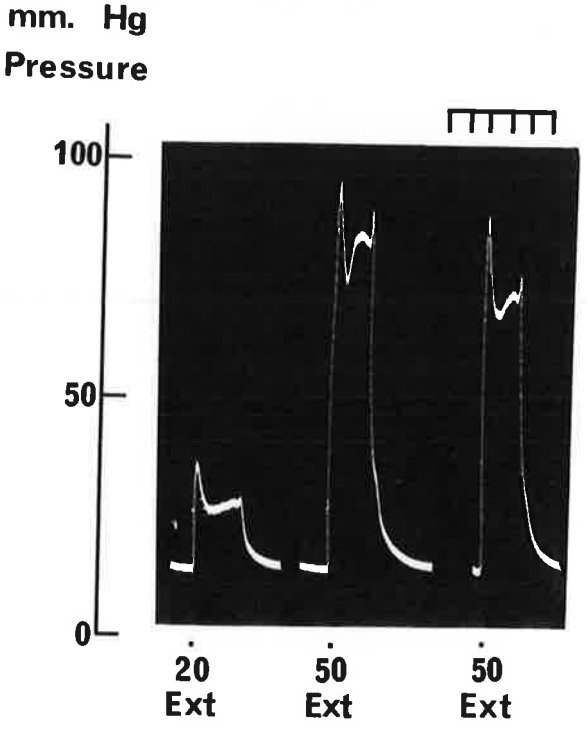
The responses of the artery to noradrenaline applied intraluminally or extraluminally were essentially similar and comprised a rapid rise in perfusion pressure, indicating constriction. The constriction commenced within 60-100 seconds for intraluminal, and 3 to 8 seconds for extraluminal application, and reached a maximum value after a further 10 to 180 seconds. The subsequent shapes of the responses conformed to one or other of the following:

a. The constriction was well maintained at or near its maximum level. (figure 3.7).

b. There was a transient decline from the first peak of the response, followed by a slower increase to a sustained maximum value. (figure 3.8).

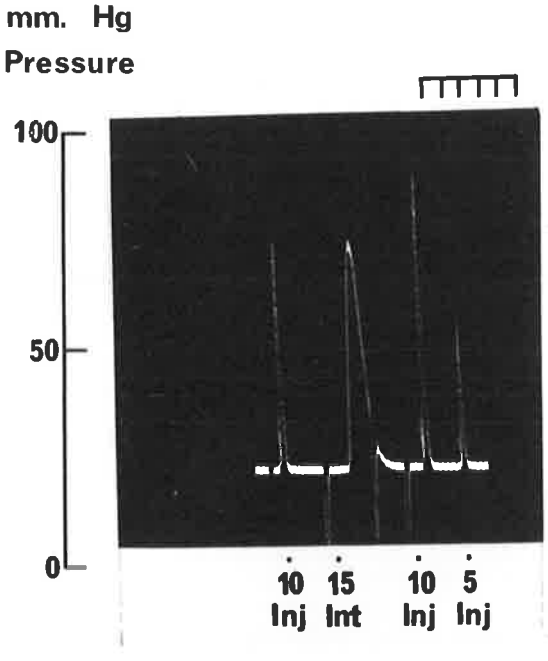
c. The response faded rapidly from its maximum value, despite continued contact of drug and artery. (figure 3.9). This was observed only after prolonged contact, or after storage of the artery overnight at $4^{\circ}C$.

Figure 3.8



Extraluminal noradrenaline, ng/ml.
Decline from first peak of response, followed by a slower increase to a sustained response.
Time trace minutes.

Figure 3.9



Intraluminal perfusion of noradrenaline 15 ng/ml, showing rapid fade of response despite continued contact of drug.
Other numerals indicate injections of noradrenaline in ng.
Time trace minutes.

Individual arteries tended to display the same pattern of response throughout an experiment. Typical time courses of the responses are shown in table 3.3.

Intraluminal noradrenaline showed less tendency to produce a diphasic response than extraluminal noradrenaline. However, this may have reflected a slower attainment by intraluminal noradrenaline of its final concentration. Tests with Evans blue dye added to the perfusion reservoir showed that once dye had reached the lumen of the vessel, a further 30 to 60 seconds was required to reach the maximum sustained value. In the case of extraluminal noradrenaline, gassing of the extraluminal fluid ensured extremely rapid mixing of the drug and the fluid in the organ bath. The particular type of response occurring in an artery was reproduced throughout an experiment, with the qualification that the artery showing diphasic responses to high concentrations of noradrenaline often tended to display only a monophasic response at low concentrations. (figure 3.10).

In view of the diphasic constriction, the magnitude of the response to noradrenaline could in theory be expressed in two ways; a response measured at the transient peak value, or at the sustained value. Usually this problem did not occur with

TABLE 3.3

Time course of action of noradrenaline.

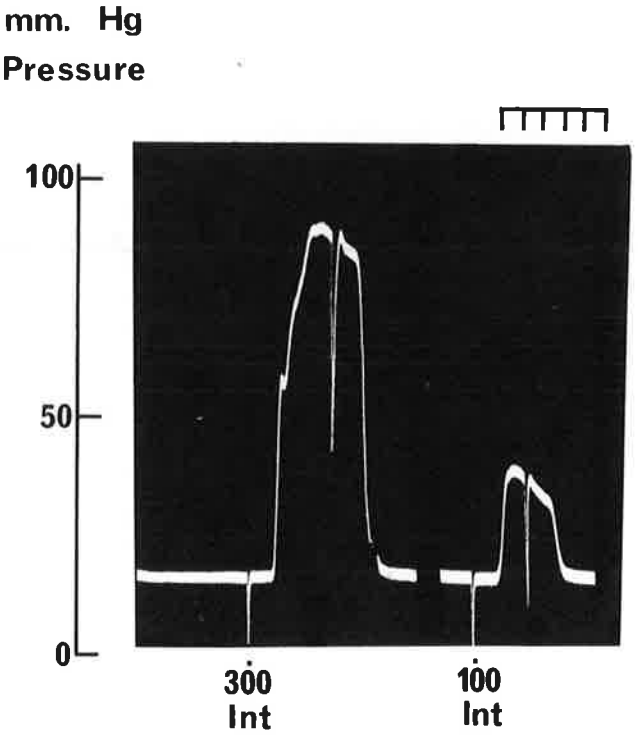
		Noradrenaline (NA)	
		Intra-luminal	Extra-luminal
Expt. 1	Onset of response	17,17	3,3
Flow rate= 8 ml/min	Onset to maximum	107	11* & 102
		122	12* & 232
Expt. 2	Onset of response	21,23	4,6
Flow rate=5.6 ml/min	Onset to maximum	204,188	12* & 120
			20* & 110

1. Numbers are times in seconds.
2. *denotes first peak of a diphasic response.
3. To allow for perfusion of "dead space" fluid, the time of onset of action of intraluminal noradrenaline is calculated from the difference between the observed time of onset and the time taken for perfused dye to reach the artery. However, the intraluminal times of onset will be underestimated since perfused dye did not reach the peak sustained concentration in the intraluminal outflow for a further 10 to 20 sec for experiment 1, and 30 seconds for experiment 2.

Experiment 1. Intraluminal noradrenaline 5 ng/ml, extraluminal noradrenaline, 10-200 ng/ml.

Experiment 2 Intraluminal noradrenaline 50 ng/ml, extraluminal noradrenaline 200-1000 ng/ml.

Figure 3.10



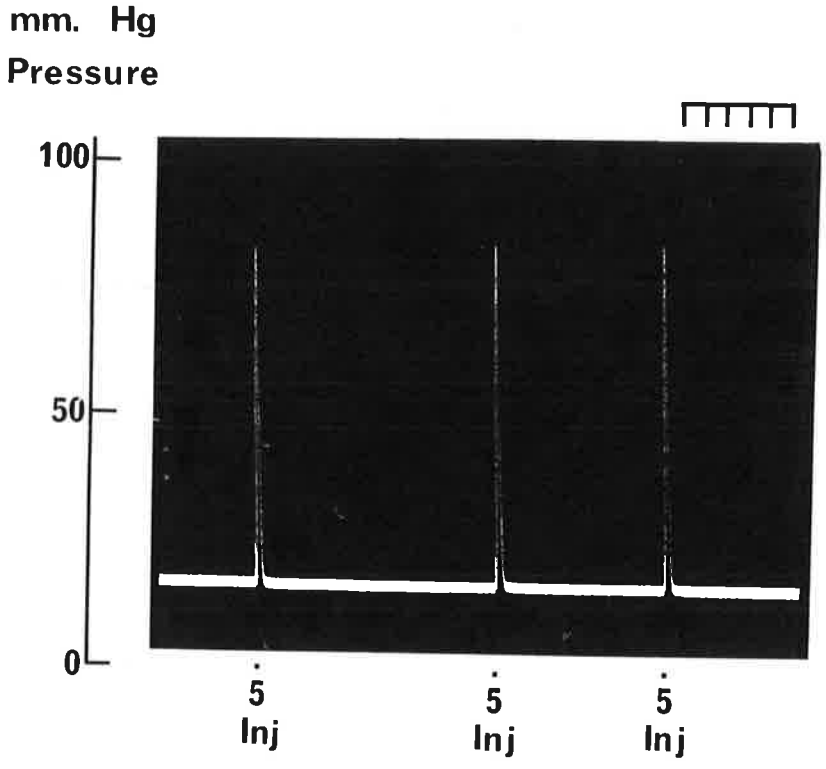
Responses to perfusions of noradrenaline, showing a diphasic response to 300 ng/ml, and a monophasic response to 100 ng/ml.
Time trace minutes.

small responses of the magnitude of 50 mm of mercury or less, as only the sustained response was evident at such levels. However, even with higher responses there was often little difference between the height of the first transient peak, and the sustained value, and in most arteries the sustained value was higher. For this reason, the maximum value of the increase in perfusion pressure occurring during sustained contact of drug and artery was taken as the response to noradrenaline.

When injected intraluminally into the artery in a small volume (0.4 ml) over a period of approximately one second, the response to noradrenaline was a rapid transient constriction with a return to resting perfusion pressure within a maximum of one minute. An example is shown in figure 3.11.

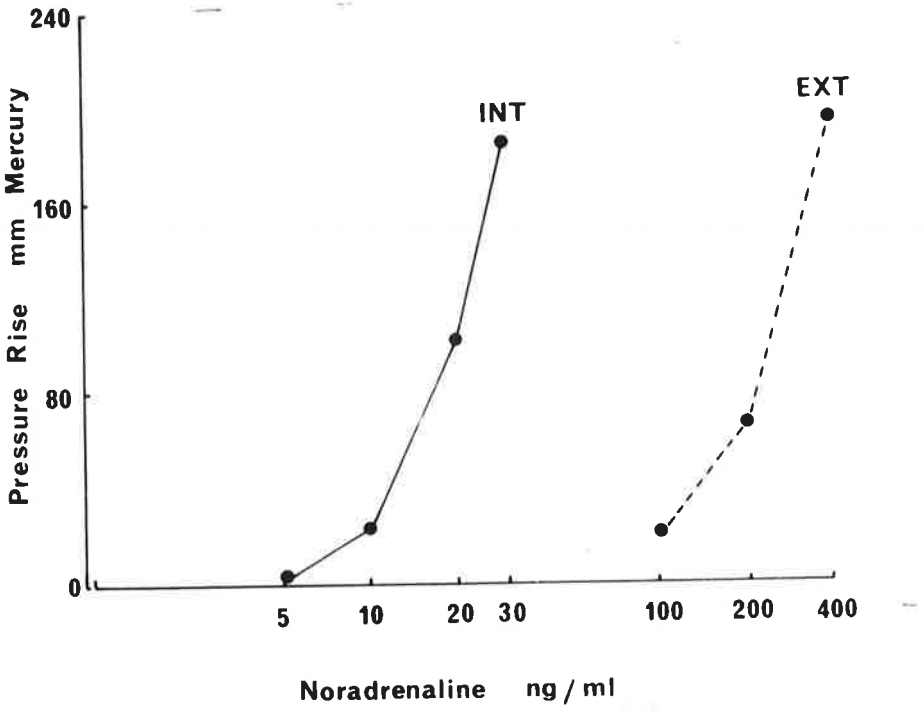
Concentration response curves to noradrenaline illustrated the marked difference between intraluminal and extraluminal noradrenaline. In the example shown in figure 3.12, intraluminal noradrenaline was 10 to 15 times more active. The sensitivity ratios in 16 arteries ranged between 0.03 and 0.5, with a mean value of 0.13 $\pm$ the standard error of 0.03.

Figure 3.11



Responses to injections (5 ng) of noradrenaline.
Time trace minutes.

Figure 3.12



Concentration response curves to intraluminal noradrenaline (continuous line) and extraluminal noradrenaline (broken line). Each point is the mean of at least two responses.

2. The effect of cocaine

i. The effect of cocaine on noradrenaline sensitivity was examined in more than 30 arteries. Cocaine, when present in the intraluminal or extraluminal fluids in concentrations from 0.1 to 10 µg/ml, caused no change in perfusion pressure, but increased the sensitivity of the artery to noradrenaline. The effect on extraluminal noradrenaline was much more marked than on intraluminal noradrenaline, The sensitising action was manifest when cocaine was added either before the response to noradrenaline or during this response. The cocaine effect was reversible, responses to noradrenaline returning to their pre-cocaine level within ten minutes of cocaine washout. Figure 3.13 shows extraluminal and intraluminal responses to noradrenaline before, during and after cocaine. The ability of cocaine to cause marked and selective enhancement of extraluminal noradrenaline is illustrated in Figs. 3.14 and 3.15, and by quantitative data on nine arteries summarised in table 3.4. Figure 3.14 shows the effects of applying cocaine during the sustained constrictor response to noradrenaline and Fig. 3.15 the effect of cocaine on the concentration-response curves to noradrenaline. It will be observed that, in each of the experiments in table 3.4, extraluminal cocaine caused only a small increase in sensitivity to intraluminal noradrenaline compared with that

LEGEND TO TABLE 3.4

In experiments 1-8 the sensitivity ratios were determined by comparing responses to intraluminal and extraluminal noradrenaline before and during the presence of cocaine, applied to one or both surfaces of the artery. In experiment 9, cocaine was applied to the artery during the sustained phase of the constrictor response to noradrenaline, and the further increase recorded. A ratio of 5 means that cocaine caused a fivefold increase in sensitivity to noradrenaline. The ratio is expressed as a range where the concentration response curves under comparison differed in shape.

Cocaine was applied to the same surface as the noradrenaline; the ratio obtained when cocaine was applied to the opposite surface as the noradrenaline is shown in brackets.

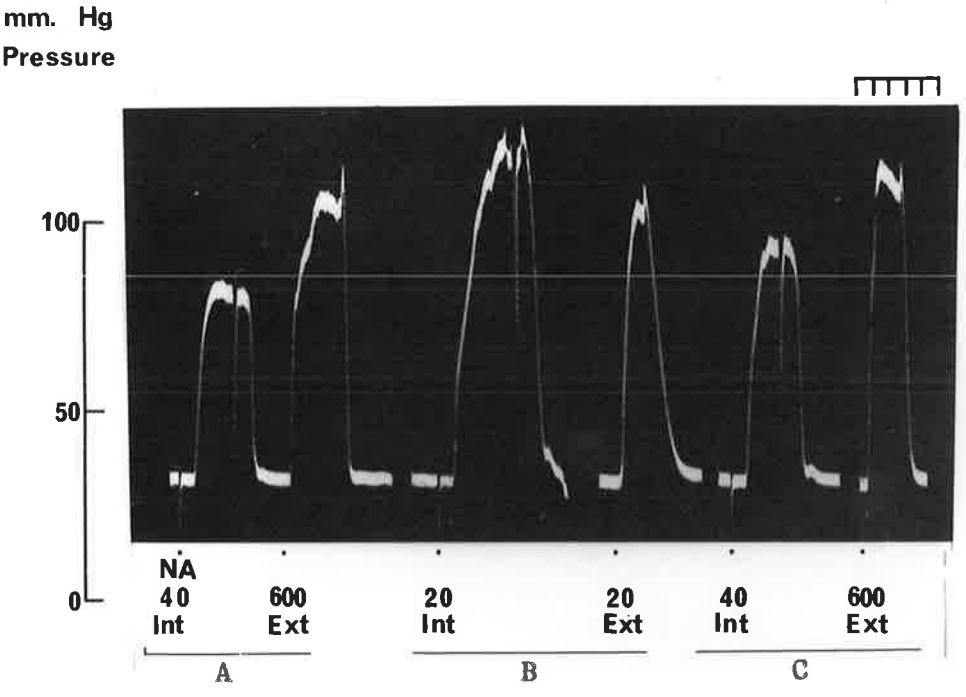
The lower table refers to the ratios of the sensitivity of extraluminal to intraluminal noradrenaline in the absence of cocaine (top row) and the maximum ratio observed in the presence of cocaine (bottom row), for each of the experiments 1 to 9. A ratio of less than one means that extraluminal noradrenaline was less active than intraluminal noradrenaline.

TABLE 3.4

Effect of cocaine on noradrenaline sensitvity

Admin of noradren.	Concn. of cocaine (µg/ml)	Ratio of sensitivities: NA during cocaine / NA without cocaine — Experiment No. 1	2	3	4	5	6	7	8	9
Intra-luminal	1	1.1	1-1.5	1.2		1.32			1.8-2.2	1.3
	5	1.1		1.7		1-4.2 (1-1.2)	1.8 (2.2-2.5)	1.7-2.0 (1.4)		
	10				1.5-1.8				2.2-2.8 (1.5-1.6)	
Extra-luminal	1	4-9.8	4.8-5.3	6.6-13		3.6-5.1			9.2-9.4	7-10 (7-10)
	5	18				4.5-7.0 (7.8)	10 (10)	6.3-6.6 (8.4)		
	10		9.6-12.0	16.5	4.7-5.2)				9-12.8 (3.8-4.2)	
		Ratio of sensitivities: NA extraluminal / NA intraluminal								
Cocaine absent		0.03	0.02	0.12	0.2	0.5	0.14	0.23	0.09	0.025
Cocaine present		0.4	0.3	1.2	0.6	1.0	0.8	1.2	1.4	0.15

Figure 3.13



Responses to intraluminal (perfusion) and extraluminal noradrenaline,

A. Before cocaine.

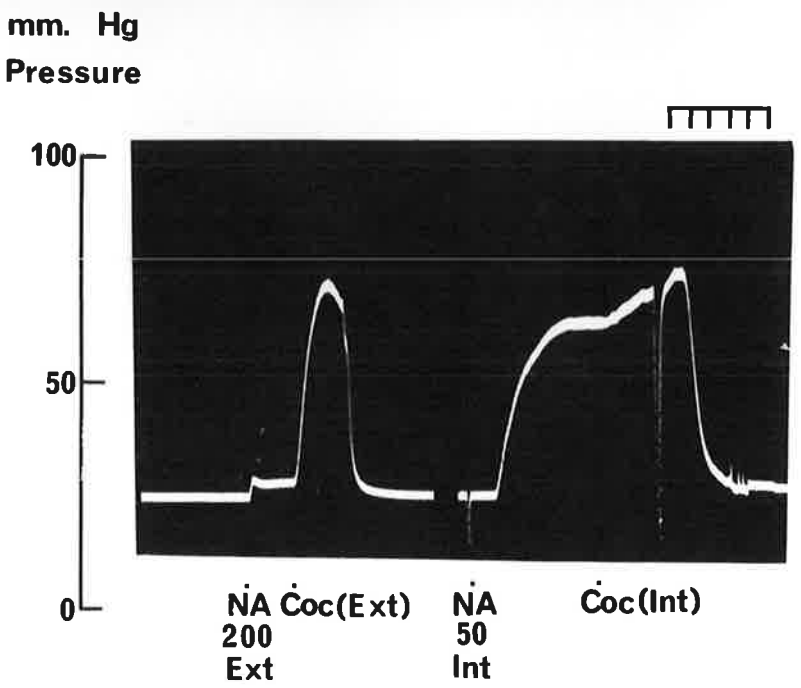
B. During cocaine 5 μg/ml (Int & Ext).

C. After cocaine washout.

Numerals ng/ml.

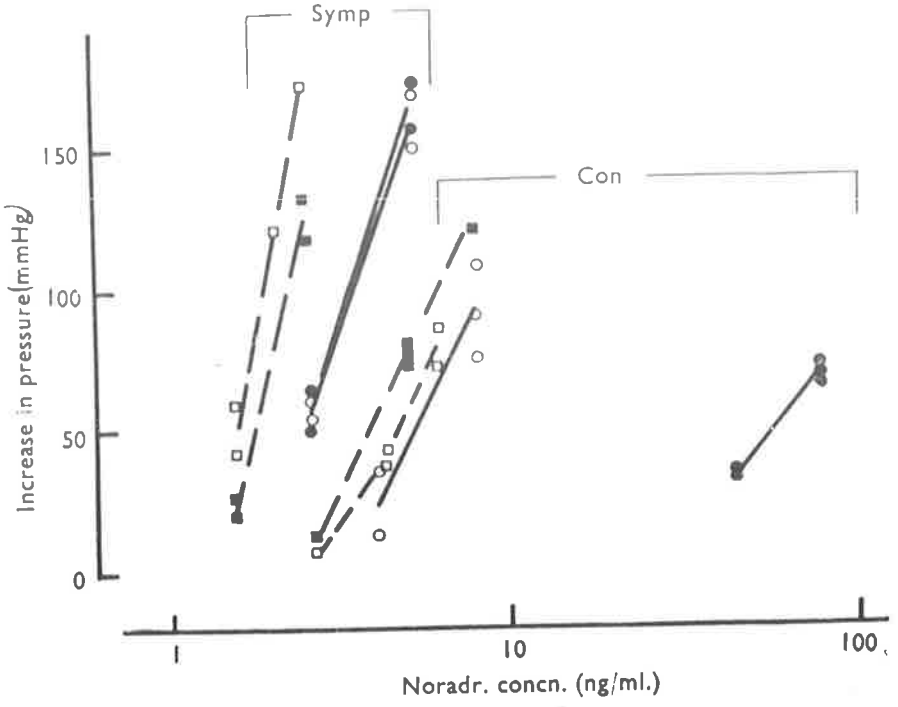
Time trace minutes.

Figure 3.14



Showing cocaine (1 μg/ml) added during the constrictor response to extraluminal noradrenaline (200 ng/ml) and intraluminal noradrenaline (50 ng/ml).
Time trace minutes.

Figure 3.15



Concentration response curves to noradrenaline in a denervated artery (Symp) and a normal innervated artery (Con) from the opposite ear. Closed symbols refer to extraluminal noradrenaline and open symbols to intraluminal noradrenaline. The broken line indicates the presence of cocaine (10 μg/ml) and the continuous line indicates the absence of cocaine.

to extraluminal noradrenaline, and that intraluminal cocaine, although less active in one artery (number 8), exerted a quantitatively similar action to that of extraluminal cocaine. A tendency for cocaine to be less active by the intraluminal route was also observed in three of a further seven arteries in which the relative sensitising potencies were estimated solely by the magnitudes of the increased response resulting from the application of cocaine during noradrenaline-induced constriction. This tendency was evident, not only by the greater response to extraluminal noradrenaline, but in two arteries by depression of the response to intraluminal noradrenaline by intraluminal but not extraluminal cocaine.

However, the striking and consistent feature of cocaine's action on all arteries was the selective enhancement of extraluminal noradrenaline. The net effect was that, regardless of its route of application, cocaine reduced or abolished the difference between the intra- and extra- luminal sensitivities to noradrenaline. This effect of cocaine is evident also in the control arteries used in the studies on denervation (table 3.5).

TABLE 3.5

Effect of denervation on sensitivity to noradrenaline

Artery	Route of application of noradrenaline	Experiment No. 1	2	3	4	5	6
Control	Intraluminal	1.0	1.0	1.0	1.0	1.0	1.0
	Extraluminal	.07	.09	.1	.18	.09	.16
Control + Cocaine	Intraluminal	1.3	2.0	1.8	3.1	4.4	2.3
	Extraluminal	1.4	0.9	1.3	1.8	1.9	0.9
Denervated	Intraluminal	1.9	2.4	9.5	1.3	1.0	5.2
	Extraluminal	2.0	1.5	6.0	0.3-1.0	0.2-0.5	3.8
Denervated + Cocaine	Intraluminal	3.7	-	-	1.8	-	13
	Extraluminal	3.0	-	-	0.8	-	7

Each figure is the ratio of extraluminal noradrenaline sensitivity to intraluminal noradrenaline sensitivity. In each control artery the latter sensitivity is arbitrarily assigned a value of one. Only the mean value of the ratio is shown, except in experiments 4 and 5 where the ratio is expressed as a range to include an increase in sensitivity to extraluminal noradrenaline which occurred spontaneously during perfusion.

Values for cocaine in the sympathectomised artery in experiments 2,3 and 5 are not shown, since the arteries progressively decreased in sensitivity to intra- and extraluminal noradrenaline once perfusion with cocaine was commenced.

Concentration of cocaine 10 µg/ml.

ii. The effect of changes in concentration of cocaine

The potentiation of extraluminal noradrenaline by cocaine in concentrations of from 0.1 to 10 µg/ml was recorded in five arteries perfused by the double cannula method (page 3.9). The cocaine was applied to the extraluminal surface only in 3 arteries, and to both intraluminal and extraluminal surfaces in 2 arteries. The potentiation was expressed as the value for the shift to the left of the dose response curve to noradrenaline if the dose response curves were parallel, or expressed as a range of values if the curves were not parallel. The results are summarised in table 3.6. In two experiments the potentiating effect of cocaine was maximal in a concentration between 1.0 and 10 µg/ml. In three experiments a maximum potentiation was not demonstrated. Cocaine caused comparable potentiation of extraluminal noradrenaline irrespective of the surface to which the cocaine was applied,

iii. Applying the drug during sustained constriction to noradrenaline permitted analysis of cocaine's time course of action. The onset and attainment of maximum sensitisation was always rapid (figure 3.14) being complete within 2 to 4 min in all arteries examined. The onset of action of cocaine was slower than that of noradrenaline but the lag was never greater than 60 sec,

TABLE 3.6

Expt. No.	Route of application cocaine	Concentration of cocaine µg/ml		
		0.1	1	10
1	Extraluminal	3.5-4.5	10-12.5	25-22
2	Extraluminal	3.5-4	13-18	32-40
3	Extraluminal	3.2-3.6	6.3-13	10.0
4	Extraluminal & Intraluminal	2.8-4	8.2-10	7.1-8
5	Extraluminal & Intraluminal	4.8-5.2	14-17	21-25

The figures indicate the shift to the left in the dose response curves to noradrenaline after cocaine in the concentrations shown. Where the dose response curves are not parallel, the shift is expressed as a range.

and was only of the order of 5 to 20 sec in the case of extraluminal applications. These features of cocaine's action are illustrated by data from two experiments shown in table 3.7. Attention is drawn to the speed of onset of intraluminal cocaine's sensitising action on extraluminal noradrenaline, which in the two experiments was only 9 and 15 sec slower than that of extraluminal cocaine.

Denervation

A constant finding in each of six rabbits in which one superior cervical ganglion had been removed at operation 14 days previously was that (i) in contrast to the contralateral artery, the homolateral artery did not possess noradrenergic fluorescent structures; and (ii) each denervated artery showed greatly enhanced sensitivity to extraluminal noradrenaline, but a smaller increase in sensitivity to intraluminal noradrenaline. Comparison was made in each case with the artery from the opposite ear which had not been denervated. An example of the concentration-response curves to noradrenaline in the two arteries is shown in figure 3.15. The results are summarised in table 3.5, which also shows the effect of cocaine on each control and sympathectomised artery. It will be noted that the denervated artery closely resembles the cocaine-treated artery in its relative sensitivities to intra-

TABLE 3.7

Time course of action of cocaine.

		Cocaine during intraluminal NA		Cocaine during extraluminal NA	
		Intra-luminal	Extra-luminal	Intra-luminal	Extra-luminal
Expt. 1	**Onset of response**	34	15	30 (20)	5
Flow rate= 8 ml/min	Onset to maximum	102	85	90	65
Expt. 2	Onset of response	78	37	42 (29)	20
Flow rate=5.6 ml/min	Onset to maximum	170	160	213	125

1. Numbers are times in seconds.
2. To allow for perfusion of "dead space" fluid the time of onset of action of intraluminal noradrenaline is calculated from the difference between the observed time of onset and the time taken for perfused dye to reach the artery. However, the intraluminal times of onset will be underestimated, since perfused dye did not reach the peak sustained concentration in the intraluminal outflow for a further 10 to 20 seconds for experiment 1, and 30 seconds for experiment 2. For this reason the onset of action of intraluminal cocaine was also estimated (shown in parentheses). The drug injection was made proximal to the artery so that maximum concentration was reached within 2 seconds.
Concentrations of cocaine; Experiment 1 Cocaine 1 µg/ml. By injection 2µg.
Experiment 2 Cocaine 0.4 µg/ml. By injection 2 µg.

and extra- luminal noradrenaline, that is, denervation, like cocaine, tends to abolish the difference between these sensitivities. The effect of cocaine on the sensitivity of the denervated arteries to noradrenaline varied between relatively slight enhancement (three arteries) and depression (three arteries).

Another trend observed in these experiments was that the sympathectomised artery was more sensitive to intraluminal noradrenaline than the control artery. This effect is apparent in figure 3.15 and table 3.5.

Histamine

The effect of cocaine on histamine-induced constriction was examined in three arteries. The response to histamine, perfused intraluminally or applied extraluminally, resembled that to noradrenaline (figure 3.16) in that it was prompt in onset, reached a maximum within 1 to 4 min, and was well-sustained. The ratios of the sensitivity of the arteries to histamine, before, and in the presence of cocaine (1 µg/ml) were derived from concentration-response curves and are shown in table 3.8. The data point to a slight depressant action of cocaine on intraluminal histamine. However, the main feature is cocaine's lack of effect

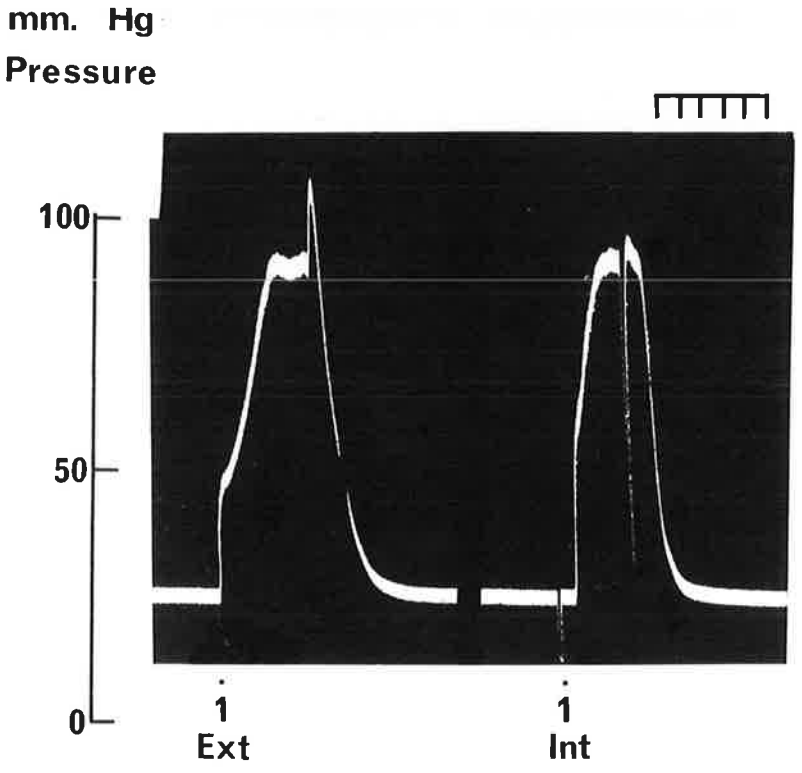
TABLE 3.8

Effect of cocaine on histamine.

	Ratio of sensitivities		
Route of application	Expt. 1.	Expt. 2	Expt. 3
Intraluminal histamine	0.46-0.56	0.6-0.9	.66-1.2
Extraluminal histamine	1.25	.7-1.0	1-1.3
Extraluminal noradrenaline	10	6-10	20
Sensitivity ratio in the absence of cocaine;			
$\frac{\text{Extraluminal histamine}}{\text{Intraluminal histamine}}$	0.5	0.5	0.9-1.0

The ratios refer to the sensitivity to histamine, or noradrenaline (extraluminal only), in the presence of cocaine compared with the sensitivity in the absence of cocaine. Concentrations: cocaine 1 µg/ml; histamine 0.1-1 µg/ml.

Figure 3.16



Responses of the perfused artery to histamine, 1 μg/ml extraluminally and 1 μg/ml intraluminally.

on extraluminal histamine which contrasts with the marked potentiation of extraluminal noradrenaline. The latter was measured in each artery following the observations on histamine, by applying noradrenaline immediately before, and after, cocaine wash out. Attention is drawn alao to the relatively small difference between the sensitivity to intra- and extra- luminal histamine. The maximum difference was two fold, compared with the five- to ten- fold difference commonly observed with noradrenaline.

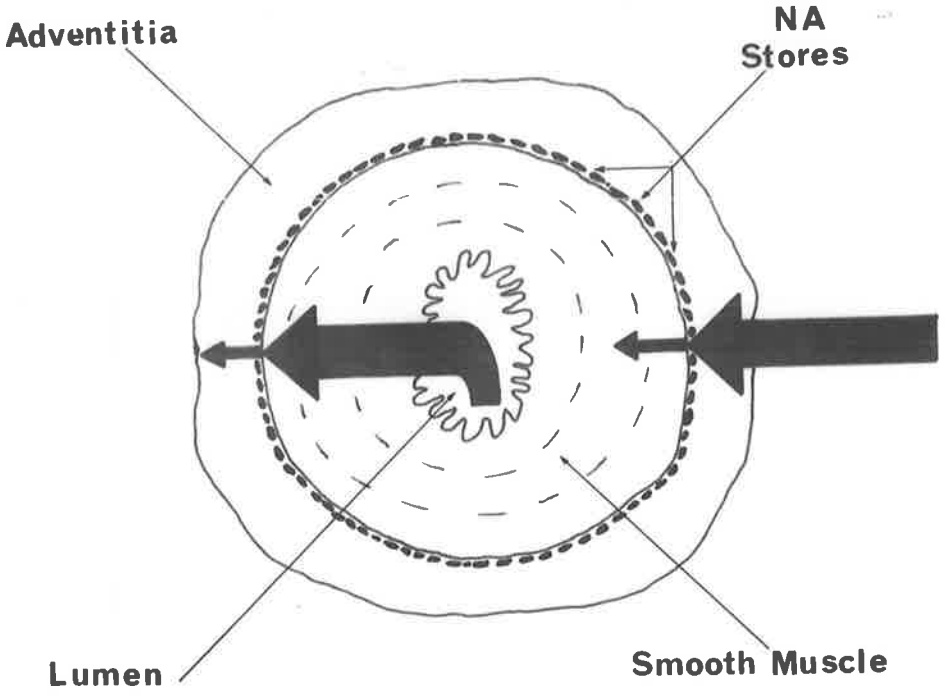
DISCUSSION

In other tissues, particularly heart and cat nictitating membrane, it has been demonstrated that cocaine prevents uptake of noradrenaline into the storage sites, and that chronic denervation achieves the same effect by causing the storage sites to deteriorate (Trendelenburg, 1963). Hence the ability of cocaine and denervation to increase the sensitivity of the artery to extraluminal noradrenaline to a level approaching that to intraluminal noradrenaline indicates that the marked differences between these sensitivities in the normal untreated artery is probably related to uptake of noradrenaline into storage sites and not simply to diffusion barriers in the artery. The observations that the sensitising action of serotonin (reported in this thesis, page 7.7) and the constrictor potency of histamine are little affected by their routes of application to the artery are further evidence that diffusion barriers are unlikely to play a major role in the differences in sensitivity to intra- and extra- luminal noradrenaline.

It has been shown (page 2.18) that the noradrenaline storage sites in this artery are closely packed structures located in the adventitia immediately outside and completely

surrounding the outer border of the smooth muscle layer. Evidence that the fluorescent structures observed at the medial-adventitial border of the artery are sites of uptake of noradrenaline has been provided earlier in this study (page 2.22). The position of the storage sites is consistent with the hypothesis that noradrenaline applied to the adventitia undergoes considerable loss by uptake into the storage sites before it reaches the underlying smooth muscle, and that cocaine and denervation exert their dramatic effect on sensitivity to extraluminal noradrenaline by preventing this loss. The relatively slight potentiation of intraluminal noradrenaline may be explained in two ways, either low uptake or an inability of uptake to influence the concentration of noradrenaline in the smooth muscle. The second explanation is favoured by the speed with which intraluminal cocaine exerts its potentiating and inhibitory effects on extraluminal noradrenaline. The effect of cocaine commences within 15 to 30 sec of application to the perfused artery, and is maximal or near maximal within a further 2 to 4 min. The time course implies rapid penetration of cocaine from the intima to the storage sites in the adventitia, and it is a reasonable assumption that intraluminal noradrenaline penetrates to the storage sites at a rate at least comparable with that of cocaine. The hypothesis is presented diagrammatically in figure 3.17.

Figure 3.17



Diagrammatic representation of the influence of the sites of uptake (shown as NA stores) on the concentration of noradrenaline in the smooth muscle of the artery. The direction of the arrow indicates the direction of diffusion of noradrenaline, and the thickness of the arrow indicates the concentration of noradrenaline.

The storage sites are assumed to represent major sites of loss for both intraluminal and extraluminal noradrenaline, so that loss of intraluminal noradrenaline occurs, but only after it has diffused through the smooth muscle - that is, after it has exerted its physiological action. Hence the extremely high sensitivity of the artery to intraluminal noradrenaline, which has provided the basis for its application to catecholamine bioassay (de la Lande and Harvey, 1965). is explained not by the absence of uptake, but by the inability of uptake to affect this sensitivity; similarly the sensitivity is little affected when uptake is prevented or abolished by cocaine or denervation.

However, the first explanation - that is, low uptake - is not excluded by the data since, despite rapid penetration to the adventitia of intraluminally applied drugs, it is possible that their concentration is markedly reduced in the outer region of the artery wall by a diluting effect of the extraluminal bathing solution. If such an effect extends to the region of the adventitial storage sites, the concentration of noradrenaline in this region may be considerably less than in the lumen, and uptake of noradrenaline will be correspondingly reduced. Parallel studies in progress in this department on the rate of penetration of

labelled noradrenaline across the artery wall may assist to distinguish between the two explanations.

SUMMARY

1. The sensitivity of the isolated rabbit ear artery to extraluminal noradrenaline, but not to intraluminal noradrenaline, is greatly enhanced by cocaine. The net effect is that the difference between the intraluminal and extraluminal sensitivity to noradrenaline is greatly reduced or abolished.

2. The effects of denervation closely resemble those of cocaine.

3. Cocaine has little effect on the constrictor response to histamine.

4. It is concluded that the position of the noradrenaline stores in the medial-adventitial border of the artery determines the low sensitivity of the artery to extraluminal noradrenaline. Cocaine and denervation, by eliminating the effects of uptake, reduce the loss of noradrenaline by uptake into the storage sites as it diffuses from the adventitia to the media.

CHAPTER 4

THE ROLE OF STORAGE STRUCTURES IN RESPONSES TO ENDOGENOUS NORADRENALINE

INTRODUCTION

The results presented in chapters 2 and 3 provided evidence firstly that noradrenaline is localised in nerve terminals in the medial-adventitial border of the rabbit ear artery, secondly that exogenous noradrenaline is taken up by these nerves, and thirdly that the uptake is responsible for the differences in sensitivity between intraluminal and extraluminal noradrenaline. The purpose of the study presented in this chapter was to explore the role which uptake by neural storage sites played in the fate of endogenous noradrenaline, that is, noradrenaline released when the sympathetic nerve endings are excited electrically.

Outschoorn (1952) noted a substance resembling noradrenaline in the outflow from the rabbit ear perfused by the method of Gaddum and Kwiatowski (1938), in which the cervical sympathetic nerve was stimulated pre-ganglionically. During peri-arterial stimulation of sympathetic nerves in the central artery of the rabbit ear, de la Lande, Paton and Waud (1964) found traces of a substance which resembled

noradrenaline in that it relaxed the electrically stimulated rat colon, and constricted the rabbit ear blood vessels. In 1965, de la Lande and Rand provided pharmacological evidence that electrically induced vasoconstriction in the perfused isolated rabbit ear artery was probably mediated by release of noradrenaline from sympathetic nerves.

The question considered in this chapter is whether uptake plays a significant role in the responses of the ear vessels to the liberated noradrenaline. Cocaine, which was shown in chapter 3 to block uptake of noradrenaline, was employed in an attempt to answer this question. Furchgott (1955), in an early review, summarised the experimental evidence that cocaine potentiated the responses to sympathetic nerve stimulation and to exogenous catecholamines. Although at that time the cat nictitating membrane had been widely employed as the test preparation, Furchgott quoted several studies on perfused vascular preparations and isolated blood vessels, showing cocaine potentiation of the effects of exogenous catecholamines. Trendelenburg (1959) noted potentiation of the response of the electrically stimulated cat nictitating membrane after cocaine. The potentiation was more evident with sub-maximal stimulation than with supra-maximal stimulation. Trendelenburg found little difference in the potentiation by cocaine of injected noradrenaline and of electrical stimulation, as judged by volume changes in the cat spleen. Bentley (1966) found that responses to exogenous noradrenaline were

potentiated far more than those to nerve stimulation when cocaine was added to the perfused guinea pig and rat vas deferens.

The only studies on the ear artery were those of de la Lande and Rand (1965) who observed that cocaine enhanced the constrictor responses to field stimulation and to noradrenaline. Their records indicate slightly greater potentiation of field stimulation than of noradrenaline, but the significance of their findings is not clear in view of the subsequent demonstration (described in chapter 3) of the importance of distinguishing between the responses to intraluminal and extraluminal noradrenaline.

Hence the present study was concerned with comparing the effects of cocaine on field stimulation with its effects on responses to intraluminal and extraluminal noradrenaline.

METHODS

A. The isolated rabbit ear artery was perfused in two ways:

1. In early studies, by the single cannula method, so that intraluminal fluid, after passing through the lumen, bathed the adventitia (page 3.10).
2. In later studies, by the double cannula method, so that intraluminal and extraluminal fluids did not mix (page 3.9).

B. Nerves in the artery wall were stimulated electrically in two ways:

1. The two platinum electrodes were placed in the extraluminal fluid (page 3.14).
2. One electrode was placed in the intraluminal perfusion stream and the other placed in the extraluminal fluid (page 3.14).

C. Methods of adding drugs intraluminally and extraluminally were as described on page 3.10.

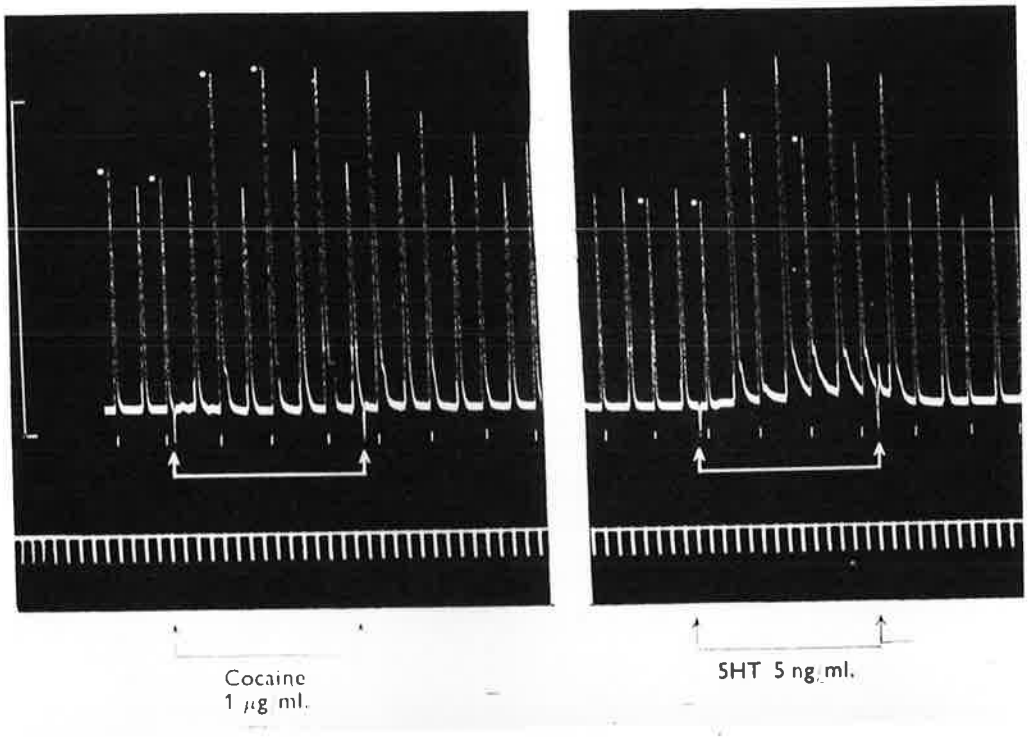
RESULTS

A. Brief application of pulses

The single cannulated artery was stimulated alternately with injections of noradrenaline and brief applications (4 to 10 seconds) of periarterial stimulation, each applied at four minute intervals. Cocaine 1 µg/ml was then added to the perfusion fluid and its effect recorded on the response to nerve stimulation and to noradrenaline.

The response to nerve stimulation was a rapidly occurring rise in perfusion pressure, which was essentially similar in nature to the response to injected noradrenaline in that it rapidly reached a peak and just as rapidly returned to the resting perfusion pressure at the termination of stimulation. The cocaine caused a slight increase in the height of the responses to injections of noradrenaline, but caused a greater increase in the response to nerve stimulation. Responses typical of four experiments are illustrated in figure 4.1, which shows for comparison the effect of adding another agent known to increase sensitivity to noradrenaline, namely serotonin. In contrast to cocaine, serotonin in a concentration of 5 ng/ml caused more marked potentiation of the response to injected noradrenaline than of the response to nerve stimulation.

Figure 4.1



The effect of cocaine (1 μg/ml) and serotonin (5 ng/ml) on responses of a single cannulated artery to 10 ng injections of noradrenaline (unmarked) and peri-arterial nerve stimulation (vertical white lines, 1 msec, 20 pulses per sec for 4 sec at 30 volts). The height of the responses to electrical stimulation before and during perfusion of the drugs is shown by white dots.
Time trace in minutes.
Pressure scale 100 mm mercury.

B. Sustained application of pulses

Although cocaine caused greater potentiation of brief application of nerve stimulation than of injected noradrenaline, the increase in the response to nerve stimulation was much less than the increase produced by cocaine on the response to extraluminal noradrenaline. In order of magnitude the potentiated responses to nerve stimulation resembled those of intraluminal noradrenaline rather than extraluminal noradrenaline.

A possible explanation of the surprisingly small effect of cocaine on nerve stimulation in the above experiments was that the period of application of pulses (of the order of a few seconds) was too brief for the effects of noradrenaline uptake into the nerve terminals to become manifest. Hence it was considered advisable to apply stimulation which would cause responses comparable with those elicited by sustained contact of the artery with noradrenaline. In the following experiments the period of stimulation was sustained so that the constriction reached its maximum and/or steady value. The further increase in the height of the responses which occurred when cocaine was added, indicated the ability of cocaine to block uptake at a time when the artery was being continually exposed to the endogenous (released) noradrenaline.

The responses to cocaine were compared with those elicited by cocaine during the sustained phase of the responses to intraluminal and extraluminal noradrenaline.

In most arteries the response to sustained nerve stimulation was a rapid rise in perfusion pressure followed by a transient decline in pressure to a steady value. In six arteries steady responses of approximately equivalent magnitude were elicited by field stimulation, intraluminal noradrenaline and extraluminal noradrenaline. During the sustained phase of the response cocaine was applied intraluminally or extraluminally, and its effect measured by the increase in constriction. The results of the six experiments are presented in table 4.1. Typical responses of the artery are shown in figure 4.2. The main feature of the results was that in five of the arteries potentiation of field stimulation was much less marked than that of extraluminal noradrenaline and, as in the case of brief application of pulses, corresponded more closely to the potentiation by cocaine of intraluminal noradrenaline. These findings indicated that the period of application of pulses was not a major factor responsible for the relatively small effect of cocaine on field stimulation.

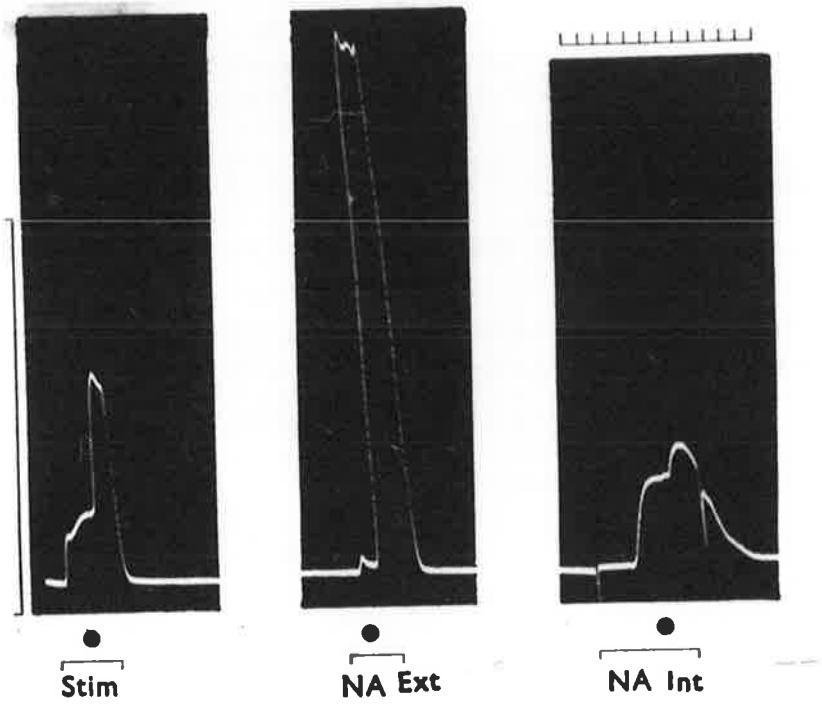
TABLE 4.1

Effect of cocaine on noradrenaline and on field stimulation.

	Expt. No.	1	2	3	4	5	6
Intra-luminal NA	Int. cocaine	0/3 0/34		8/12 20/12	20/30	8/38	10/33
	Ext. cocaine	8/22 7/13 30/85	20/55 8/26		36/45	10/45	
Stimulation	Int. cocaine	32/20		20/18 10/15	15/5 20*/25	105/25	30/30 30/28
	Ext. cocaine	35*/20 25/5	14/10 12*/21		59*/20 40**/36	136*/17	
Extra-luminal NA	Int. cocaine	41/9 115/12		128/12 150/10	170/20	180/10	48/40
	Ext. cocaine	66/4 136/2	135/5		160/20	180/10	
Stimulation characteristics	Pulse duration (msec) /pulse frequency per sec	0.3/2 0.3/0.4*	1/3 1/8	1.0/0.8	0.3/5 *1/3 **1/1.5	1/1 *1/5	1/0.35

1. Each ratio is the $\frac{\text{Increase in response (in mm) elicited by cocaine}}{\text{Response (in mm) prevailing immediately before adding cocaine.}}$
2. Stimulation characteristics shown at foot of table marked* refer to the response marked* in the same column.
3. Concentration of cocaine: Experiment 1: 0.5 µg/ml; 2: 2 µg/ml; 3: 5 µg injection; 4: 1 µg/ml (extraluminal), 5 µg injection; 5: 0.5 µg/ml extraluminal, 5 µg injection; 6: 5 µg injection.

Figure 4.2



Comparison of the action of cocaine (0.5 µg/ml applied extraluminally at the black dot) on field stimulation (0.3 msec 0.4 pulses/sec) extraluminal noradrenaline (200 ng/ml) and intraluminal noradrenaline (10 ng/ml). Time trace in minutes.
Pressure scale 100 mm mercury.

DISCUSSION

The finding that cocaine's enhancement of the response to field stimulation was very much less than that to extraluminal noradrenaline, and was only a little greater than that to intraluminal noradrenaline, was unexpected. It had been reasoned that if release of noradrenaline occurred at or close to the sites of uptake and storage, uptake might be expected to play a major role in terminating the effects of the liberated noradrenaline. Nevertheless, the findings are consistent with those on other sympathetically innervated tissues. Bentley (1966) observed little if any potentiation of the effects of sympathetic nerve stimulation (on the guinea-pig and rat vas deferens) by cocaine, despite potentiation of exogenous noradrenaline of the order of 30 to 40 fold. Since, unlike their position in the artery, the storage sites of noradrenaline in the vas deferens occur throughout the smooth muscle layer, it must be assumed that the relative position of the storage sites and smooth muscle cells is not in itself the major factor responsible for the relatively small effect of cocaine on the nerve mediated response.

Recently, Trendelenburg (1966) examined changes in uptake of noradrenaline which occur during excitation of the nerve terminal. Using the cat nictitating membrane, pretreated with reserpine to minimise the contractile response to stimulation, he showed that sensitivity to noradrenaline was enhanced during stimulation. Trendelenburg also

observed that despite the uptake of labelled noradrenaline into nerves during their excitation, there was no "net uptake", that is, there was exchange of noradrenaline as though its retention within the nerve terminal was impaired. Hence Trendelenburg's study pointed to a possible explanation of the relatively minor effect of cocaine on the response to electrically released noradrenaline in the artery. This was that if net uptake of noradrenaline did not occur during excitation of the nerve terminal, superimposed inhibition of uptake into the excited nerve terminal by cocaine might not lead to the further accumulation of noradrenaline outside the terminal.

Another possible explanation may be saturation of the uptake mechanism. In view of the evidence of Dengler, Michaelson, Spiegel and Titus (1962) and Iversen (1967) that in brain and heart respectively, the noradrenaline transport mechanism is saturable, it is possible to argue that the endogenous noradrenaline which is released from the storage structures attains concentrations in the immediate extracellular environment of the nerve terminals which are in large excess of the transport maximum for noradrenaline re-uptake. Under these circumstances, cocaine would be unlikely to cause a marked increase in the extracellular concentration of noradrenaline by inhibiting its uptake.

CHAPTER 5

THE ACTION OF TYRAMINE

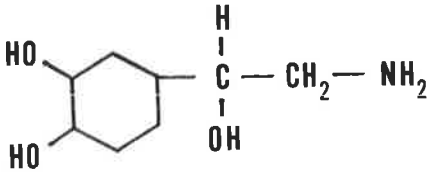
INTRODUCTION

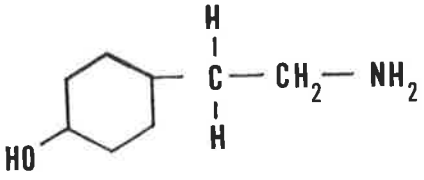
Trendelenburg (1963) reviewed the evidence that the sympathomimetic action of tyramine is indirect, mediated largely by release of noradrenaline from neural storage sites, and the following evidence supported this view. Tyramine caused release of noradrenaline from perfused tissue (although the amount of noradrenaline recovered in the effluent was usually small). Tyramine perfusion greatly reduced tissue content of noradrenaline, and at the same time the sympathomimetic action of tyramine was reduced. The action of tyramine was restored by perfusion of noradrenaline.

The hypothesis (page 3.29) that the distribution of sympathetic nerves in the rabbit ear artery determines intraluminal and extraluminal noradrenaline sensitivity differences has implications in the action of tyramine on the artery. If, as is assumed in the hypothesis, noradrenaline readily penetrates to the storage sites from either surface of the artery, it might be anticipated that the close structural analogue tyramine (figure 5.1), would penetrate equally readily from either surface. It follows that, if the noradrenergic storage structures are the primary site of action of tyramine, the drug should display the same constrictor

Figure 5.1

Noradrenaline





Tyramine

Formulae of noradrenaline and tyramine.

activity regardless of the surface to which it is applied. The constrictor potencies of intraluminal and extraluminal tyramine have been examined in the following study, as well as the contribution which a direct action on noradrenergic receptors may make to the constrictor action of tyramine. In this connection Farmer (1966) had previously shown that intraluminal injections of tyramine in the artery caused a biphasic constrictor response, the first phase of which was attributed to an effect on the smooth muscle (a direct effect), and the second phase to the release of noradrenaline from sympathetic nerves (an indirect action of tyramine). The conditions of perfusion employed by Farmer were those in which the intraluminally applied drug escaped into the extraluminal fluid bathing the artery (the single cannula method described on page 3.10). Hence, in the course of the present study the possibility was investigated that the two constrictor phases described by Farmer reflected separate intraluminal and extraluminal actions of tyramine.

MATERIALS AND METHODS

Perfusion of the artery

The method of perfusion of the artery was the double cannula method described on page 3.9. Arteries were tested for leakage as described on page 3.12 and those displaying leaks were rejected.

The use of the double cannulated artery permitted the vasoconstrictor response to tyramine added to the extraluminal bathing medium to be compared with that to tyramine added to the intraluminal perfusing medium. Since the sensitivity of perfused arteries to noradrenaline changed considerably during the first hour of perfusion (see page 3.13) after which it remained constant for at least four hours, experiments on tyramine were routinely commenced after the artery had been perfused for one hour. Intraluminal and extraluminal tyramine were applied alternately to the artery at intervals of ten to thirty minutes, following wash-out of the previous dose. Tyramine was allowed to remain in contact with the artery until constriction was maximal; in most arteries periods of 5 to 6 minutes sufficed.

Measurement of sensitivity changes

The ratio of sensitivities of the artery to intraluminal and extraluminal tyramine was established in a similar fashion to that described for noradrenaline (page 3.13). Concentration response curves to

intraluminal and to extraluminal tyramine were obtained, and the ratio of the concentrations producing constriction of equal magnitude (i.e. the dose ratio) was determined. The inverse of the dose ratio is referred to as the sensitivity ratio; for example, a sensitivity ratio of 10 for $\frac{\text{extraluminal tyramine}}{\text{intraluminal tyramine}}$ means that extraluminal and intraluminal tyramine produce responses of the same magnitude when the former is present in one-tenth the concentration of the latter. The ratio of the sensitivities of two separate arteries, for example a denervated and control artery, to the one stimulant, for example extraluminal tyramine, was also expressed by the corresponding sensitivity ratio. Where the curves under comparison differed in slope, the sensitivity ratio was expressed as the range of the maximum and minimum values of the ratio. In some arteries, the sensitivity ratio was obtained from the concentration-response curve to intraluminal (or extraluminal) tyramine, and the response to one level of concentration only of extraluminal (or intraluminal) tyramine.

Denervation

In three experiments, the responses of arteries which had been denervated by removal of the superior cervical ganglion two to three weeks previously were compared with the artery from the opposite ear which had not been denervated. Denervation was carried out by the method described on page 3.15 and the effectiveness of denervation was routinely

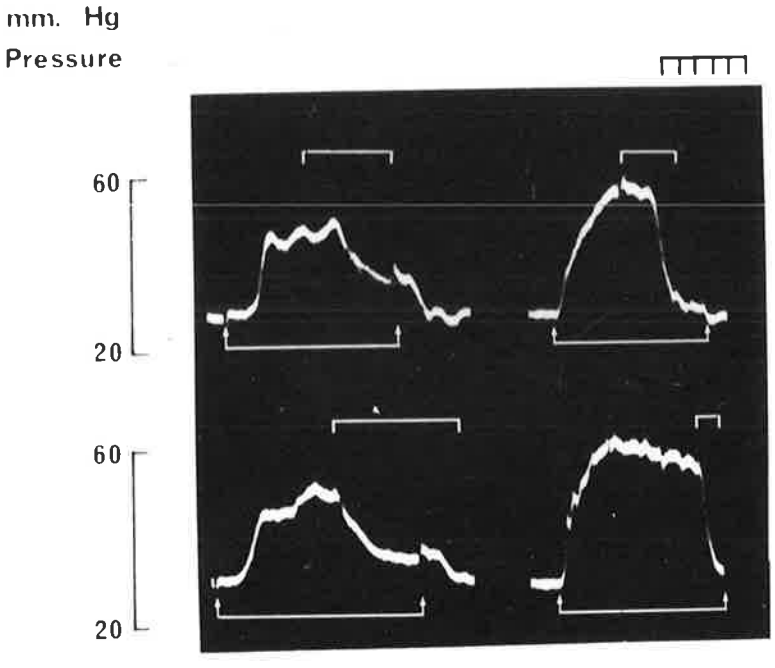
tested by comparing the responses of the denervated and control artery to periarterial electrical stimulation.

RESULTS

Nature of responses

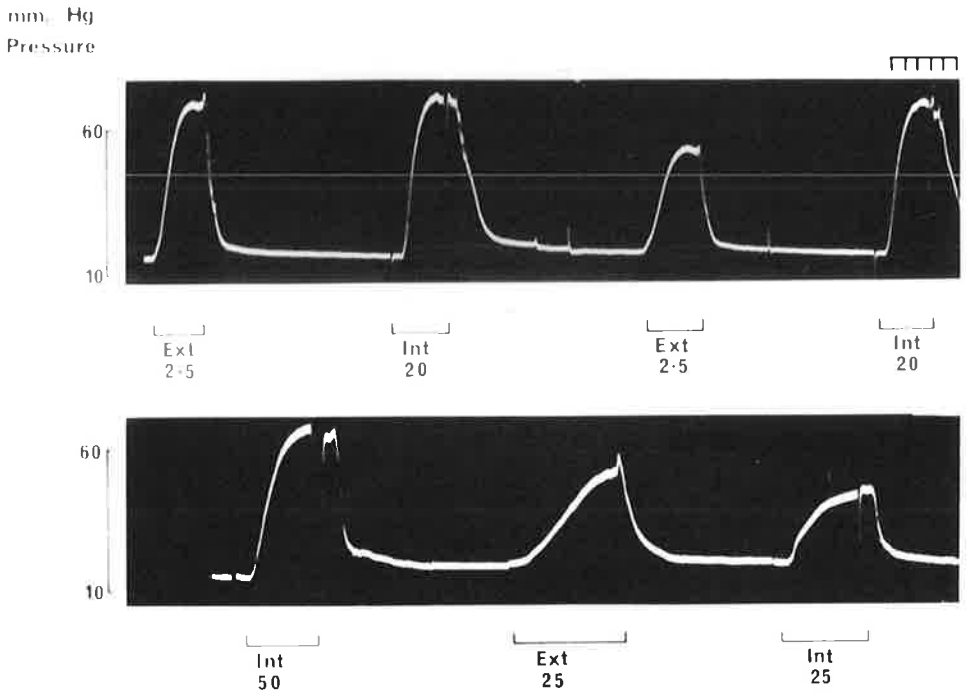
The responses to intraluminal and extraluminal tyramine were examined in 30 arteries. In the majority of arteries, the responses in the range of 0-100 mm mercury were similar in shape and time course and comprised an increase in perfusion pressure which reached its maximum value within 30 seconds to 3 minutes according to the magnitude of the response. The constriction was sustained at the maximum value or declined slowly for the period of contact of drug and artery; the perfusion pressure returned to its resting value promptly on washout of drug and only in rare instances did the time for recovery exceed 3 minutes. Occasionally the shapes of the responses varied in that the sustained phase of large responses was interrupted by rapid oscillations in the perfusion pressure. This type of response was observed consistently in five arteries, and was more prominent with intraluminal than extraluminal tyramine. However, the shapes and time courses of the responses illustrated in figures 5.2 and 5.3 were typical of those observed in most arteries. Compared with the responses to noradrenaline, which were described in earlier studies (page 3.19), those to tyramine were characterised by more gradual attainment of the peak level of constriction.

Figure 5.2



Responses of an artery to intraluminal tyramine 5 μg/ml (top left and bottom left) and extraluminal tyramine 1.5 μg/ml (top right and bottom right). The white bars indicate cocaine 1 μg/ml added intraluminally (top left and right) and extraluminally (bottom left and right). The double arrows indicate the period of application of tyramine.
Time trace in minutes.

Figure 5.3



Responses of a denervated artery (bottom panel) and its control artery (top panel) to tyramine extraluminally (Ext) and intraluminally (Int).
The figures indicate µg/ml of tyramine, and the brackets show period of application.
Time trace in minutes.

Sensitivity to tyramine

The responses to repeated applications of intraluminal and extraluminal tyramine at various levels of concentration were examined in 24 arteries. In each artery the sensitivity to extraluminal tyramine was considerably greater than that to intraluminal tyramine. The difference in sensitivity was particularly marked in the early stages of perfusion (within the first and second hours of perfusion) at which stage it was a common finding that 2 µg/ml of extraluminal tyramine caused constriction of the order of 40-100 mm mercury, whereas intraluminal tyramine failed to elicit a response in concentrations of 5 µg/ml. Spontaneous changes in sensitivity occurred in most arteries. These consisted of a decline in sensitivity to extraluminal tyramine, and either no change or an increase in sensitivity to intraluminal tyramine. Decline in sensitivity to extraluminal tyramine is illustrated in figure 5.3. As a result, the difference between intraluminal and extraluminal sensitivity often tended to become less marked during the course of an experiment. In some arteries, the spontaneous changes in sensitivity nullified attempts to establish concentration-response curves. In others the sensitivity to one of the stimulants, for example intraluminal tyramine, was sufficiently steady throughout the perfusion to enable the mean of the first few responses to constant concentrations of the second stimulant (extraluminal tyramine) to be used as a guide to the sensitivity ratio

prevailing early in the experiment. However, in 12 arteries, spontaneous changes were minor and it was possible to measure sensitivity ratios based on concentration-response curves to both intraluminal and extraluminal tyramine. Curves which included a wide range of responses in two arteries (numbers 9 & 10 in table 5.1) are shown in figure 5.4. It will be noted that the greater potency of extraluminal tyramine is prominent at all levels of response, despite differences between the slopes and shapes of the curves under comparison. The sensitivity ratios determined in each artery are summarised in tables 5.1 and 5.2. Sensitivity ratios based on one response level in a further 6 arteries are also included in tables 5.1 and 5.2. The latter ratios refer, not only to arteries where spontaneous changes in sensitivity vitiated one of the concentration-response curves, but also to arteries in which the response to intraluminal tyramine in the highest concentration tested failed to exceed the level of the smallest response to extraluminal tyramine (figure 5.5).

Although the ratios quoted in tables 5.1 and 5.2 range from two to more than fifty it will be noted that most fall within the range of 4 to 12. Since the levels of response on which the ratios were based ranged from barely threshold i.e. of the order of 2 to 3 mm of mercury to 168 mm of mercury increase in perfusion pressure, and differences between the slopes of the curves under comparison in individual arteries

TABLE 5.1

SENSITIVITY RATIOS

Extraluminal tyramine / Intraluminal tyramine

Expt. No.	1	2	3	4	5	6
Ratio	1.3-2.8	2.6-3.0	3.1	3.3-4.9	3.3-5.1	2.6-5.0
Responses range (mm of mercury)	4-50	16-40	76-168	8-82	4-30	3-100
Expt. No.	7	8	9	10	11	
Ratio	3.3-5.3	4.2-4.8	9-12.4	12.5-20	30-37.5	
Response range	24-116	17-76	76-116	5-68	3-20	
Expt. No.	12	13	14	15		
Ratio	5	7.1	>10	12.5		
Response range	20	26	8	12		

NOTE A sensitivity ratio of 10 means that a tenfold greater concentration of intraluminal tyramine was required to produce the same response as extraluminal tyramine. Maximum and minimum values of the ratios are expressed as a range, together with the range of the responses over which the ratios were measured.

TABLE 5.2

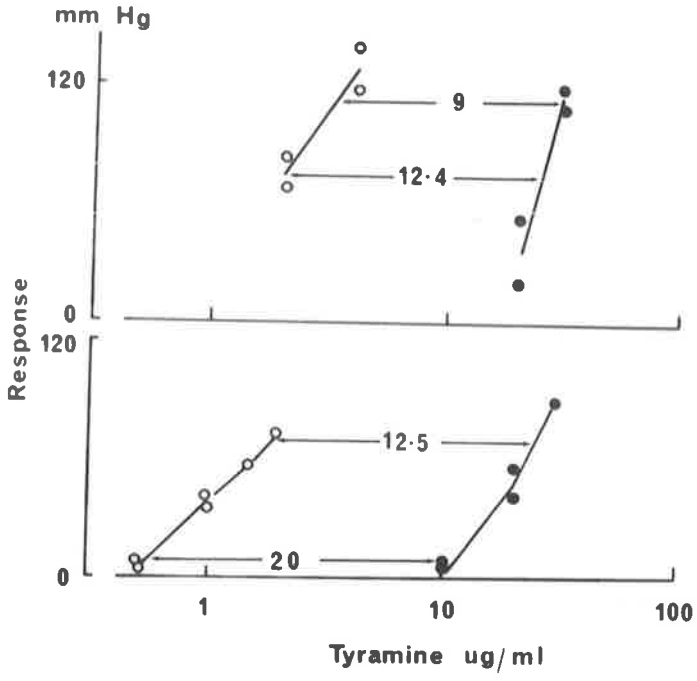
SENSITIVITY RATIOS IN CONTROL AND DENERVATED ARTERIES

(Figures in brackets refer to level of response or range of responses at which sensitivity ratios measured)

	Extraluminal tyramine / Intraluminal tyramine		
	Expt. 1	Expt. 2	Expt. 3
Control	>50 (63)	3.9-6.4 (20-50)	10 (22)
Denervated	1.1-1.8 (15-110)	1.1 (40-130)	0.74-0.9 (18-52)
	Denervated artery / Control artery		
Intralum. tyramine (20-100 µg/ml)	2.3-2.4 (12-36)	0.25-0.64 (35-52)	0.95-1.1 (20-70)
Extralum. tyramine (1-10 µg/ml - Control)	0.03-0.05 (63-135)	0.11-0.14 (40-85)	0.08 (22)
Intralum. noradrenaline (5-10 ng/ml, Expt 3) (5-10 ng inj, Expt 1+2)	1.8 (40)	1.6-2.1 (30-77)	1-1.3 (41-59)
Extralum. noradrenaline (50-400 NG/ml)	28-34 (25-42)	42-51 (10-60)	12-12.1 (37-70)

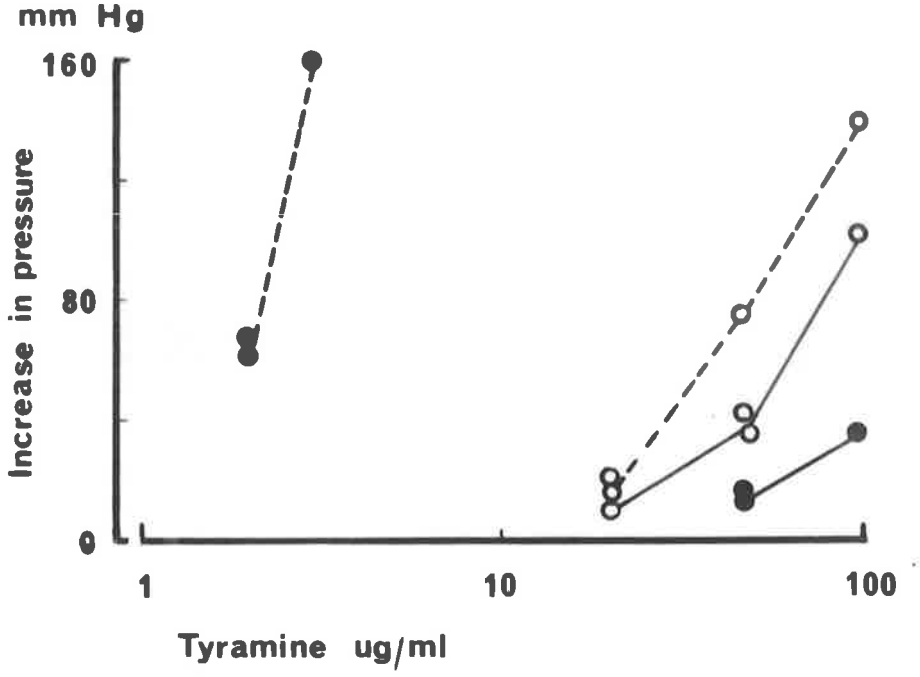
The sensitivity ratios are based on concentrations of the drugs producing responses equal in magnitude with the exception that in experiments 1 and 2, noradrenaline was given by injection and the ratios here refer to doses instead of concentrations.

Figure 5.4



Concentration response curves to intraluminal tyramine (closed symbols) and extraluminal tyramine (open symbols). The upper and lower graphs refer to arteries 9 and 10 in table 5.1. The minimum and maximum values of the sensitivity ratios of each artery are shown.

Figure 5.5



Concentration response curves to tyramine for a denervated artery (open symbols) and its control artery (closed symbols). The unbroken line indicates intraluminal tyramine and the broken line extraluminal tyramine.

rarely caused the ratio to differ by more than a factor of two, it may be concluded that the sensitivity ratios reflect a major difference between the constrictor potencies of intraluminal and extraluminal tyramine. The mean value of the sensitivity ratios (10.4) is the guide to the magnitude of the greater potency of extraluminal tyramine.

Denervation

In three experiments, the effects of tyramine on an artery denervated by removal of the superior cervical ganglion 2-3 weeks previously were compared with its effects on the (control) artery from the opposite ear which had not been denervated. Concentration-response curves are illustrated in figure 5.5 and the results are summarised in table 5.2. The main feature is that, compared with its action on the corresponding control arteries, extraluminal tyramine is much less active in the denervated arteries so that there is now little difference between sensitivity to intra- and extra- luminal tyramine. Another characteristic difference between the denervated and control arteries was the relative slowness of the response of the former to extraluminal tyramine; the slow response is evident in figure 5.3. In accord with previous observations, the denervated arteries also displayed greatly enhanced sensitivity to extraluminal noradrenaline, but only a small increase in sensitivity to intraluminal noradrenaline compared with the control arteries (table 5.2). In order to avoid interaction between the

two drugs, noradrenaline was applied to the artery only after sensitivity to tyramine was established.

Effect of cocaine

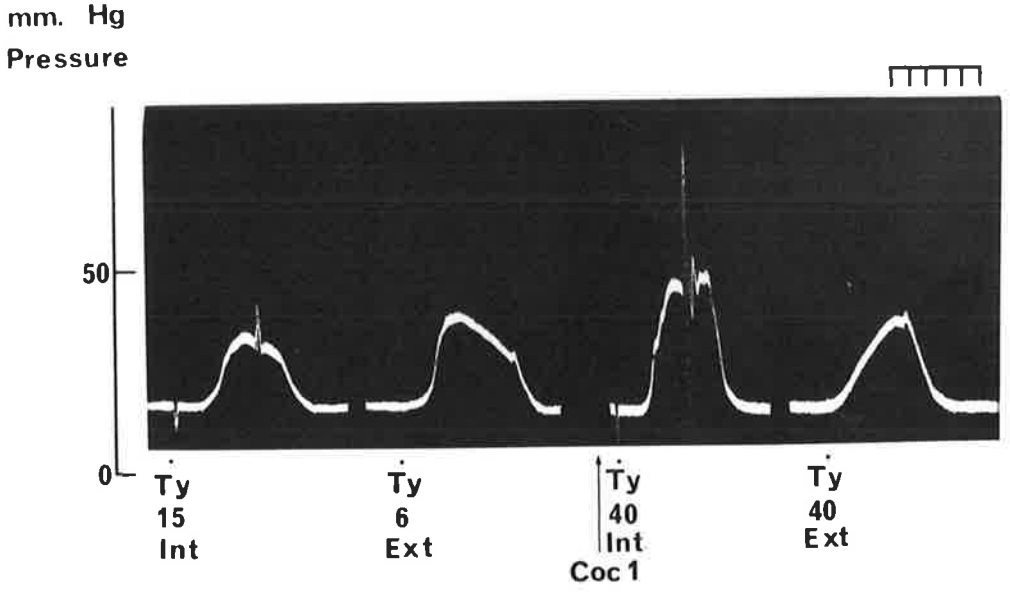
In four arteries cocaine 1 µg/ml was added prior to tyramine, or during the sustained constrictor response to tyramine. In each artery cocaine greatly reduced or abolished responses to extraluminal tyramine, but had either a much smaller inhibitory effect, or no effect on those to intraluminal tyramine. The more marked effect on extraluminal tyramine occurred regardless of the route of application of cocaine (figure 5.2).

In two arteries, the responses to extraluminal and intraluminal tyramine were measured in the presence of cocaine 1 µg/ml. The sensitivity to tyramine was greatly reduced, particularly to extraluminal tyramine. Typical responses before and after cocaine are shown in figure 5.6.

Effect of reserpine

Two rabbits were pretreated with reserpine, 2.5 mg/ml as described on page 3.18, and the effects of tyramine were observed on a reserpinised artery and a control unreserpined artery from the opposite ear. The effect of reserpine was to greatly reduce sensitivity to both intraluminal and extraluminal tyramine, the effect being more marked for extraluminal tyramine. The tyramine induced constriction in both control

Figure 5.6



Showing responses to tyramine before and after cocaine 1 µg/ml applied intraluminally and extraluminally at the point indicated by the arrow.

Figures indicate µg/ml of tyramine.

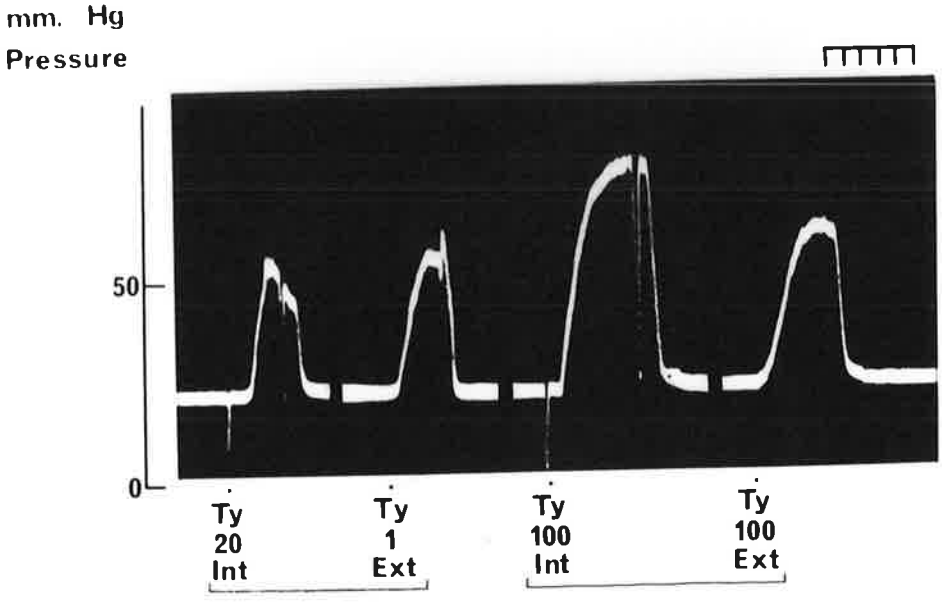
Time trace in minutes.

and reserpinised arteries is compared in figure 5.7.

Response to injected tyramine

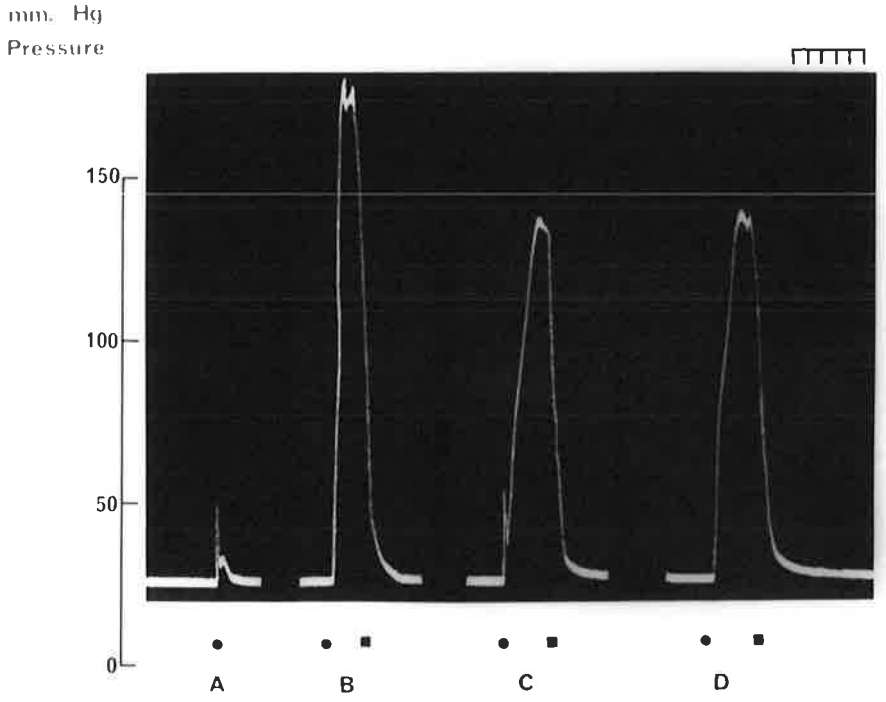
Farmer (1966) presented evidence that the response of the ear artery to an injection of tyramine comprised an immediate transient constriction due to a direct effect of the drug on arterial smooth muscle, followed by a prominent secondary constriction which was mediated by the release of noradrenaline (indirect effect). Under the conditions employed by Farmer, drug escapes into the extraluminal fluid following its passage through the lumen of the artery. In view of the high sensitivity of the artery to extraluminal tyramine observed in the present study, it seemed likely that the secondary constriction observed by Farmer was due to the response of the artery to the tyramine which had escaped into the extraluminal fluid. The following observations indicated that this was the case. In each of three arteries which were double cannulated to avoid mixing of intraluminal and extraluminal fluids the response to an intraluminal injection of tyramine was diphasic, the main feature being the initial transient constriction. This was extremely rapid in its onset and in its rate of decline from the peak value, and was followed by a small, slower constriction (secondary response shown in figure 5.8a). The same amount of tyramine added to the extraluminal fluid caused sustained constriction which was much greater in magnitude (figure 5.8b). After washout of tyramine the

Figure 5.7



Comparison of intraluminal and extraluminal tyramine in a normal artery (2 left-hand responses) and the opposite artery after reserpine treatment (2 right-hand responses). The figures indicate µg/ml of tyramine.
Time trace in minutes.

Figure 5.8



Effect of tyramine in an artery where mixing of intraluminal and extraluminal fluid was prevented (A and B) and permitted (C and D).

A & C Injection of 100 µg of tyramine

B & D Addition of 100 µg of tyramine to the extraluminal fluid.

Time trace in minutes.

outflow of the intraluminal perfusion fluid was returned to the extraluminal fluid by appropriate adjustment of the outflow cannula or by removing the latter cannula entirely. An intraluminal injection of tyramine now caused a diphasic response in which the rapid transient constriction was little different in magnitude to that previously obtained (figure 5.8c). Finally, extraluminal addition of the same amount of tyramine produced a response (figure 5.8d) which did not possess an initial transient phase but was otherwise identical in magnitude and time course with the marked secondary response previously produced by intraluminal tyramine in figure 5.8c.

DISCUSSION

The results indicate that the constrictor potency of tyramine is influenced by its route of application to the artery. Thus extraluminal tyramine is considerably more potent than intraluminal tyramine and is highly sensitive to the effects of cocaine and of denervation. These findings are consistent with an assumption that the noradrenergic storage sites play a major role in the constrictor response to extraluminal tyramine. This assumption also receives support from Farmer's earlier evidence, based on the effects of cocaine and denervation, that the secondary phase of the constrictor response to an intraluminal injection of tyramine is indirectly mediated (Farmer, 1966). It has been shown that the secondary phase is greatly reduced when escape of tyramine into the extraluminal fluid is prevented and that the secondary response is reproduced by the same quantity of tyramine added directly to the extraluminal fluid i.e. the "secondary" phase is largely the response to extraluminal tyramine. The quantitative importance of the action of tyramine on the storage sites is indicated by the findings that denervation was associated with a reduction in sensitivity to extraluminal tyramine of the order of tenfold or greater (table 5.2). The residual constrictor activity in the denervated artery presumably represents the direct effect of tyramine i.e. constriction mediated by receptors in the smooth muscle.

It is of interest that the denervated arteries displayed little difference in their sensitivity to intraluminal and extraluminal tyramine. Since intraluminal tyramine was less sensitive to the effects of denervation and cocaine than extraluminal tyramine it seems likely that the direct effects are of greater quantitative importance in the constrictor action of intraluminal tyramine. However, the most significant feature of the observations on intraluminal tyramine is that its potency was consistently less than that of extraluminal tyramine. This implies that the concentration which tyramine achieves in the region of the noradrenergic storage sites i.e. in the medial-adventitial border, depends on the particular surface of the artery to which the tyramine is applied. Since the sensitivity ratios ranged up to values of 50, it would appear that the gradient in concentration of tyramine between the lumen and the medial-adventitial border may be up to 50 fold greater than that which exists between the adventitial surface and the medial-adventitial border.

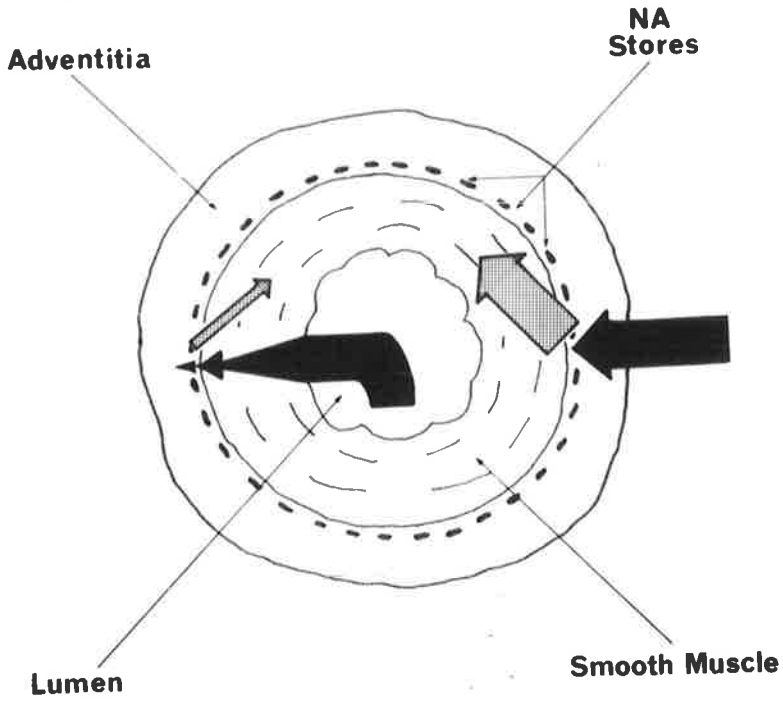
A possible explanation for this difference is that as the tyramine diffuses across the artery from the lumen and approaches the outer border of the media, it is diluted by the tyramine-free solution bathing the adventitia. Such a diluting effect must inevitably occur at some point between the surface of the artery to which the drug is applied and the opposite surface. Other factors contributing to the low activity of intraluminal tyramine may be the presence of permeability or enzymic

barriers. The presence of a permeability barrier seems unlikely for several reasons. In the denervated arteries there is little difference between the potencies of intraluminal and extraluminal tyramine, suggesting that the drug attains the same concentration in the media of the denervated artery irrespective of the surface to which it is applied. It has been shown earlier (page 3.25) that denervation has the same effect on the action of noradrenaline i.e. it tends to eliminate the effect of the route of application to the artery on constrictor potency, again pointing to the probability that when the influence of the nerve terminals is eliminated, drugs diffuse equally well from either surface into the media. It is noteworthy also that, when applied intraluminally, cocaine potentiates the constrictor action of extraluminal noradrenaline to approximately the same extent as does extraluminal cocaine, and the rapid onset of action of intraluminal cocaine is consistent with its rapid penetration from the intima to the noradrenaline storage sites. However, the possibility that an enzymic barrier exists between the lumen and the adventitia cannot be excluded. It has been demonstrated recently that extra-neuronal monoamine oxidase is present throughout the media of the ear artery (de la Lande and Jellett, unpublished) i.e. monoamine oxidase occurs in the region where it may be expected to exert a major influence on the amount of tyramine reaching the medial-adventitial border from the lumen of the vessel. Current studies in this department

on the interaction between tyramine and monoamine oxidase inhibitors may shed further light on this possibility. In figure 5.9 this hypothetical action of tyramine is presented diagrammatically.

The relation between these findings and those of Luduena (1963) on the whole ear is not clear. Luduena found little difference between the constrictor activity of tyramine in rabbit ears before and after reserpine perfusion, or in ears from control and reserpine pretreated rabbits and concluded that the constrictor activity is direct. However, the ear is a complete vascular bed, and it is possible that areas beyond the central artery are more sensitive to the direct effect of tyramine. It is shown in the next chapter (page 6.4) that the sensitivity of the whole ear and that of the central artery may differ markedly to vasoconstrictor drugs. Furthermore, Luduena used single injections of tyramine, and it is possible that under these conditions very little tyramine escaped into the extracellular space to act on the noradrenergic storage structures. Hence it is possible that the response used by Luduena as a measure of tyramine's action corresponded more to that elicited by intraluminal perfusion in the present study.

Figure 5.9



Diagrammatic representation of the indirect (noradrenaline releasing) action of tyramine. The black arrows represent tyramine; the shaded arrows represent noradrenaline released from neural storage sites (NA stores) by tyramine. The direction of the arrows indicates the direction of diffusion of the amines, and the thickness of the arrows indicates concentrations of the amines. The diagram indicates that intraluminal tyramine is reduced in concentration as it diffuses through the artery wall, releasing less noradrenaline than the extraluminal tyramine, which has not been subjected to diffusion through the artery wall.

SUMMARY

1. The vasoconstrictor potency of extraluminal tyramine on the isolated perfused ear artery is approximately ten times that of intraluminal tyramine.

2. Chronic denervation, which caused the noradrenergic storage structures in the medial-adventitial border of the artery to disappear, greatly reduced the potency of extraluminal tyramine so that it approached that to intraluminal tyramine. The latter was relatively little affected by denervation.

3. The effects of denervation indicate that the action of intraluminal tyramine may be largely directly mediated, and that of extraluminal tyramine indirectly mediated. This conclusion is supported by analysis of the diphasic response of the artery to intraluminal injection of tyramine under perfusion conditions which permit intraluminal fluid to mix with the extraluminal fluid. Evidence is presented that the first phase of the response is mediated by intraluminal tyramine, and the second phase by extraluminal tyramine.

4. The results are interpreted in the light of an hypothesis (page 3.29) that the position of the noradrenergic storage sites in the adventitia of the artery plays a major role in determining the relative intra- and extra-luminal sensitivities of the artery to sympathomimetic drugs.

CHAPTER 6

COMPARISONS OF THE ISOLATED EAR AND THE ISOLATED CENTRAL ARTERY

INTRODUCTION

The high sensitivity to noradrenaline of the central artery of the rabbit ear has been shown earlier in this study (page 3.19). As the isolated rabbit ear is usually perfused through its cannulated central artery (page 3.7), the possibility exists that constriction of the artery alone is responsible for many of the responses observed during perfusion of the whole ear. However, the rabbit ear is a complex vascular bed, and the many smaller resistance vessels could also be involved in vasoconstrictor responses to drugs. To test this possibility, the vasoconstrictor potencies of noradrenaline, histamine, and angiotensin were compared by testing responses in the isolated perfused ear, the proximal one-third of the ear, and the isolated central artery.

Constrictor responses to drugs were qualitatively similar in the whole ear and in the perfused artery, and no further description is given in this chapter of the characteristics of responses to drugs which have been considered earlier in the thesis.

MATERIALS AND METHODS

The methods used were those described for the perfusion of the whole rabbit ear (page 3.7), and both methods described for perfusion of the isolated artery (pages 3.9 and 3.10).

Comparisons between the ear and the artery were made in two ways. In the first method, the sensitivity of one ear was compared with that of the artery from the opposite ear. To allow for spontaneous changes in sensitivity with time, comparisons were always based on responses measured at the same time on the two preparations. The relative sensitivities of two preparations to the vasoconstrictor substance was expressed as the inverse ratio of the concentrations of the latter which gave responses of equal magnitude. For example, a sensitivity ratio of 10 for $\frac{\text{artery}}{\text{ear}}$ means that a tenfold greater concentration of the drug was required in the ear to achieve constriction of the same magnitude as that observed in the artery. Since the dose response curves of the two preparations to a vasoconstrictor substance were not always parallel, measurements were usually restricted to responses which were between 10 and 100 mm Hg increases in perfusion pressure. In the second method, the sensitivity of the perfused ear to the vasoconstrictor under investigation was recorded, after which the distal two-thirds of the ear were cut off. The response of the remaining part

of the ear (proximal one-third) which was still being perfused, was then recorded. Finally, the artery from the proximal one-third of the ear was excised, perfused by the method described above, and its sensitivity recorded.

In several experiments, serotonin (20 ng/ml) was present in the perfusion fluid in order to increase the sensitivity of the ear and the artery to the vasoconstrictor under investigation (de la Lande, Cannell and Waterson, 1966).

RESULTS

Noradrenaline

The relative sensitivities of the ear and the artery to noradrenaline are summarised in table 6.1. The ear was less sensitive than the artery in four experiments, and more sensitive in four experiments. However, in seven of the comparisons, the difference between the sensitivities was minor, the ratio of the two ranging between 0.5 and 1.6. The wide range of the ratio in experiment 7 (.07-1.6) occurred because the sensitivity of the artery increased spontaneously to a marked degree during the comparison. The effect of reducing the size of the vascular bed by two-thirds ranged between approximately 50% reduction in sensitivity (two experiments), and slight enhancement of sensitivity (two experiments). Removal of the rest of the ear so that the artery alone was perfused caused no further change in sensitivity in two arteries and a further reduction (which was, however, less than 50%) in another artery.

Histamine

Histamine was more active on the artery in four experiments, and less active in two (table 6.2). Removal of two-thirds of the ear, followed by removal of the rest of the ear caused in each instance a small reduction in sensitivity.

TABLE 6.1

SENSITIVITIES OF ARTERY AND EAR TO NORADRENALINE

Expt. No.	1-6	7	8	9	10	16
Opp. artery / ear	1-1.4, 0.6-0.8 0.6-1.0, 1.5-1.6 7-7.9, 1-1.6	0.07-1.6	0.5-0.9			0.3
Cut ear / ear	Not measured	0.47-0.52	1.2-1.6*	0.4-0.5	0.9-1.5*	
Artery / cut ear			0.8-1.2*	0.5-0.8	1.0*	
Artery / ear						0.7-0.8

* 5HT, 20 ng/ml present

NOTE: The sensitivity ratios in this and tables 6.2 and 6.3 are expressed as a range to include departures from parallelism of the two dose response curves under comparison (see methods). The lower value corresponds to the closest distance apart of the two curves, and the higher value to the furthest distance apart.

TABLE 6.2

SENSITIVITIES OF ARTERY AND EAR TO HISTAMINE

	Experiment No.					
Sensitivity Ratio	2	3	4	5	11	12
Opp. artery / ear	2.5	0.7-1.0*	4.6	2.1-3.7	0.45-0.56	2.8-4.5
Cut ear / ear					0.64	0.4-0.56
Cut ear / artery					0.8-0.95	0.68-0.8

* 5HT, 20 ng/ml present

Angiotensin

The responses of the ear and of the artery to injections of angiotensin were qualitatively similar to those following noradrenaline and histamine. They comprised a sudden rise in perfusion pressure, which rapidly reached a peak, and quickly returned to the resting perfusion pressure. Both the ear and the artery displayed tachyphylaxis to angiotensin. Tachyphylaxis was particularly marked on the artery. For this reason, dose-response curves which permitted estimation of sensitivity ratios were difficult to obtain. Nevertheless, angiotensin always caused greater constriction of the ear than of the artery when applied in the same dose for the first time, and by subsequently reducing the dose applied to the ear, it was possible to establish that the maximum value of the sensitivity ratio $\frac{\text{opposite artery}}{\text{ear}}$ was much less than that prevailing with noradrenaline and histamine. The greater activity of angiotensin in the ear was particularly marked in experiment 13, where the opposite artery failed to respond to angiotensin in all doses tested; the latter were up to 100 times those which caused definite constriction of the ear. Removal of the major portion of the ear also caused a dramatic reduction in sensitivity to angiotensin; there was no further reduction when the artery alone was excised and perfused. The results are summarised in table 6.3.

TABLE 6.3

SENSITIVITIES OF ARTERY AND EAR TO ANGIOTENSIN

	Experiment No.						
Sensitivity Ratio	2	4	5	13	14	15	16
Opp. artery / ear	<0.2	<0.25	<0.25	<0.01	0.07	<0.02	0.1-0.14
Cut ear / ear				<0.01	0.12	0.04-0.05	
Artery / cut ear					0.12		
Artery / ear				0.15-0.24			0.07-0.24

DISCUSSION

The main feature of the results is that the magnitude of the increases in resistance to flow through the ear caused by histamine and noradrenaline are affected to a surprisingly small extent when most of the vascular bed is removed so that only portion of the central artery remains. In the case of histamine, these observations may be interpreted in terms of its ability to constrict the major artery of the ear while at the same time dilating the smaller vessels (Feldberg, 1927; Armin and Grant, 1953). Hence the response of the ear to histamine is a balance between the dilator and constrictor components, and removal of the major part of the vascular bed containing the smaller vessels may be expected to enhance the constrictor component. This tendency was observed in some, but not all of the experiments. However, it may be noted that the observations of Feldberg, and Armin and Grant, were made in the intact ear *in vivo*, and the present experiments were carried on *in vitro* preparations perfused with artificial media i.e. under conditions where blood vessel tone is low and where it is difficult to demonstrate dilator components of drug action. Hence the significant feature of the findings on histamine is to emphasise the major role which the central artery alone plays in the vasoconstrictor response of the whole ear to histamine. A similar argument may be applied to noradrenaline, although a search of the literature failed to reveal an account of its actions on

the small and large vessels of the ear in vivo. Nevertheless, it has been shown that adrenaline constricts small arteries including arterioles in the rabbit ear in vivo (Abell and Page, 1942) as well as the central artery (Armin and Grant, 1953), and there is no reason to suppose that noradrenaline's constrictor action is less extensive than that of adrenaline, particularly in view of the well-documented evidence that the former constricts both large and small arteries including arterioles in the dog forelimb in vivo (Haddy, Fleishman and Emanuel, 1957; Haddy, Molnar, Borden and Texter, 1962). Hence the greater sensitivity of the ear to noradrenaline observed in some of the experiments may reflect an added component arising from constriction of small resistance vessels including arterioles. However, the main conclusion to be drawn from the comparisons between ear and artery is that, as in the case of histamine, constriction of the central artery is a major factor underlying the constrictor response of the whole ear to noradrenaline.

The observation that angiotensin is far less active on the artery than on the ear highlights the probable location of its constrictor action on small resistance vessels beyond the central artery. This is in accord with the observations of Abell and Page (1942) that angiotensin constricts arterioles in the blood perfused ear. Although these authors do not comment on comparative changes in the central artery it has been shown that unlike the action of noradrenaline, angiotensin's

action on the dog forelimb vessels is localised to the small arteries (less than 50 microns in diameter) and arterioles (Haddy, Fleishman, and Emanuel, 1957).

The above considerations suggest that ear-artery comparisons provide a useful and simple guide to the major sites of action of vasoconstrictor drugs in the ear. The results of the comparisons also have implications to the mechanism of blood flow control in the ear, as well as to the application of the ear and its vessels to the bioassay of constrictor agents. Thus it would seem likely that the rapid and large shifts in blood flow which are desirable in an organ whose primary function is that of temperature regulation are mediated to a large extent by changes in diameter of the central artery. The dense sympathetic innervation of the artery (page 2.30) and the high sensitivity of the artery to sympathetic nerve stimulation (de la Lande and Rand, 1965) are in accord with such a role of the central artery. The implications of the findings to bioassay are that, despite a tendency to be less sensitive to noradrenaline, the isolated artery is probably more useful for bioassay of noradrenaline since it is subject to less interference by angiotensin than is the whole ear. On the other hand, the whole ear, or preparations of its smaller resistance vessels may provide a better test object for the bioassay of angiotensin and other polypeptides than the isolated artery, and it is noteworthy that the whole ear, but not

the central artery, has proved to be an extremely sensitive test object for the bioassay of vasoactive proteinaceous components of the venom of Myrmecia pyriformis (Lewis and de la Lande, 1967).

SUMMARY

1. The vasoconstrictor potency of angiotensin was greatly reduced by sectioning the rabbit ear so that only the proximal portion remained, or by removal of the entire vasculature other than a small segment of the central artery immediately distal to the site of cannulation.

2. The above procedure had much less effect on the vasoconstrictor potencies of noradrenaline and histamine.

3. The findings indicate that angiotensin's action on the ear vessels is mediated largely beyond the larger resistance vessels such as the central artery, whereas noradrenaline and histamine constrict both large and small resistance vessels.

4. It is concluded that comparison of drug effects on the whole ear, and on the central artery alone provides a useful guide to the sites of action of these drugs on the ear vasculature.

CHAPTER 7

VASOCONSTRICTION BY BARBITURATES

INTRODUCTION

The introduction of thiopentone as an agent for rapid induction of general anaesthesia was followed by many reports of tissue damage after intra-arterial injection, although the incidence of serious tissue damage diminished rapidly when less concentrated solutions of thiopentone came into common use. Burn and Hobbs (1959) observed the constrictor effect of thiopentone on spiral strips of rabbit aorta and on the blood vessels of the rabbit ear perfused by a constant pressure technique. This constrictor effect on blood vessels was unexpected, as thiopentone was known to cause a fall in blood pressure in the intact animal. The investigation showed that the constriction was not due to the alkaline pH of the solution, as a buffered solution adjusted to the same pH as the thiopentone did not cause vasoconstriction. Burn and Hobbs suspected that released noradrenaline may have caused the constriction and therefore investigated the effects of cocaine, which was known to potentiate noradrenaline, on thiopentone vasoconstriction. Cocaine in a concentration of 5 µg/ml increased the constrictor activity of thiopentone, as well as that of noradrenaline. The results were viewed as consistent

with an hypothesis that thiopentone caused constriction by an indirect action in liberating noradrenaline from stores in or near the blood vessel walls. Further evidence that noradrenaline was involved was obtained from experiments in which tolazoline antagonised the constrictor action of noradrenaline and of thiopentone. In the reserpinised ear noradrenaline was found to be constrictor, but in this case thiopentone produced not constriction but dilatation. These observations also supported the view that thiopentone caused constriction by releasing noradrenaline. Burn and Hobbs also found that hexobarbitone, in contrast to thiopentone, failed to cause constriction except in very high concentrations.

Kinmonth and Shepherd (1959) were interested in the vascular damage caused by thiopentone, and studied the pathology and treatment of the sequelae of the injection of thiopentone into arteries. They used the rabbit ear in vivo for intra-arterial injections, observing the artery during injection through a dissecting microscope. The vessels constricted soon after the injection but returned rapidly to the original state, the thiopentone effect concluding with a brief dilatation. However, at no time did Kinmonth and Shepherd observe any resemblance to the spasm which follows mechanical trauma. They found that injections of buffered alkaline solution (pH 10.9) produced no change in the arterial diameter. Because the period of vasoconstriction was so short-

lived, Kinmonth and Shepherd postulated that the occurrence of necrosis could not be related to the immediate constrictor effects of thiopentone. They also produced necrosis in the rabbit ear by injections in vivo, measuring the area of necrosis which resulted, and employing a number of counter measures to modify the necrotic area. It was found that prior heparin treatment and sympathetic denervation both reduced the area of gangrene following thiopentone injection. Kinmonth and Shepherd concluded that there was no evidence that prolonged arterial spasm contributed to the incidence of necrosis, but that direct vascular damage from thiopentone was responsible for the later development of ischaemia. They also concluded that the injury was not due to the alkalinity of the thiopentone.

Burn and McDougal (1961) investigated the effects of thiopentone injected subcutaneously into the tail of the mouse. Gangrene frequently resulted, its occurrence being reduced by pre-treating the mice with noradrenaline-depleting doses of reserpine. Burn and McDougal considered that the period of thiopentone vasoconstriction in the experiments of Burn and Hobbs, and of Kinmonth and Shepherd was too brief to result in gangrene. Also, in the mouse tail study the thiopentone concentrations were 80 times greater than those used by Burn and Hobbs, and the thiopentone was given subcutaneously and was probably not subject to the rapid dilution and removal from the tissues likely to occur

with intravascular administration.

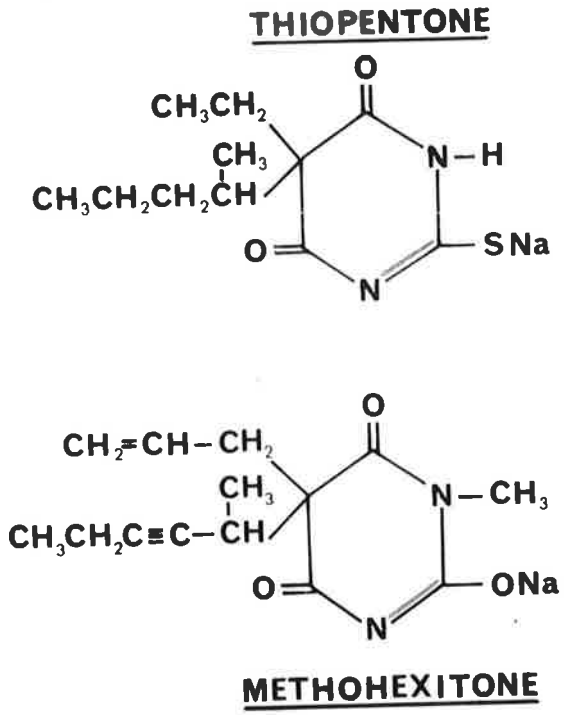
Burn (1960) reviewed the available evidence and concluded that thiopentone did in fact cause gangrene by local release of noradrenaline from artery walls. His evidence for this was that when noradrenaline was either reduced in its effect or potentiated then the thiopentone effect was correspondingly reduced or potentiated.

Stone and Donnelly (1961) further reviewed the available information about thiopentone necrosis and concluded that the vasoconstriction itself was not responsible for the gangrene associated with thiopentone damage, but that changes in the blood vessel wall with subsequent thrombosis caused tissue destruction.

Methohexitone (see figure 7.1), a drug having similar clinical uses to thiopentone was compared with thiopentone by Francis (1964), and Mather and Goodhead (1966) who produced necrosis in the rabbit ear with equivalent doses and concentrations of the two drugs. It was not known whether methohexitone was also vasoconstrictor, and the present study was commenced in order to provide a precise comparison of the relative constrictor activity of this drug and thiopentone. Therefore, two aspects of barbiturate vasoconstriction were investigated:

A study of the mechanism of thiopentone vasoconstriction.

Figure 7.1



Formulae of thiopentone and of methohexitone.

A comparison of the vasoconstrictor effects of thiopentone and methohexitone.

The opportunity was taken of exploring further the mechanism of the vasoconstrictor action of thiopentone proposed by Burn (1960). Apart from clinical implications of this action of thiopentone on sympathetic nerve endings, the possibility existed that analysis of thiopentone's constrictor action would reveal further information on noradrenaline binding and release in the artery wall.

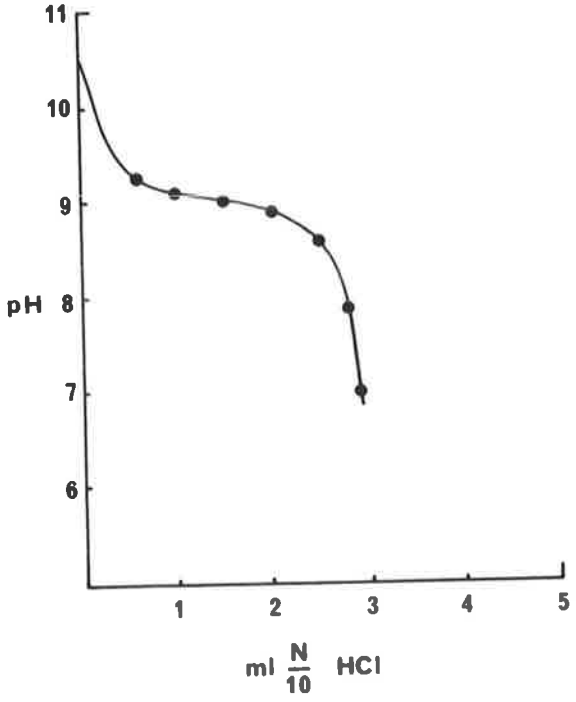
MATERIALS AND METHODS

The effects of barbiturates were studied on the perfused ear, and the isolated perfused central artery. The preparations were perfused as described on pages 3.7 to 3.10. The sensitivity of the blood vessels was tested by repeated application of noradrenaline, and by stimulation of the sympathetic nerves in the artery wall using peri-arterial electrodes as described on page 3.14.

Drugs

Solutions of thiopentone were prepared by dilution of the solid with distilled water to an initial strength of 100 mg/ml. Further dilutions were made with isotonic NaCl solution. Both the solid and the distilled water were in the standard ampoules provided by May and Baker for therapeutic use. It was established in preliminary experiments that solution strengths in the range of 1 to 10 mg/ml were required to produce vasoconstrictor responses by injection in the perfused rabbit ear. These solutions had a high pH, and in the case of the 10 mg/ml solution were up to pH 10.4. Furthermore, they possessed considerable buffer capacity. The buffer capacity is shown in figure 7.2, and was a matter of concern, as it meant that such a solution when injected into the perfusion stream of Krebs bicarbonate solution, would retain its alkalinity while passing through the artery. Hence, it became important to use as a control a solution which not only possessed the same pH as

Figure 7.2



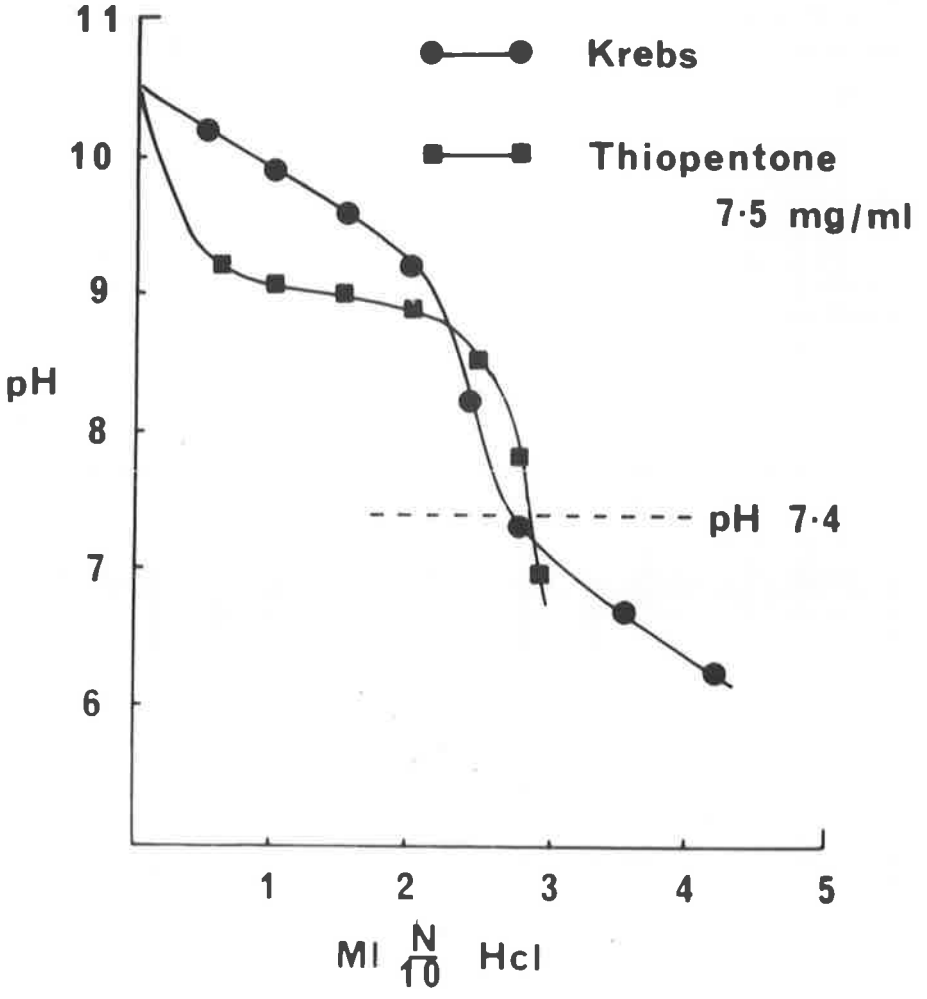
Buffer curve for thiopentone 7.5 mg/ml, commencing at p^H 10.4.

that of the thiopentone, but also equivalent buffer capacity. Krebs bicarbonate solution which had been adjusted to pH 10.4 with NaOH, was selected on the basis that the same amount of HCl was required to reduce its pH to 7.4 as in the case of a solution of thiopentone of 7.5 mg/ml. The buffer curves are shown in figure 7.3. However, there was some doubt that the full buffer capacity of thiopentone was measured by this procedure of acid titration, as the thiopentone began to precipitate as the pH was reduced. For this reason the constrictor action of prolonged perfusions of thiopentone (and methohexitone) was also measured. In these experiments the drug was added to the perfusion reservoir. The pH of the solution in the reservoir was measured, and a control perfusion was carried out with Krebs bicarbonate solution adjusted to the same pH. This procedure avoided the problem of the influence of the buffer capacity on the pH of the solution perfusing the ear.

In a number of experiments serotonin creatinine sulphate 20 µg/litre was maintained in the Krebs solution to sensitise the ear to the vasoconstrictor action of thiopentone. This enabled the concentration of the drug to be reduced to a level which still gave constriction, but which reduced the changes in pH produced by thiopentone in the perfusion fluid. These changes were now of the order of 0.1 of a pH unit.

To examine the role of noradrenaline in thiopentone's vasoconstrictor action, the effect of thiopentone was examined under

Figure 7.3



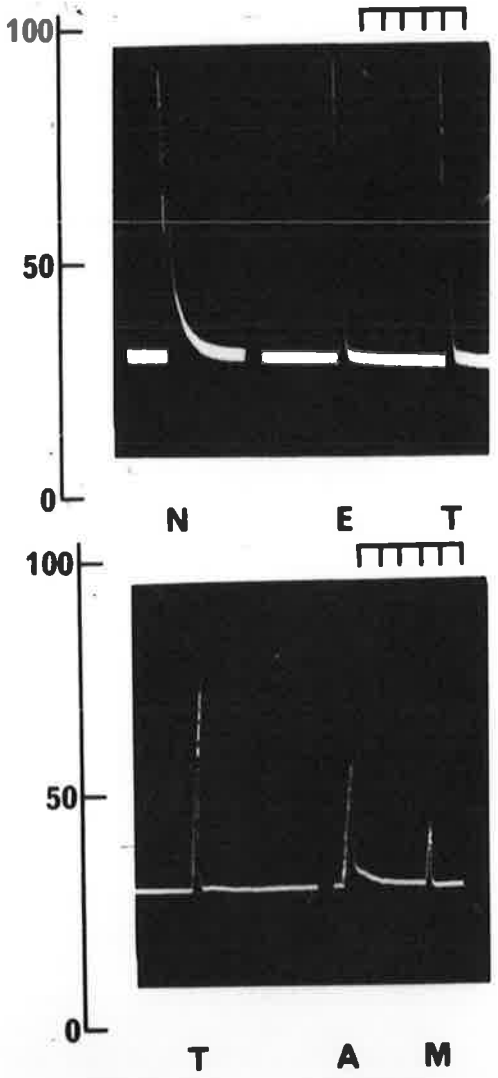
Comparison of the buffer curves for thiopentone 7.5 mg/ml (square symbols) and alkaline Krebs solution (round symbols) both commencing at pH 10.4. Approximately the same volume of $\frac{N}{10}$ HCl is required to reduce the pH of the two solutions to pH 7.4.

conditions which are known to modify noradrenaline activity, nerve activity, or both.

RESULTS

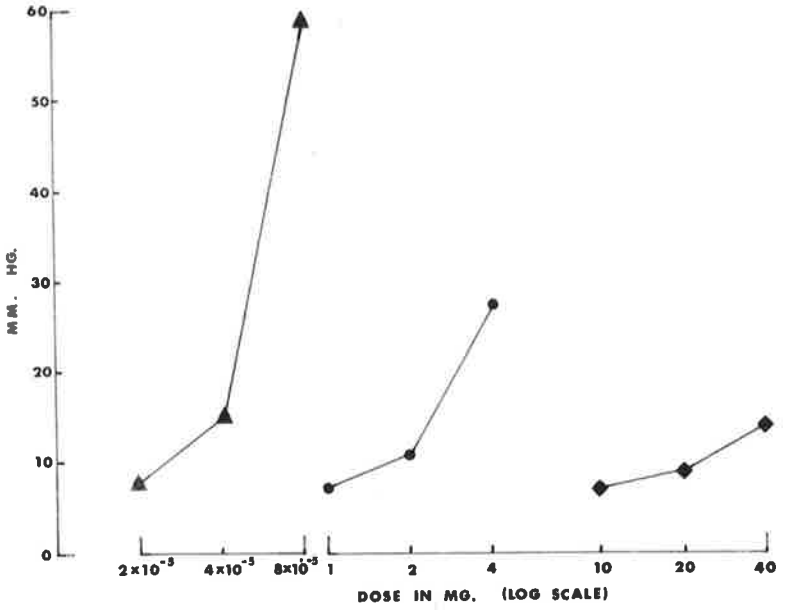
The responses to thiopentone and methohexitone injected into the fluid perfusing the ear were essentially similar, and comprised a rapid but quite transient rise in perfusion pressure. The return to the resting pressure occurred within 30 to 60 seconds. The responses were similar to those caused by noradrenaline (by injection), brief (10 seconds) peri-arterial application of pulses, and injections of buffered alkaline solutions. Examples of each type of response are shown in figure 7.4. Following the return to resting pressure after each of the barbiturates there was a phase of depressed excitability lasting from 10-20 minutes as judged by depression of the constrictor responses to noradrenaline. A depression was not observed after alkali, electrical stimulation or after noradrenaline itself. Responses to noradrenaline and thiopentone were reproducible and were dose-dependent. However, the dose response curve to thiopentone was generally less steep than that to noradrenaline while constrictor responses to methohexitone showed little change with dosage. Comparison of the three curves on an ear which was typical of those observed in 10 experiments, is shown in figure 7.5. The doses of the drugs giving comparable constriction in the experiment shown in figure 7.5, were noradrenaline 0.02 µg, thiopentone 10^{-3} µg, and methohexitone 10^{-4} µg, and this relative order of activity was observed in each of 6 experiments. The data from the latter are summarised in

Figure 7.4



Typical responses to noradrenaline 40 ng (N) electrical stimulation 30 volts (E) thiopentone 4 mg (T in upper block) 0.75 (T in lower block) buffered alkali (A) and methohexitone 0.75 mg (M). The solutions were all injected and were at pH 10.3.
Time trace in minutes. Pressure mm mercury.

Figure 7.5



Dose response curves to noradrenaline (triangular symbol), thiopentone (circular symbol), and methohexitone (diamond shaped symbol), in the perfused rabbit ear.

table 7.1, where the doses of thiopentone and methohexitone giving increases in perfusion pressure in the range of 9-35 mm of mercury are shown. The magnitude of the response of the ear vessels to an injection of methohexitone was either of the same order or less than the response of the ear to an injection of Krebs solution in the same volume, and at the same pH. The response to thiopentone was in most experiments greater than that to the alkaline solution. Hence it was assumed that most if not all the constrictor response to methohexitone was due to its inherent alkalinity and buffer capacity, whereas thiopentone possessed a true constrictor action. This assumption was tested by comparing the effects of perfusions of methohexitone and thiopentone in ears which were sensitised with serotonin (described in methods, page 7.7).

Methohexitone produced only vasodilatation under these conditions (figure 7.6), whereas perfusion with thiopentone produced a diphasic response which comprised an initial transient but definite constriction which was quickly followed by prolonged dilatation for the duration of the perfusion (figure 7.6). The concentrations of the two barbiturates used in these experiments increased the pH by less than 0.2 units. Increasing the pH of the Krebs solution perfusing the artery by the same amount produced either no detectable change, or a slight and slowly developing constriction. It is of interest that the dilator response in the arteries perfused by thiopentone and methohexitone persisted after

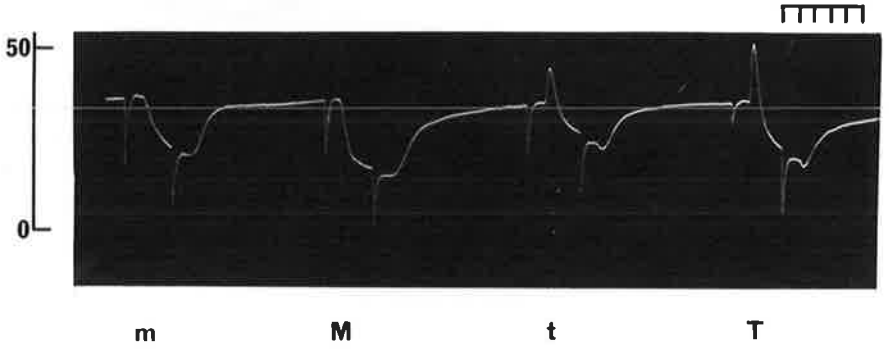
TABLE 7.1

Relative activity of thiopentone and methohexitone

Expt. No.	Thiopentone		Methohexitone		Activity of thiopentone relative to methohexitone
	Dose mg	Response	Dose mg	Response*	
1	0.5-2	9-27	4	8	More than 8
2	1-2	28-35	4	27	" " 4
3	1-4	17-27	4	12	" " 4
4	1-2	27-35	10	17	" " 10
5	1-4	6-26	10	6	" " 10
6	2-6	20-33	6	16	" " 3

* The response to methohexitone quoted for each experiment is the maximum observed during the period of comparison with thiopentone.

Figure 7.6



Responses of a rabbit ear to three minute perfusions of methohexitone 100 μg/ml (m) and 200 μg/ml (M), and thiopentone 100 μg/ml (t) and 200 μg/ml (T). The descending lines indicate the beginning and end of each perfusion.

Time trace in minutes.

Pressure mm mercury.

termination of the perfusion, and corresponded in time with the phase of depressed excitability after injection of the drugs. Since normally the vascular tone is extremely low in the perfused ear, it is likely that dilatation was more apparent because the tone of the vessels in the ear had been increased by serotonin.

Mechanism of thiopentone's constrictor action: role of noradrenaline

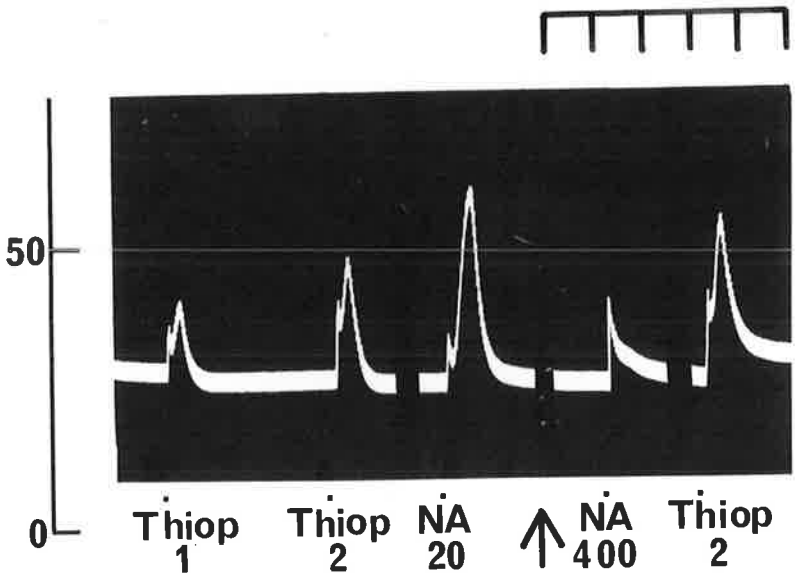
1. Phenoxybenzamine

The constrictor responses to both injected and perfused thiopentone were compared in 7 ears before and after the injection of phenoxybenzamine in amounts ranging from 1 to 100 µg. In no case was the constrictor response to thiopentone reduced, despite the fact that phenoxybenzamine reduced the sensitivity to noradrenaline up to 10,000 fold. An example of the lack of effect of phenoxybenzamine on thiopentone's action is shown in figure 7.7.

2. Reserpine

The effects of thiopentone were examined in ears from 18 rabbits which had previously received reserpine to deplete their tissues of noradrenaline. The doses of reserpine ranged from 0.1 mg/kg daily for 10 days (3 rabbits) to 5 mg/kg 24 hours before use (10 rabbits). Methods of reserpine pretreatment are described on page 3.17.

Figure 7.7



Responses of a rabbit ear to injections of thiopentone in mg and noradrenaline in ng before and after an injection of 1 μg of phenoxybenzamine (marked by the arrow).

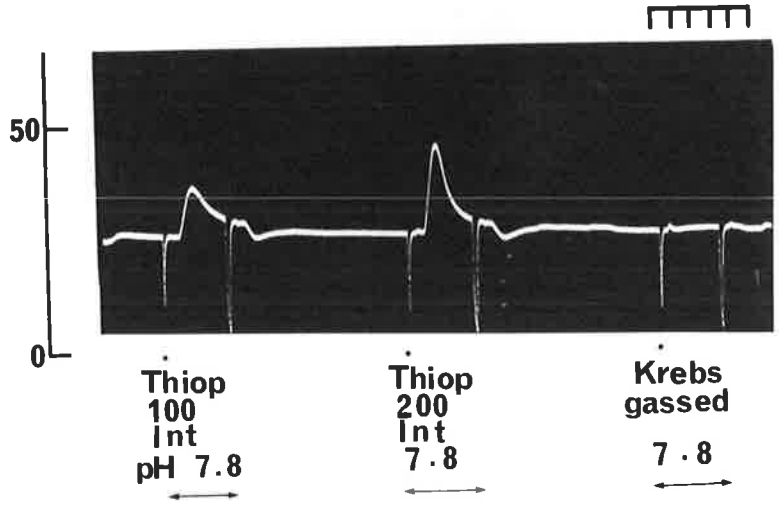
Time trace in minutes.

Pressure mm mercury.

The absence of response of the perfused ear to peri-arterial nerve stimulation was taken as evidence that reserpine had depleted the nerves of noradrenaline. The responses to injections and perfusions of thiopentone were qualitatively similar to those previously observed in normal ears (figure 7.8). There was no indication of a major quantitative change in sensitivity to injections of thiopentone. In the case of perfusions, the interpretation was complicated by the tendency of the perfusion pressure to slowly increase in an erratic fashion after the first few applications of the drug. However, it was significant that the characteristic constriction followed by dilatation always occurred in the early stages of perfusion.

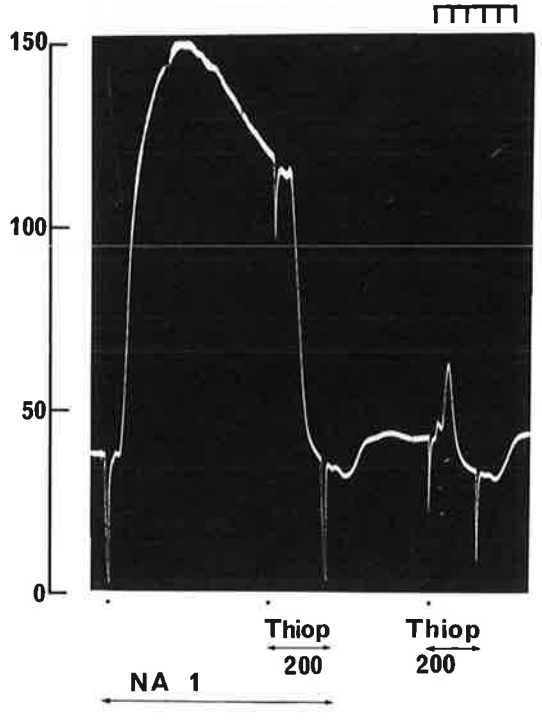
As perfusion continued and the resting perfusion pressure rose and became unsteady, the constrictor effect, but not the dilator effect of thiopentone was sometimes reduced in magnitude or absent. That this was simply a reflection of the raised perfusion pressure was indicated by an experiment in which the perfusion pressure of a normal ear was raised with noradrenaline, and the effects on thiopentone's constrictor action examined. The constrictor phase of the thiopentone action was no longer apparent, but the dilator phase was prominent (figure 7.9).

Figure 7.8



Responses of a rabbit ear to perfusions of thiopentone 100 µg/ml and 200 µg/ml and Krebs solution (all at pH 7.8). The rabbit was pretreated with reserpine 2.5 mg/ml intraperitoneally 24 hours previously.
Time trace in minutes.
Pressure scale mm mercury.

Figure 7.9



Infusions in the rabbit ear of thiopentone 200 µg/ml during and after the constrictor phase produced by noradrenaline 1 ng/ml.
Pressure scale mm Hg.
Time trace minutes.

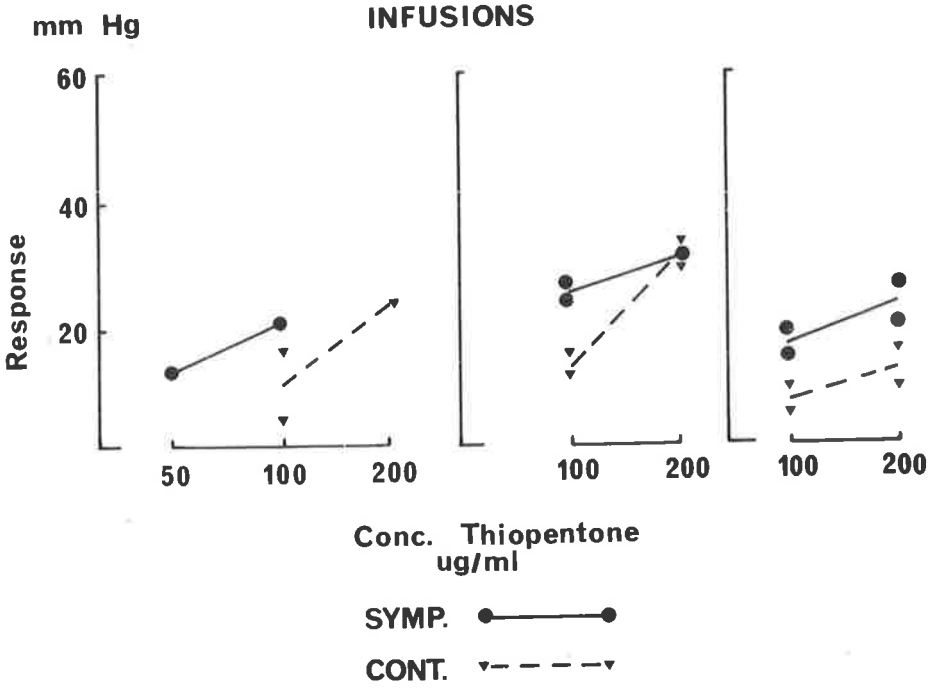
Sympathetic denervation

The effect of sympathetic denervation was examined in 10 rabbits in each of which one superior cervical ganglion was removed 1-3 weeks previously. The sympathectomised ears were perfused and in each case the opposite ear was used as a control. In each of the 10 rabbits the sympathectomised ear showed little or no response to peri-arterial stimulation compared with the control ear. Sensitivity to noradrenaline was enhanced in 5 of the sympathectomised ears compared with their controls. The constrictor response to thiopentone was of the same magnitude in the control ears as in each of the sympathectomised ears. Serotonin was present in the perfusion medium in 5 of the ears and was absent in 5. The results of three of the experiments are summarised in figure 7.10.

Cocaine

The effects of cocaine were examined in 4 ears. The responses to noradrenaline were increased in all of the ears, although the magnitude of the increase was small. Cocaine in concentrations of 1 µg/ml and 10 µg/ml caused enhancement of constrictor responses to peri-arterial stimulation. The constrictor responses to injections of strong solutions of thiopentone (10 mg/ml) or to weaker solutions (0.1 to 0.2 mg/ml in the presence of serotonin 20 µg/litre) were unchanged in three experiments, but slightly enhanced in a fourth experiment. In one experiment the transient constrictor response to infusions of thiopentone

Figure 7.10



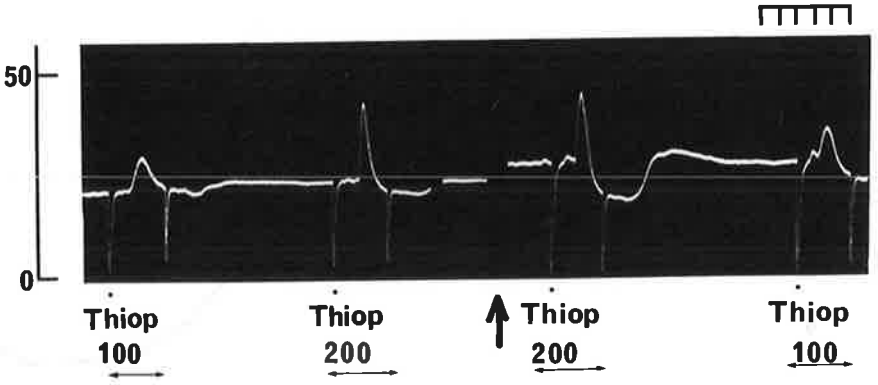
Comparison (for three experiments) of the dose-response curves for denervated (Symp.) and control (Cont.) artery for infusions of thiopentone. The figure indicate μg/ml thiopentone.

was unchanged in the presence of cocaine. Figure 7.11 illustrates the effect of cocaine on responses to thiopentone and to noradrenaline.

Studies on the artery

The isolated ear artery was perfused using the double cannulation method described on page 3.9. In three arteries the intraluminal infusion of thiopentone produced either no constriction, or constriction which was very much less than that observed in the whole ear. In each of four arteries the vessels constricted to thiopentone added to the extraluminal bathing solution. The magnitude of the constriction was reduced in the presence of cocaine 1 µg/ml, but the reduction was slight and equivocal (figure 7.12). The important finding was that in no case was constriction enhanced by cocaine, as had been reported by Burn and Hobbs (1959) in the whole ear.

Figure 7.11

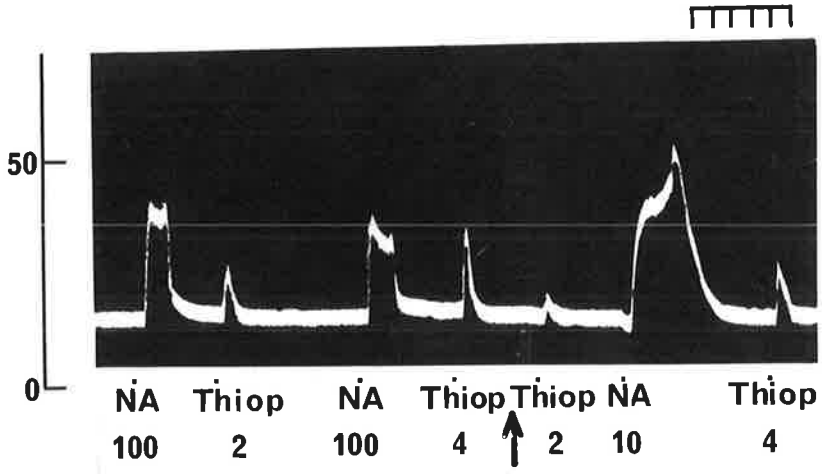


Responses to infusions of thiopentone in the rabbit ear before and after cocaine 5 µg/ml. The commencement of the cocaine infusion is indicated by the arrow. The figures represent µg/ml of thiopentone.

Pressure mmHg.

Time trace in minutes.

Figure 7.12



Responses in the double cannulated rabbit ear artery to noradrenaline (extraluminally) and thiopentone (by injection) before and during cocaine (intraluminally and extraluminally) 1 µg/ml (commencing at the arrow). Figures indicate ng/ml of noradrenaline and mg of thiopentone.
Pressure scale mm Hg.
Time trace minutes.

DISCUSSION

Comparison of the effects of thiopentone and methohexitone

The responses of the ear to thiopentone and methohexitone indicate that when given by injection both drugs produce a constrictor action, but thiopentone is much more potent in this respect than methohexitone. The results suggest that the weak vasoconstrictor action of methohexitone may be due entirely to content of buffered alkali, since solutions of the latter give equivalent or greater constriction, and constriction is not observed under conditions where the effect of methohexitone on the pH of the perfusion medium is minimal. By the same criteria thiopentone may be viewed as possessing a constrictor action enhanced by its buffered alkali nature. Within a range of doses of 0.5 mg - 6.0 mg and when given by injection thiopentone had a constrictor potency at least 3-10 fold greater than that of methohexitone. As thiopentone has been shown to have one-third the anaesthetic potency of methohexitone (Green and Jolly, 1960) it is possible that in clinical use thiopentone could have a constrictor effect at least 9 times greater than that of methohexitone. Since Francis (1964) demonstrated a similar incidence of necrosis with equivalent amounts of thiopentone and methohexitone, results of this study indicate that the ability of the two drugs to produce necrosis in the rabbit ear is unlikely to be related to their vasoconstrictor actions; if in fact the necrosis is related

in any way to an initial vascular action of the drugs, vasodilatation or reduced vascular excitability appears more likely to be involved, as this change is at least a common effect of the two drugs.

Mechanism of thiopentone vasoconstriction

The main feature of the results is the inability of a number of procedures which reduce or modify the responses of the ear vessels to noradrenaline to cause a substantial modification of the responses to thiopentone. The rapid speed of onset and the transient nature of thiopentone's action (de la Lande and Waterson, 1967b) is in itself difficult to reconcile with an indirect mechanism of action. It is concluded that under these conditions of perfusion the constrictor action of thiopentone is a direct effect not mediated by the release of noradrenaline.

This conclusion that the action of thiopentone is direct, appears at variance with that of Burn (1960) who assumed an indirect action on the part of thiopentone, namely that of noradrenaline release. Burn's conclusion was based on the marked reduction of thiopentone constriction by reserpine pretreatment, and by the noradrenaline antagonist tolazoline, and the potentiation of constriction by cocaine in concentrations which also potentiated the effects of noradrenaline. The effects of similar procedures, and also of sympathectomy in the experiments reported in this chapter, were slight and equivocal. The

two sets of findings seem difficult to reconcile. The relatively small increase in sensitivity to noradrenaline produced by cocaine in the whole ear, and reported in the present study, compared with the definite potentiation described by Burn, suggests a possible explanation of the cause of the discrepancy. The results in the earlier section of this thesis indicate that the sensitising potency of cocaine depends on the particular surface of the artery to which noradrenaline is applied, and is very much greater in the case of extraluminal noradrenaline.

It is possible that under Burn's conditions of perfusion a greater proportion of the drug injected into the perfusion stream was able to reach the noradrenergic storage structures via the extracellular fluid bathing the adventitia. In other words, the constant pressure technique may have permitted more effective diffusion of solutes across the artery wall. There is no direct evidence on this point. However, it is noteworthy that the study of thiopentone's action on the isolated artery gave no clear-cut evidence that there was a major difference between its intraluminal and extraluminal vasoconstrictor potency.

Waters (1966) has made the interesting suggestion that since thiopentone is insoluble at pH 7.4, clogging of arterioles by the drug in a particulate form may be the factor causing discharge of noradrenaline and eventual damage. This mechanism provides a second possible

explanation of the difference between these results and those of Burn. With a constant pressure technique vasoconstriction is associated with a reduced flow rate and even temporary cessation of flow. Hence a favourable situation is created for interaction between the blood vessels and particulate matter in the perfusion stream. With the constant flow technique the time of contact of the particle with the vessel wall should be less than with the constant pressure technique; in addition the increased driving pressure which follows vasoconstriction with the constant flow method may dislodge particles adhering to the small arterioles. Hence the probability of precipitation occurring during the time of passage of drugs through the ear would be less, and it is conceivable that the presence of the particulate matter is in some way connected with discharge of noradrenaline from nerve endings.

However, the above explanations are tentative and highly speculative, and the results of this study do not indicate that thiopentone is a suitable agent for the study of noradrenaline release mechanisms in the rabbit ear blood vessels.

CHAPTER 8

GENERAL DISCUSSION

The major finding presented in this thesis is that the position of the layer of sympathetic nerves in the wall of the rabbit ear artery has a profound influence on the sensitivity of the vessel to noradrenaline. The hypothesis which has been developed to explain the comparative effects of intraluminal and extraluminal noradrenaline, and the effects of cocaine and of denervation, is presented again in figure 8.1.

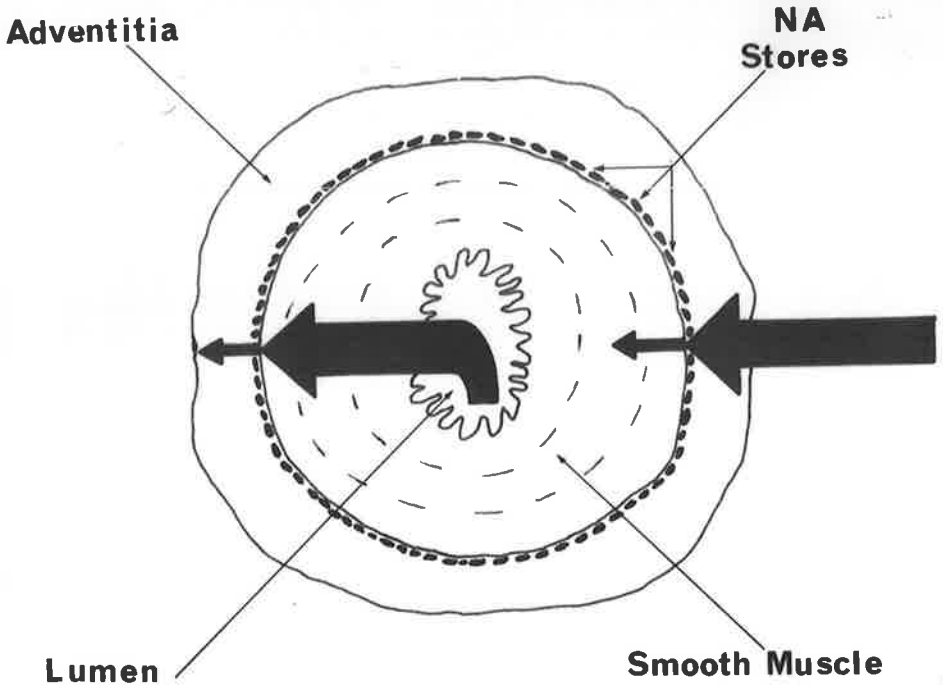
The validity of the hypothesis depends largely on the following points:

1. That uptake of noradrenaline in the rabbit ear artery is restricted mainly to the sympathetic nerves.
2. That noradrenaline can diffuse readily in the smooth muscle of the artery.

Uptake of noradrenaline

Since the suggestion by Burn (1932) that exogenous

Figure 8.1



Diagrammatic representation of the influence of the sites of uptake (shown as NA stores) on the concentration of noradrenaline in the smooth muscle of the artery. The direction of the arrow indicates the direction of diffusion of noradrenaline, and the thickness of the arrow indicates the concentration of noradrenaline.

catecholamines could accumulate in peripheral tissues, many workers have demonstrated tissue uptake of noradrenaline (Pennefather and Rand, 1960; Whitby, Axelrod and Weil-Malherbe, 1961; Stromblad and Nickerson, 1961; Muscholl, 1961; Bhagat, 1963). A relationship between sympathetic innervation and uptake of exogenous noradrenaline was proposed by Whitby, Axelrod and Weil-Malherbe, following studies on the cat heart. As the rabbit ear artery is rich in sympathetic innervation, a similar relationship could be expected. In chapter 3, evidence was presented that exogenous noradrenaline restored fluorescence at the medial-adventitial border in arteries which had been previously depleted of noradrenaline by reserpine. The experiments were conducted both in vivo and in vitro, and there was no notable change in the pattern of the restored fluorescence, although changes in distribution of restored noradrenergic fluorescence had been noted by Malmfors (1965). In Malmfors' experiments, rat iris muscle was depleted of noradrenaline by reserpine, and fluorescence restored by administering noradrenaline in vivo. The irises were examined as stretched preparations, and the pattern of the restored fluorescence differed from the fluorescence in untreated animals in that the brightness of the varicosities was much reduced, suggesting that noradrenaline had accumulated in the axoplasm rather than in the intra-neuronal granules.

There is also evidence that noradrenaline is taken up into non-neural binding sites in rat heart (Bhagat, Bovell and Robinson, 1967) and in rat salivary gland (Hamberger, Norberg and Olsson, 1967). In the denervated rat salivary gland, Anden, Carlsson, and Waldeck (1963) found that uptake of H^3 noradrenaline was of the same order as for normal glands, suggesting that in this tissue, extraneuronal uptake may be of functional importance. Recently, Avakian and Gillespie (1968) demonstrated extraneuronal binding of noradrenaline in smooth muscle cells, collagen and elastic tissue in the rabbit ear artery. The threshold concentration of noradrenaline for this fluorescence was 10 µg/ml, whereas in the present study this concentration was the upper limit, rather than the lower limit, of the concentrations of noradrenaline employed. Therefore, extraneuronal binding of noradrenaline (demonstrable by formaldehyde induced fluorescence) was unlikely to have been shown in the present study. An interesting point discussed by Avakian and Gillespie was the relationship of their work with that of Iversen (1965). Iversen proposed that noradrenaline uptake could be divided into two stages, an "uptake 1" occurring at low concentrations of noradrenaline, and "uptake 2" evident only at much higher concentrations of noradrenaline. Although Iversen stated that both forms of uptake were uptake into nerves, evidence provided by Avakian and Gillespie refutes the

suggestion that "uptake 2" is solely uptake into neural tissue, and indicates that extraneuronal tissue may be involved. Because of the lower concentrations used (not more than 10 µg/ml) it may be considered that the uptake of noradrenaline demonstrated in the present study, both by direct (chapter 2) and indirect (chapter 3) evidence, corresponds to the "uptake 1" proposed by Iversen, that is, uptake into sympathetic nerves.

Another approach to the question of the role of the nerve endings in sensitivity to noradrenaline was that of Bevan and Verity (1967). These workers found that denervation of rabbit aortic strips by mechanical destruction of the layers containing the sympathetic nerves, resulted in a slight reduction in the maximum response to noradrenaline of the denervated strip, compared with the control strip. The effectiveness of denervation was tested by the lack of response to transmural electrical stimulation, and to small doses of tyramine and nicotine. There was no significant difference in the median effective dose and the slope of the dose response curves of the control strips compared with the nerve free strips. This finding is at first sight at variance with the finding in the present study that denervation caused a considerable increase in the sensitivity of the artery to extraluminal noradrenaline. However, in

Bevan and Verity's experiments, the preparation used was a vascular strip, and noradrenaline was applied to both intraluminal and extraluminal surfaces simultaneously. Because of this, noradrenaline should reach receptor sites in the smooth muscle without being subjected to uptake by sympathetic nerves, although the concentration of noradrenaline in the immediate vicinity of the sympathetic nerves in the normal strips would be subject to alteration by uptake. The point of difference between Bevan and Verity's method and the method in the present study is that extraluminal noradrenaline (applied to the double cannulated artery) could reach the receptors in smooth muscle only after being subjected to uptake by the layer of sympathetic nerves, whereas in the vascular strip preparation, noradrenaline could stimulate receptors without first being subjected to uptake by the sympathetic nerves. For this reason, the results of Bevan and Verity do not necessarily contradict the general theme of this thesis that the distribution of the sympathetic nerves in arteries controls their sensitivity to noradrenaline.

Associated with the phenomenon of extraneuronal uptake of noradrenaline is the possibility that cocaine may inhibit extraneuronal uptake as well as uptake into nerves. In the present study, histological evidence has been presented that uptake of

exogenous noradrenaline into sympathetic nerves in the rabbit ear artery *in vitro* is blocked by cocaine (page 2.23). The evidence was based on the inability of exogenous noradrenaline to restore fluorescence at the medial-adventitial border (compared with a control), when cocaine 10 µg/ml was present. A similar procedure (under *in vivo* conditions) was used by Malmfors (1965) to demonstrate cocaine's ability to block uptake of noradrenaline in the rat iris. However, the possibility that cocaine may possess actions in addition to those of block of neuronal uptake, was suggested by Bevan and Verity (1967). These workers found that cocaine 3 µg/ml increased the maximum response to noradrenaline in both the denervated and the normal rabbit aortic strips, although cocaine's potentiation of noradrenaline was much greater for normal than for denervated strips. They concluded that the potentiation by cocaine in normal aortic strips is a dual process, a presynaptic event (block of neuronal uptake of noradrenaline) and a post synaptic event (a direct action by cocaine on vascular smooth muscle). A direct action by cocaine as a cause of supersensitivity of rabbit aortic smooth muscle cells was also proposed by Maxwell, Wastila and Eckhardt (1966). However, the only evidence obtained in the present study pointing to a second action of cocaine was obtained from denervated arteries, where in some arteries cocaine caused potentiation, and in others depression. These effects were

usually small, and were not further analysed. A possible explanation of the different effects is that the concentration of cocaine (10 µg/ml) may have approached those concentrations which produce local anaesthetic effects.

Diffusion of noradrenaline

That noradrenaline can diffuse through vascular smooth muscle has been shown histochemically by Gerova, Gero and Dolezel (1967) and Avakian and Gillespie (1968). Gerova, Gero and Dolezel showed formaldehyde induced fluorescence in the media of the dorsal pedal artery of the dog, immediately following stimulation of sympathetic nerves in the artery. The distribution of the fluorescence suggested that noradrenaline had emanated from the nerve endings (in this artery within the media) and had spread for some distance into the smooth muscle. The authors presented the results as evidence that the transmitter diffused from nerve terminals to layers of smooth muscle remote from the sites of release. Avakian and Gillespie electrically stimulated segments of rabbit ear artery, which were frozen in isopentane during the stimulation. Formaldehyde treatment revealed fluorescence of smooth muscle cells adjacent to the nerve terminal, and this finding was interpreted as being due to diffusion of transmitter substance.

An important consideration is the distance over which released transmitter substance must diffuse to reach and produce an effect on smooth muscle cells. The rabbit ear artery contains a layer of some 10 smooth muscle cells across a transverse section of the artery wall (a distance which in this study was found to vary from about 50 to more than 100 microns), and diffusion of transmitter substance across this distance was not shown in either of the two studies reported above. However, Dolezel (1966) has shown that exogenous noradrenaline administered intravenously diffused throughout the media of the rat femoral artery *in vivo*. Avakian and Gillespie (1968) showed strong fluorescence throughout the smooth muscle layer of the rabbit ear artery after perfusion *in vitro* of 10^{-4} g/ml of noradrenaline for periods of between 4 and 16 minutes.

Recent experiments in this department have demonstrated that noradrenaline in lower concentrations can diffuse from the intimal surface of the artery to the medial-adventitial border. In experiments with double cannulated arteries *in vitro*, which had been depleted of noradrenaline by reserpine, fluorescence at the medial-adventitial border was restored after the intraluminal perfusion of noradrenaline 200 ng/ml for approximately 10 minutes (Campbell, 1968, personal communication). In the present study, noradrenaline

(0.5 mg/kg intravenously) in vivo restored fluorescence in the rabbit ear artery (previously depleted by reserpine) five minutes after administration of the noradrenaline.

The above evidence indicates that noradrenaline can diffuse into the smooth muscle layer of arteries, but the question of importance is whether or not the diffusion can occur in the brief interval (1 to 2 minutes) required for the steady sustained response to noradrenaline to be achieved. This diffusion should be sufficiently rapid and unrestricted to allow the same concentration to reach the medial-adventitial border, irrespective of the surface to which the noradrenaline is applied. If, however, intraluminal noradrenaline does not reach the same concentration, its uptake will be less than that of extraluminal noradrenaline. Future experiments in arteries depleted by reserpine, and which have been briefly exposed to graded concentrations of noradrenaline, may provide more information on this point.

Another assumption implicit in the hypothesis is that the constriction of the artery produced by exogenous noradrenaline is mediated entirely by noradrenergic receptors on the (individual) smooth muscle cells. However, the possibility that there may be electrical transmission between smooth muscle cells has been

considered by a number of workers. Burnstock and Holman (1963) in a review of autonomic nerve transmission, considered the possibility that intercellular transmission of excitability occurs in smooth muscle and discussed the evidence available at that time for the close contact of smooth muscle cells necessary to facilitate transmission of an electrical impulse. Recently, Tomita (1966) supported such a concept, and Holman (1967) postulated that in a number of "muscular" blood vessels, individual smooth muscle cells may have connections which allow changes in membrane potential to spread electrotonically to adjacent parts of the tissue. Burnstock and Holman (1963) found the evidence for the existence of intercellular linkage to be inconsistent and unconvincing, and despite many reports of such connections between smooth muscle cells, the subject remains contentious. However, Rhodin (1967) has shown close contact of neighbouring smooth muscle cells in arterioles of rabbit fascia.

Some of the results of the present study do not support the concept that electrotonic spread of excitation is essentially part of transmission of the contraction impulse in vascular smooth muscle.

In arteries which were depolarised with high concentrations of potassium ions, responses to the lower range of doses of noradrenaline were unchanged (de la Lande, Cannell and Waterson, 1966).

In these arteries it may be assumed that electrical activity of the smooth muscle membrane is absent.

In arteries where the influence of uptake of noradrenaline is removed by cocaine or denervation, noradrenaline tends to be equi-active intraluminally and extraluminally, suggesting that the proportion of noradrenergic receptors in the smooth muscle layer being stimulated is the same, regardless of the surface from which the amine is diffusing.

Another point is that intraluminally applied cocaine potentiates extraluminal noradrenaline in a time course not very different from that required by noradrenaline to produce a sustained constriction of the artery.

Some physiological implications of the hypothesis

In clinical practice, and particularly in dentistry, adrenaline and noradrenaline are used extensively, and are mixed in solution with drugs which share at least one property in common with cocaine in that they are local anaesthetics. The widely used local anaesthetics procaine and lignocaine are known to potentiate responses to exogenous and endogenous noradrenaline in isolated preparations (Bentley, 1965, 1966), and potentiation by these drugs of the pressor

response to noradrenaline *in vivo* has been noted in an earlier study in this department (Fotheringham and Waterson, unpublished). The results of the present study have indicated that the presence of the noradrenaline uptake mechanism is extremely important in determining the potency of noradrenaline, and of related amines, which are applied to tissues so that they reach the blood vessels via the extracellular fluid. If the potentiation of noradrenaline by other local anaesthetics reflects another property shared with cocaine, that is, block of neural uptake of noradrenaline, then the administration of the mixture of vasoconstrictor and local anaesthetic drug to the adventitial surface of the blood vessels, could result in considerable enhancement of the vasoconstrictor potency of some local anaesthetic solutions.

The restriction of uptake of noradrenaline to the region of the artery wall outside the smooth muscle layer may also help to explain some features of vascular responses in the human. Duff (1955) and Parks, Skinner and Whelan, (1961) noted comparatively small changes in the magnitude of the responses to catecholamines in sympathectomised human limb vessels *in vivo*. Cooper, Fewings, Hodge and Whelan (1963) noted similarly small changes after sympathetic blockade with bretylium and guanethidine. However, in these experiments the catecholamines were applied intraluminally,

and it is possible that, as in the rabbit ear artery, changes in the rate of uptake of noradrenaline have little effect on the concentration reaching the receptors in the smooth muscle from the intimal surface of the artery.

Evidence that in the human forearm vessels, neurotransmitter is released from sites remote from the lumen of the vessel, is provided by Frewin and Whelan (1967), who found differences between the times of onset of the actions of tyramine and noradrenaline infused into the brachial artery. They reasoned that infused noradrenaline exerted its action directly on the smooth muscle coat, whereas tyramine diffused through the smooth muscle to reach the nerve plexus at the outer part of the vessel, and that the transmitter then released by tyramine could be subject to uptake and to enzymic degradation before exerting its effect on the smooth muscle. In addition, Frewin and Whelan found that tyramine produced no effect (in the doses employed) in sympathectomised vessels.

The above findings in the human are consistent with the mechanisms of release and re-absorption proposed in the artery model hypothesis, and offer support for the view that the hypothesis may at least be applied to studies of the actions of sympathomimetic drugs on human limb vessels.

APPARATUS

A. Perfusion experiments

1. The roller pump (designed by O. Saxby, Department of Pharmacology, Oxford University was manufactured in the Medical School Workshop, University of Adelaide. A photograph of the pump is shown in appendix figure 1.

2. A Braun circulating pump (Thermomix model) with thermostat, was used to circulate heated water through the warming coil of the perfusion apparatus.

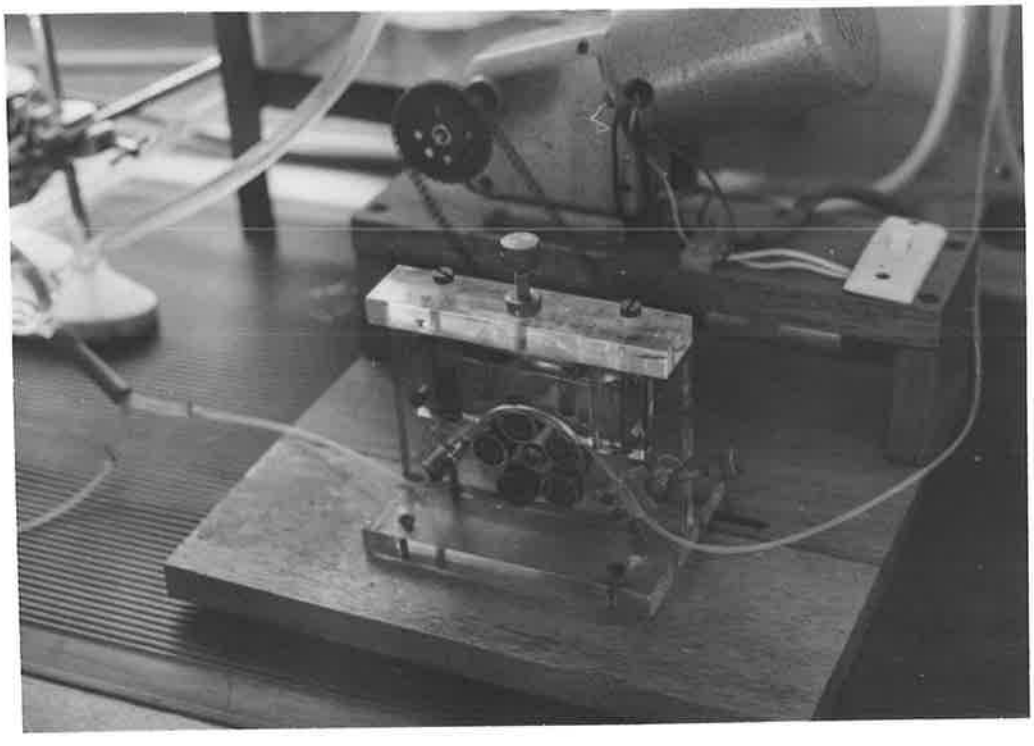
3. Pressure changes were recorded on a Palmer kymograph (Model Super-Ten) using a 12 inch diameter drum.

4. A Palmer mercury manometer (Condon Model) was used to measure perfusion pressures.

5. Electrical stimulation was provided from a Grass Stimulator Model S4. Platinum electrodes were employed.

6. The organ bath (for the isolated artery) was of 10 ml capacity, with a ground glass insert plug at the bottom, and open at the top (appendix figure 2).

Appendix Figure 1

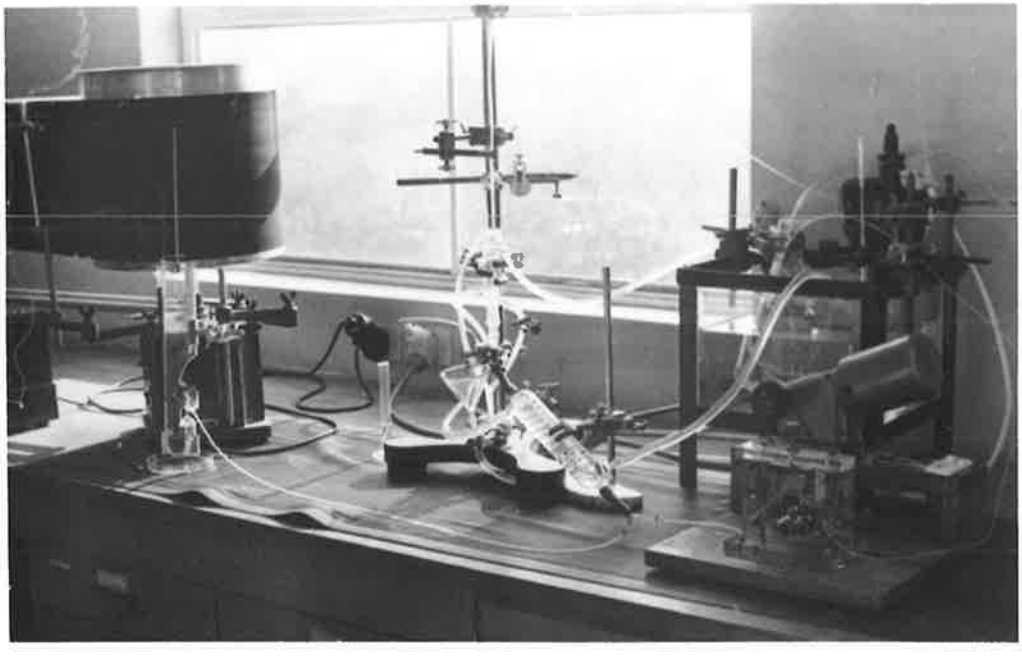


The constant volume pump used for ear and artery perfusion.

Appendix Figure 2

Organ bath (10 ml capacity) used for the artery perfusion experiments.

Appendix Figure 3



Arrangement of the apparatus for a typical artery perfusion experiment.

7. The rabbit ear was perfused in a perspex chamber designed by M.J. Tyler, in which perspex tubing of 3" diameter was lined with warming coils of polythene tubing ($\frac{3}{8}$" diameter.

B. Histochemical experiments

1. Freeze dryer - Thermovac Model FD-3.
The pump was not provided with gas ballast. The freezing chamber was of large capacity (several litres) and the apparatus was not specifically designed as a small tissue freeze dryer. No dessicant was used in the chamber.

2. Vacuum infiltration.
The apparatus used was manufactured by the National Appliance Company, and was operated using a water vacuum pump.

3. The microtome was a Leitz Model 1212, and sections were usually cut at 5 to 7 microns.

4. Microscope - Leitz Ortholux Model, with Orthomat Camera and exposure meter attachments. Dry and oil immersion dark field condensers were used.

C. Miscellaneous

1. The pH meter used was Beckman Model E350B.

PERFUSION SOLUTION

The perfusion solution was Krebs bicarbonate solution of the following composition.

	grams/litres	Concentration mM
NaCl	6.9	120
KCl	0.35	4.7
$CaCl_2$	0.28	2.5
$MgCl_2$	0.1	1.1
$NaHCO_3$	2.1	25.0
KH_2PO_4	0.16	1.0
Glucose	1.0	5.5

The pH of the solution after saturation with 5% carbon dioxide in oxygen was 7.4. The pH before gassing was 7.8.

DRUGS USED IN STUDY

The following drugs were used in the study. The weight of all drugs except noradrenaline refer to weight of the salt. The weight given for noradrenaline refers to weight of the base.

Noradrenaline bitartrate (Sigma)

Tyramine hydrochloride (Koch-Light)

Cocaine hydrochloride

Reserpine (Serpasil, Ciba)

Nialamide (Pfizer)

Histamine acid phosphate (B.D.H.)

Thiopentone (Intraval, May and Baker)

Methohexitone (Brietal, Lilly)

Angiotensin (Hypertensin, Ciba)

Atropine Sulphate (B.D.H.)

Serotonin creatinine Sulphate

PREPARATION OF DRUGS

1. Noradrenaline bitartrate was dissolved either in ascorbic saline (ascorbic acid 1 in 10,000 in 0.9% NaCl) or in normal HCl. Further dilutions were made with ascorbic saline, the pH of which had been adjusted to pH 5.5 with NaOH.

2. Nialamide was dissolved in a minimal amount of 2 N HCl and further diluted with 0.9% NaCl. The pH of the solution was then adjusted to pH 5 with NaOH.

3. Thiopentone and methohexitone were dissolved in distilled water, and further diluted with 0.9% NaCl solution.

All other drugs were made up in 0.9% sodium chloride solution. Lignococaine hydrochloride 2% with adrenaline 1 in 80,000 was the local anaesthetic used for extraction of human teeth from which the pulps were removed prior to histochemical treatment. The anaesthetic was infiltrated around the teeth in some patients, and applied at a site remote from the teeth (nerve block) in other patients.

MATERIALS USED IN THE STUDY

1. Paraformaldehyde powder (Merck) was stored (in 5 gram lots) over sulphruic acid in 10 inch dessicator jars. The acid was of varying concentrations (in different jars) to provide relative humidities of from 50% to 90%. Most of the paraformaldehyde used in the study was stored at a relative humidity of 70%. The acid was changed weekly, irrespective of the quantity of paraformaldehyde used.

2. Kodak Photoflure film was used throughout the study for both phase contrast and fluorescence photographs.

The film was developed in Ilford ID2 developer, and prints of fluorescence photographs were made on No. 5 Kodak paper to obtain the necessary contrast. Fluorescence photographs in this thesis were of necessity printed on Kodak projection soft paper, which does not provide (for these photographs) prints of the same contrast as Kodak No.5 paper.

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