THE ACTIONS AND INTERACTIONS OF NORADRENALINE, DOPAMINE AND L-DOPA

THESIS SUBMITTED TO THE UNIVERSITY OF ADELAIDE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

BY

MARGARET ANN LAZNER

DEPARTMENT OF HUMAN PHYSIOLOGY
AND PHARMACOLOGY
THE UNIVERSITY OF ADELAIDE
SOUTH AUSTRALIA

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SUMMARY

1. In this thesis the vascular actions in the rabbit of dopamine and L-dopa are examined. The tissue used throughout was the isolated central artery of the rabbit ear.

2. Dopamine was found to exert its action in the rabbit ear artery by stimulation of the alpha adrenergic receptors causing constriction of the artery. Dilatation due to the action of dopamine could not be demonstrated in this preparation. The sensitivity to dopamine was not modified by chronic denervation, by cocaine, or by reserpine. It was thus concluded that neuronal mechanisms play little part in the response to dopamine. Fluorescence histochemistry showed that dopamine is taken up into nerve terminals, but uptake reached saturation level at concentrations of dopamine which are threshold concentrations for the constrictor response. L-dopa was found to have no constrictor effect on the artery.

3. The role played by the metabolizing enzymes in the response to dopamine, L-dopa and tyrosine was studied in nialamide treated arteries. It was found that nialamide treatment caused an increase in sensitivity and a change in the response to L-dopa and dopamine, the response and the recovery after washout of the drugs becoming much prolonged. These effects were not decreased by prior treatment with cocaine, or reserpine, but were reduced by chronic denervation. Cocaine applied during the response, or during the recovery phase caused a rapid further constriction of the artery. This evidence indicated that the changes induced by nialamide treatment were in the most part mediated by neuronal mechanisms and intraneuronal MAO, but did not depend on the presence of intragranular stores of noradrenaline.

4. No response due to tyrosine was detected in nialamide treated arteries, nor was there any response to cocaine

after tyrosine.

5. In view of the similarity between the effects of nialamide on the response to dopa, dopamine and noradrenaline, a role for noradrenaline in the secondary sensitization response to dopa and dopamine is hypothesized. The role of noradrenaline was assessed by blockade of the enzyme dopamine-β-hydroxylase. It was shown that prior treatment with diethyldithiocarbamate (DDC) largely prevented the occurrence of secondary sensitization to dopamine in reserpine and nialamide treated arteries; however, no effect of DDC was observed in arteries treated with nialamide alone.

6. The effect of lowering the temperature from 37°C to 25°C was studied. It was found that in untreated arteries lowered temperature produced no change in the response to dopamine and noradrenaline; however, in nialamide treated arteries a profound effect of cooling on the parameters of altered response and increased sensitivity to dopamine, noradrenaline and L-dopa was seen. It was also shown that cooling reduced the potentiation of noradrenaline due to cocaine and the uptake and retention of ^{3}H noradrenaline.

7. It is concluded that there is a role for noradrenaline, and the $Uptake_1$ process, in the secondary sensitization response to L-dopa and dopamine in the nialamide treated artery. However, other mechanisms may be involved, such as release of endogenous noradrenaline, or noradrenaline newly formed from the exogenous dopamine, by the dopamine.

8. Histochemical evidence from the perfused nialamide and reserpine pretreated ear artery segment showed that uptake by smooth muscle and metabolism by COMT in the media were factors in the failure of intraluminal noradrenaline to penetrate across the media to the nerve endings, and to cause secondary sensitization to noradrenaline. Inhibition of COMT alone had little effect on the sensitivity to intraluminal noradrenaline,

or dopamine. However, in nialamide treated arteries inhibition of COMT caused a two fold increase in sensitivity to intra-luminal noradrenaline.

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 any other person, except where due reference is made in the text. Material from or related to this thesis has formed part of the following publications:

CIRCULATION RESEARCH (1970) 26-27 Supp. 2, 41-48
PROCEEDINGS AUSTRALIAN PHYSIOLOGICAL AND PHARMACOLOGICAL SOCIETY (1971) 2, 33
ibid. (1972) 3, 183
AUSTRALIAN JOURNAL OF EXPERIMENTAL BIOLOGY AND MEDICAL SCIENCE (1974) 52, 193-200
JOURNAL OF PHARMACY AND PHARMACOLOGY (1974) 26,62-65
JOURNAL OF DENTAL RESEARCH (1971) 50, 743-744

Results from this thesis have also been presented to meetings of the Australasian Society of Clinical and Experimental Pharmacologists, and the International Association for Dental Research.

Margaret Ann Lazner,
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CHAPTER ONE

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

It is the purpose of this thesis to examine the vascular actions in the rabbit of the precursors of noradrenaline, i.e. L-dopa and dopamine, and in particular to examine the part played by the sympathetic nerves and the metabolising enzymes, monoamine oxidase and catechol-O-methyltransferase, in the responses to these substances. In addition, the relative role of uptake by the sympathetic nerves and enzymic metabolism in the fate of noradrenaline applied to the intimal surface of the rabbit ear artery has been studied.

As an introduction to the studies described in this thesis, it is useful to trace briefly the current status of knowledge of the vascular pharmacology of L-dopa and dopamine. Early workers found that, in the intact animal, dopamine has both pressor and depressor effects depending on the species and the dose used (Barger and Dale 1910; Tainter 1930; Hamet 1931; Gurd 1937; Holtz and Credner 1942). It was found early that the pressor effects were mediated through the α-adrenergic system (Tainter 1930; Hamet 1931), but a full explanation of the depressor effects was not possible until more recent times. Dilator effects due to stimulation of β-adrenergic receptors were shown to occur in some vascular beds after complete blockade of α-receptors, notably the femoral and brachial vascular beds; however, large doses of dopamine were required (Allwood and Ginsberg 1964; McNay and Goldberg 1966). Certain of the depressor effects to dopamine were, however, neither blocked by β-adrenergic blocking agents, nor atropine nor antihistamines (McDonald and Goldberg 1963; Vanov 1963), so the concept was formed that dopamine has, in addition, an intrinsic effect in some vascular tissue. It was found that dopamine causes vasodilatation in the renal vascular bed in man (McDonald and others 1963, 1964), and in the renal, mesenteric and coronary vascular beds in the dog (McNay and others 1963, 1964; Eble 1964;

Schuelke and others 1971). The dilator effect in dogs, resistant to β-adrenergic blocking drugs, was later found to be selectively attenuated by haloperidol (Yeh and others 1969; Schuelke and others 1971), phenothiazines (Goldberg and Yeh 1971) and bulbocapnine (Tseng and Walaszek 1970). The evidence for a direct intrinsic effect of dopamine in certain vascular beds has been recently reviewed by Hornykiewicz (1971) and Goldberg (1972).

Although dopamine causes a fall in blood pressure in the intact rabbit (Holtz and Credner 1942), in isolated rabbit vascular tissue dopamine is usually constrictor. The relative constrictor potency of dopamine to noradrenaline has been shown to be 1/50 in the rabbit ear artery (de la Lande and Harvey 1965; Campbell and Farmer 1968) and the rabbit aortic strip (Kohli 1969). However, dilator effects to dopamine, which were blocked by propranolol, have been shown in the carbachol contracted rabbit aortic strip (Kohli 1969). Other dilator effects have been shown in isolated, phenoxybenzamine and sotalol treated rabbit renal and mesenteric artery strips (Toda and Goldberg 1973). These latter effects may have been due to activation of specific dopamine receptors in these arteries.

The part played in the dopamine response by the sympathetic nerves has been a subject of debate for many years. Much evidence has been presented to show that, at least in some tissues, dopamine depends in part for its action on release of endogenous noradrenaline from the sympathetic nerves (Bulbring and Burn 1938; Bejrablaya and others 1958; Stromblad 1960; Farmer 1965, 1966; Spiers and Calne 1969). Reports which show the effects of cocaine and denervation on responses to dopamine have been inconsistent, and show a spectrum of effects ranging from unaltered responses to potentiation, depending on the tissue used (Tainter 1930; Hamet 1931; Gurd 1937; Hamilton 1972). Tsai and others (1967) summed up the evidence in the suggestion that dopamine has three actions, manifested more or less prominently

in different tissues:

(1) a direct action on the α or β-adrenergic receptors,

(2) a release of endogenous noradrenaline,

(3) an impairment of the reuptake of the released noradrenaline. (To these must now be added the direct intrinsic effect of dopamine mentioned above.)

The part played in the response to dopamine by its metabolizing enzyme, monoamine oxidase (MAO), was first noted by Helmer (1957). He found that inhibition of MAO by iproniazid caused a marked potentiation of the response to dopamine in the rabbit aortic strip. Studies in the dog (Goldberg and Sjoerdsma 1959; Gatgounis 1965) showed that concomitant administration of MAO inhibitors and dopamine caused marked augmentation and prolongation of the cardiovascular effects of dopamine.

In studying the importance of MAO in the response to dopamine, it is pertinent to note the observations made on the effect of MAO inhibition on the responses to noradrenaline. Furchgott and Sanchez Garcia (1968) showed that, in the guinea pig atria, inhibition of MAO produced a slow, gradual secondary augmentation of the response to noradrenaline, and a slow decline of the response after washout of the noradrenaline. A similar effect was shown in the rabbit ear artery (de la Lande and Jellett 1969, 1972) and the isolated cat nictitating membrane (Tsai 1968; Trendelenburg 1971). The phenomenon was called 'secondary sensitization' by Furchgott and Sanchez Garcia (1968), and was attributed by them to free noradrenaline leaking out from structures in which it had accumulated to levels exceeding the removal capacity of the storage granules, and cytoplasmic binding sites. Trendelenburg (1971) found that in the cat nictitating membrane, the degree of sensitization was greater after pargyline and reserpine than after pargyline alone. He suggested that any impairment of the intraneuronal mechanisms of inactivation leads to supersensitivity by causing a decrease in the neuronal net uptake of the amines and that the prolonged time course of the response

reflects a slowly developing exhaustion of the capacity of the nerve endings to inactivate the amines after their uptake. Graefe and others (1971) showed that a time dependent decrease of removal of amines from fluid perfused through isolated pargyline treated rabbit heart was due to an increased efflux of the amine from the nerve endings. They concluded that "any impairment of the intraneuronal mechanisms of inactivation (vesicular storage and MAO) leads to an increase in the axoplasmic concentration of free noradrenaline which causes an increased efflux of the amine, while the influx remains unchanged. The axoplasmic concentration of free noradrenaline seems to rise more after block of MAO than after pretreatment with reserpine and is most pronounced after both." In studies on the MAO inhibited rabbit ear artery, de la Lande and Jellett (1972) found that secondary sensitization to noradrenaline was a neuronal phenomenon, since it occurred only on extraluminal application of the drug to the artery, and could be abolished by chronic denervation or cocaine pretreatment. It was found to be independent of the intraneuronal storage granules, since pretreatment with reserpine did not decrease the response. During the delay in recovery after washout of noradrenaline, cocaine and phentolamine caused constriction and dilatation respectively at a stage when the constrictor tone of the artery was still high; the cocaine constriction being enhanced in the reserpine pretreated arteries. This evidence thus supports the idea that the delayed recovery was associated with the presence of free extragranular noradrenaline, and that influx of noradrenaline as well as efflux is a contributing factor to the prolonged nature of the delay in recovery.

The aim of the present study was:

(1) to examine the nature of the vascular response to dopamine in a small muscular artery,

(2) to examine the effects of inhibition of monoamine oxidase and catechol-O-methyltransferase on the response to

dopamine and its precursor substances L-dopa and tyrosine.

Previous studies of this type have been restricted to the larger non-muscular arteries such as the aorta (Kohli 1969); however, the extent to which these studies can be generalized to other types of artery is open to question. The rabbit ear artery, as a representative of small muscular arteries, has several advantages that make it well suited for pharmacological studies.

(1) It is sufficiently large to be easily isolated and readily susceptible to pharmacological studies, and there is evidence that it subserves much of the changes in vascular resistance in the whole ear.

(2) There are few side branches in the artery at the base of the ear, and a length suitable for perfusion can be easily dissected. Thus the effects of a drug may be analysed separately according to whether it is applied to the intimal or adventitial surface of the artery. The studies of de la Lande and co-workers (de la Lande and others 1966; de la Lande and Waterson 1967, 1968) have demonstrated that analysis of these separate actions can provide much useful information about the relative roles which the sympathetic nerves and the smooth muscle may play in the vascular actions.

(3) The artery at the base of the ear is innervated via the homolateral superior cervical ganglion (Feldberg 1926), and so denervated preparations may be easily prepared and compared with the contralateral innervated artery.

(4) Strip preparations may be made of the same tissue, and comparisons made between the action of a drug on the perfused segment, and the action of the same drug on a strip preparation. Complete dose response curves may also be obtained on the strip preparation for convenient quantitation of effects.

Abbreviations used throughout this thesis:

DA	dopamine {β-(3,4-dihydroxyphenyl) ethylamine}
NA	l-noradrenaline {(-) 2-(3,4-dihydroxyphenyl)-2-hydroxyethylamine}
MAO	monoamine oxidase
COMT	catechol-O-methyltransferase
DDC	sodium diethyldithiocarbamate
L-dopa	L-β-(3,4-dihydroxyphenyl) alanine

Tests of significance:

Where statistical tests of significance have been applied to groups of data, the result is considered to be significant if the probability lies below the 5% level. This is expressed as, for example, (t-test, $P<0.05$).

CHAPTER TWO

GENERAL METHODS

CHAPTER 2

METHODS

Experimental methods described in this chapter are:

(a) preparation of rabbits;
(b) perfusion of the isolated central ear artery;
(c) helical strip preparation of the central ear artery;
(d) pretreatment of rabbits;
(e) pretreatment of isolated arteries;
(f) techniques used in experiments with radio isotopes.

The most commonly employed materials and methods for each of the above procedures are described; modifications of the methods appear in later chapters and 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 Appendix 1, together with their manner of preparation for use in the study.

PREPARATION OF RABBITS.

The male and female semi-lop-eared rabbits used were bred at the Central Animal House of the University of Adelaide. The weights of the animals varied from 1.5 to 2.5 Kg, although most of the rabbits used for this study were in the range 1.5 to 2.0 Kg. The rabbits were not starved before use. The animal house temperature was generally maintained at about 21°C, however some variation of this temperature occurred under winter conditions.

Anaesthesia was induced with urethane, 10 ml/Kg of a 25% solution being injected intraperitoneally, with increments given as required. The animals were pretreated with heparin 1,000 units/Kg intravenously into an ear vein. Sometimes the

animals were stunned and bled before use.

PREPARATION OF THE ISOLATED EAR ARTERY FOR PERFUSION.

Polythene or glass cannulae were made by heat drawing fine polythene tubing or glass tubing in such a way that a slight bulge near the tip of the cannula prevented the cannula from slipping out of the artery, once tied in.

The prepared rabbit was placed on an operating table so as to make prominent the cartilage near the base of the ear. Pulsation of the central artery could be usually seen or felt at this point. The fur was moistened with Krebs bicarbonate solution and an incision made in the skin. About 1.5 to 2.5 cm of the central ear artery was exposed by blunt dissection beginning at the base of the ear and was gently cleared of adherent tissue; the tissue being kept moist at all times with Krebs bicarbonate solution. A cotton thread was placed under the artery and the artery tied as close as possible to the proximal end of the ear. A polythene or glass cannula was then inserted into the lumen of the artery via an incision, and firmly tied into position.

A second, finer cannula was inserted into the distal part of the selected segment of artery proximal to the first major branch of the artery (Fig. 1). The double cannulated artery was then transferred to a dish containing warm gassed Krebs bicarbonate solution. The artery segment was gently perfused with Krebs bicarbonate solution, with a glass syringe, to remove any debris and to ascertain if the artery was intact or perforated in any way. It was set up without delay in a double-jacketed organ bath and perfused at constant temperature (usually 37^{0}C but sometimes varied according to the nature of the experiment). The perfusion fluid entered by the proximal cannula, pumped at constant flow rate by a roller pump, and the effluent passed out through the fine distal cannula. A small

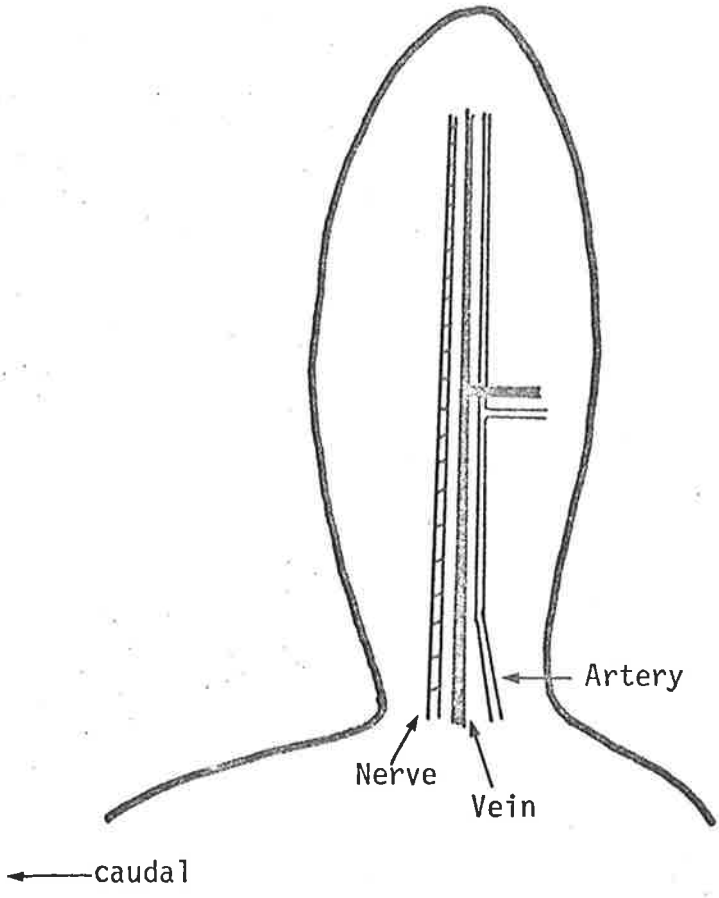


Figure 2.1

Diagram of the left ear of a semi-lop eared rabbit. The convex surface of the ear is shown as seen from the midline of the skull, showing the relative positions of the ventral (great) auricular nerve, the central vein and the central artery of the ear.

tension (1 g) was applied to the distal cannula to prevent the artery kinking on elongation during constriction.

The method of artery perfusion using double cannulation was described by de la Lande, Cannell & Waterson (1966). Using this method, drugs can be applied intraluminally by injection into the perfusion fluid or into the perfusion reservoir; or extraluminally into the fluid bathing the external surface of the artery (Fig. 2).

A mixture of 5% CO_2 and 95% O_2 bubbled into the perfusion reservoir and, by a separate lead, into the extraluminal fluid in the organ bath. Tests for leakage in the artery perfused by the double cannula method was as follows:

(i) observation of the level of extraluminal fluid in the organ bath during perfusion of the artery;

or occasionally

(ii) by perfusion of Evans blue dye through the artery with photometric comparison of intraluminal and extraluminal solution with normal Krebs bicarbonate solution.

Arteries were allowed to equilibrate at 37°C in Krebs bicarbonate solution while being perfused at constant rate for 1 hour before any drug treatment. De la Lande & Waterson (1968) found that steady responses to noradrenaline were not usually obtained in the first hour; however, after this, reproducible steady responses were obtained for 4 hours.

Changes in vascular resistance were recorded as changes in perfusion pressure by means of a mercury manometer with a floating pointer writing on smoked Kymograph paper.

For each experiment, the concentration of DA or NA producing a response of 60 mmHg was calculated from the dose-response curves, and the ratio of these taken as follows:

$$\frac{\text{concentration of DA in untreated artery}}{\text{concentration of DA in treated artery}}$$

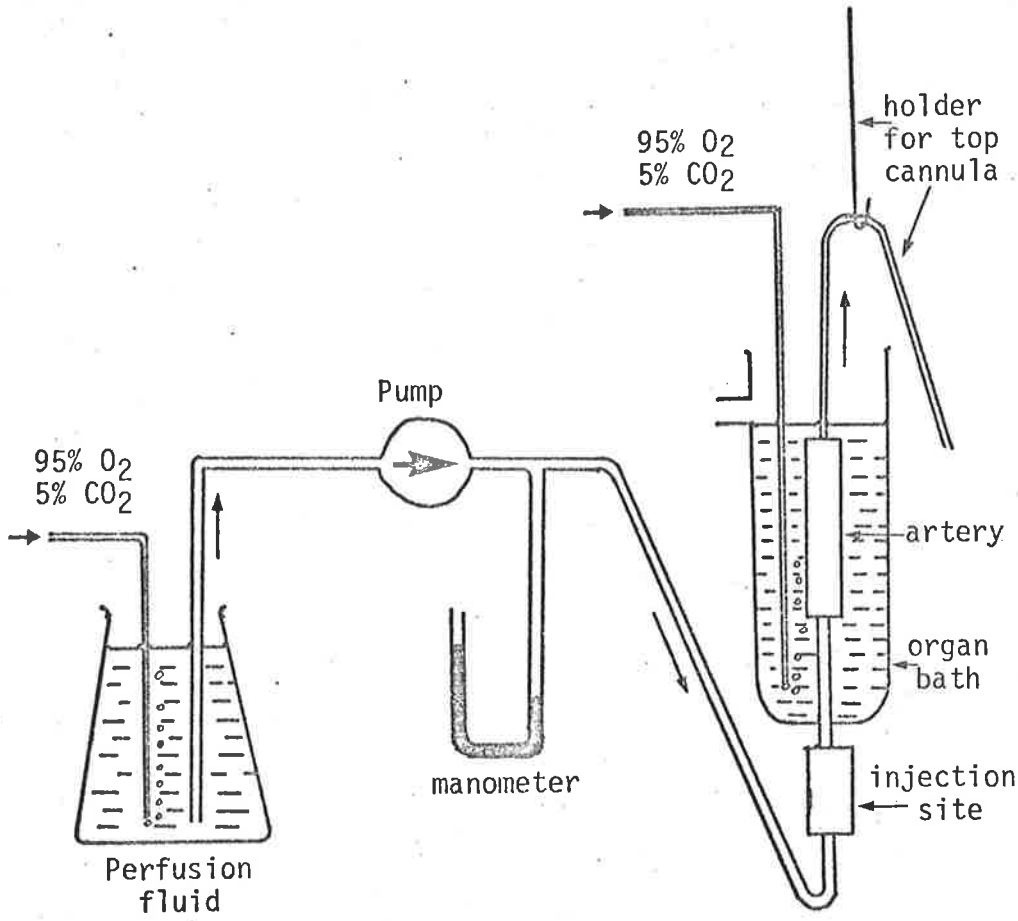


Figure 2.2

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

Hence a value greater than 1 refers to potentiation, and less than 1 to depression.

HELICAL STRIP PREPARATIONS.

The rabbits were prepared as for perfusion experiments and the artery isolated by blunt dissection as before. Adherent tissue was removed as far as possible. A double cotton ligature was applied to the proximal part of the artery as close as possible to the cartilage at the base of the ear and a second double ligature applied about 2 cm distal to the first. The segment of artery was excised so that one ligature remained on each end of the segment which was then placed in a dish of warm gassed Krebs bicarbonate solution. It was sometimes necessary to go higher in the ear to remove more than one strip. The artery segment was then laid on a Krebs-wet fore-finger and cut spirally with fine scissors into a helical strip. Care was taken to keep the artery wet, and also to keep a constant angle of cut, about 30^0-45^0, at all times. The proximal end of the artery strip was tied without delay into a stainless steel holder (fixed in a clamp) and immersed in warm gassed Krebs solution in a jacketed organ bath of about 10 ml capacity. The strips were untwisted and the distal end attached to the lever of a Harvard Heart/Smooth Muscle transducer by the distal cotton tie. A tension of about 1 g was always applied to the strip by a weight attached to the opposite end of the Harvard lever. The organ baths were about 10-12 ml in volume; this volume was measured accurately.

The solution used was Krebs bicarbonate solution gassed with 5% CO_2 and 95% O_2. Gassed Krebs bicarbonate solution was kept in a reservoir at room temperature, but was heated at 37^0C (or other predetermined temperature) by passage through a large (approx. 70 ml capacity) heating coil before passing into the organ bath. Drugs were added with a glass syringe into the top of the bath and were washed out by overflow of fresh warmed

Krebs solution, through a side arm at the top of the organ bath.

Recordings were made from the transducer through a Rikadenki chart recorder. The tension on the artery strip was adjusted slightly before each experiment so that it was possible to record full scale deflection on the recorder. The amplification of the system was obtained for each individual experiment by recording the movement of the transducer lever through 1 cm at the point of attachment of the artery. Strips tended to relax for the first hour after setting up, so to facilitate recording, the tension was altered from time to time during this period to maintain a constant base line. No drugs were added during the first 60-90 minutes to allow for equilibration. Strips were measured in the organ bath at base line tension (approx. 1 g) at the beginning of the experiment after equilibration.

Cumulative dose response curves were used since the maximum contraction obtained cumulatively for this preparation is the same as that obtained with a single supramaximal dose.

Responses were recorded as centimetres of shortening and two parameters were then calculated from this raw data.

i) % of maximum

Responses were calculated as percentages of the maximum responses obtained either with DA or NA, usually at the end of the experiment. The maximum response obtained with DA was the same as that obtained with NA.

ii) % shortening of strip

The percentage shortening of the strip was calculated from the raw data by use of the following formula:

$$\% \text{ shortening of strip} = \frac{(\text{deflection in cm}) \quad (100)}{(\text{amplification of transducer})\ (\text{strip length in cm})}$$

PRETREATMENT OF RABBITS.

(a) Sympathetic denervation

(b) Reserpine

(c) Nialamide

Rabbits were pretreated to modify the function of neurotransmitter sites in the blood vessels of the ear by sympathetic denervation or by pretreatment with reserpine, and MAO was inhibited with nialamide.

(a) Sympathetic denervation

The ear blood vessels of rabbits were sympathetically denervated by surgical removal of the superior cervical ganglion on the homolateral side. The method for sympathetic denervation was that followed by de la Lande & Rand (1965). Sterile technique was used, anaesthesia was usually induced with halothane, and maintained with ether. No premedication was necessary with this regime. In some rabbits anaesthesia was induced with pentobarbital (Sagatal) intravenously through an ear vein. After skin shaving and preparation of the area with a mixture of cetrimide and chlorhexidine, and before incision, 1 ml of 2% lignocaine containing adrenaline 12.5 µg/ml was infiltrated into the area.

A midline incision was made in the neck of the rabbit, the trachea exposed by blunt dissection, the carotid artery exposed and the cervical sympathetic nerve tissues identified. The superior cervical ganglion was located and held in mosquito forceps, while the preganglionic and postganglionic nerve fibres were cleared as far as possible. The ganglion was then excised with 1-2 cm of afferent and efferent fibres. After removal of the ganglion, an antibiotic powder containing polymixin B sulphate (5000 u/g) and neomycin base (10 mg/g) was insufflated into the wound. The wound was then closed with running sutures. After skin closure, a plastic film was sprayed over the suture line.

The effectiveness of superior cervical ganglionectomy was assessed by constriction of the pupil and early vasodilatation of the vessels of the ear on the operated side, under conditions of temperature which caused constriction of the vessels of the contralateral ear. Some animals were used two days after denervation, but most were used from 4-28 days after operation.

Effectiveness of the denervation procedure was further tested by

(i) Electrical stimulation of the isolated artery.

Field stimulation (1-5Hz 1 msec. duration, supra maximal voltage) was applied to the isolated perfused artery. Occasionally a slight response to stimulation occurred but this was always considerably smaller than the response in the control artery taken from the contralateral (untreated) ear.

(ii) Examination by fluorescence microscopy after formaldehyde treatment. (see page 2.8 for method used)

This was done routinely on segments and strips from denervated arteries. In general, segments and strips taken from the proximal part of the ear (below the first branch of the artery) showed no fluorescence. However, in some cases where more than one segment was removed from the same ear, it was necessary to remove artery from above this point. In this case sections were routinely examined at both their proximal and distal ends for fluorescence and in many such cases some fluorescence was seen. Where fluorescence was detected, the results were discarded.

(b) Reserpine.

Reserpine was used to deplete tissue stores of catecholamines. An intraperitoneal injection of 2.5 mg/Kg was given as a single dose 17-24 hrs before use. The effectiveness of the reserpine pretreatment was assessed by the methods used after denervation, i.e., by fluorescence studies, and by the effects

of electrical stimulation. Pretreatment with reserpine effectively eliminated specific catecholamine fluorescence, and the response to electrical stimulation was either eliminated, or was very much less than that seen in untreated arteries.

(c) Nialamide.

Some rabbits were pretreated with a single injection of nialamide 100 mg/Kg intraperitoneally 16 hrs before use, to inhibit the enzyme monoamine oxidase. This was an alternative method to treating the isolated artery with nialamide, and arteries from rabbits treated in this way showed no difference to those perfused for 1 hour with nialamide (see below).

PRETREATMENT OF ISOLATED ARTERIES.

Nialamide.

Arteries were perfused for 1 hour at 37°C with nialamide 100 µg/ml by adding the inhibitor to the Krebs bicarbonate bathing solution. The solution was replaced by fresh nialamide solution after 30 minutes and by drug-free Krebs bicarbonate solution after a further 30 minutes. The artery segments or strips were perfused or incubated for a further 15 minutes to 2 hours before drug treatment. This method has been shown by de la Lande and others (1970) to produce a complete disappearance of histochemically detectable MAO activity in the ear artery. In many cases one ear artery was treated in this way while the opposite ear artery was perfused with drug-free Krebs bicarbonate solution for the same time interval and used as a control.

FLUORESCENCE HISTOCHEMISTRY.

The technique for showing the location and distribution of the sympathetic nerve terminals in the central ear artery of the rabbit, under the various experimental conditions used in these studies, is based on the method developed by Falck (1962), modified by Waterson & Smale (1967).

The following is a description of the method used for the histochemical location of amines and L-dopa in the present studies.

A fresh artery segment at least 1 cm long was quickly plunged into an acetone and dry ice mixture in a glass container so that the artery was rapidly frozen. It was then placed in a numbered space in a previously chilled aluminium holder (Waterson & Hume, 1973). Several arteries were then quickly transferred to the previously cooled freeze drying apparatus so that the specimens did not thaw. The arteries were freeze-dried for 16-20 hours at temperatures from -50°C to -40°C and at pressures of 15 to 50 microns of mercury. After this procedure, the specimens were removed and placed in a glass jar containing 5 g of paraformaldehyde powder which had been stored over 34% V/V sulphuric acid at a relative humidity of 70% for at least 7 days. The glass jar was sealed and placed into an oven which had been preheated to 80°C, and allowed to remain for 1 hour.

After this time, the artery specimens were removed and vacuum infiltrated for 30 minutes with paraffin wax at a temperature of 60°C. They were then embedded in paraffin wax. Subsequently, tissue sections were cut at a thickness of 7 microns and mounted in an Entellan (Merck) and xylol mixture. The tissue sections were examined with a Leitz microscope with a dry dark field condenser. Fluorescence was produced with an HBO 200W mercury vapour lamp using a 3mm Schott BG 12 excitation filter and 490 to 530 millimicron barrier filter. A Leica camera with microscope and exposure meter attachments and Kodak Photoflure film were used for photography.

Specific catecholamine fluorescence was assessed by two independent observers, both of whom were unaware of the particular treatment of the artery. The following arbitrary system of assessment was used:

0 specific fluorescence absent

\+ specific fluorescence definitely present but sparse compared with that found in a normal artery (i.e. not treated with reserpine).

++ specific fluorescence comparable with that observed in freshly excised arteries from untreated rabbits.

+++ specific fluorescence of greater intensity than that commonly observed in freshly excised arteries from untreated rabbits.

TECHNIQUES USED IN EXPERIMENTS WITH RADIOISOTOPES.

i) Radio labelled compounds

Radio labelled compounds used were ^{3}H DA [3'-4' dihydroxyphenyl (ethylamine 1-2T) hydrochloride], ^{14}C DA [3'-4' dihydroxyphenyl (ethylamine-1-^{14}C) hydrochloride] ^{3}HNA [(±)2-(3'-4' dihydroxyphenyl) 2-hydroxyethylamine-7 T hydrochloride].

All labelled material used was supplied by the Radiochemical Centre Amersham.

Purity of labelled DA was checked before use, after dilution from the manufacturer's sample, by paper chromatography using two systems (i) Whatman P81 cellulose phosphate ion exchange paper developed with isopropanol 1.5 : ammonium acetate (pH 6.8) 1 and (ii) Whatman No. 1 paper developed with n-butanol : water : acetic acid 12:5:3. Co-chromatography with the authentic unlabelled compound was taken as evidence of purity.

The ^{3}HNA was purified by adsorption onto alumina, elution with perchloric acid (0.05N), and passage through a Dowex 50 ion exchange column eluted with 1.0N hydrochloric acid. (This further purification was found necessary after evidence was found that a labelled impurity was present in the commercial ^{3}HNA on arrival at the laboratory) (Head, 1973).

ii) Incubation with labelled compounds.

Artery segments were excised as previously described, and tied at one end to a polythene cannula approximately 3" long to act as support. Sometimes the free end was also tied off. Arteries were not perfused. Drug pretreatments were carried out by suspending the artery segments in a reservoir of Krebs bicarbonate solution bubbled with CO_2/O_2 and kept at a constant temperature (usually 37°C). Incubation with radioactive solutions was carried out by suspending the artery segment in a conical tube in approximately 1 ml of a gassed solution of Krebs bicarbonate containing labelled material so that the segment was submerged at all times in the solution.

iii) Washout methods.

Segments were transferred from the incubation medium to a series of 1 ml volumes of gassed Krebs bicarbonate solution maintained at a predetermined temperature, usually 37°C. The segment was moved from one wash bath to the next at predetermined intervals.

In some experiments this method was varied so that the initial two wash baths contained approximately 100 ml Krebs bicarbonate solution.

iv) Extraction of catecholamines from the tissue.

Catecholamines were extracted from arteries with 0.1N hydrochloric acid. Segments were extracted with 3 volumes of acid at 37°C with shaking. Total extraction time was 2 hours. Preliminary experiments showed that this method provided an extraction efficiency of 98-99%, as opposed to 94% obtained by grinding the tissue with perchloric acid.

v) Chromatography.

Chromatography of labelled and unlabelled catecholamines and metabolites was carried out on 3 x 57 cm strips of Whatman P81 chromatography paper (cellulose phosphate ion exchange) (Roberts, 1962). Tissue extracts of pure DA, NA and

metabolites, dissolved in ethanol or ethanol/acetone, were applied to the paper and allowed to dry without heat. Papers were equilibrated with the developing solvent for 1 hour, then developed with isopropanol 0.2 M : ammonium acetate pH 6.5, 1.5 : 2 (solvent modified from Roberts, 1962) over 12-16 hours to a distance of 46 cm, at room temperature, in the dark. The air in the developing tank was previously replaced with nitrogen gas. The papers were then allowed to dry in air at room temperature. Spots were visualized by U.V. light or by a light spraying with a diazo-p-nitroaniline reagent (Appendix 2). A pencil tracing was then taken of the chromatogram.

Fig. 7.1 shows the relative positions of the catecholamines and metabolites using diazo-p-nitroaniline reagent. The sensitivity of the method for non-radioactive compounds was 10-20 µg. A source of variation in Rf values was found; this was due to the high salt concentration present in some extracts, which caused high Rf values for some of the compounds. To avoid inaccuracy due to these effects pure compounds were added to radioactive tissue extracts, to act as markers.

vi) *Radio counting methods.*

All samples were counted in a Packard Tri-Carb Liquid Scintillation Counter in precounted pots at 16°C. Counting time for most samples was 5 minutes, precounting time for pots was also 5 minutes.

vii) *Preparation of samples for counting.*

(a) Direct counting. 0.5 to 1 ml samples were added to 20 ml volumes of Brays Scintillant (see Appendix 2).

(b) Counting of samples from radiochromatograms.

(i) ^{14}C DA. Dried chromatograms were sectioned transversely into 1 cm or 0.5 cm segments which were placed directly into toluene Scintillant (Appendix 2).

(ii) ^{3}H DA, ^{3}HNA and ^{3}H metabolites.
The dried chromatogram was sectioned transversely as above. The segments were further cut into small pieces and were eluted by shaking overnight in 1 ml 0.01N hydrochloric acid at room temperature in small vials. The eluate and paper was then added to 20 ml Brays scintillant for counting.

Preliminary experiments showed that neither the presence of the spray reagent (diazotised p-nitro-aniline) nor the 1 ml of 0.01 N acid affected the efficiency of counting in this scintillant.

(c) Counting of tissue samples.

Tissue residues after acid extraction (see above) were solubilised in 0.3 ml NCS reagent in a glass counting vial overnight at 37°C. 10 ml toluene scintillant (Appendix 2) was added and the sample counted as before.

CHAPTER THREE

ACTIONS OF DOPAMINE IN THE RABBIT EAR ARTERY

CHAPTER 3

INTRODUCTION

Previous work in this laboratory by de la Lande and co-workers has established that the central artery of the rabbit ear is richly supplied with sympathetic nerves, a knowledge of the morphology of which is important in the understanding of the response to exogenous NA. The sympathetic nerve endings lie in a plexus at the media-adventitia border of the artery. When NA is applied intraluminally, the sensitivity is approximately 10-20 times greater than when NA is applied by the extraluminal route of application. De la Lande and Waterson (1967) have suggested that this is due to uptake of extraluminally applied NA by the plexus of sympathetic nerve endings before it can reach the receptors in the smooth muscle in the media. This explanation is supported by the evidence that cocaine, which prevents uptake into sympathetic nerves, or chronic denervation, which causes the nerve endings to degenerate, both potentiate extraluminal NA 10-20 times, but affect sensitivity to intraluminal NA very little, the result being that the extraluminal to intraluminal difference tends to disappear.

It was considered that, since DA, the immediate precursor of NA (Blaschko, 1939) is taken up by sympathetic nerves (Burgen and Iversen, 1965), it may have similar characteristics to NA on this preparation. DA has been found to activate both α and β adrenergic receptors in many tissues, to produce excitatory and inhibitory effects respectively. There is also evidence to suggest that it has a physiological action in its own right, not mediated by the adrenergic receptors. This subject has been reviewed extensively by Hornykiewicz (1971).

Responses of the rabbit ear artery to DA were studied by Campbell and Farmer (1968). They found that neither

denervation nor cocaine had any significant potentiating effect on DA. They attributed this result to the method of administration, since it was by injection of a small volume into the stream of perfusion fluid. The resulting exposure may have been too short to allow equilibrium conditions to occur.

However, there is other evidence that DA like NA is taken up by sympathetic nerves. Burgen and Iversen (1965) using inhibitor kinetics, showed that DA had a high affinity for the catecholamine uptake system of the sympathetic neurone in the rat heart, and Peskar and others (1968) showed that the uptake of DA followed Michaelis-Menten kinetics with a Km of 0.68×10^{-6}M and Vmax of 1.45×10^{-9} mol/g/min which compared with the figure for NA (Iversen, 1963) Km (-) NA 0.27×10^{-6}M, Vmax 1.16×10^{-9} mol/g/min. Nevertheless, conflicting reports of the effects of cocaine and denervation on responses to DA have appeared in the literature over a number of years (Tainter, 1930; Hamet, 1931; Gurd, 1937; Campbell and Farmer, 1968; Tsai and others, 1967; Birmingham and others, 1970; Hamilton, 1972). In some tissues or systems responses to DA were potentiated by cocaine or denervation, in some they were unchanged, in others they were depressed. Tsai and others (1967) explained these conflicting effects by suggesting that at least in the cat nictitating membrane, DA has three actions: (i) a direct action on α or β adrenergic receptors; (ii) a release of endogenous NA (i.e. indirect effect); (iii) an impairment of the reuptake of the released NA (i.e. cocaine type effect).

This suggested combination of direct and indirect effects, one of which may be more important than the other in a particular tissue, could account for some of the differing effects of cocaine and denervation reported above, since cocaine has been known to potentiate direct effects of many amines, and to depress

indirect effects. Campbell and Farmer (1968), found no evidence of an indirect component in the action of DA in the rabbit ear artery. However, their study does not take into account the possibility that the route of application (intraluminal or extraluminal) of DA to the artery may influence the extent to which its action is direct or indirect. Thus Campbell and Farmer's results might be explained simply on the basis that DA applied intraluminally, failed to reach the nerve terminals located at the media-adventitia border.

In the present study, an attempt was made to elucidate the action of DA in the rabbit ear artery, in this case using (i) isolated perfused segments cannulated at both ends such that drugs could be applied separately to either the intraluminal (intimal) surface, or the extraluminal (adventitial) surface (de la Lande and others, 1966), or (ii) helical strips cut from an isolated segment (de la Lande and Urquilla, 1969). In this latter preparation no attempt was made to distinguish between drugs applied to the intimal or adventitial surface.

The aim of the work was to:

(a) Investigate the nature of the response to DA in the normal artery, and compare this with the response to NA in this preparation.

(b) To evaluate the influence of the sympathetic nerves on the response to DA by studying the effects on the response of chronic denervation, cocaine and reserpine.

METHODS

The techniques used in the experiments described in this chapter are fully annotated in Chapter Two, General Methods.

RESULTS

1. *RESPONSES to DOPAMINE*

(a) Perfused segments.

The response to sustained application of DA in the untreated rabbit ear artery was found to be essentially the same by either the extraluminal or the intraluminal route of application. It comprised a dose-dependent, rapid rise in perfusion pressure, indicating constriction. The constriction commenced within 2 minutes of intraluminal application, and reached its steady state level after a further 2-6 minutes. The constriction was usually well maintained at or near its maximum level, (Fig. 1a) but occasionally the response faded rapidly from its maximum value despite continued contact of drug and artery (Fig. 1b). Individual arteries tended to display the same pattern of response throughout the experiment. After washout of the dopamine, the return to resting perfusion pressure was relatively rapid; i.e. of the order of 5-7 minutes or less. These responses were abolished or greatly depressed in the presence of the α-adrenergic antagonist, phentolamine, in a concentration of 0.2 - 1.0 μg/ml.

Attempts to demonstrate a dilator action of DA in the perfused segment were made by injecting DA into the perfusion fluid of segments, the tone of which was previously raised and maintained by histamine present in the extraluminal bathing solution. In all preparations, the injection of dopamine caused further constriction (Fig. 2a). This constriction was abolished by phentolamine. In 3 arteries there was no further response to DA after the phentolamine. However in another 2 arteries, it was possible that DA did cause slight dilatation, but it was difficult to distinguish this effect

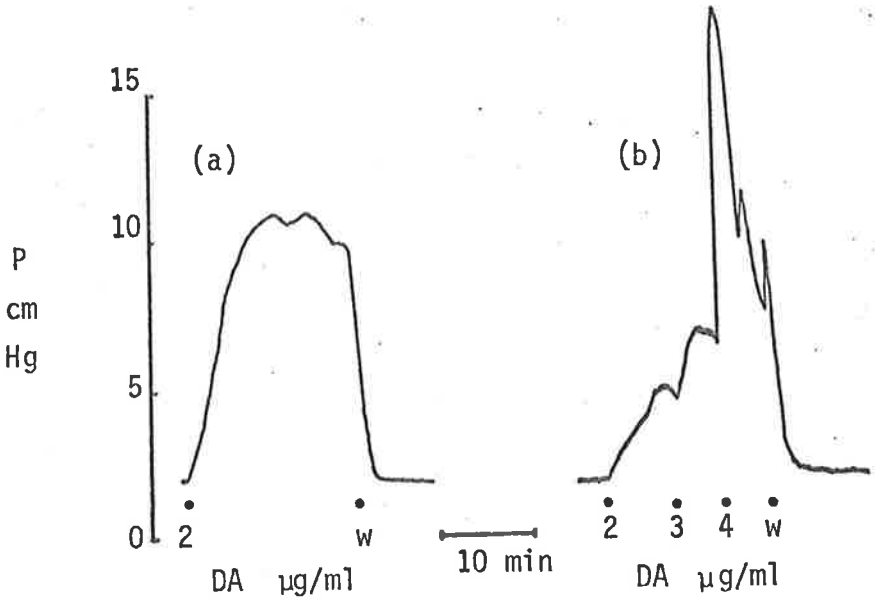


Figure 3.1

Types of response to extraluminal dopamine in perfused artery segments.

(a) Shows a response which was well maintained near its maximum level.

(b) A response which faded rapidly from its maximum value despite continued contact with the drug.

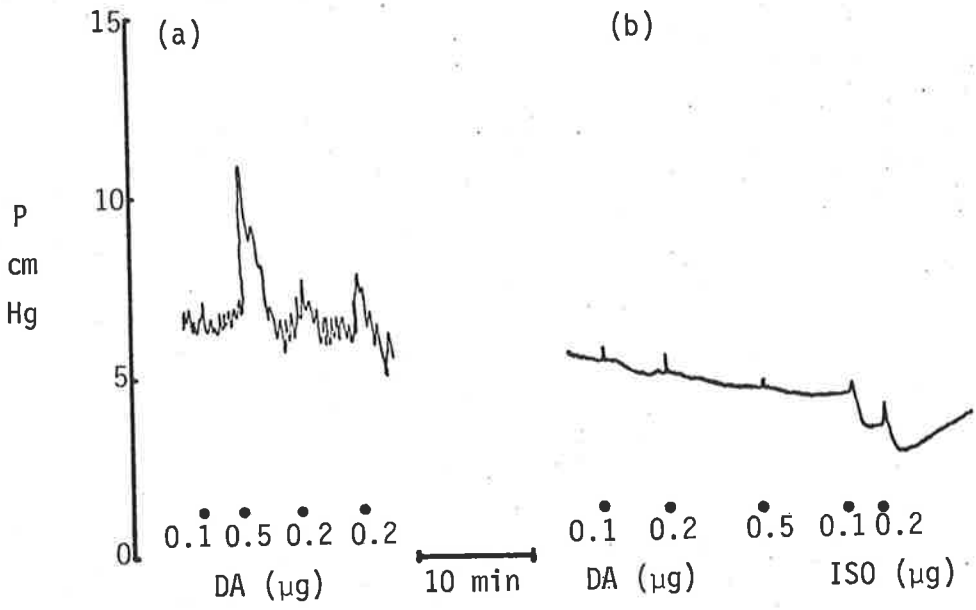


Figure 3.2

Responses to dopamine and isoprenaline injected intraluminally into perfused artery segments, the tone of which was previously raised with histamine (0.3 - 0.4 µg/ml).

(a) Constrictor responses to low doses of dopamine.

(b) Response to similar doses of dopamine after phentolamine 0.25 µg/ml. Dilator responses to isoprenaline (ISO) are also shown.

from the spontaneous fluctuations in tone. Wherever tested, isoprenaline (0.1 - 0.2 µg) caused an unequivocal and pronounced vasodilator response under the same conditions (Fig. 2b). From these results it was concluded that a dilator effect of DA, if present was small compared with its constrictor action; i.e. the latter action would provide a reasonable guide to the concentration of DA at its receptors. For this reason, the measure of the sensitivity of the artery to DA was based on the magnitude of the constrictor response.

Cumulative dose - response curves were obtained to both extra and intraluminal DA on the same arteries; threshold doses being obtained in the dose range 0.3 - 1.0 µg/ml. Ratios of equipotent doses were assessed at an arbitrarily chosen response level of 60 mm Hg, since it was not possible to obtain complete dose response curves in the perfused segment. This was because the responses became erratic at high perfusion pressures (e.g. pressures greater than 200 mm Hg) as previously noted by de la Lande and Jellett (1972).

Table 1 shows the mean extraluminal to intraluminal ratios for DA in 13 arteries, and also for NA in 5 of these. It can be seen that the artery is approximately equisensitive to DA whether the route of administration is extraluminal (EL) or intraluminal (IL) i.e. the EL/IL ratio approaches 1. In contrast to this, the EL/IL ratios for NA showed a mean of 14.4 confirming earlier reports that NA is considerably more potent when applied intraluminally (de la Lande and Waterson, 1967).

(b) Helical strips.

Since the above data indicated that the sensitivity to DA in the rabbit ear artery was independent of its route of administration, it was decided to use the helical

TABLE 3:1

Effect of denervation on the extraluminal (EL) to intraluminal (IL) ratios of dopamine and noradrenaline in the rabbit ear artery segment.

	NORMAL	DENERVATED
DOPAMINE EL/IL Ratio	0.99* 0.87 - 1.12 (13)	0.96* 0.77 - 1.2 (9)
NORADRENALINE EL/IL Ratio	14.4 11.6 - 18.0 (5)	1.00 0.83 - 1.2 (5)

Values quoted are the geometric means of the potency ratios. A separate ratio was determined for each artery. The values immediately below the mean refer to the mean ± s.e.; and the figure in brackets refers to the number of experiments.

Potency ratios were calculated at a response level of 60 mm Hg.

* Means do not differ significantly (t-test, P >0.05)

strip preparation for much of the quantitative work in this study. The advantage of the helical strip preparation is that a maximum response, and hence full dose response curves, can be obtained. This permits the sensitivities of the different arteries to DA to be expressed in terms of the concentrations producing a fixed percentage of the maximum response (e.g. ED 50).

The response to sustained application of DA in the helical strip was a contraction which commenced within a few seconds of application of the DA, and reached steady state after a further 1-4 minutes. In a manner similar to the perfused segment the contraction was either well maintained at or near its maximum level or, less frequently, faded rapidly from its maximum level despite continued contact of drug and strip. After washout of the DA, the artery relaxed quickly at first, and then more slowly as the resting length was approached. Sometimes the artery strip continued to relax to below its previous resting level. At 3 μg/ml (approx. ED 50-70) the mean time to reach 5% residual response was approx. 6 minutes. Threshold doses of DA were in the range 0.1 - 0.3 μg/ml, and maxima occurred at 100-300 μg/ml. Responses were recorded in two ways, firstly, as a percentage of the maximum response, and secondly as the percentage shortening of the strip (details of these are given in Chapter 2.5). The latter method gave an index of the contractile performance of each artery strip. When two dose response curves were elicited in the same strip it was often found that during the second (cumulative) application of the agent there was a loss of sensitivity. This was reflected in a diminution of the maximum response (Fig. 3), and a shift to the right of the dose response curve even when the two curves were calculated in terms of their separate maximum responses

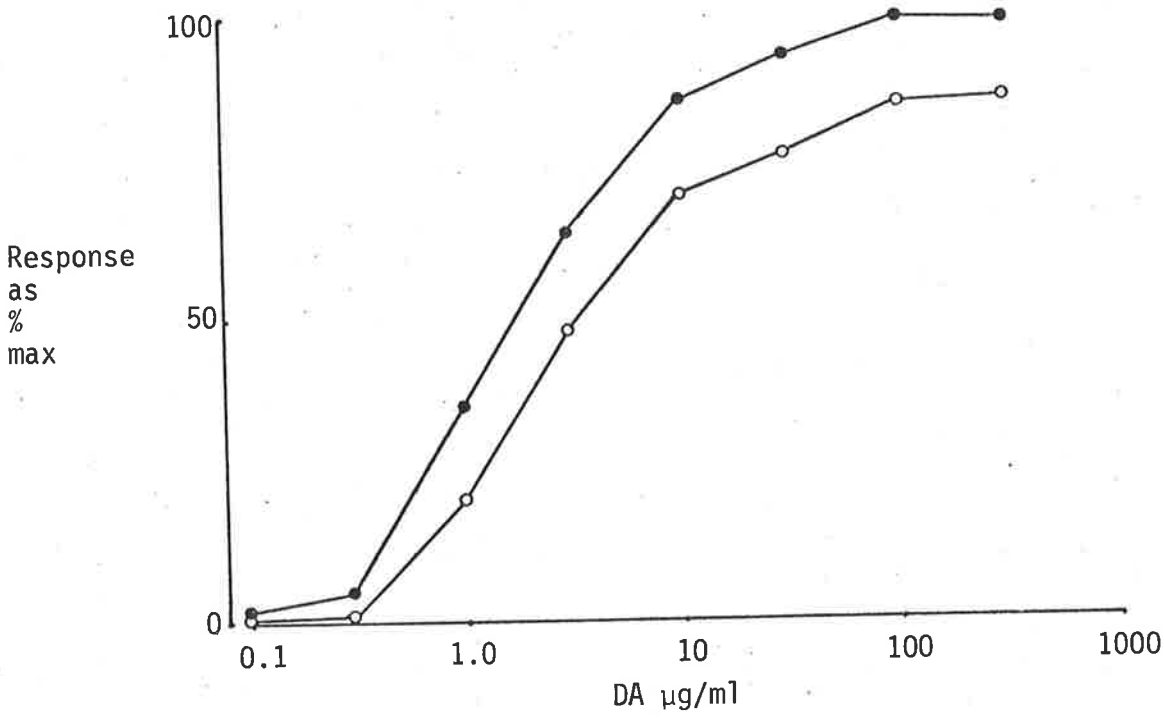


Figure 3.3

Effect of repeated cumulative dose response curves to dopamine in the artery helical strip.

(a) Means of 4 experiments in which the second cumulative dose response curve (open circles) was calculated as a percentage of the first cumulative dose response curve (closed circles), showing the loss of sensitivity seen after the first maximum response.

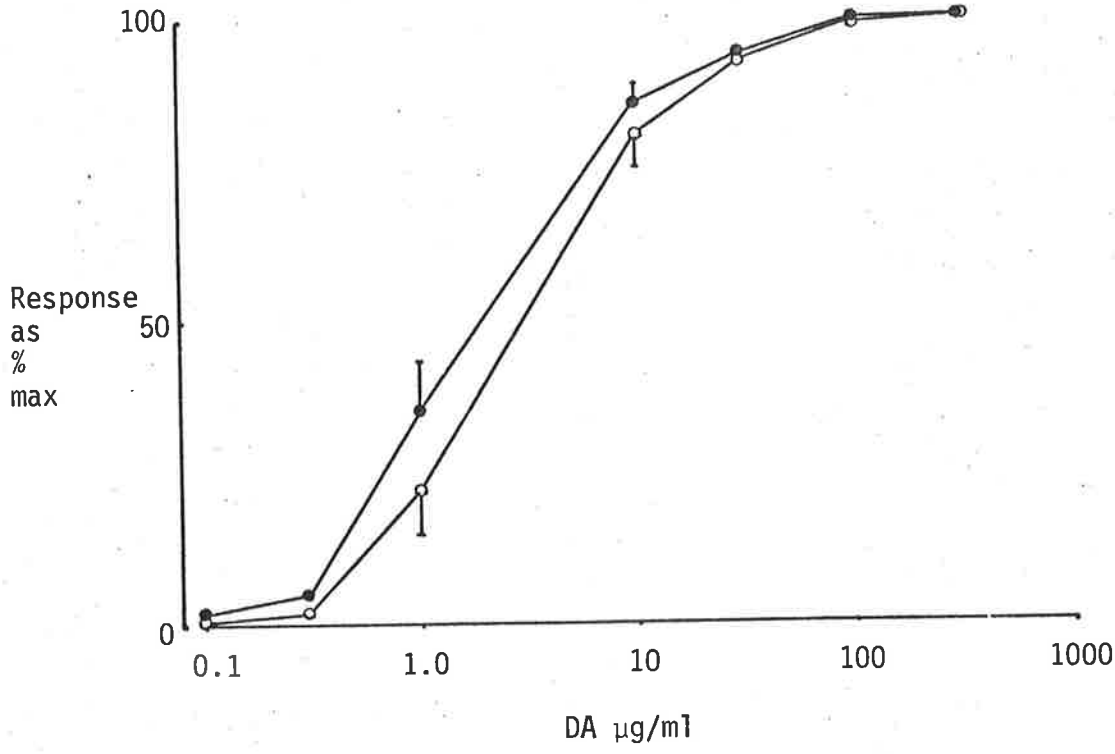


Figure 3.4

Effect of repeated cumulative dose response curves to dopamine in the artery helical strip.

(b) Means ± s.e. of four experiments in which the first (closed circles) and second (open circles) cumulative dose response curves to dopamine were each calculated as percentages of their separate maximum responses.

The mean values of the responses are significantly different at 1 and 10 µg/ml dopamine (t-test for paired observations $P<0.05$).

(Fig. 4). Thus in 5 experiments, the mean percentage shortening at maximum of the second dose response curve was 88.9% of that of the first; and in 4 experiments the geometric mean of the ratios of $\frac{\text{ED 50 1st application DA}}{\text{ED 50 2nd application DA}}$ was 0.66.

In many experiments, such a decrease in sensitivity was small compared with the effect of the particular treatment e.g. the effects of nialamide as shown subsequently. However, wherever practicable, paired arteries were employed, one of which served as the control for the treated artery, and the maximum response to DA was not elicited until the end of the experiment.

2. *EFFECT OF CHRONIC DENERVATION.*

The effect of chronic denervation on the sensitivity to DA was studied in both perfused segments and helical strips from rabbits the left ears of which were denervated by removal of the homolateral superior cervical ganglion 5 to 33 days prior to the experiment (see Methods 2.6).

Table 2 shows that in 6 perfused segments, measured at 60 mm Hg, the denervated arteries were slightly more sensitive to extraluminal and intraluminal DA than their contralateral controls, however, statistical tests showed that this increase in sensitivity was not significant (t-test, $P > 0.05$). The sensitivity ratio of extraluminal DA to intraluminal DA in the denervated arteries (0.96), was not significantly different to that in the innervated control arteries (0.99). However, in 5 of the preceding experiments, the increased sensitivity in the denervated artery to NA was 26 fold for extraluminal and 1.8 for intraluminal NA.

In helical strips, chronic denervation shifted the DA response curve to the left and decreased its slope

TABLE 3:2

Effect of denervation and cocaine on the extraluminal and intraluminal potencies of dopamine and noradrenaline in the rabbit ear artery segment.

	DENERVATION			COCAINE	
	STRIP	SEGMENT		SEGMENT	
		EXTRALUMINAL	INTRALUMINAL	EXTRALUMINAL	INTRALUMINAL
DOPAMINE	2.2 1.7-2.7 (5)	1.55 1.07-2.24 (6)	1.47 1.01-2.14 (6)	1.2 1.1-1.3 (9)	1.8 1.5-2.2 (6)
NORADRENALINE		26.1 23.4-29.2 (5)	1.81 1.41-2.32 (5)	11.9 10.5-13.3 (8)	

The values quoted are the geometric means of the potency ratios for paired arteries (denervated) and the same artery (cocaine).

Potency ratio = $\frac{\text{ED60mm untreated}}{\text{ED60mm treated}}$ (SEGMENTS) $\frac{\text{ED50 Untreated}}{\text{ED50 treated}}$ (STRIP

ED 60 mm refers to the dose which produces a response of 60 mm Hg in the perfused segment. In normal arteries these were: extra- and intraluminal DA 13 ± 2 and 14 ± 4 x 10^{-6}M respectively, and extra- and intraluminal NA 1.9 ± 0.5 and 0.087 ± 0.017 x 10^{-6}M respectively. The ED 50 in strips was 16 ± 4 x 10^{-6}M for DA and 0.83 ± 0.31 x 10^{-6}M for noradrenaline.

The figures immediately below the mean of the potency ratios are the mean ± se; and the figure in brackets indicates the number of experiments.

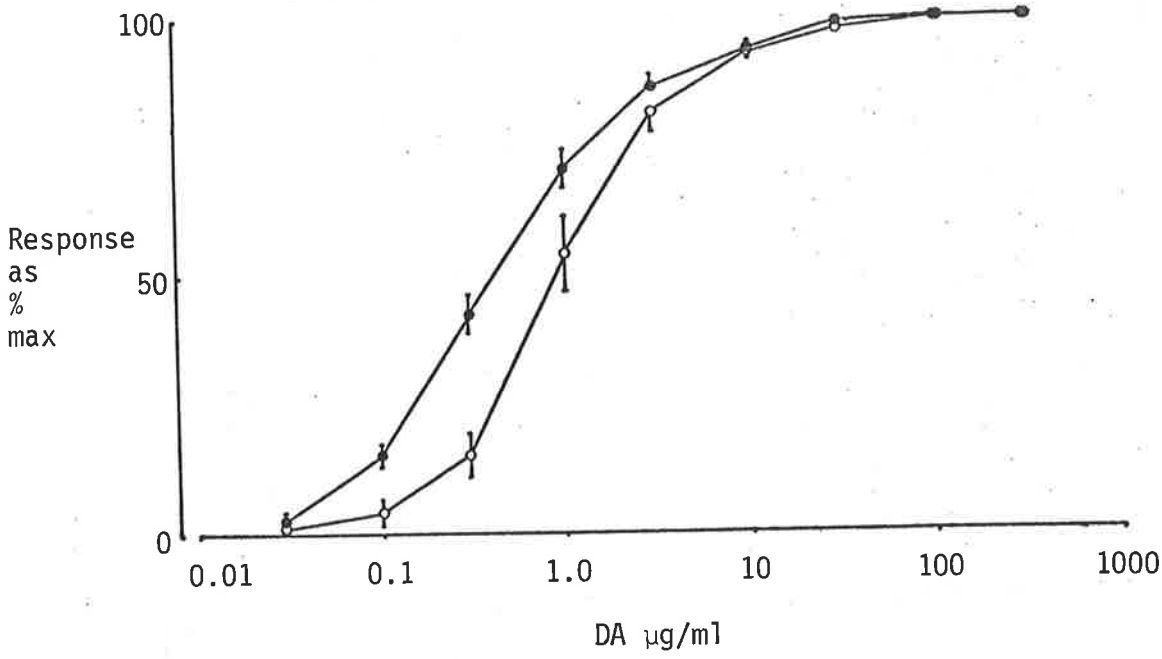


Figure 3.5

The effect of denervation on the sensitivity to dopamine in artery helical strips.

Values shown are the means ± s.e. of five experiments in denervated (closed circles) and innervated (open circles) paired arteries. The slopes of the dose response curves are 51.5 ± 4.1 (denervated) and 69.5 ± 3.6 (innervated). The difference between slopes was found to be significant by paired t-test ($P<0.001$). The slopes were calculated from the formula of Langer and Trendelenburg (1969) where

$$\text{Slope} = \frac{50}{\log \text{ED } 80 - \log \text{ED } 30}.$$

(Fig. 5). The increase in sensitivity was 2-3 fold (Table 2) and was statistically significant when estimated in the range ED 5 to ED 70. However, there was no difference between the maximum responses for the two groups of arteries.

3. *EFFECT OF COCAINE.*

The effect of cocaine on DA sensitivity was tested in perfused segments only. Dose response curves to intraluminal DA and extraluminal DA were obtained in the absence and presence of cocaine 1 µg/ml, and the sensitivity ratio estimated at a response level of 60 mm Hg. Fig. 6 shows an example of dose response curves to DA in the presence and absence of cocaine. The results, summarised in Table 2, show that the mean increases in sensitivity due to cocaine, to both extraluminal DA (in 9 arteries) and intraluminal DA (in 6 arteries) were very slight (less than 2 fold). In contrast, the effects of cocaine on the sensitivity to extraluminal NA, estimated in 8 of these arteries, were marked, amounting to a 12 fold increase, in agreement with results previously obtained in this laboratory (de la Lande and Waterson, 1967).

Since the effects of cocaine on the sensitivity to DA were slight, a further test was applied. In two additional arteries, a steady state response to DA was obtained at a dose level which gave a response of about 60 mm Hg. Cocaine 1 µg/ml was then added to the bath. Potentiation was manifested as a further increase in perfusion pressure (Fig. 7). Cocaine caused a definite increase in perfusion pressure, from which it was estimated that it had increased the sensitivity to extraluminal DA by factors of 1.15 and 1.04, and to intraluminal DA by factors of 2.08 and 1.25. Since the results in

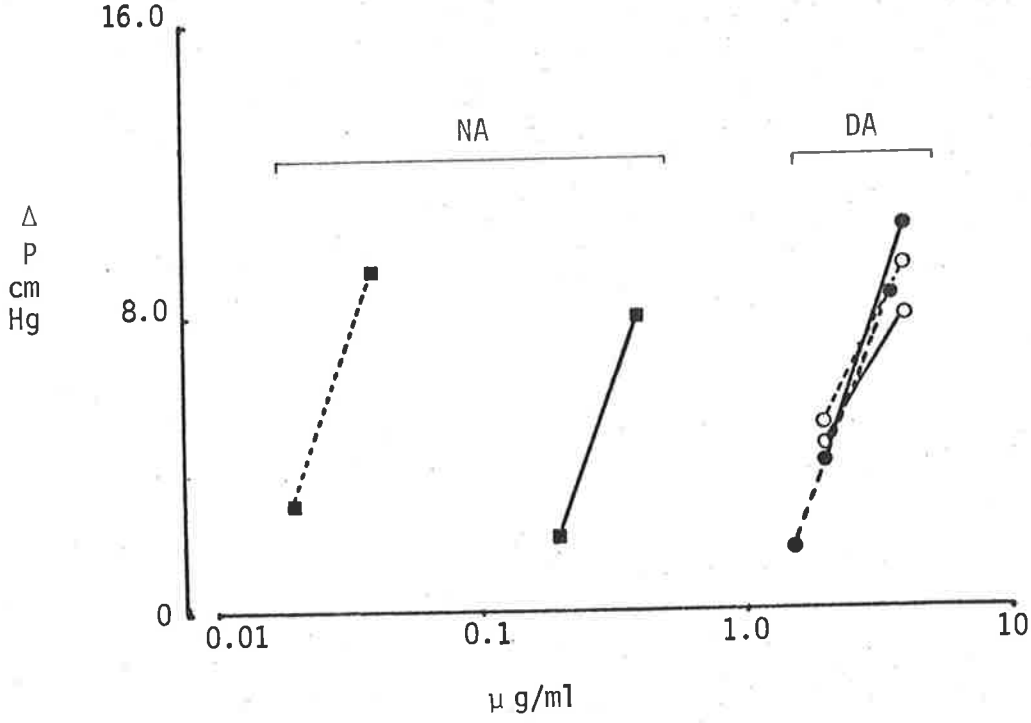


Figure 3.6

The effect of cocaine on the sensitivity to dopamine and noradrenaline in perfused artery segments.

Typical dose response curves to extraluminal noradrenaline (squares) and intra- and extra-luminal dopamine (open and closed circles respectively), in the absence (solid line) and presence (dotted line) of cocaine (1 μg/ml).

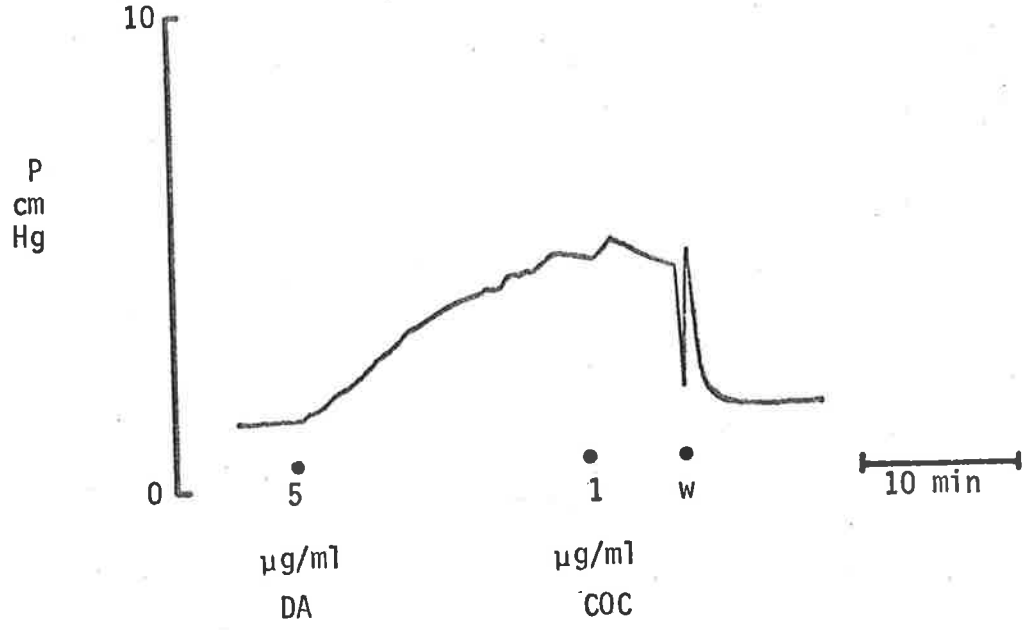


Figure 3.7

The effect of cocaine on the sensitivity to dopamine in the perfused artery segment.

Recording of an experiment in which cocaine 1 µg/ml was added to the perfused fluid and bath solution during the steady state response to extraluminal dopamine.

these two experiments were essentially in agreement with those described above, no further experiments of this nature were carried out.

Histochemical observations on the effect of cocaine on DA uptake into nerve terminals.

In these experiments, artery segments from rabbits pretreated with reserpine and nialamide were incubated with varying concentrations of extraluminal DA. In some experiments, cocaine 1 µg/ml was present 10 minutes prior to, and during the exposure to DA. After incubation for 30 minutes, the drugs were washed out for 10 minutes, after which the segments were frozen for histochemistry. The technique used for the drug pretreatment and histochemical analysis is described in Methods 2.7 and 2.8.

Table 3 shows that in 2 preliminary experiments, specific monoamine fluorescence was faint in arteries which had been incubated with DA 0.01 µg/ml for 30 minutes, and appeared to increase with increasing concentrations of DA up to 0.5 µg/ml. Further experiments showed that the fluorescence achieved by incubation with 0.3 µg/ml DA could not be distinguished from that achieved by incubation with 3 µg/ml DA, suggesting that the uptake had become maximal. In the cocaine treated arteries, the fluorescence appeared less intense when the DA concentration was below 0.5 µg/ml, but could not be distinguished from that in untreated arteries when the concentration of DA was greater than 1 µg/ml. Thus it appeared that cocaine exerted an inhibitory effect on the reappearance of monoamine fluorescence in reserpine and nialamide treated arteries when these were incubated with DA at concentrations at or below 0.3 to 0.5 µg/ml, but had little effect at the higher concentrations of DA (1-3 µg/ml).

TABLE 3:3

The effect of cocaine on the reappearance of catecholamine fluorescence, after exposure to DA in nialamide and reserpine pretreated arteries.

	DA µg/ml							
EXPERIMENT	0.01 - 0.02		0.05 - 0.1		0.3 - 0.5		1 - 5	
	Control	Cocaine	Control	Cocaine	Control	Cocaine	Control	Cocaine
1	0-+		+		++		++	
2	+-++	0-+	++	+	+++	+-++	+++	+++
3					++-+++	+	+++	++
4					+++	+-++	+++	+++
5					+++	+	+++	++
6					+++	+	+++	+++

Key to Symbols Used:

0 No specific catecholamine fluorescence.
\+ Fluorescence present but not equally distributed around plexus.
++ Catecholamine fluorescence in nerve terminals equivalent to that in normal arteries.
+++ Catecholamine fluorescence visible in adventitia as well as in nerve terminals.

Data presented is from 6 experiments in which pairs of arteries, one of which was treated with cocaine, were incubated with varying concentrations of dopamine for 30 minutes, followed by 10 minutes washout before histochemical treatment. All arteries were pretreated with reserpine and nialamide. Assessment of the degree of fluorescence was carried out by two independent observers, both of whom were unaware of the experimental protocol.

4. *EFFECT OF RESERPINE PRETREATMENT.*

This was tested in helical strips only. Dose response curves to DA were obtained in strips from 9 rabbits which had been pretreated with reserpine. This data was compared statistically with data from 10 untreated strips from a different group of animals. The latter 10 experiments were carried out over the same time period, using similar animals to those used for reserpine experiments.

Responses to DA in the reserpine pretreated group were similar in shape and time course to those in the untreated group. The sensitivity to DA in the reserpine pretreated group did not significantly differ at any of the dose levels tested from that in the untreated group (t-test, $P > 0.05$) (Fig. 8).

5. *RELATIVE POTENCY DA TO NA.*

In the course of the experiments described in this chapter, sufficient data was accumulated to provide a precise estimate of the ratio of the potencies of DA to NA. Since the relevant data has formed only an incidental part of the results presented to date, it is represented in a concise form at this stage. In all, NA and DA were compared in a total of 9 artery segments using extraluminal application, and in 5 of these using intraluminal application, 5 chronically denervated artery segments using both extra and intraluminal application of the drugs, and in 12 strips from untreated arteries. Dose response curves for DA and NA were approximately parallel both in the segments, and in the strips.

The relative potencies of DA and NA in denervated and control segments and strips are summarized in Table 4. It will be seen that (a) in the innervated artery, although NA was always more potent than DA, the extraluminal

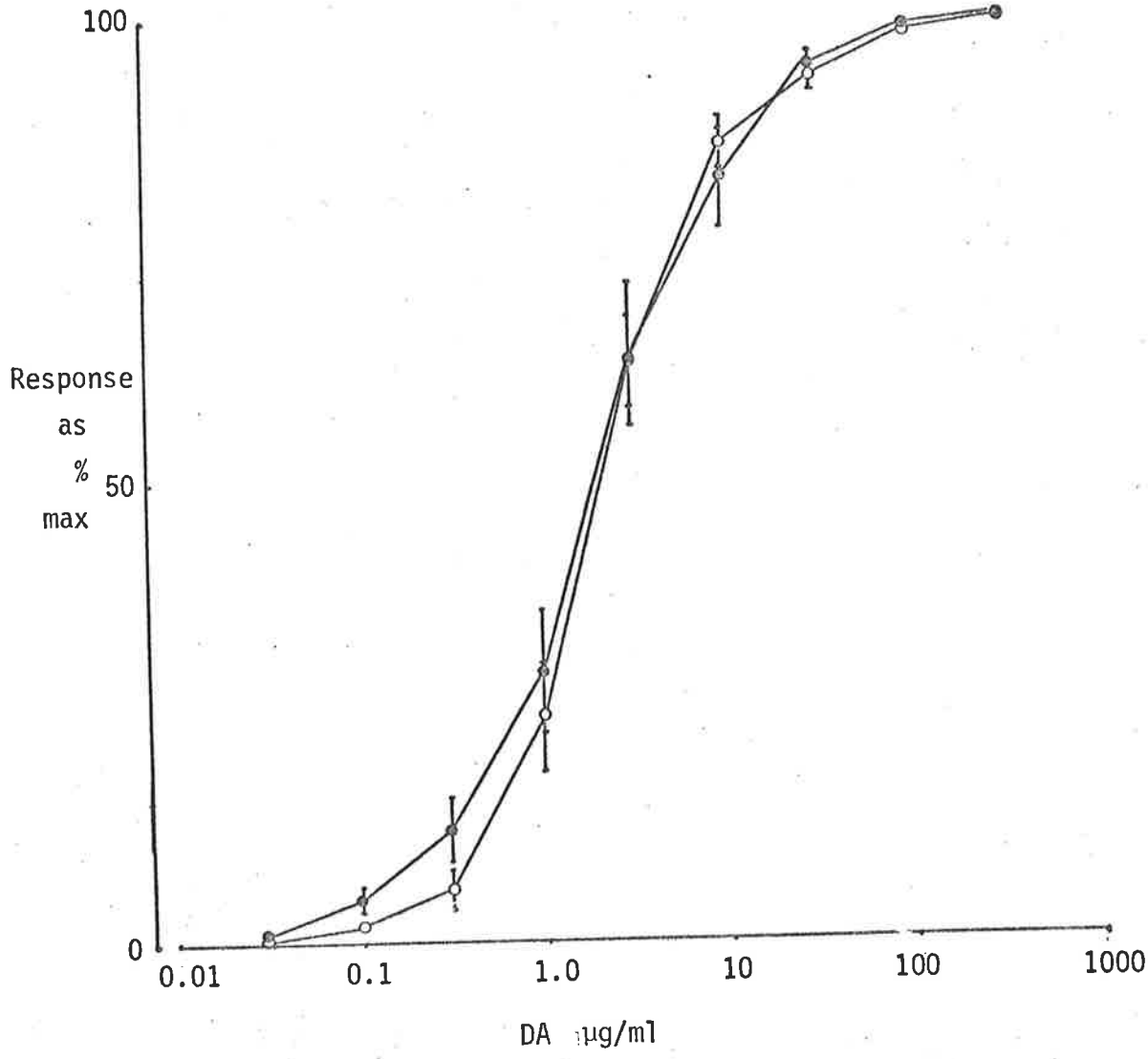


Figure 3.8

The effect of reserpine on the sensitivity to dopamine in the artery helical strip.

Values shown are the mean responses ± s.e. for 10 untreated arteries (open circles) and 9 reserpine pretreated arteries (closed circles). The values at each dose level do not differ significantly (t-test, $P > 0.05$).

TABLE 3:4

Relative potencies of dopamine and noradrenaline in the normal and denervated rabbit ear artery segment and strip.

	SEGMENT		STRIP
	EXTRALUMINAL	INTRALUMINAL	
NORMAL	7.8 6.7 - 9.0 (9)	142* 102 - 198 (5)	32.9 23.6 - 45.8 (12)
DENERVATED	110* 90 - 134 (5)	150* 118 - 189 (5)	

*Values do not differ significantly from one another (Analysis of Variance, $P > 0.05$)

Values quoted are the geometric means of the potency ratios determined at a response level of 60 mm Hg for segments, and at the ED 50 in strips. A separate ratio was determined for each artery. The number of arteries is shown in brackets. The values immediately below the mean refer to mean ± se. See legend for Table 3:2 for mean ± se of the equieffective concentrations of DA and NA which give a response of 60 mm Hg and ED 50 in strips.

potency ratio was much less than the ratio of intraluminal potencies, and (b) this difference did not occur in the denervated arteries, where the intraluminal ratio of potencies (150) was not significantly different from the extraluminal ratio of potencies (110). These latter ratios did not differ significantly from the ratio of the intraluminal potencies (142) in the innervated preparation when analysed by Analysis of Variance ($P > 0.05$).

D I S C U S S I O N

The main response of the rabbit ear artery to DA is constriction. This is probably mediated by α adrenergic receptors, since it can be blocked by the α adrenergic blocking agent, phentolamine. In contrast, attempts to demonstrate a dilator response under conditions where isoprenaline caused enequivocal dilatation were generally unsatisfactory. It would seem that the ear artery contains relatively few of the DA receptors which are believed to mediate dilatation in the dog renal and mesenteric arteries (McNay and others, 1963, 1965).

In contrast to NA, the action of DA on the rabbit ear artery appears to be relatively independent of the sympathetic nerves. Firstly, there is no significant difference in the potency to DA whether it is applied intraluminally or extraluminally. This is in contrast to NA which is 10-20 times less potent extraluminally. Elimination of the influence of the sympathetic nerves, by chronic denervation or by cocaine treatment has little effect on the sensitivity to extraluminal or intraluminal DA, whereas with NA, these measures have a selective effect on the extraluminal potency, causing it to increase about 10-20 times. These results for NA have been interpreted by de la Lande and Waterson (1967) to mean that the sympathetic nerves, which are situated in this preparation

at the media-adventitia border, act as a pre-receptor site of uptake (and therefore loss) of extraluminal NA, and play a large part in the determination of the concentration of extraluminal NA which finally reaches the receptors. It is of interest that in the helical strip, the mean relative potency of DA to NA was 32.9, estimated at the ED 50 level. This is little different from the geometric mean (35.0) of the extraluminal and intraluminal ratios of the potencies of the two amines (i.e. 7.8 and 142 respectively) in the innervated segment. On the other hand, the nerves have little influence on the receptor concentration of intraluminal NA. The data suggests therefore, that the same conclusion should apply to DA, i.e. that the sympathetic nerves play little part in the determination of the concentration of DA at the receptors, whether it is applied intra- or extraluminally.

Although the above data suggests that neuronal uptake is unimportant in the pharmacological action of DA, the histochemical evidence presented in this chapter does indicate that neuronal uptake of DA can occur. This has also been shown by other workers, (Burgen and Iversen, 1965; and Peskar and others, 1968). Monoamine fluorescence first appears at a DA concentration of about 0.01 µg/ml, and steadily increases with increasing concentration up to a level of about 0.5 µg/ml. Beyond this concentration it is difficult to assess further increases in fluorescence intensity. In the presence of cocaine (1µg/ml), the fluorescence intensity is less at DA concentrations up to about 0.3 - 0.5 µg/ml. However, at higher levels (1-3 µg/ml) the fluorescence intensity is similar in cocaine treated or untreated preparations. From the evidence it would appear that uptake is saturated at concentrations of DA exceeding about 0.5 µg/ml. The perfused artery segment is sensitive to DA at a threshold concentration of about 0.3-1.0 µg/ml

and thus at the concentrations where DA is effective as an agonist, it is at a concentration above the saturation level for the $Uptake_1$ system. It follows, therefore, that uptake by nerves will have little effect on the concentration of DA which reaches the receptors. This argument is borne out by the evidence that, in helical strips where the threshold sensitivity is 10 fold greater than in perfused segments, denervation caused a selectively greater increase in sensitivity at the lower end of the dose response curve, than at higher levels, and changed the slope of the curve. Langer and Trendelenburg (1969) showed that the neuronal uptake of a drug has a profound effect on the slope of its dose response curve, when the drug in question is at a concentration where the uptake is functional but not saturated.

The failure of reserpine to alter the sensitivity to DA in the ear artery would imply that in this tissue, the action of DA is largely direct, i.e. not dependent on the release of endogenous NA from neuronal sites. However, in some other tissues the action of DA is mixed (Tsai and others, 1967) or largely indirect (Spiers and Calne, 1969). It is possible that in this tissue also, a small indirect component to the DA response may exist, but is not revealed by the methods used. Finally, the data provides an opportunity for defining the relative potencies of NA and DA. Reported values show that DA has a potency on the rabbit ear artery 1/50th that of NA (Campbell and Farmer, 1968; de la Lande and Harvey, 1965). However, in both studies the amines were injected intraluminally into the isolated artery, which was perfused in such a way that the dopamine then escaped into the extraluminal solution. In the data presented here, intraluminal and extraluminal relative potencies have been determined separately and it is evident that where uptake of NA occurs, the differences between potencies are much less than when the uptake is prevented or

minimised e.g. by chronic denervation or by intraluminal application. The mean value for relative potency under these conditions is 2-3 times greater than the previous published values.

CHAPTER FOUR

THE EFFECT OF INHIBITION OF MAO ON RESPONSES TO DOPAMINE

CHAPTER 4

INTRODUCTION

In the preceding chapter it was shown that dopamine exerts a direct effect on adrenergic receptors in the ear artery. The failure of cocaine, denervation, or reserpine to modify its action would indicate that the sympathetic nerves play little role in its pharmacological action in the normal artery.

The present chapter deals with the changes in the response to DA produced by inhibition of the metabolising enzymes, especially MAO. This was done in view of the evidence that DA is a substrate for MAO (Blaschko and others, 1937), and it was thought possible that the potency of the vaso-constrictor action may be limited by the fact that DA was inactivated by MAO in the artery wall.

Previous studies in this laboratory confirmed the presence of MAO in the rabbit ear artery. Histochemical methods revealed that the enzyme was distributed largely extra-neuronally, throughout the media, however, this technique did not reveal intra-neuronal enzyme (de la Lande and others, 1970). Subsequently pharmacological techniques revealed that (i) the extra-neuronal enzyme did not play an important role in the response to NA, and (ii) the intra-neuronal enzyme was present despite the negative histochemical findings and that inhibition of this enzyme produced a marked effect on the response to NA (de la Lande and Jellett, 1972). This effect comprised a slow attainment of the steady state response and delayed recovery after washout; the effect was abolished by cocaine, and by chronic denervation, indicating that it required uptake of NA by the sympathetic nerves for its occurrence. Furchgott and Sanchez Garcia (1968) had independently shown that inhibition of MAO produced a similar effect on the response of the guinea pig atria to NA and referred to this effect as

"secondary sensitization".

There have been a number of studies on the interaction between MAO and DA. Helmer (1957) reported that iproniazid markedly potentiated the contraction produced by DA in the rabbit aortic strip. Other workers found enhancement, and sometimes prolongation of the pressor response to DA by MAO inhibitors in the dog *in vivo* (Goldberg & Sjoerdsma, 1959; Gatgounis, 1965) and also in man (Horwitz and others, 1960). In contrast to these, nialamide failed to potentiate the pressor effects of DA in the cat (Balzer and Holtz, 1956) and failed to augment the pressor effects of DA in the conscious rat (Rubenson, 1971). Tsai and others (1967) showed that pargyline treatment increased the sensitivity of guinea pig atria to DA more than ten times, while the increase with alpha-methyldopamine, which is not a substrate for MAO was only three fold. In the same study, a slow increase in tone with time after infusion of DA was also noted in untreated animals. The slow increase in tone was abolished by denervation or cocaine treatment, but not by reserpine, or reserpine and disulfiram, and was considered to be related to an impairment of the uptake of DA. The effect was more prominent when alpha-methyl DA was used instead of DA. These studies indicated both a role of MAO in the response of the cat nictitating membrane to DA, and the probability that the MAO was neuronal rather than extraneuronal.

The aims of the study described in this chapter were to examine the effect of inhibition of MAO on the response of the ear artery to DA, and if an effect were observed, to explore the respective roles of intraneuronal and extraneuronal MAO using denervated, cocaine treated, and reserpine pretreated arteries.

Both isolated perfused segments, and isolated helical strip preparations of the artery have been employed. The advantage of the segment is that it permits the responses to intraluminal DA to be distinguished from those to extraluminal DA.

The separate analysis of the intraluminal and extraluminal responses to NA had already proved to be useful in elucidating the role of MAO in the response of this artery to NA (Jellett and de la Lande, 1969; de la Lande and Jellett, 1972). However, the majority of the quantitative studies described in this chapter have been carried out on strip preparations. As mentioned already, it has not been possible to obtain satisfactory maximum responses on the segment; this difficulty does not apply to the strip.

METHODS

The methods employed in the experiments described in this chapter are described in detail in Chapter 2 - General Methods.

RESULTS

The effect of MAO inhibition on the responses to DA has been studied in:

ARTERIES WITHOUT PRETREATMENT:
CHRONICALLY DENERVATED ARTERIES:
RESERPINE PRETREATED ARTERIES:
COCAINE TREATED ARTERIES:

1. ARTERIES WITHOUT PRETREATMENT.

(a) *Shapes of Responses.*

Segments: As described in Chapter 3, the response of the untreated artery to sustained application of both intraluminal and extraluminal DA comprised a rapid increase in perfusion pressure to a well sustained steady level, and a rapid return to the resting perfusion pressure on washout of the DA.

In nialamide treated arteries, the response to dopamine

differed from the untreated artery in two respects.

(i) At low concentrations of dopamine the time required to attain the steady state response was greatly prolonged. Sometimes a short lag phase preceded the response. However, at higher concentrations, the steady state was attained much more rapidly, but at the concentrations studied this was still slower than the untreated artery.

(ii) The return to resting perfusion pressure after washout of the drug was prolonged.

The two phenomena, (i) and (ii), will be described together as secondary sensitization, since Furchgott and Sanchez Garcia (1968) have already used this term to describe similar effects of inhibition of MAO on the response of the isolated guinea pig atria to NA.

Secondary sensitization to DA occurred after both intraluminal and extraluminal application of DA, although the effect was more pronounced with extraluminal application. These differences are illustrated in Fig. 1. These effects of nialamide on the response to DA resembled those on the response to extraluminal NA in the rabbit ear artery described by de la Lande and Jellett (1972). However, with NA, the sensitization was not observed when the NA was applied intraluminally.

Artery helical strip: As described in Chapter 3, the response of the untreated artery helical strip to sustained application of DA was characterised by rapid attainment of a steady state level followed by rapid recovery on washout.

Following nialamide treatment, the responses to DA were as follows:

(i) Low doses of DA caused a slowly increasing contraction of the strip which often took 30-60 minutes to reach a steady state value. Before the response, a lag of 1-4 minutes sometimes occurred especially for the first dose of DA after nialamide.

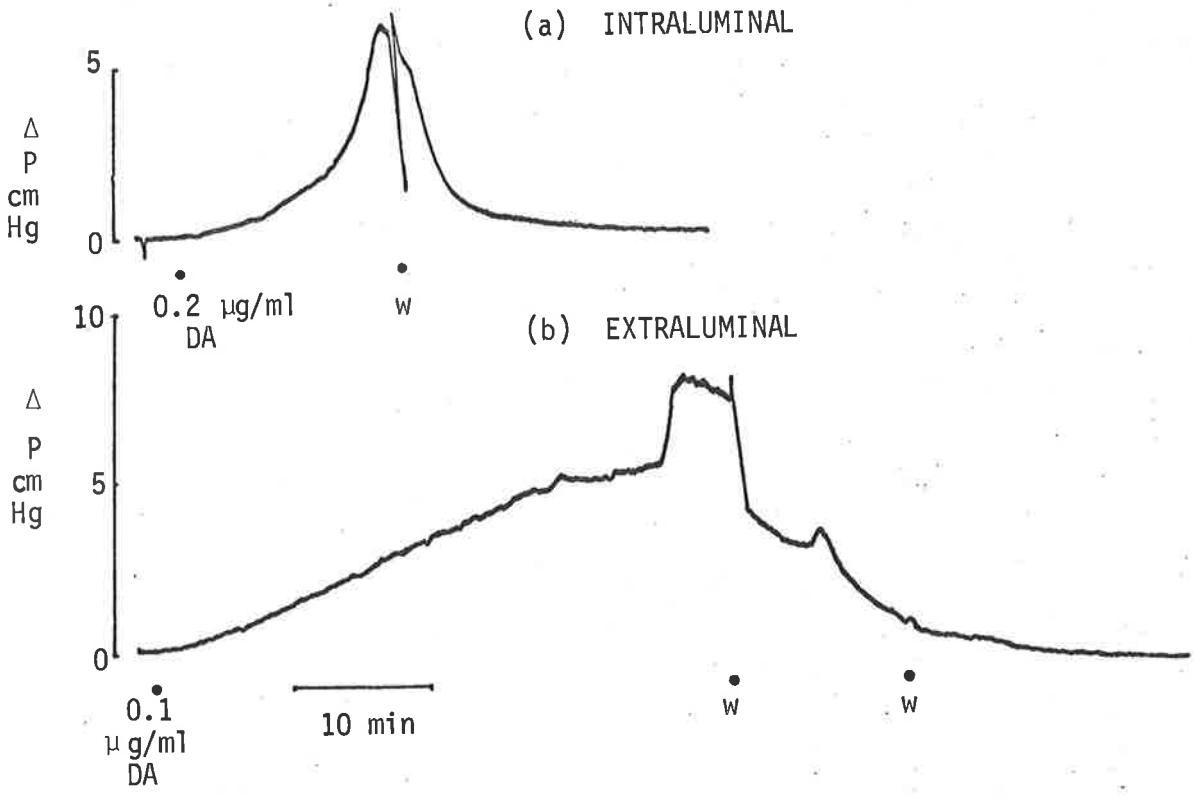


Figure 4.1

Responses to dopamine after nialamide treatment in a perfused artery segment.
(a) intraluminal DA
(b) extraluminal DA
showing the features of secondary sensitisation. i.e. prolonged nature of the response before steady state is reached, and the delay in return to normal resting pressure after washout of the DA.

(ii) At higher concentrations of DA, responses became more rapid in onset and in attaining their steady state level.

(iii) The recovery after washout of the DA was very slow. Hence the effect of nialamide on DA responses in the strip was similar to that in the segment. Examples of the above responses are illustrated in Fig. 2.

(b) *Kinetics of the responses.*

The relationship between the concentration of DA and the kinetics of the response of nialamide-treated arteries was studied in helical strips only. Fig. 3 summarises the results of 6 experiments in which the time required to attain the steady state response was measured at different concentrations of DA. The concentration was increased cumulatively. It will be seen that the rate of onset of steady state was slow at low concentrations and that as the maximum concentration was approached, the rate increased until it was little different from that prevailing in untreated arteries.

Studies on the effect of nialamide treatment on recovery after washout of the DA were confined to a submaximal and to a supramaximal concentration of DA. The submaximal response was achieved by applying DA 3μg/ml for 15 minutes or for 30 minutes; the maximal by applying DA 300 μg/ml until the steady state value of the maximal response was achieved. Following washout of the DA, the height of the response was recorded at various intervals until the preparation had returned to its resting length. Fig. 4 shows that recovery following nialamide treatment was markedly slowed compared with untreated arteries. The mean times to reach a residual response of 10% of the maximum was 34 minutes and more than 60 minutes for 3 and 300 μg/ml DA respectively as compared with 4 and 8 minutes respectively for the untreated arteries.

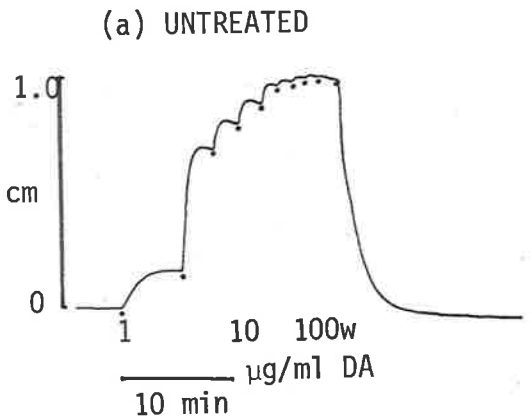


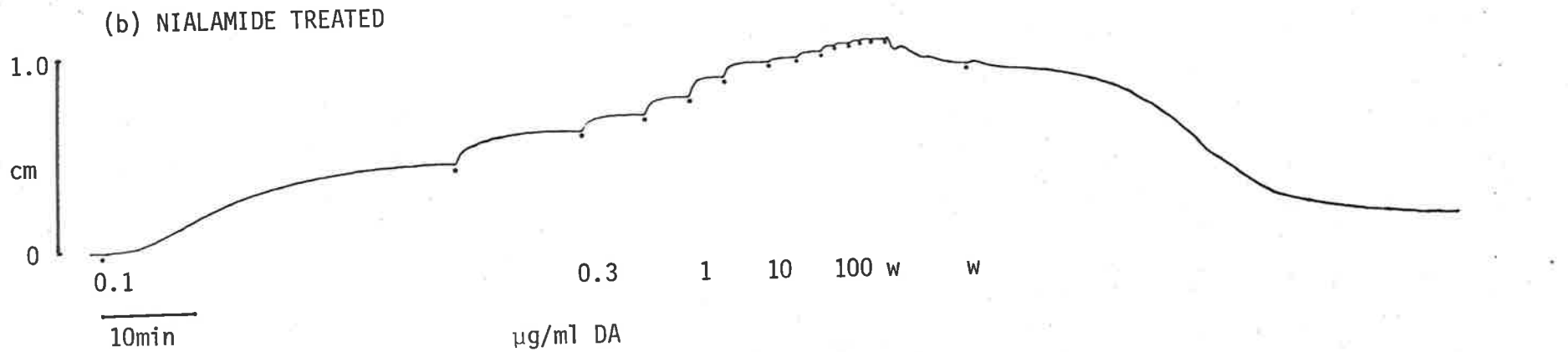
Figure 4.2

Cumulative responses to dopamine in an artery helical strip.

(a) before nialamide treatment,
(b) after nialamide treatment,

showing the nature of the response to dopamine in the untreated strip and the features of secondary sensitization in the nialamide treated strip; i.e. the slow rise of the response to low doses of dopamine and the slow recovery after washout of the dopamine.

The concentration of dopamine was increased cumulatively when the response to the previous concentration reached a plateau.



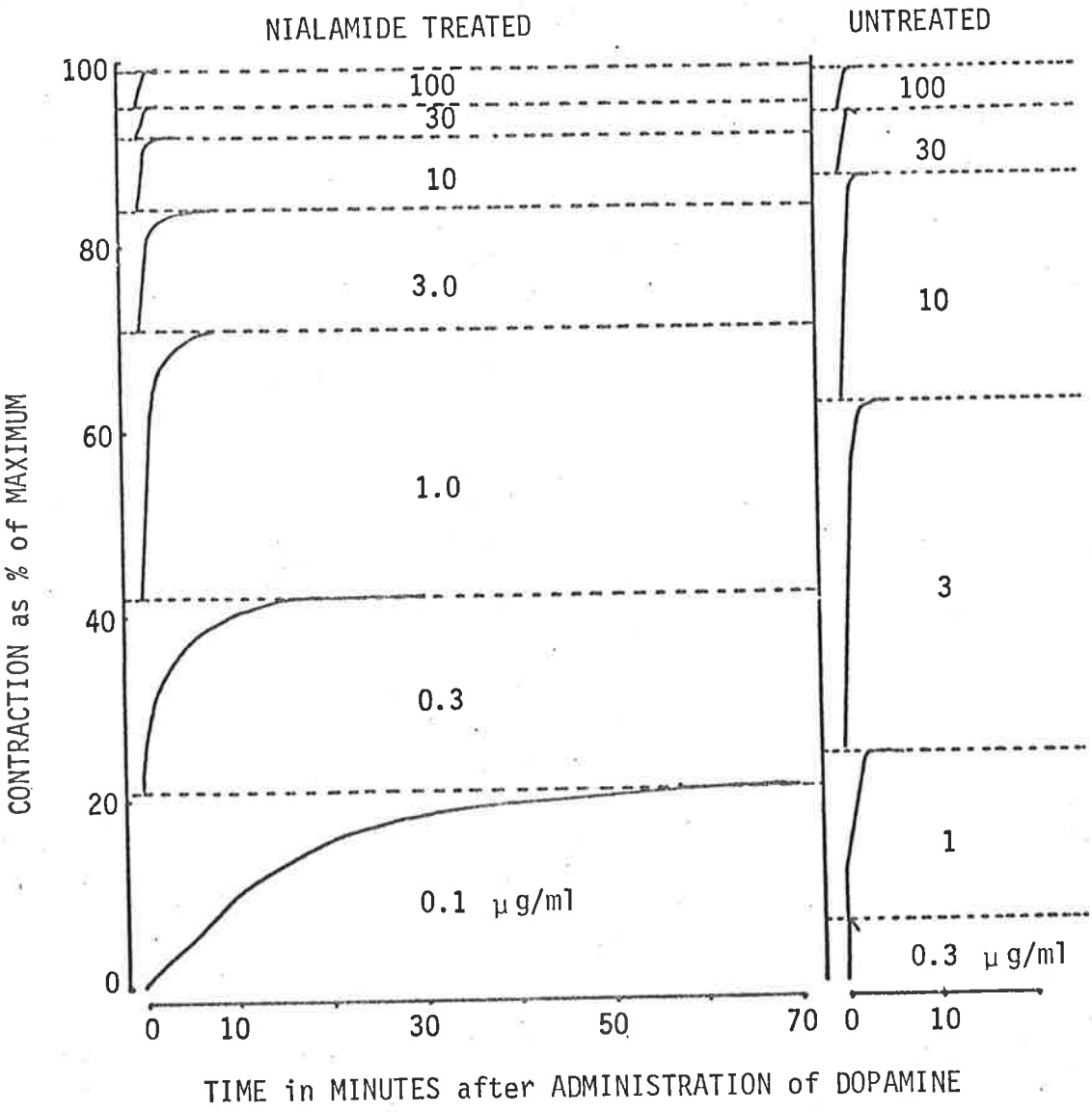


Figure 4.3

Time course of responses to dopamine in nialamide treated and untreated arteries.

Values shown are the mean responses of six experiments at each level of dopamine concentration, from cumulative dose response curves. After administration of each dose, the strip was allowed to reach steady state response, after which the next higher dose was added.

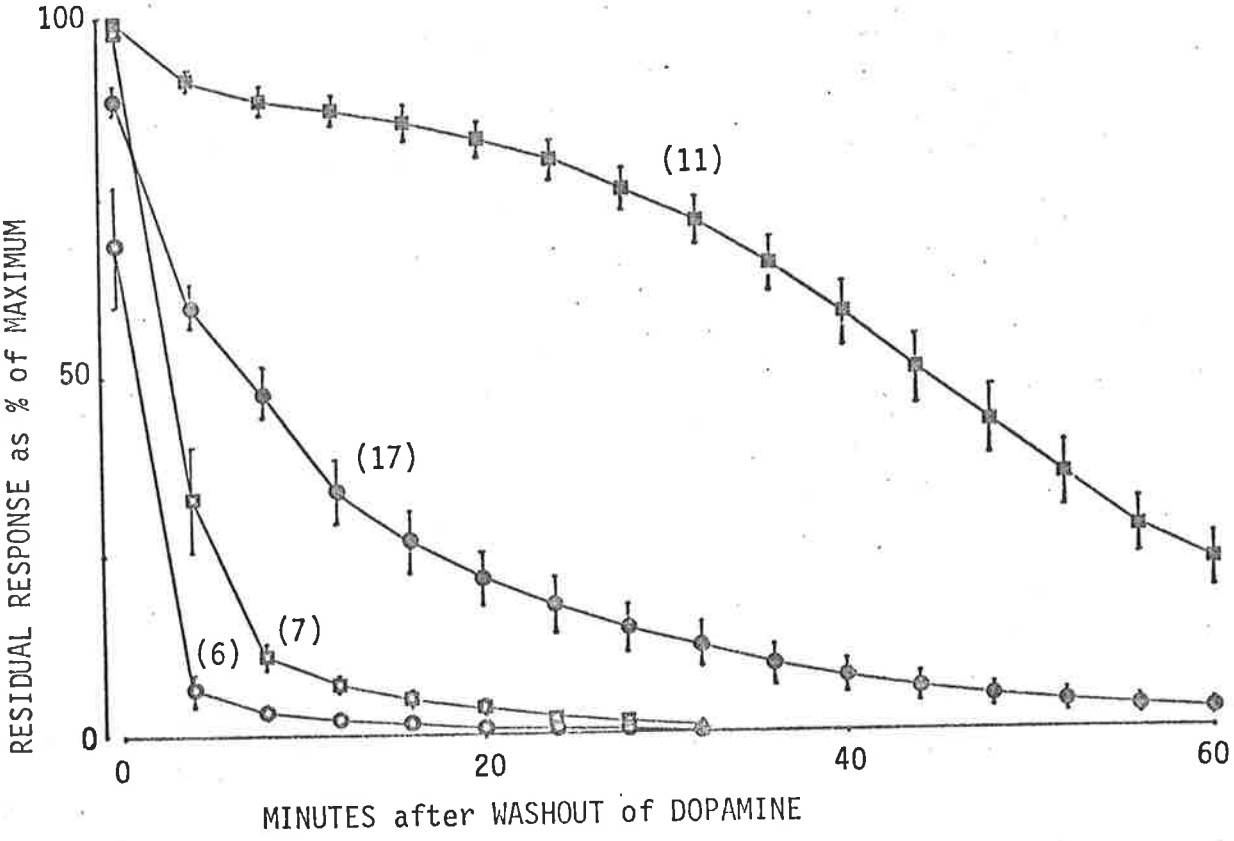


Figure 4.4

Recovery after submaximal and supramaximal doses of dopamine in nialamide treated and untreated strips, demonstrating the slow recovery phase of secondary sensitization in the nialamide treated strip.

Means ± s.e. of the residual responses at time intervals after washout of dopamine are shown for nialamide treated (closed symbols) and untreated (open symbols) artery strips. Supramaximal (300 μg/ml) doses of DA are represented by square symbols, and submaximal (3 μg/ml) by circles. The number of experiments is in brackets over the respective curve.

(c) *Effect of nialamide on magnitude of steady state responses.*
Segments: Besides altering the shapes of the responses, nialamide also enhanced the sensitivity to DA as assessed by the ratio of the concentrations required to produce the same steady state level of response (40 mm Hg) in untreated and nialamide-treated arteries. The ratio was estimated from concentration response curves at a minimum of two concentrations of DA added cumulatively. The results are presented in Table 1. It will be noted that the mean potentiation of extraluminal DA (15 fold) tended to be greater than that of intraluminal DA (6.5 fold). However, the results between experiments varied considerably and the difference between the potentiations of intraluminal DA and extraluminal DA was not significant (t-test, $P > 0.05$). This was undoubtedly caused by the difficulty of deciding the level of the steady state response due to the slow incremental nature of the response. A typical dose response curve is shown in Fig. 5.
Helical strips: Cumulative dose response curves to DA were determined on helical strips before and after nialamide treatment, each dose being applied after the preceding response had reached its steady state value. The dose response curve to DA in the untreated artery was S-shaped (Fig. 6). Treatment with nialamide caused a shift to the left which was more pronounced in the lower region of the curve. As a result, the dose response curves before and after nialamide were not parallel, as indicated by the different slopes of the curves.

In this series of experiments, dose response curves were obtained from the same artery before and after nialamide treatment. It was shown in Chapter 3.6 that when two cumulative dose response curves were carried out on the same artery, the maximum contraction attained by the artery during the second cumulative drug addition was usually less. The same phenomenon occurred in some of the nialamide treated arteries; however, overall the maximum

TABLE 4:1

The effect of nialamide on the sensitivity of the untreated and denervated ear artery to dopamine.

	SEGMENTS		STRIPS		
	INTRALUMINAL	EXTRALUMINAL	ED 30	ED 50	ED 80
UNTREATED	6.5 5.0 - 8.5 (7)	14.7 11.5 - 18.8 (7)	6.7 4.9 - 9.0 (14)	5.3 4.2 - 6.6 (14)	4.3 3.3 - 5.8 (14)
DENERVATED	1.3 1.0 - 1.7 (6)	1.1 0.9 - 1.4 (6)		0.53 0.36 - 0.77 (5)	

Values quoted are the geometric mean of ratios $\frac{\text{Conc. of DA before NIAL}}{\text{Conc. of DA after NIAL}}$

In the case of segments the concentrations are those which were equipotent in producing a response to 40 mm Hg. The figures below the means are the mean ± s.e., and the number in brackets refers to the number of experiments.

The differences between denervated arteries and their contralateral control arteries were highly significant ($P < 0.005$ t-test). However the difference between intraluminal and extraluminal DA was not significant at the 5% level. In the untreated artery this was due to the large variation in values obtained, stemming from the extreme difficulty in gauging steady state response in these arteries.

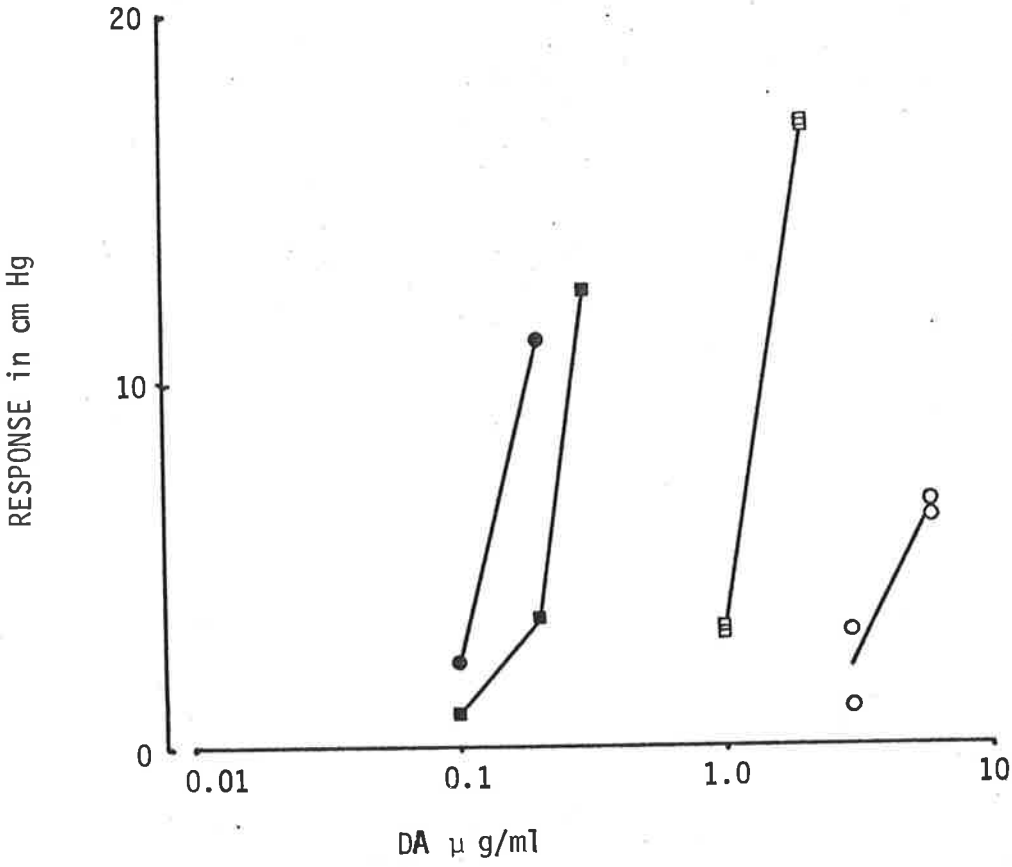


Figure 4.5

Effect of nialamide on the sensitivity to dopamine in the perfused artery segment.

Typical dose response plot of extraluminal (circles and intraluminal (squares) dopamine before (open symbols) and after (closed symbols) nialamide showing an increase in sensitivity of 36 fold and 5.fold for extra- and intra-luminal dopamine respectively.

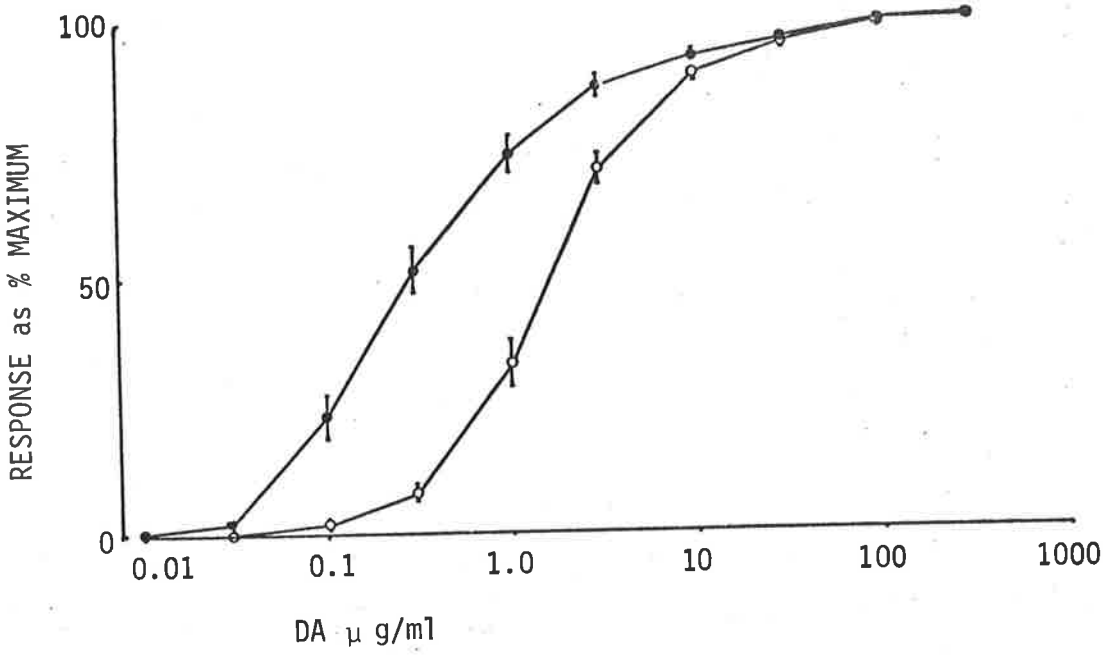


Figure 4.6

Effect of nialamide on the sensitivity to dopamine and the slope of the dose response curve in the artery helical strip.

The values shown are the means ± s.e. of cumulative responses to dopamine in 21 untreated (open circles) and 18 nialamide treated (closed circles) strips. The slopes of the curves are 63.3 and 45.3 for untreated and nialamide treated respectively. The slopes were determined by the formula of Langer and Trendelenberg (1969) where the slope = $\frac{50}{\log ED\ 80 - \log ED\ 30}$ using the mean ED 80 and ED 30.

response to the second application of drug in these arteries was not significantly different from that attained in the untreated arteries (t-test, $P > 0.05$). This is shown in Table 2.

Table 1 shows the potentiation of DA responses due to nialamide at various equieffective dose levels in 14 strips. As stated above, the potentiation at lower dose levels is greater than at the higher dose levels. Maximum potentiation was at ED 30 level. It will be seen that the potentiating effect of nialamide on the helical strips was approximately equal to its effect on the responses of segments to intraluminal DA, but only about one-half the effect on the responses of segments to extraluminal DA.

2. CHRONICALLY DENERVATED ARTERIES.

Left ear arteries of rabbits were sympathetically denervated (see Chapter 2.6) 4-12 days prior to the removal of the left (denervated) and right (control) arteries for use as isolated perfused segments, or as helical strips. The shapes of responses, shapes of dose response curves and changes in sensitivity were observed in both segments and strips, and the time course of responses and relaxation after standard doses of dopamine were also observed in the strip preparation.

Responses to submaximal DA in chronically denervated arteries (both segments and strips) were similar before and after nialamide treatment. i.e. responses were rapid in onset, and in the attainment of steady state, and were followed by rapid recovery on washout of DA. Fig. 7a and b shows responses typical of 8 experiments in the denervated segment. Table 3 summarises data on the times required to attain steady state responses to DA in nialamide treated strips. The main feature is the relatively rapid attainment of the steady state response to low concentrations of DA in the denervated artery compared with the innervated artery. Table 1 shows that nialamide did not

TABLE 4.2

Effect on maximum response height of repeated cumulative application of dopamine to arterial strips.

EXPERIMENTAL CONDITIONS DURING		2nd maximum response as % of 1st maximum response.
1st Response	2nd Response	
DA	DA	88.9 s.e. ± 6.4 (5)
DA	DA after nial	94.8 s.e. ± 2.6 (14)

The effect of a previous cumulative response to maximum on the maximum response in the next dose response curve is shown above.

The height of the maximum response in the second cumulative response was calculated as a percentage of the height of the maximum in the first cumulative response. A time lag of at least 1½ hours occurred between the two cumulative responses in all cases. The mean ± s.e. of the findings is presented above. The means of the two groups did not differ significantly. (t-test, $P > 0.05$).

Figures in brackets refer to the number of experiments.

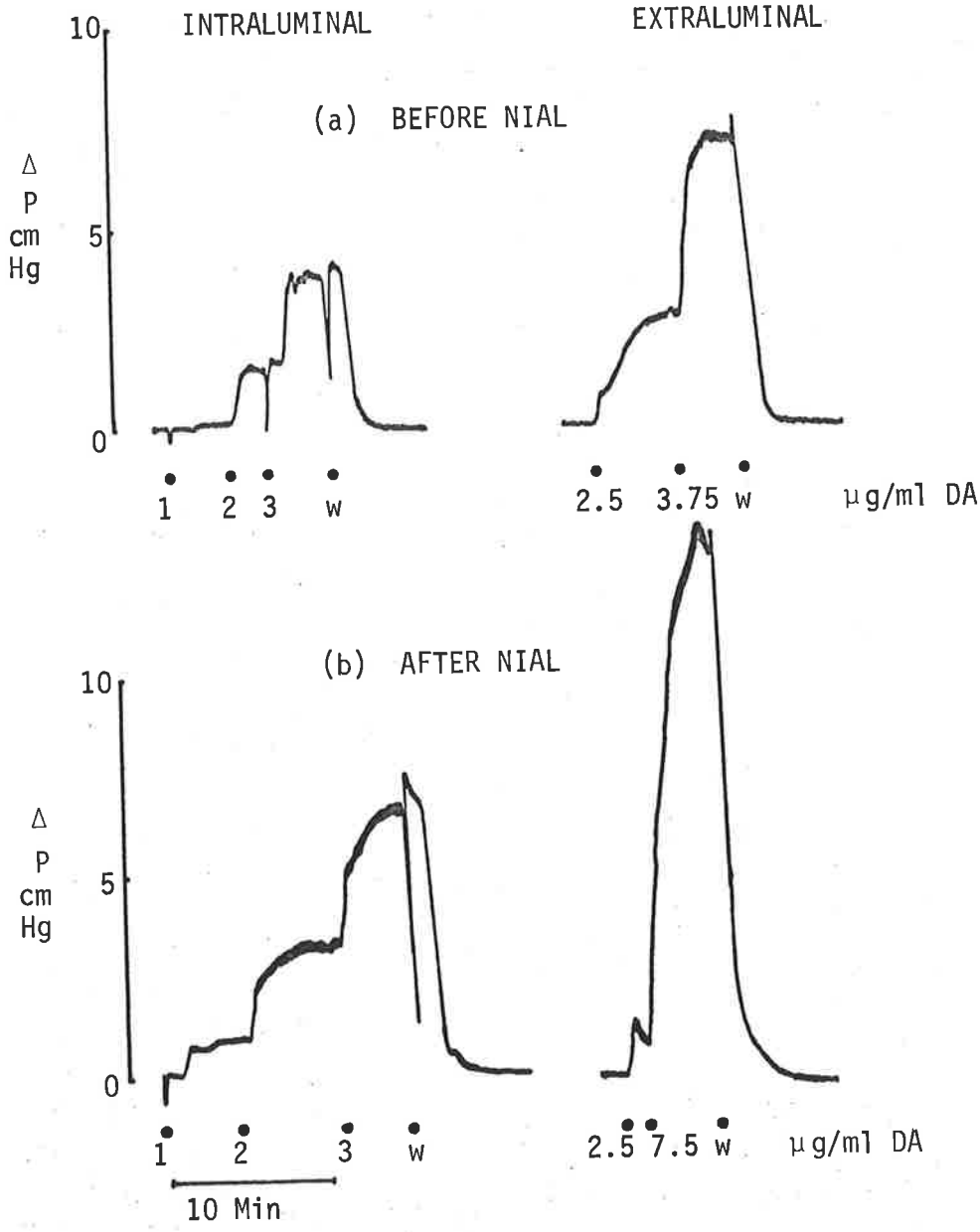


Figure 4.7

Responses to extra- and intra-luminal dopamine in denervated artery segments, typical of those in 8 experiments

(a) before nialamide,

(b) after nialamide treatment,

showing the lack of the features of secondary sensitization in the denervated preparation after nialamide treatment.

TABLE 4.3

The effect of denervation on the time required to attain the steady state response to DA in nialamide treated artery strips.

CONC OF DA (µg/ml)		0.1	0.3	1.0	3.0	10	30	100
TIME (MIN)	DENERVATED	2.4	2.4	3.3	1.6	1.4	0.6	0.6
	CONTROL	41	6.8	3.0	1.8	1.3	0.9	0.7

The data refers to the means of results in 5 pairs of arteries. In each artery, the concentration of DA was increased cumulatively and the intervals between the additions of dopamine and the attainment of the steady state response measured.

increase the sensitivity to DA in the denervated artery segment. In the strip a slight decrease in sensitivity of about 2 fold was seen. Fig. 8 shows the mean recovery curves for submaximal (3μg/ml) concentrations of DA. It will be seen that recoveries from the submaximal concentration of DA (3μg/ml) are substantially faster in the nialamide treated denervated artery compared with the innervated artery (Fig. 4), however, the rate of recovery is still slower than the non-nialamide treated control. This slow recovery in the denervated artery after nialamide is much more pronounced after the supramaximal dose of DA (300 μg/ml). Nevertheless it should be noted that the effect of nialamide was significantly greater in the control arteries than in the denervated arteries (t-test $P < 0.001$).

3. RESERPINE PRETREATED ARTERIES.

Rabbits were pretreated with reserpine 24 hours prior to sacrifice (see Methods 2.7).

Experiments with perfused segments were limited to qualitative observations on the shape and time course of the response. Fig. 9 shows a response to DA typical of that in reserpine pretreated arteries following nialamide treatment. It can be seen that the slow rise to the steady state level and slow recovery after washout, characteristic of secondary sensitization, still occurred both in reserpine pretreated artery segments and strips. Quantitatively the potentiation of DA sensitivity by nialamide was unaltered in the reserpine pretreated artery strip. The sensitivity to DA in 5 reserpine and nialamide treated arteries did not differ significantly from that in 9 nialamide treated arteries (Fig. 10). Analysis of the rates of recovery from DA in nialamide treated strips showed that these rates were not significantly different in arteries from reserpine pretreated rabbits and arteries from untreated rabbits (Fig. 11). Thus it was concluded that the phenomenon of

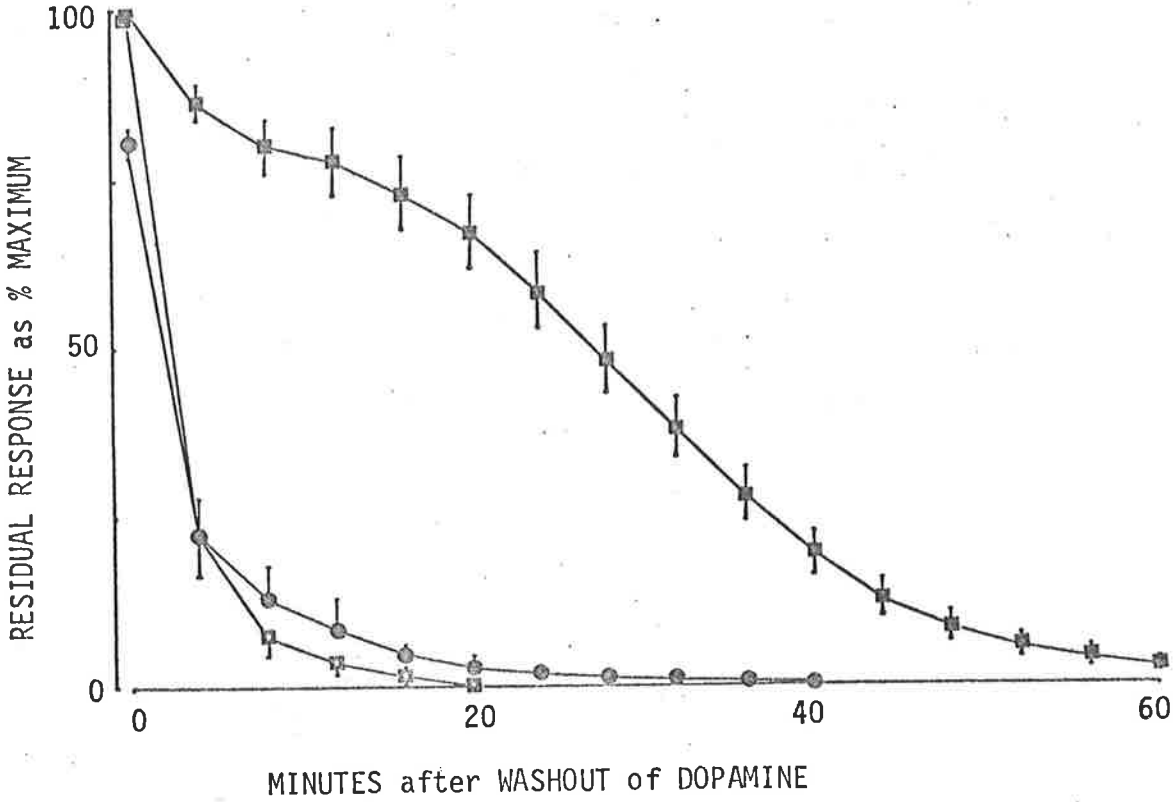


Figure 4.8

The effect of nialamide on recovery from supramaximal (300 µg/ml) and submaximal (3 µ g/ml) concentrations of dopamine in 5 denervated artery strips.

Symbols used: squares 300 µ g/ml
circles 3 µ g/ml
open - untreated
closed - nialamide treated

Values shown are the mean residual responses ± s.e. of 5 experiments.

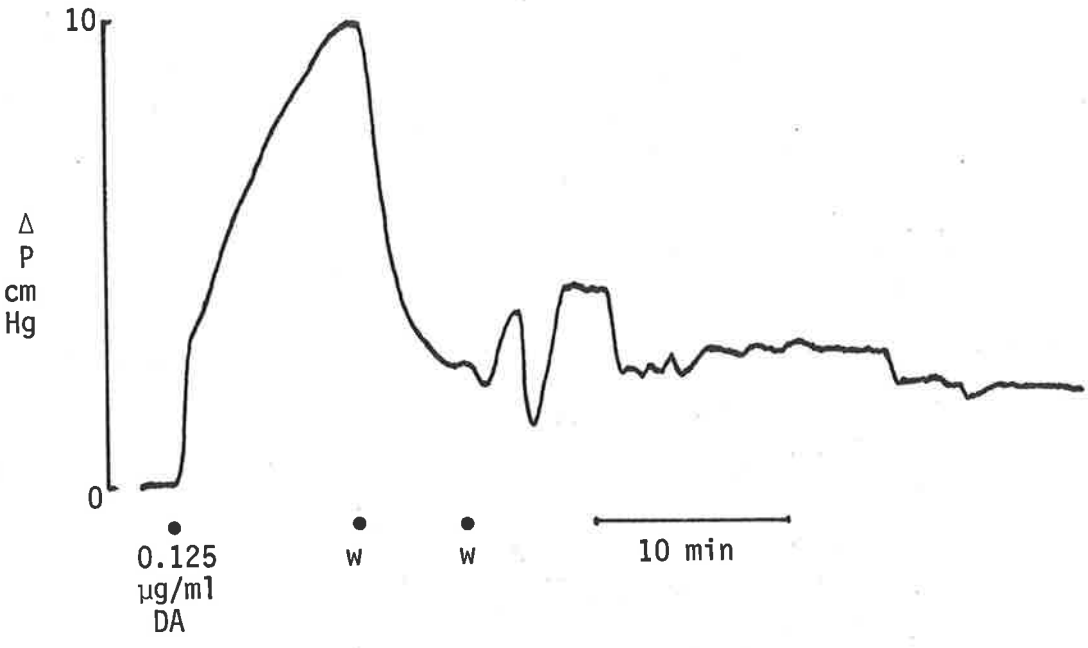


Figure 4.9

Response to dopamine after nialamide treatment in a reserpine pretreated artery segment.

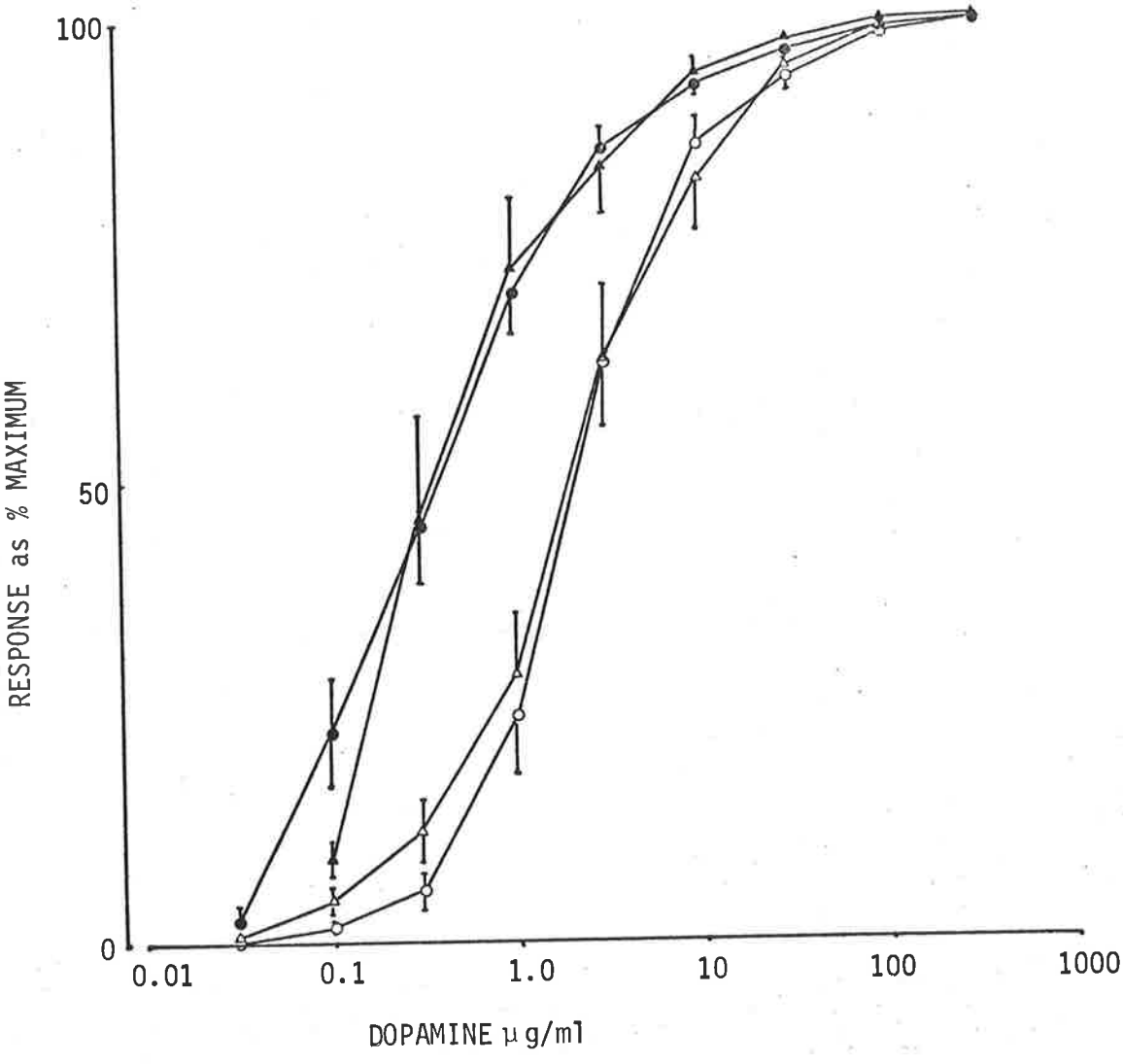


Figure 4.10

The effect of reserpine pretreatment on the potentiation of dopamine responses by nialamide in artery helical strips.

Values shown in the nialamide treated (closed symbols) strips were from 5 reserpine pretreated and 9 untreated strips, and those in the control groups (open symbols) were from 9 reserpine pretreated and 10 untreated strips. Reserpine treated strips are denoted by triangles, and untreated by circles.

Mean values in the reserpine pretreated groups did not vary significantly from those in the corresponding reserpine -

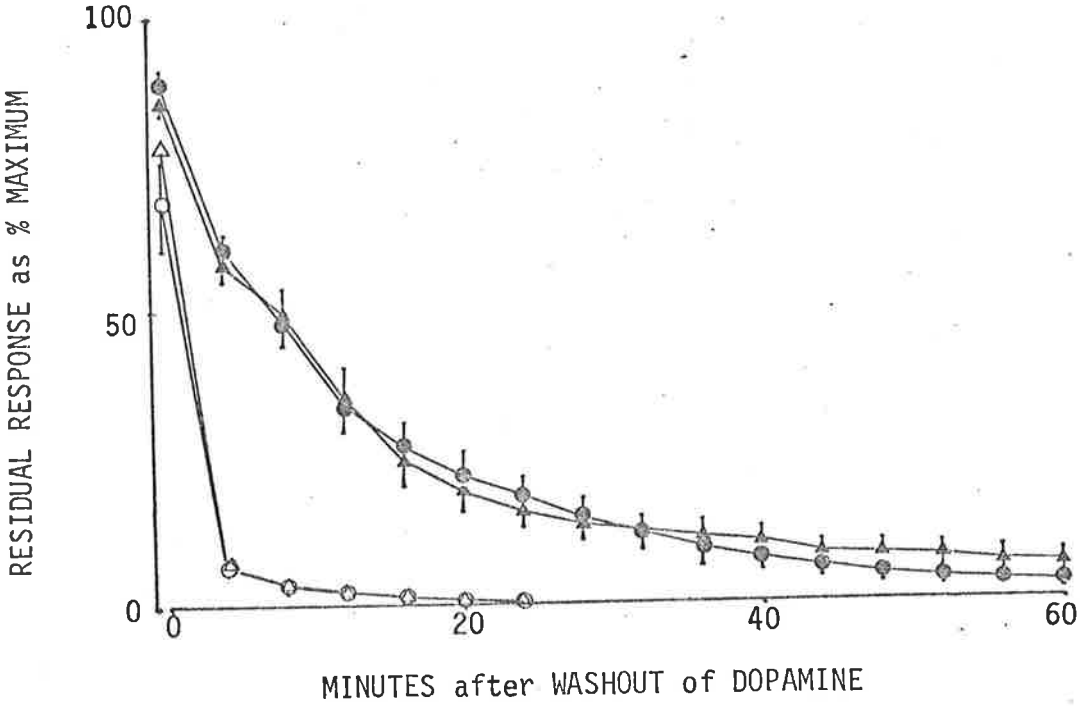


Figure 4.11

The effect of reserpine pretreatment on recovery from a submaximal concentration of dopamine (3 μg/ml) in nialamide treated and untreated artery strips.

Values shown are the means ± s.e. of the residual responses in 7 reserpine pretreated (triangles) and 17 untreated (circles) strips. Nialamide treated strips are denoted by closed symbols, control strips by open symbols.

Mean values in the reserpine pretreated groups did not vary significantly from those in the corresponding reserpine-untreated groups. (t-test, $P > 0.05$)

secondary sensitization to DA was not appreciably modified by reserpine.

4. COCAINE TREATED ARTERIES.

Studies on cocaine were restricted to perfused segments. The segments were treated with nialamide, and cocaine 1 µg/ml was then applied in one of the following ways:

(i) 5 minutes before DA was added and retained throughout the period of application of DA;

(ii) at some stage during the course of the response;

(iii) at some stage during recovery from the response after washout of DA;

(iv) at periods of up to 20 minutes afer the preparation had recovered from DA, i.e. after perfusion pressure had returned to its resting level.

Procedures (iii) and (iv) were also carried out on reserpine pretreated arteries.

In the course of these experiments, the route of application of cocaine varied, i.e. it was applied intraluminally, extraluminally, or to both surfaces of the artery simultaneously. However, there was no difference in the results obtained. The different routes of application of cocaine are not distinguished in the following account of the results.

(i) Dose response curves obtained to DA in the absence and presence of cocaine were approximately parallel. Comparisons in 6 arteries indicated that cocaine did not significantly affect the sensitivity to DA in the nialamide treated preparation. This lack of effect of cocaine parallels its lack of effect on the sensitivity to DA in the untreated artery. Furthermore, cocaine also failed to alter the shape of the response and its slow time course, typical of secondary

sensitization to DA in these arteries (Fig. 12).

(ii), (iii), (iv) With each of these procedures, cocaine (1 μg/ml) caused an increase in the perfusion procedure which was rapid in onset and in attaining a maximum, and then declined slowly towards the resting pressure, despite the continued presence of the cocaine (illustrated in Fig. 12). This constrictor response to cocaine occurred in 31 of 33 arteries, with the qualification that usually only one response could be elicited to cocaine after the perfusion pressure had returned to its resting level (iv. above). Cocaine had a similar effect in reserpine pretreated arteries. However, the constrictor response to cocaine in this group of arteries tended to be greater in magnitude and more prolonged than in the untreated arteries, and responses to cocaine could be elicited by repeated application of the drug during the recovery period.

5. *THE EFFECT OF PHENTOLAMINE.*

Phentolamine (1 μg/ml), when applied to segments during the phase of delayed recovery, caused a rapid return of the perfusion pressure to its resting level (or, in the case of strips, a rapid return to its resting length). Also, phentolamine added prior to cocaine prevented the constrictor action of cocaine.

D I S C U S S I O N

The results in this chapter show that secondary sensitization to DA occurs after treatment with nialamide, i.e. the sensitivity to DA is increased, and the response to DA shows slow attainment of steady state and slow recovery after washout. This change in shape and time course is most marked in responses to small doses of DA, and occurs on both intra- and extraluminal application of the drug. These changes are virtually abolished by chronic sympathetic denervation,

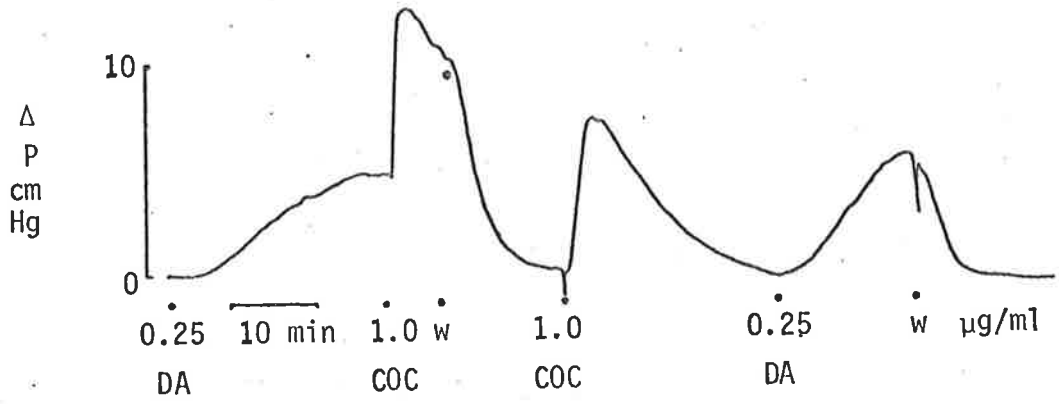


Figure 4.12

Response to DA in nialamide treated perfused artery segment in which cocaine was added
a) during the steady state response to dopamine,
b) during the delayed recovery from the response.
showing the constrictor response to cocaine which occurred in these arteries.

It may be seen also that when a second dose of dopamine was administered in the presence of cocaine no increase in sensitivity to dopamine occurred.

indicating that they are largely neuronal in origin.

The failure of reserpine to decrease secondary sensitization suggests that neither the presence of endogenous NA in the nerve terminal, nor the binding of amines to intraneuronal storage granules is essential for its occurrence.

The above features of the response to DA after nialamide, resemble many of those described for NA after MAO inhibition by Furchgott and Sanchez Garcia (1968) in guinea pig atria, by Trendelenburg (1971) in the isolated cat nictitating membrane, and by de la Lande and Jellett (1972) in the rabbit ear artery. Points of similarity in the rabbit ear artery were: (i) the changes in shape and time course of the response were similar for both NA and DA; (ii) reserpine pretreatment failed to decrease secondary sensitization to NA and DA; and (iii) cocaine applied during the recovery from both DA and NA caused further constriction of the artery. This response to cocaine was also more pronounced in reserpine pretreated arteries, both in the case of DA and NA. There are, however, points of difference thus: (i) In the rabbit ear artery, secondary sensitization was observed only in response to extraluminal application of NA, whereas, in the case of DA, the phenomenon was observed with both extraluminal and intraluminal application; and (ii) concurrent treatment with cocaine prevented secondary sensitization to NA, but not to DA.

The latter failure of cocaine to significantly modify the secondary sensitization, suggests that uptake by the nerves is not involved in this phenomenon. However, the rabbit ear artery is much less sensitive to DA than to NA, and histochemical evidence presented in the previous chapter suggests that in the range of concentrations of DA commonly used in the pharmacological studies, the $Uptake_1$ system may be saturated. If so, uptake of DA might still proceed in the presence of cocaine and thus account for the lack of effect of cocaine on secondary sensitization.

The above effects of nialamide treatment on the response to DA may be explained in two ways. One possibility is that nialamide, by inhibiting intraneuronal MAO, leads to a decline in the net uptake of DA, thus increasing the concentration of exogenous DA available at receptors in the smooth muscle. This type of sensitization would be analogous to presynaptic supersensitivity. The difficulty with this explanation is that neither cocaine, nor chronic denervation, enhance sensitivity to DA. The second possibility is that DA increases the rate of synthesis of NA in the nerve terminal. Normally the excess NA is inactivated by intraneuronal MAO. However, following inhibition of MAO by nialamide, the excess NA accumulates, and overflows from the nerve terminal, and as a result of its reaction with the noradrenergic receptors in the smooth muscle, gives rise to the secondary sensitization to DA. This possibility is supported at this stage by the observation that cocaine causes a further constriction when added during the phase of delayed recovery from DA. As reported in Chapter 3, cocaine does not enhance the response to DA itself in the absence of nialamide treatment. However, it markedly potentiates the response to NA (de la Lande & Waterson, 1967). Hence the constrictor effect of cocaine is readily explained if this is due to inhibition of reuptake of the NA which is escaping from the nerve terminal. This explanation assumes that the escape is a dynamic process, involving a higher rate of egress from, than ingress of NA into, the nerve terminal. The latter process (ingress) is inhibited by cocaine, so that the net "escape" of NA from the terminal is increased, leading to constriction of the artery.

The results presented in this chapter also provide some evidence on the role of extraneuronal metabolism on the DA response. In chronically denervated arteries, nialamide did not increase the sensitivity to DA and caused only a small

increase in the rate of recovery from a submaximal concentration of DA. Hence extraneuronal MAO must be considered unimportant in the pharmacological response to DA. However, nialamide did cause a marked delay in recovery from supramaximal doses of DA (300 µg/ml). Thus inactivation by extraneuronal MAO does appear to become important with high concentrations of DA. This is similar to the situation with NA (de la Lande and Johnson, 1972).

Hence the results at this stage suggest that intraneuronal metabolism by MAO plays a significant role in the response to DA, whereas the role of extraneuronal metabolism by MAO is unimportant, at least when the concentration of DA is at submaximal response levels.

CHAPTER FIVE

ACTIONS OF PRECURSORS OF DOPAMINE ON THE RABBIT EAR ARTERY

CHAPTER 5

INTRODUCTION

In the previous chapter, a possible role for NA in the response to DA after nialamide treatment has been proposed. Thus it was decided to test whether other substrates besides DA in the synthetic pathway for NA produced vascular changes, and whether these were modified by inhibition of MAO in a similar fashion to DA. The substrates used were (a) tyrosine (b) L-dopa.

Early evidence showed that L-dopa had no pharmacological effect in animals (Blaschko and Crusciel, 1960), but subsequently conflicting reports as to the effects of L-dopa have appeared in the literature. After administration of L-dopa in man, various cardiovascular side effects have been reported; changes in blood pressure are common, and these may be hypertensive, but are mostly hypotensive. This subject has been recently reviewed by Goldberg and Whitsett (1971). Concomitant administration of MAO inhibiting drugs with L-dopa has produced severe hypertensive episodes in man (Hunter and others, 1970; Pollin and others 1961), in cats (Balzer and Holtz, 1956), and in rats (Rubeson, 1971). Although there is evidence for an intrinsic action of L-dopa in the CNS (Henning and Rubeson, 1970) there is also much evidence, based mainly on enzyme inhibitor studies, that the effects of L-dopa are mediated in many cases by its metabolites, usually DA which may be acting as a false transmitter (Burn and Rand, 1960; Whitsett and others 1970; Liu and others 1971; Collins and West, 1968b). In the present study, the pharmacological action of L-dopa has been studied in untreated arteries, and that of tyrosine and L-dopa in nialamide treated arteries.

The role of the nerves in the response to L-dopa after nialamide has been investigated by the use of chronic denervation, cocaine, and reserpine.

The role of NA in the response to L-dopa has been further investigated by the use of dopamine β-hydroxylase inhibitors and lowered temperature. These data are reported in subsequent chapters, i.e., Chapter 6 and Chapter 7.

RESULTS

1. TYROSINE.

In 5 experiments, perfused artery segments from rabbits pretreated with nialamide 100 mg/kg (see Chapter 2.8 for details of methods) were exposed to tyrosine 20 µg/ml in Krebs bicarbonate solution for 1 hour. The solution also contained ascorbic acid (20 µg/ml)to minimise autoxidation of the tyrosine. Fifteen minutes after washout of the tyrosine, cocaine 1 µg/ml was added to the extraluminal bathing fluid for 10 minutes and any response recorded. Fifteen minutes after the cocaine was washed out, the procedure was repeated using 200 µg/ml tyrosine.

No response was seen with tyrosine in either of the above concentrations, either during the exposure to the drug or after its washout. There was also no constrictor response to cocaine (described previously for DA in Chapter 4) after tyrosine washout in any of the 5 experiments.

2. L-DOPA.

(a) Untreated Arteries.

L-dopa, in concentrations up to 200 µg/ml was applied to perfused artery segments (3) and helical strips (8) for time periods from 10 minutes up to 1 hour. Dose response curves to NA were obtained after the L-dopa treatment as an index of the sensitivity of the preparation.

Dopa in the above concentrations did not produce

either constriction of the perfused segments or contraction of the artery strips. Fig 1c shows the trace from a typical experiment.

(b) Nialamide treated arteries.

The effect of nialamide treatment on the response to L-dopa was studied in perfused segments and helical strips. In nialamide treated strips and segments both intra- and extraluminal dopa in the dose range 10 - 200 µg/ml caused a dose-dependent constrictor response. There was an initial lag period of about 3-10 minutes, followed by a slow rise in perfusion pressure in segments or slow contraction in strips over 30-60 minutes before steady state was reached. After washout of the dopa, the artery recovered slowly, the recovery times varying between 30-60 minutes. A typical response in a segment is shown in Fig. 2, and in a strip in Fig. 1a. The response and recovery closely resembled the secondary sensitization response of DA in the nialamide treated segment and strip.

In 2 experiments, in which the sensitivity to intra- and extraluminal dopa was tested on the same artery, it was found that the extraluminal sensitivity to dopa was 10 times greater than the intraluminal sensitivity. However, the response to intraluminal dopa was similar in shape and time course to that of extraluminal dopa.

Fig. 3 shows the mean dose response curve to dopa in 14 helical strips, the responses being plotted as a percentage of the response of each strip to a supramaximal dose of NA. Fig. 4 shows the mean recovery curve for 9 of these experiments.

Effect of chronic denervation.

Since the responses to dopa in segments and strips are similar, the effects of denervation on the two types of preparation will be described together.

Fourteen experiments were carried out using rabbits, the left ear of which had been sympathetically denervated by

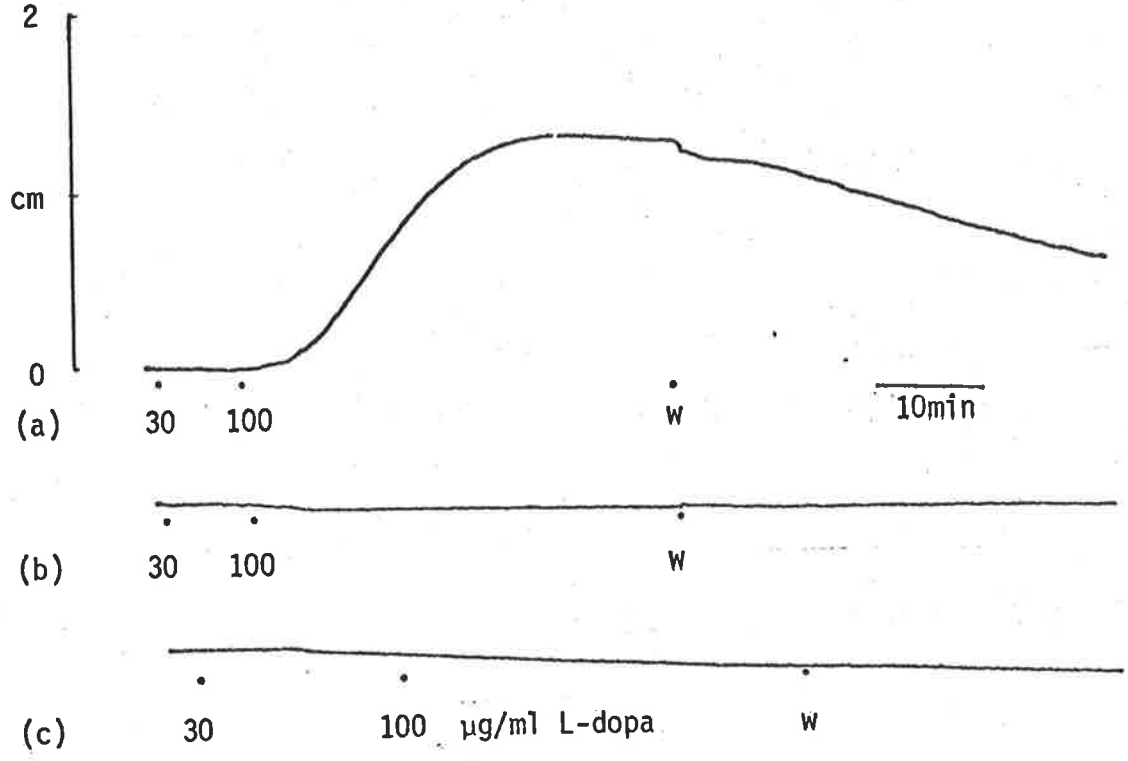


Figure 5.1

(a) Response to L-dopa in an innervated nialamide treated strip
(b) and (c) shows the failure of a denervated nialamide treated strip, and an innervated untreated strip to respond to L-dopa.

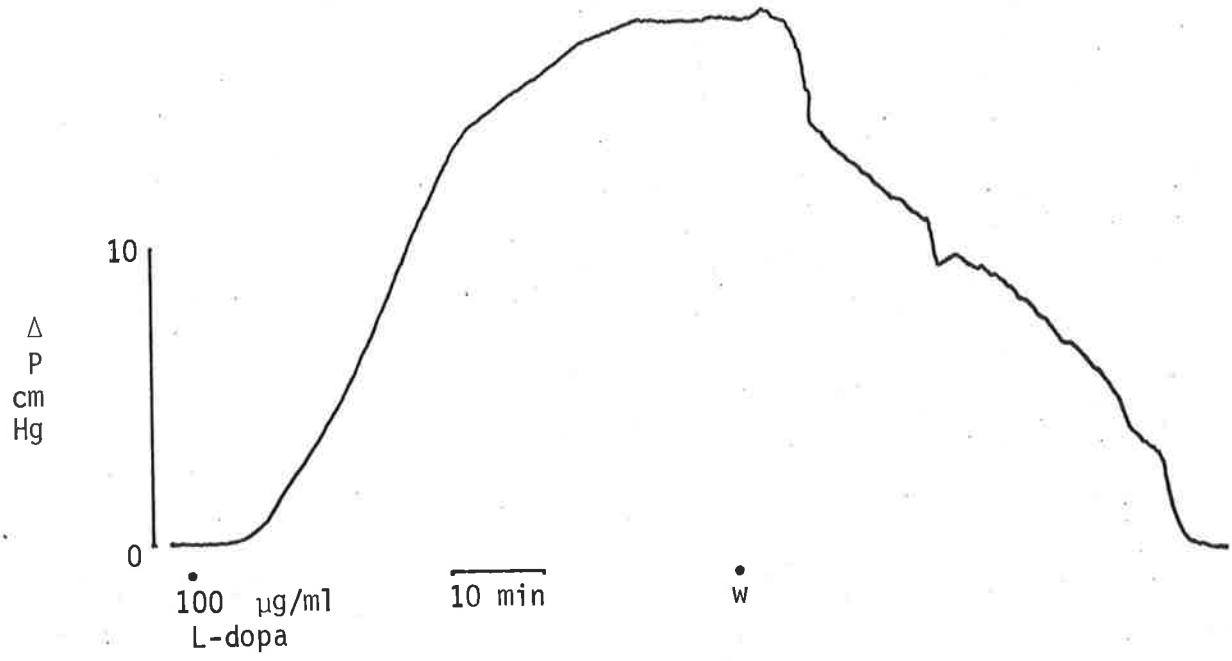


Figure 5.2

Response to L-dopa in nialamide treated artery perfused segment.

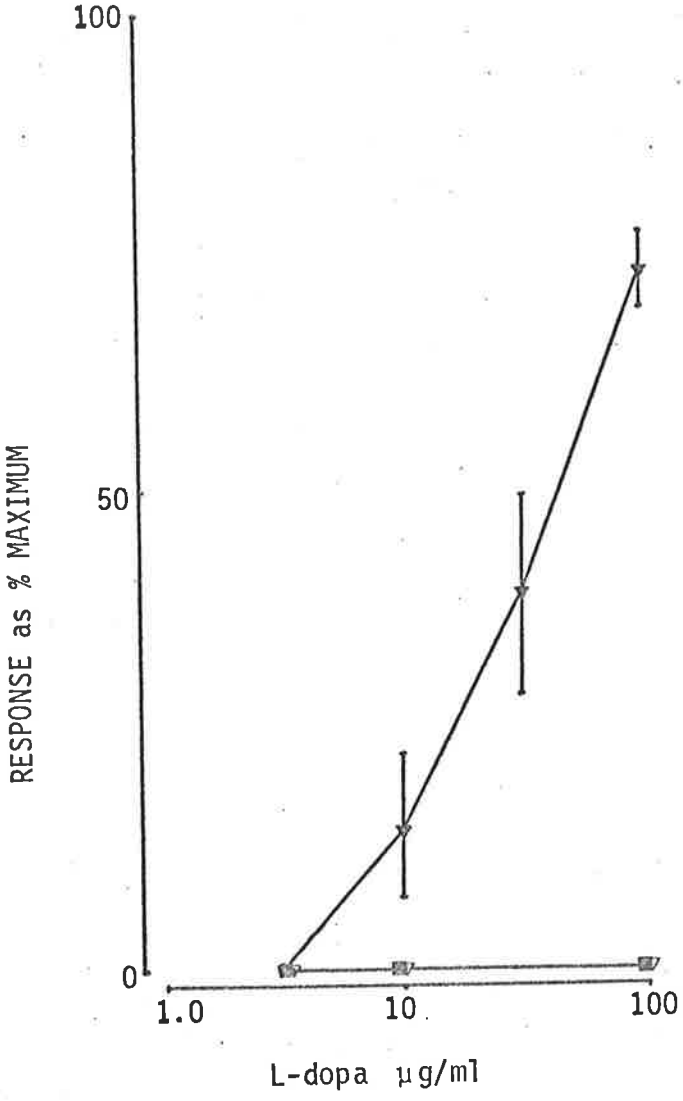


Figure 5.3

The effect of nialamide treatment on the response to L-dopa in artery helical strips.

Values shown are the means ± s.e. of responses from 14 strips.

triangles - innervated strips
squares - denervated strips
closed symbols - nialamide treatment

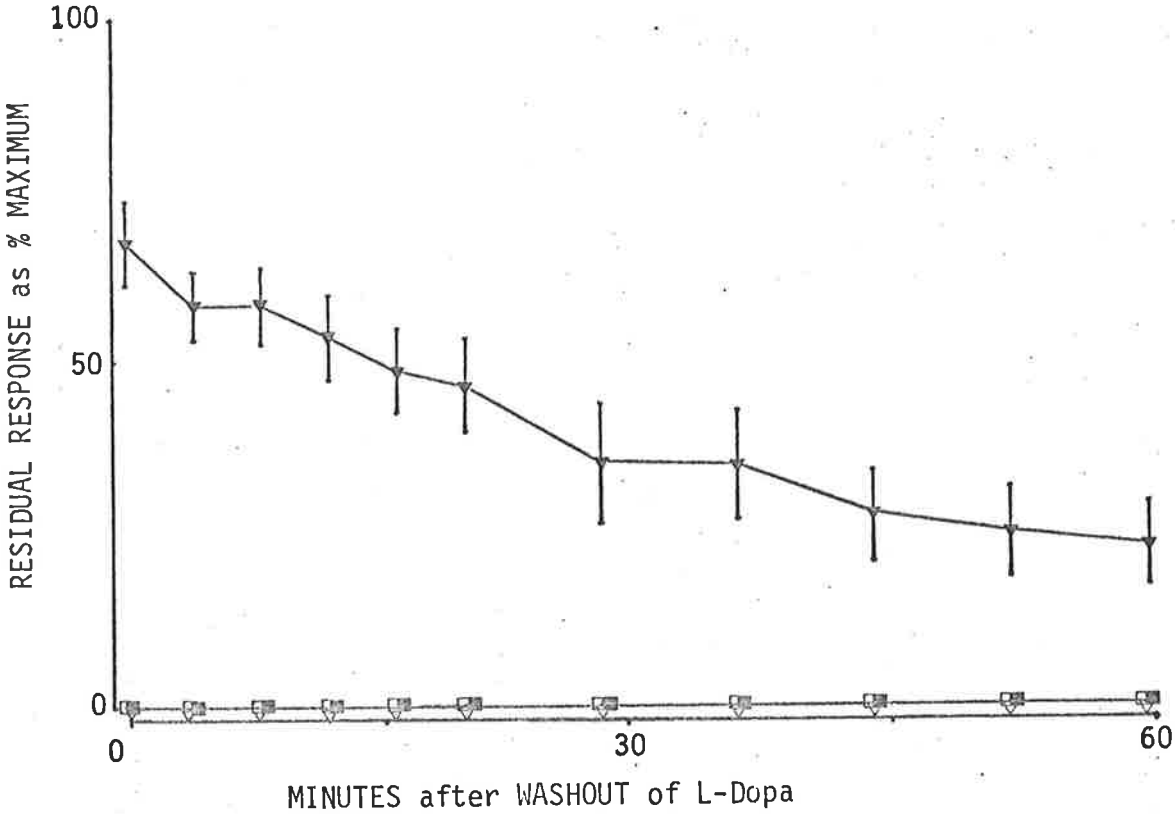


Figure 5.4

Recovery after response to L-dopa in artery helical strips.

Shown are the mean values ± s.e. of 9 experiments in which the residual response after washout of 100 µg/ml L-dopa was measured at regular time intervals up to 60 mins.

Symbols used: triangles - innervated strips
squares - denervated strips
closed symbols - nialamide treatment

removal of the homolateral superior cervical ganglion from 2 to 14 days before excision of the ear arteries. All denervated arteries were tested for efficacy of the denervation procedure by fluorescent histochemical methods and the results of any which showed incomplete denervation were discarded. (The methods used for these procedures are detailed in Chapter 2.6). The denervated and contralateral innervated arteries were treated with nialamide and then tested for response to dopa in the concentration range 10 to 200 µg/ml. Twelve of the nialamide treated denervated arteries showed no response to dopa (Fig. 1b). Two of the nialamide treated denervated arteries, however, showed responses to dopa after MAO inhibition. These were tested 5 and 7 days respectively after denervation and histochemically showed no monoamine fluorescence at the media-adventitia junction. The responses to dopa in the arteries were different in nature to the secondary sensitization response. They were dose-dependent, occurred immediately on contact with the drug, rising quickly to a well defined steady state and, on washout of the drug, recovery was rapid. One of these unusual responses in the denervated artery is shown in Fig. 5 (7 days post-denervation). The response was not seen in other denervated arteries, even as soon as 2 days after denervation. The contralateral innervated arteries in each experiment showed the characteristic secondary sensitization type of response to dopa.

Effect of reserpine.

Arteries from 2 rabbits pretreated with reserpine (see Chapter 2.7 for details of methods used) were treated with nialamide and tested for a response to dopa. A typical secondary sensitization type response to dopa occurred in each case.

Effect of cocaine.

The effect of cocaine on the response to dopa in nialamide treated arteries was assessed in perfused segments in

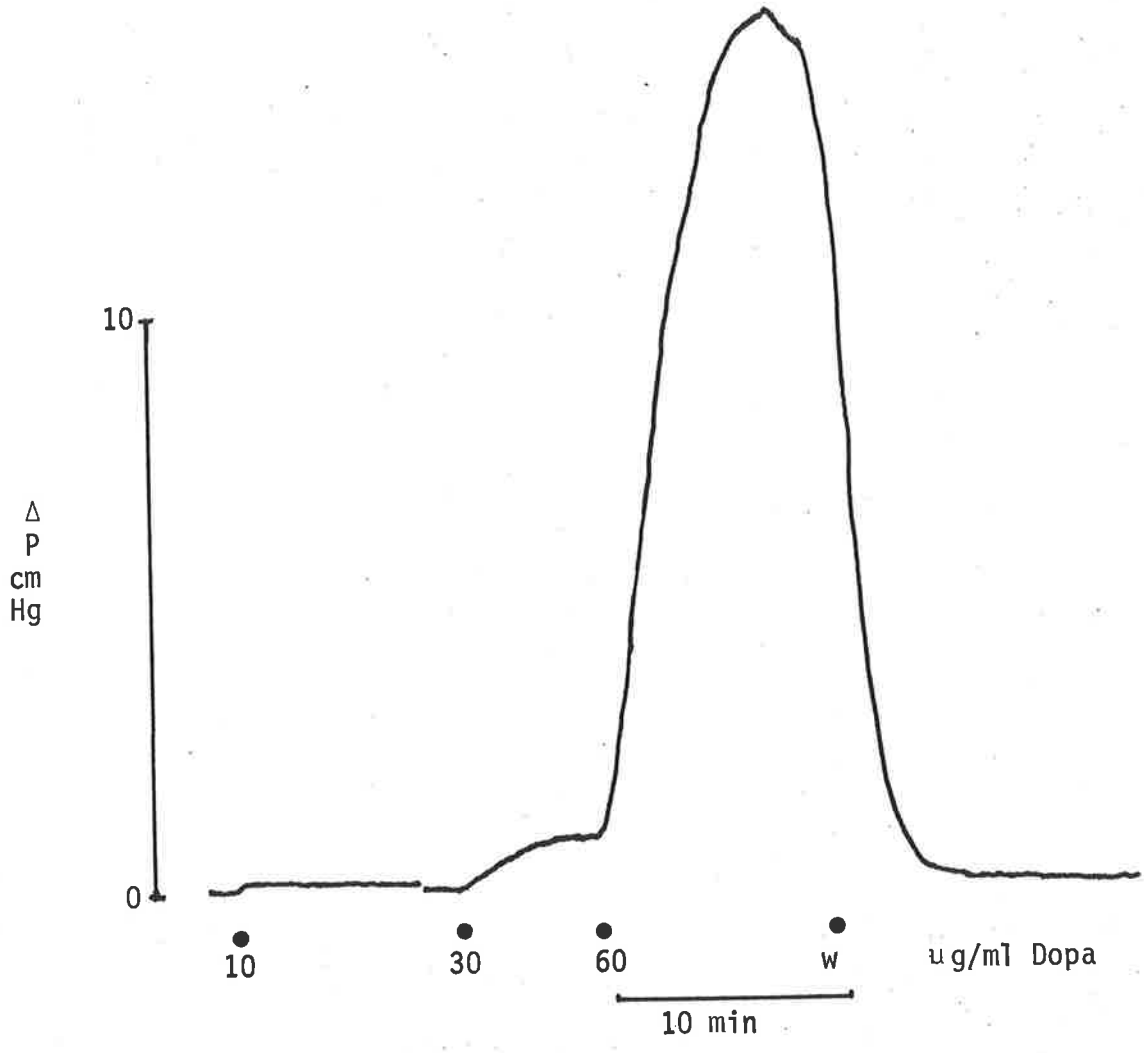


Figure 5.5

Unusual response to L-dopa in denervated artery segment (7 days post denervation) showing the lack of the characteristics of secondary sensitization in this response.

two ways:

(a) Cocaine was applied intra- and extraluminally 15 minutes before the dopa was added extraluminally and remained in contact with the artery throughout the dopa treatment;

(b) Cocaine (1µg/ml) was added during the recovery phase of the response to dopa.

In 5 experiments carried out as in (a), using concentrations of dopa 10-200 µg/ml, cocaine 1 µg/ml or 10 µg/ml did not prevent the occurrence of the response to dopa, nor did it change the characteristic shape of the response.

In 7 experiments carried out as in (b) above, cocaine produced a large constrictor response similar to the response seen in the MAO inhibited artery after DA (Chapter 4) or NA (de la Lande & Jellett, 1972). The response is immediate, rises to a maximum and declines even though the cocaine is still present. A typical response of this type is shown in Fig. 6.

Restoration of monoamine fluorescence with L-dopa.

Paired artery segments were removed from 2 rabbits pretreated with reserpine. One of each pair was then treated with nialamide for 1 hour. All arteries were incubated with dopa 100 µg/ml for 30 minutes at 37°C in Krebs bicarbonate solution. After incubation, all arteries were washed for 10 minutes, then frozen in acetone/dry ice mixture ready for fluorescence histochemistry. This was carried out as described in Chapter 2.8.

All arteries, nialamide treated and untreated, showed intense green fluorescence in the smooth muscle of the media and in the region of the nerve plexus. In one preparation the fluorescence in the nerve terminal region was difficult to differentiate from that in the media due to the brightness of the latter.

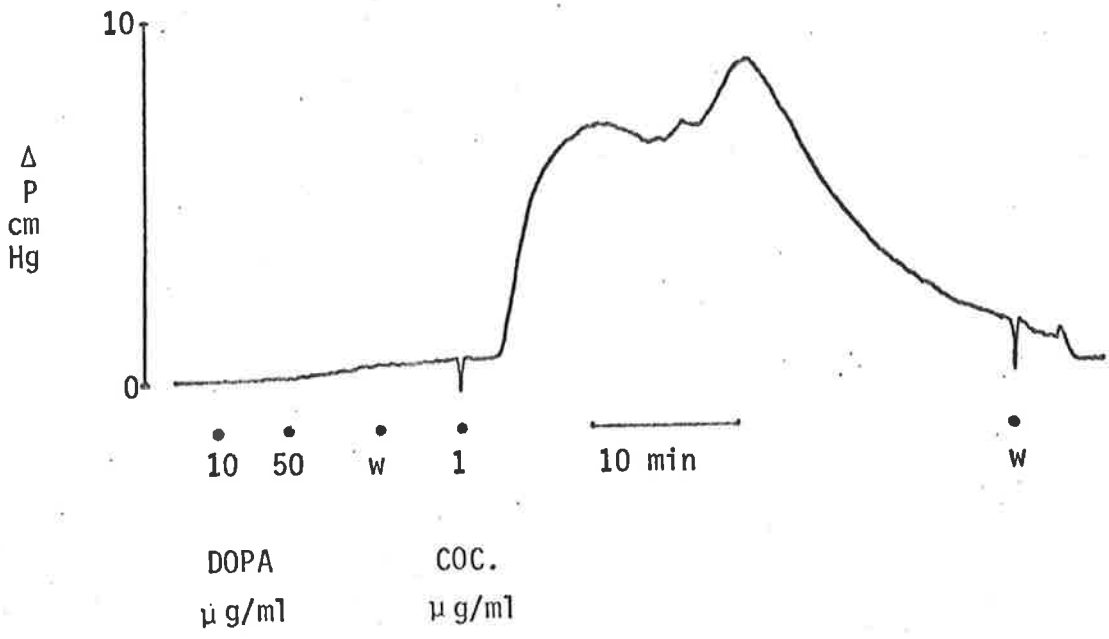


Figure 5.6

Cocaine constrictor response after exposure of nialamide treated artery perfused segment to L-dopa.

5.6

DISCUSSION

In the rabbit ear artery L-dopa caused no response in either isolated segments or helical strips. In the nialamide treated segment and strip dopa produced a prominent response after a short lag phase. The response was of the secondary sensitization type, similar to the response to DA after nialamide, and was characterised by the prolonged time course before steady state was achieved and by the prolonged recovery time. The response, like the secondary response to DA and NA, was dependent on the presence of the sympathetic nerves, since it did not occur in chronically denervated arteries. However, like DA, but in contrast to NA, blockade of $Uptake_1$ by cocaine failed to prevent the response. However, cocaine added during the slow recovery from the dopa response produced a rapid response similar in nature to that seen when cocaine was added during the slow recovery after DA and NA.

Reserpine pretreatment did not affect the secondary response to dopa (nor to NA or DA) thus indicating that the intragranular storage mechanism was unimportant in the response to dopa as well as to NA and DA.

Since L-dopa is not a substrate for MAO and produced no response in the untreated artery, it is likely that the response seen after MAO inhibition is caused by a product of dopa which is susceptible to MAO. Since the response is similar to that seen after DA, with similar effects when cocaine is added, it is probable that the formation and subsequent accumulation of metabolites of L-dopa, i.e. DA and/or NA, are the cause of the response seen after MAO inhibition.

The precursor to L-dopa, tyrosine, caused no response in the nialamide treated artery. This is not surprising, since it has been shown that excess of end-product (NA) in the cytoplasm causes inhibition of the enzyme tyrosine hydroxylase (Spector and

others, 1967). Under conditions of MAO inhibition, normal intraneuronal metabolism of NA virtually ceases and a cytoplasmic accumulation of NA can occur (Taxi and Droz, 1969). Under these conditions tyrosine hydroxylase would be inhibited, and the synthetic chain halted, thus preventing further increase in the NA concentration.

The nature of the response to L-dopa in two of the denervated nialamide treated preparations remains obscure. Fluorescence histochemistry showed no fluorescence in these arteries, nor did they respond to nerve stimulation, thus indicating that denervation was successful. The time (5-6 days postoperative) was well in excess of that in which nerve degeneration normally occurs (Van Orden and others, 1967) and in excess of that of other arteries in the same series which showed no response to L-dopa. The response was abolished by phentolamine, so was alpha-adrenergically mediated and not due to histamine release or some other mechanism. A direct action of L-dopa, not normally observed in an innervated preparation, is a possibility, though this was not seen again in any preparation denervated or not.

CHAPTER SIX

THE EFFECT OF INHIBITORS OF DOPAMINE β-HYDROXYLASE ON SECONDARY SENSITIZATION

CHAPTER 6

INTRODUCTION

Since L-dopa and DA are precursors of NA in sympathetic nerves, it was conceivable that the secondary sensitization to both L-dopa and DA was mediated by the release of NA. To test this possibility, the effects of inhibitors of dopamine-β-hydroxylase, disulfiram and its reduction product sodium diethyldithiocarbamate (DDC), on the sensitization to DA and dopa produced by nialamide, were studied. The results are described in this chapter.

Disulfiram and DDC have been shown to be potent inhibitors of dopamine-β-hydroxylase, disulfiram acting after its reduction to DDC (Goldstein and others, 1964, 1965). Disulfiram does not affect the uptake, storage, release or metabolism of NA or the uptake, storage or release of DA in the rat heart (Goldstein and others, 1964; Mussachio and others, 1966, 1969).

METHODS

The techniques used in this chapter are described in Chapter 2 - General Methods. DDC 3.5 μg/ml or disulfiram 3 μg/ml was applied to the perfused segment or strip preparation for 15 minutes prior to and continually during treatment with DA or dopa. In some preliminary experiments the period of DDC treatment prior to DA or dopa was extended to 1 hour. There was no significant difference in the results from the two series, so the lesser exposure time was used.

RESULTS

The effects of DDC on the sensitivities to NA and DA in arteries which had not been treated with nialamide was first investigated. Helical strip preparations were used.

In 9 experiments, using normal arteries and 6 experiments with reserpine pretreated arteries, it was found that DDC did not alter the sensitivity to NA for periods of up to 2 hours after application of the drug. However, in 2 of these arteries, there was a marked decrease in the maximum response to NA 6 hours after DDC treatment; hence all experiments on the effects of DDC described later in this chapter were restricted to the period within 2 hours of DDC treatment.

In a further 3 experiments, the effect of DDC on the sensitivities of artery strips to DA was tested. Following DDC, there was a small decrease in the maximum response to DA compared with the maximum response prior to DDC treatment. However, the decrease (about 6%) was comparable in magnitude to that observed in strips not subjected to DDC treatment (Chapter 3.6).

The above results indicated that DDC was unlikely to interfere with the pharmacological responses to either NA or DA if used under defined conditions, and hence would be suitable as a pharmacological agent to study the role of dopamine-β-hydroxylase in the secondary sensitization to DA and dopa.

1. THE EFFECT OF DDC ON THE RESPONSE TO DA AND DOPA IN THE NIALAMIDE TREATED EAR ARTERY.

In 9 preliminary experiments, using nialamide treated perfused segments from 6 untreated and 3 reserpine pre-treated rabbits, responses to DA were elicited before and during DDC (3.5 μg/ml), or disulfiram (3 μg/ml), treatment in paired arteries. In all of the arteries from the untreated rabbits secondary sensitization to DA was evident. However, in all 3 of the reserpine pre-treated arteries no secondary sensitization to DA occurred. This preliminary result was tested quantitatively on strips as below.

In any one experiment two helical strips were treated

according to the following plan:

Strip 1 - 1st dose response curve to DA then nialamide then 2nd dose response curve to DA.

Strip 2 - nialamide then DDC treatment commenced during which dose response curve to DA elicited.

The above plan, inadvertently, is not a strict comparison between arteries of the effect of DDC, since the latter was not the only variable. However, as indicated earlier, there was little change in the sensitivities of both the untreated and nialamide treated arteries to DA on repeated application (refer to page 3.6 and 4.6). Thus, between-arteries comparisons of the effects of DDC were valid in testing the conclusions based on the within-arteries comparisons from the preliminary experiments mentioned above.

Recovery rates were studied in 4 strips from each rabbit according to the plan below:

Strip No.	1	2	3	4
Treatment	NA DR	Nial	NA DR	Nial
	-	DDC	DDC	-
	NA DR	DA	NA DR	DA

(DR = dose response)

Arteries in groups 2 and 4 were allowed to remain in contact with a standard submaximal concentration of DA for 15 minutes. The DA was then washed out, and the arteries allowed to relax to their pretreatment resting length.

Typical cumulative responses after nialamide in the presence and absence of DDC are shown in Fig. 1; Fig. 2 shows mean dose response curves for 5 experiments. Fig. 3 shows the recovery curves after a standard dose of DA (3 µg/ml) in 5 experiments. It is apparent that DDC had little effect on any of the phenomena associated with secondary sensitization, i.e. time required to attain a steady state response, the

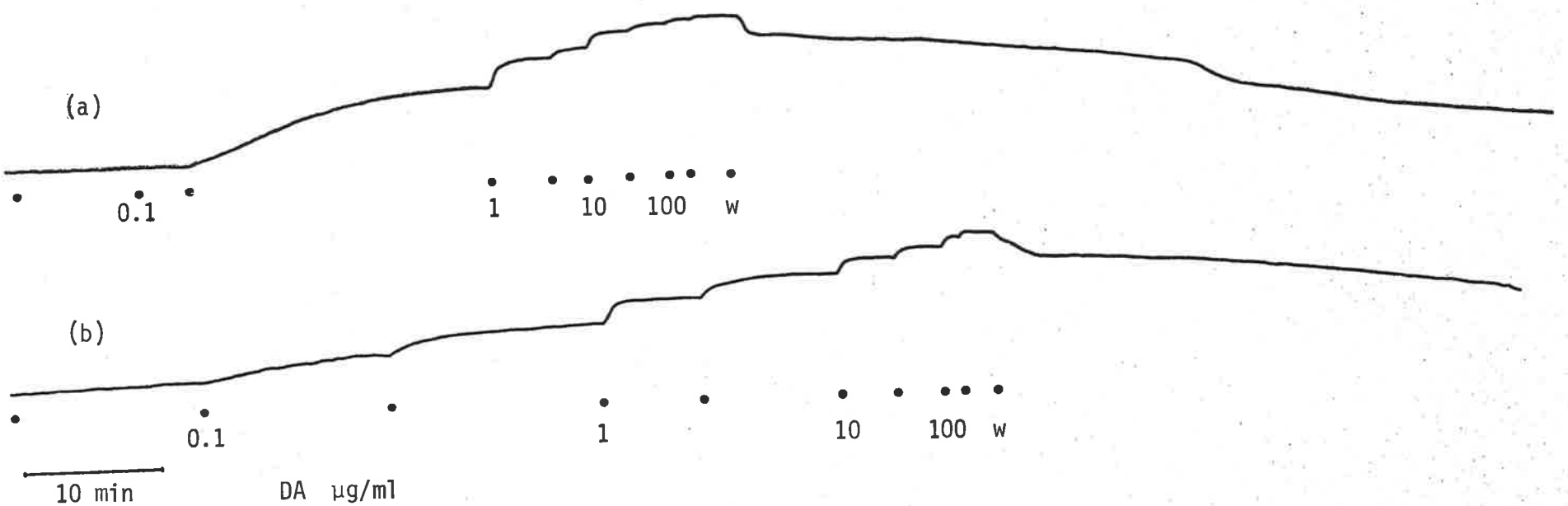


Figure 6.1

Responses to DA in nialamide treated helical strips.

(a) in presence of DDC (3.5 µg/ml)

(b) in absence of DDC

illustrating the failure of DDC to prevent the occurrence of secondary sensitization to dopamine.

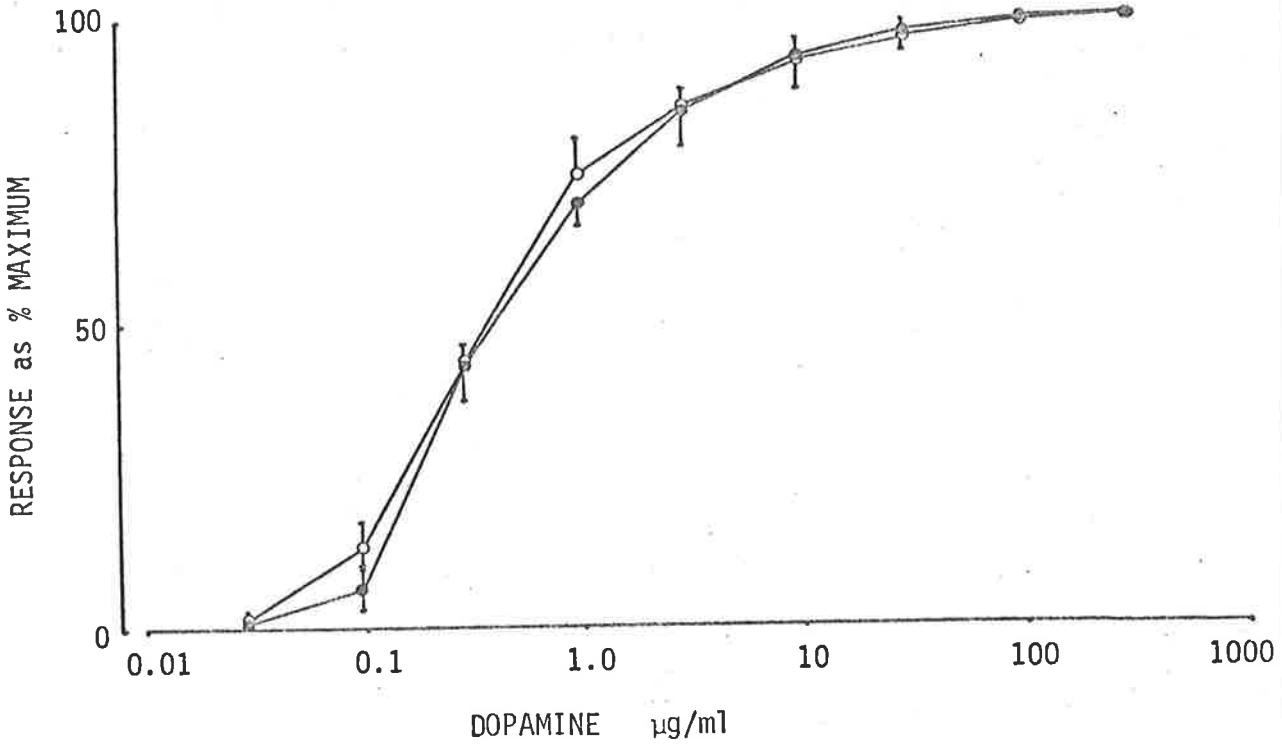


Figure 6.2

The effect of DDC 3.5 µg/ml on the sensitivity of nialamide treated artery helical strips to dopamine.

Values shown are the mean responses ± s.e. from 5 experiments. Open circles denote responses in absence of DDC, closed circles in presence of DDC.

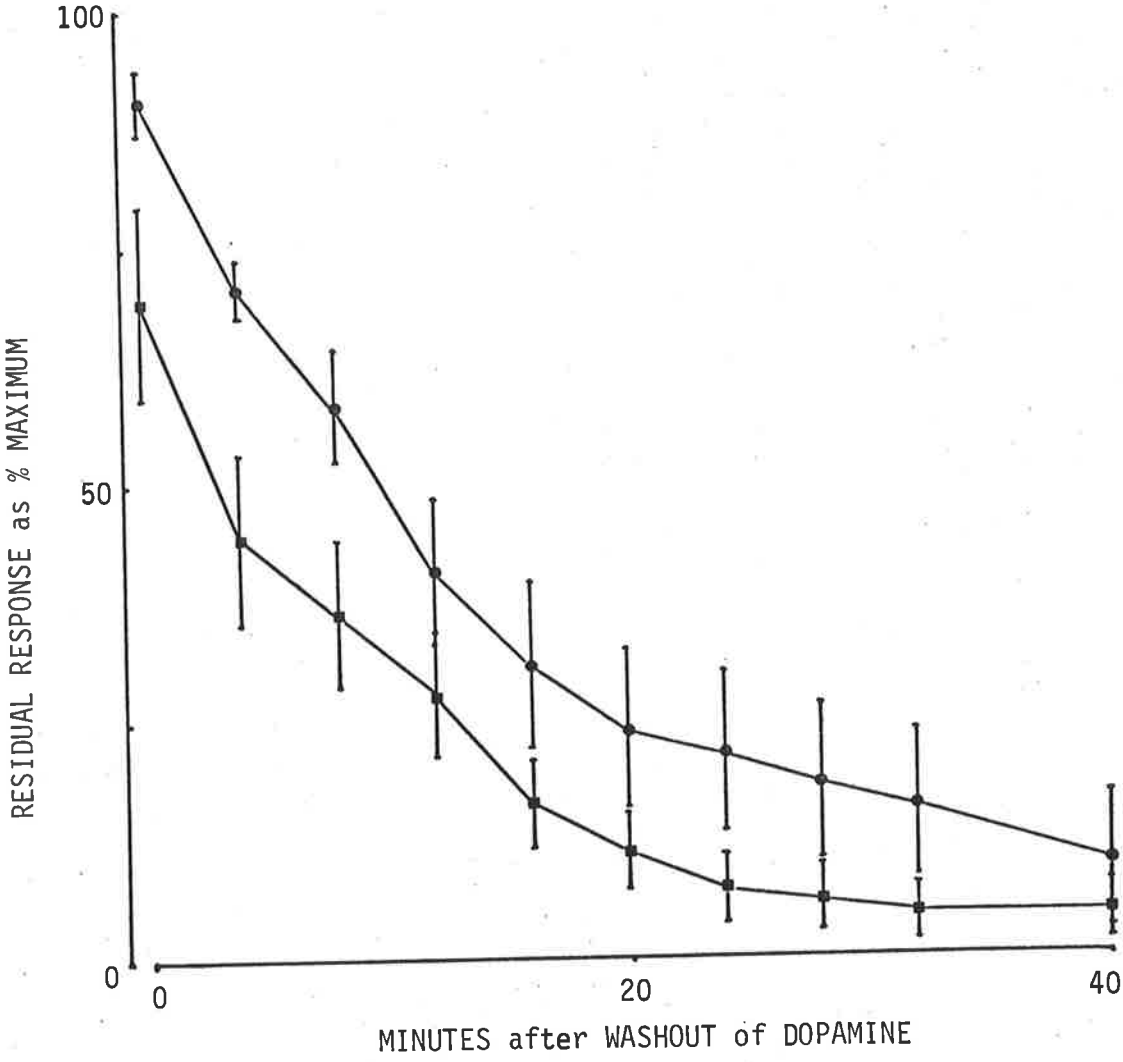


Figure 6.3

Recovery after submaximal concentration of dopamine (3 μg/ml) in nialamide treated artery helical strips, in presence of DDC (squares) or in its absence (circles).

Data shown are the means ± s.e. of 5 experiments in which strips were exposed to a submaximal concentration of dopamine (3 μg/ml) for 15 minutes, and the recovery observed after washout of the drug.

Values at each point do not differ significantly except at 4 mins. (t-test for paired observations $P > 0.05$).

magnitude of the steady state response, or the rate of recovery from this. The only significant change was a tendency for the DDC to hasten recovery during the first 4 minutes after DA washout.

However, DDC did appear to modify the effects of nialamide on artery strips from reserpine pretreated rabbits. When treated according to the plan described earlier (page 6.3), the sensitivity to DA following nialamide treatment was significantly less in the DDC treated than in the untreated arteries (Fig. 4). Furthermore, as illustrated in Fig. 5a and b, the characteristics of secondary sensitization in the reserpine and DDC treated artery were much less pronounced than in DDC treated arteries from untreated rabbits. Thus, the arteries displayed rapid attainment of the steady state level of the response and relatively rapid recoveries from these responses. The ability of DDC to increase the rates of recovery from DA was confirmed in another series of experiments where reserpine pretreated arteries were exposed to a standard dose of DA according to the above plan (Fig. 6). This figure includes data from nialamide untreated arteries, which makes it clear that, although the rates of recovery in the reserpine pretreated DDC-nialamide treated arteries are not as rapid as those prevailing in the absence of nialamide treatment, they are still significantly faster than the rates prevailing in nialamide treated arteries. If, however, the reserpine pretreated DDC-nialamide treated arteries are further compared with a group of denervated arteries after nialamide treatment (Fig. 7 from experiments described on page 4.7), it will be seen that there is no significant difference between the recovery curves, thus indicating that the retarding effect on recovery in the former group is caused by inhibition of extraneuronal MAO.

2. EFFECT OF COCAINE.

Cocaine was added to paired nialamide treated artery

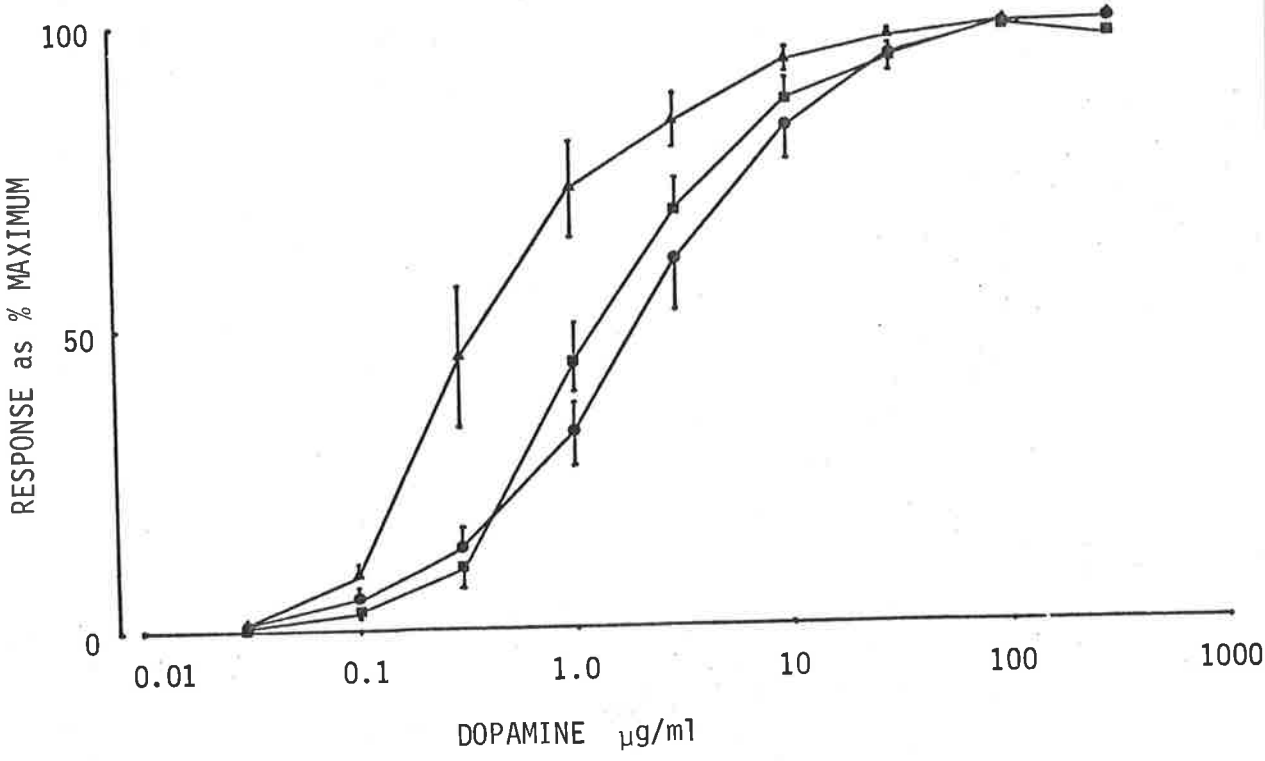


Figure 6.4

Reserpine pretreated strips: Effect of DDC on the sensitivity to dopamine in nialamide treated artery helical strips.

Shown are the means of responses ± s.e. from 5 nialamide treated (triangles), 6 DDC and nialamide treated (squares) and 8 untreated (circles) strips.

Analysis of variance shows that the responses in the DDC and nialamide treated strips did not differ significantly at any point from the untreated strips. However the nialamide treated strips varied significantly from the other two at concentration levels of 0.1 to 1.0 µg/ml DA ($P < 0.05$).

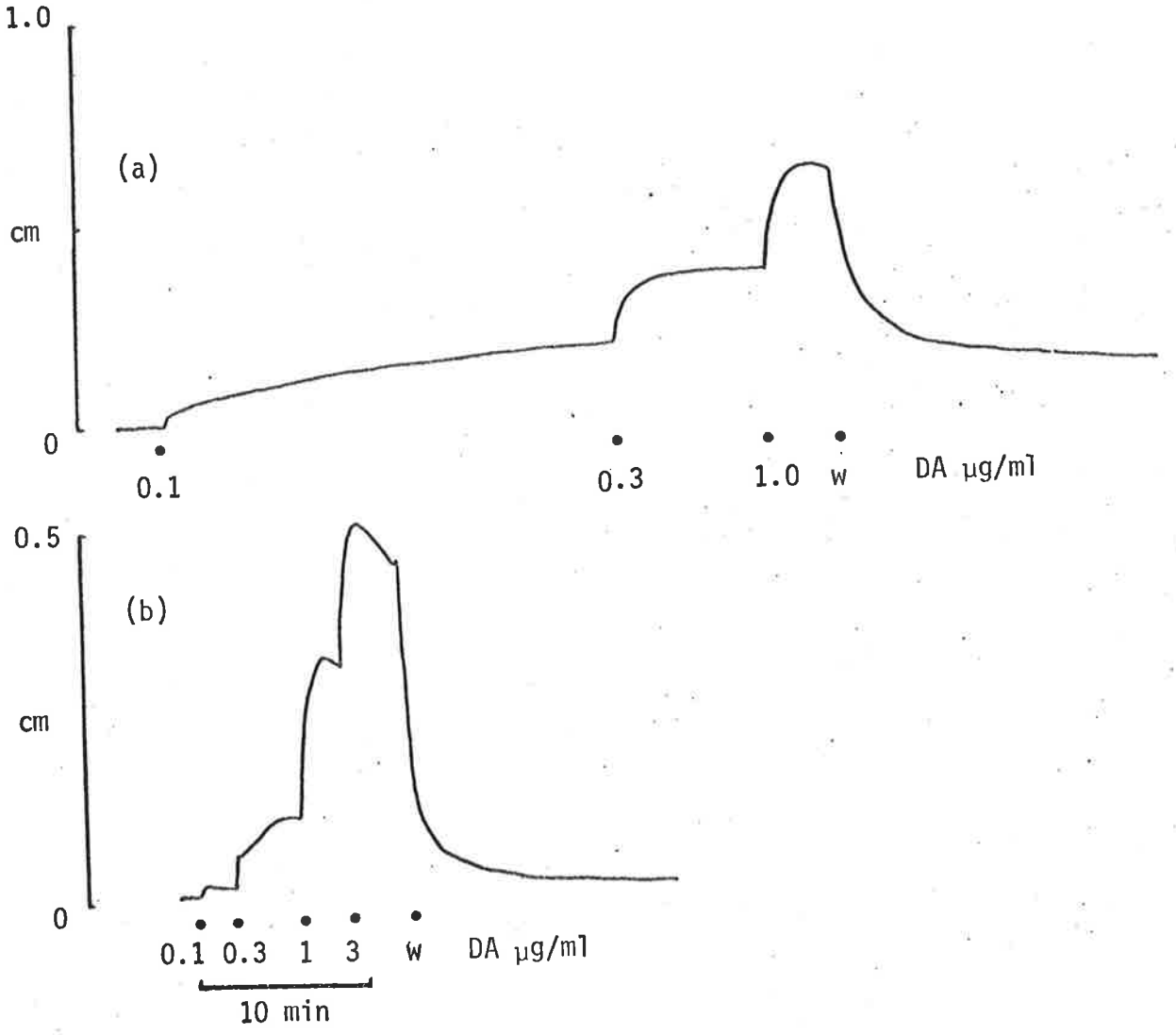


Figure 6.5

Reserpine pretreated artery: Effect of DDC (3.5 µg/ml) on the response to dopamine in nialamide pretreated artery helical strips.

(a) shows secondary sensitization to dopamine in absence of DDC,

(b) shows the lack of secondary sensitization in the presence of DDC.

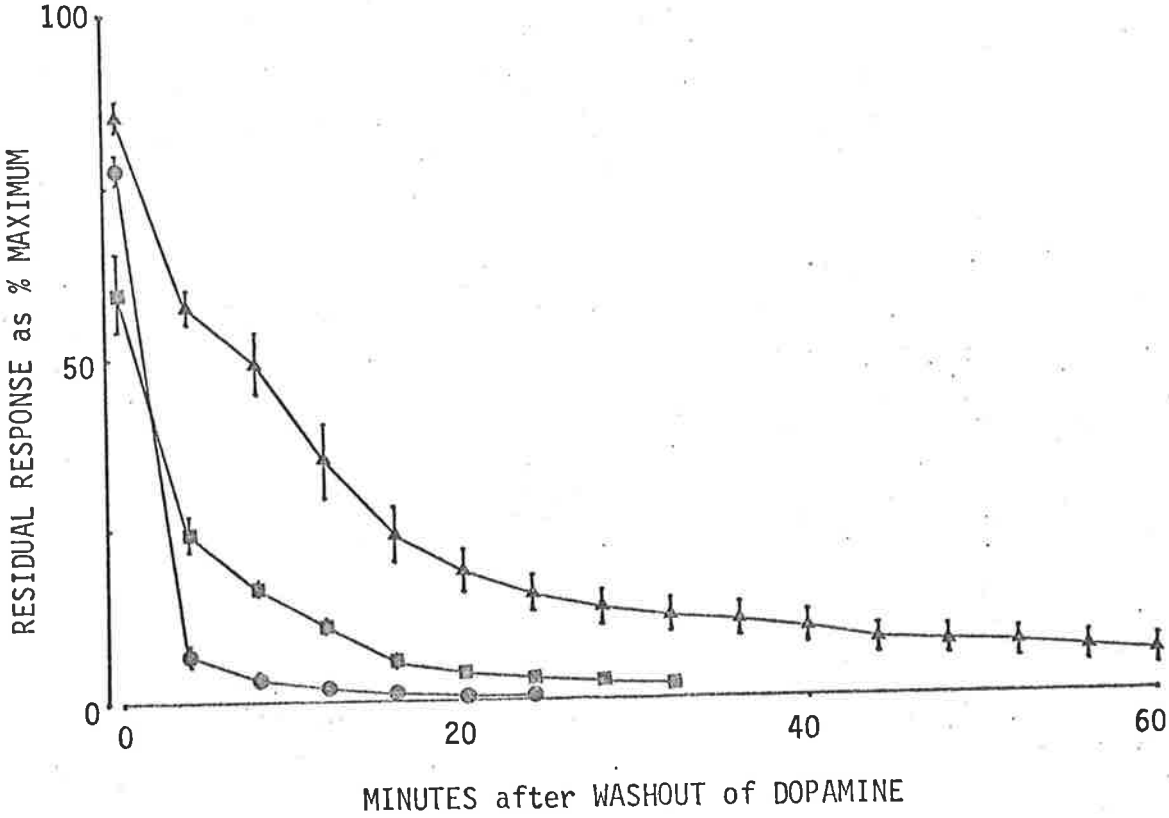


Figure 6.6

Reserpine pretreated arteries: Effect of DDC (3.5 µg/ml) on recovery from a submaximal concentration of dopamine (3 µg/ml) in nialamide treated artery helical strips.

Values shown are the mean residual response ± s.e. from 7 experiments in which recoveries were recorded for nialamide treated (triangles), nialamide and DDC treated (squares), and untreated (circles) strips.

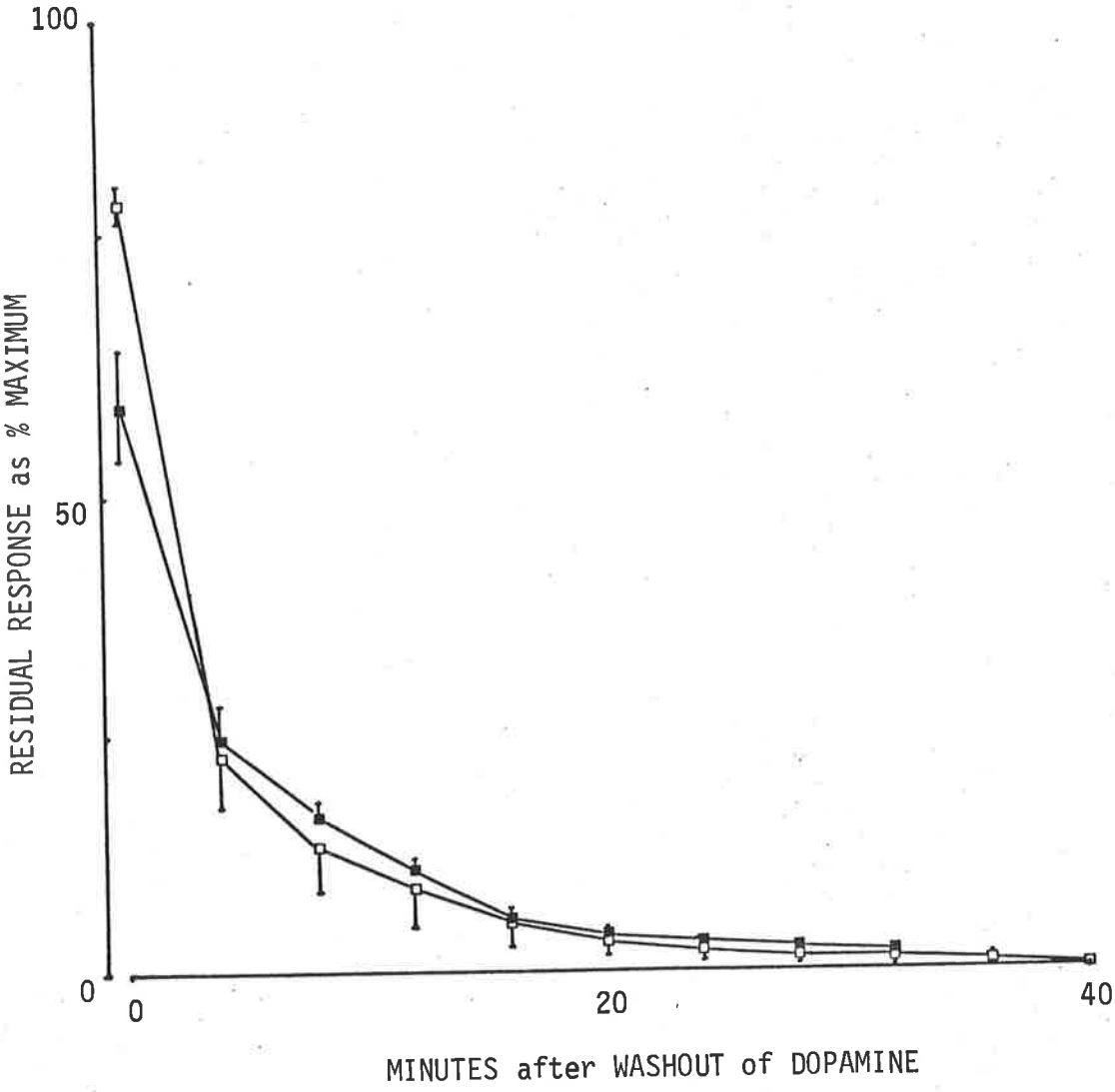


Figure 6.7

Recovery curves from 5 nialamide treated denervated (open squares) and 7 DDC, nialamide and reserpine treated (closed squares) artery helical strips.

Values at each point after 0 time do not differ significantly (t-test $P > 0.05$).

The values for the denervated nialamide treated strips were taken from experiments recorded in Chapter 4.7, and Figure 4.8.

segments 10 minutes after DA washout and the constrictor response to cocaine recorded. (This effect of cocaine was first described on page 4.9.) The paired arteries were from either untreated or reserpine pretreated rabbits; one of each pair was treated with DDC. Cocaine elicited a constrictor response from 5 of 6 arteries from untreated rabbits, but not from 3 arteries from reserpine pretreated rabbits which had been treated with DDC. As indicated earlier, the latter arteries also failed to display delayed recovery, so that the perfusion pressure was at or close to the resting level at the time of cocaine application.

In summary, the results so far presented in this chapter showed that in nialamide treated arteries an inhibitor of dopamine-β-hydroxylase, DDC, had little or no effect on the secondary sensitization to DA in arteries from untreated rabbits, but tended to prevent this phenomenon in arteries from reserpine pretreated rabbits.

3. EFFECT OF DDC ON RESPONSE TO L-DOPA.

Responses of artery strips to L-dopa were elicited under the following conditions:

Strip No.					
1		Nial	-	DOPA	NA max
2	NA DR	-	-	NA DR	NA max
3		Nial	DDC	DOPA	NA max
4	NA DR	-	DDC	NA DR	NA max

(DR = dose response; max = supramaximal dose)

The DDC was applied 60 minutes before L-dopa in 4 experiments and 15 minutes before L-dopa in one experiment. As indicated in Fig. 8, the responses to dopa (3 to 100 μg/ml, applied cumulatively) were significantly depressed by DDC. However, the characteristics of secondary sensitization, namely slow attainment of the steady state response and slow recovery on washout, remained prominent. Unfortunately, time did not permit comparisons to be made on

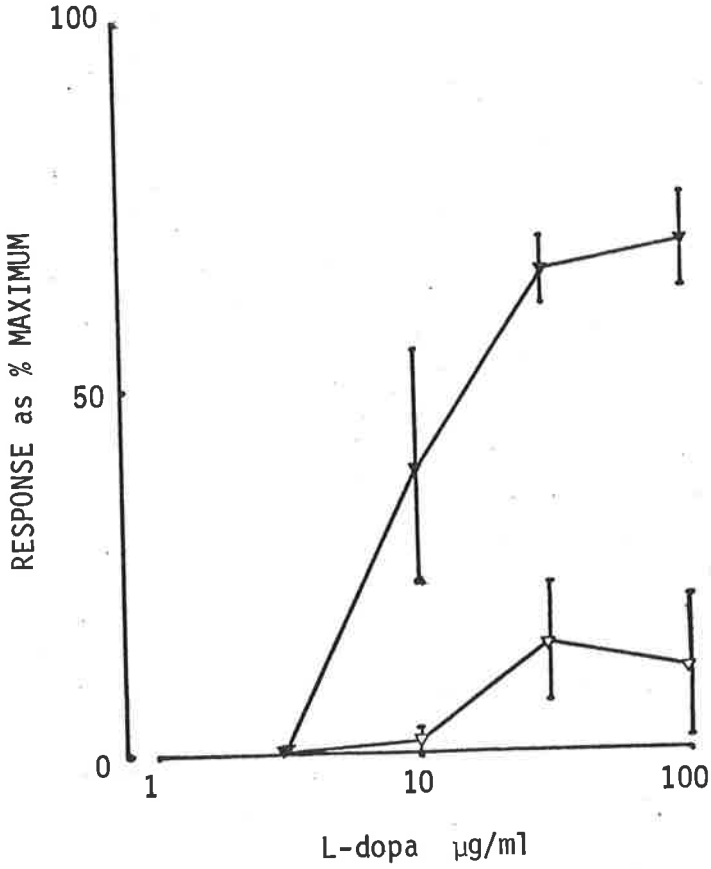


Figure 6.8

The effect of DDC on responses to L-dopa in nialamide treated artery helical strips.

The values shown are the means ± s.e. from 5 experiments in which arteries were treated with nialamide (closed triangles) or DDC and nialamide (open triangles) before exposure to L-dopa.

reserpine pretreated arteries. It was concluded that the interference by DDC with secondary sensitization to L-dopa was more marked than in the case of DA.

D I S C U S S I O N

The failure of DDC to modify the secondary sensitization to DA in normal arteries suggests that either NA is unimportant in the secondary response to DA, or that NA has a role to play in the secondary response to DA, but newly synthesised NA does not influence the response when a supply of endogenous NA is available. A third possibility is that DDC is unable to inhibit DA-β-hydroxylase under the experimental conditions used.

To consider the latter possibility first, the concentration of DDC, 3.5 μg/ml, which is equivalent to disulfiram 1 x 10^{-5}M, was that reported to completely inhibit dopamine-β-hydroxylase *in vitro* (Goldstein and others, 1964). Much higher concentrations (10-500 μg/ml) have been used by other workers using isolated tissues, e.g. Collins and West (1968a), Tsai and others (1967). However, due to the depressant effect after a time on NA sensitivity in the rabbit ear artery, it was considered unwise to use increased concentrations. More importantly, however, DDC (in the same concentration) did reduce the secondary sensitization in reserpine pretreated arteries, thus indicating that the drug did behave as though it inhibited the enzyme under these conditions. However, the kinetics of the enzyme may differ in the presence of a high concentration of end-product (i.e. NA), and the two situations may not in fact be comparable.

The first possibility mentioned above, that NA may be unimportant in the response, appears unlikely, since in the reserpine pretreated artery inhibition of its formation caused a significant reduction in the secondary sensitization to DA. This

occurred in the absence of any depressant effect of DDC on NA sensitivity in reserpine pretreated arteries or any effect of reserpine itself on secondary sensitization (Chapter 4).

The possibility that newly synthesized NA is not necessary for secondary sensitization to DA, provided a source of endogenous NA is present, is an attractive alternative explanation to the failure of DDC to reduce secondary sensitization. DA is known to be taken up by the granular uptake mechanism (Stjarne 1966) and thus may cause displacement of intragranularly bound NA into the cytoplasm. Since it has been shown that the secondary sensitization to DA (Chapter 4) and NA (de la Lande and Jellett 1972) does not depend on intragranular binding, it is probable that an increase in the cytoplasmic concentration of NA (made possible by inhibition of MAO) is responsible for the secondary effects seen. Under conditions of dopamine-β-hydroxylase inhibition, this increased level of NA may be provided from the intragranular stores.

In the case of reserpine pretreatment no stored NA is present, either in granule or in cytoplasm. However, after exogenous DA is added, rapid synthesis of NA may occur, providing levels of NA sufficient to cause the secondary sensitization effects. Synthesis of NA from DA in reserpine pretreated MAO inhibited preparations has been demonstrated by Jonsson and Sachs (1970). If this synthesis of NA from DA is inhibited by DDC, the secondary response does not occur.

In summary, from the evidence presented in this chapter, it is clear that NA plays an important role in the secondary sensitization response to DA and to dopa. It is also probable that this NA may either be made available from the endogenous stores (by displacement) or by synthesis from the exogenous DA.

CHAPTER SEVEN

THE EFFECT OF LOWERED TEMPERATURE ON THE RESPONSES TO NA, DA AND L-DOPA IN NIALAMIDE TREATED AND UNTREATED ARTERIES

CHAPTER 7

INTRODUCTION

It has been shown in the foregoing chapters that the effect of MAO inhibition on the responses to L-dopa and to DA in the rabbit ear artery was to produce a sensitization of the secondary type. A similar sensitization to NA has been seen by other workers, in this and other tissues (de la Lande and Jellett 1969, 1972; Tsai 1968; Tsai and others 1967; Furchgott and Sanchez Garcia 1968). The effects appear to be neuronal in origin and, at least in the case of NA, related to the $Uptake_1$ system.

In an attempt to further elucidate the mechanism involved in secondary sensitization, the effect of lowering the bath temperature on (a) the secondary response to dopa, DA and NA in nialamide-treated arteries, and (b) the functioning of various systems which may be involved in secondary sensitization has been investigated. To assist interpretation of the results, the effect of lowered temperature on the response to NA and DA in the untreated artery has also been studied.

In the work presented here, it was found, fortuitously, that a reduction in bath temperature to about $25^{o}C$ caused the loss of the secondary response to dopa (described in Chapter 5). The phenomenon was further studied as it seemed to offer an alternative to enzyme inhibition as a means of analyzing the mechanism of secondary sensitization. The particular questions asked were:

1) whether the effects of a decrease in temperature extended to the secondary responses to DA and NA following nialamide treatment,

2) if so, whether the loss of effect was due to a decrease in (a) effects of these agents on the target organ, (b) their uptake by the nerve terminals, or (c) the activity of the synthesising enzyme.

At the time of commencing this study, there was already a considerable amount of information available in the literature on the effect of temperature on adrenergic mechanisms in a variety of tissues. This will be considered further in the discussion. The only systematic analysis on the rabbit ear artery was that of Glover and others (1968) who studied the differential effects of cooling on the sensitivity to NA in rabbit ear and femoral arteries. They reported that cooling the vessels from 37°C to 3°C caused a steady rise in tone, and an increased sensitivity to intraluminal injection of NA between 37°C and 18°C with a peak sensitivity at 24°C. Below 18°C the sensitivity to NA diminished steadily, and disappeared at 7°C. This sensitization did not occur in the femoral artery, in which the sensitivity steadily declined, and at 18°C the response disappeared. In a later study, Glover and Wallace (1969) suggested that the sensitization in the rabbit ear artery produced by cooling was more likely to be linked with the effects of temperature on the membrane potential of the smooth muscle, rather than with neuronal mechanisms. One reason for this suggestion was that the sensitivity to NA was increased equally intra- and extraluminally by cooling. Later work showed that the increased sensitivity at lowered temperature was seen only in 'cold-acclimatised' animals (McClelland and others 1969). However, there is evidence in the rabbit ear artery that neuronal mechanisms are also affected by cooling (Herron and others 1971). They reported that the sensitivity to extraluminal l-NA in the rabbit ear artery was similar at 37°C and 3°C, but that the sensitivity to extraluminal d-NA was markedly depressed at 3°C. This was thought to be due to progressive blockade of uptake into the nerve terminals as the temperature fell, thus causing supersensitivity to the l-isomer. The d-isomer, not being so affected by uptake showed no potentiation due to blockade of uptake, but merely depression as the temperature fell. In addition to the

effect of temperature on $Uptake_1$, extraneuronal uptake mechanisms in the splenic artery have also been shown to be impaired by cooling (Gillespie and Hamilton 1967).

Thus there is evidence that, in the rabbit ear artery, several mechanisms, neuronal and extraneuronal, may be affected by reduced temperature.

The plan of the present study was to observe the effect of a reduction in temperature to 25°C on the sensitivity to NA and DA in the untreated artery, and on the secondary sensitization to NA, DA and L-dopa after nialamide treatment. As part of this study, the effect of reduced temperature has also been examined on the neuronal uptake of NA, both directly, by measuring the net uptake, retention, and efflux of ^3H at 37°C or 25°C after incubation with (±) ^3HNA, and indirectly, by assessing the amount of inhibition of cocaine potentiation of extraluminal NA at 37°C and 25°C. In addition, data is presented on the net uptake of DA and on the formation of NA from DA after incubation with ^3H- and ^{14}C-DA.

METHODS

The details of the methods used are outlined in Chapter 2. Additional details are presented below.

GENERAL.

All rabbits used in these experiments had been kept at an ambient temperature of approximately 21°C prior to use.

In the experiments described in this chapter, responses to NA and DA in arteries were compared at 37°C and 25°C. Arteries were either single preparations in which dose response curves were obtained first at one of the temperatures, and subsequently at the other temperature; or paired arteries in which one artery was maintained at 25°C and the other at 37°C

throughout the experiment. The exception to the latter regime was in nialamide treated arteries. When paired arteries were used, both were maintained at 37°C throughout the time of the nialamide treatment; the temperature being reduced to 25°C subsequently in one artery of each pair. Arteries were cooled by lowering the temperature of the external bathing solution, and in the case of perfused arteries, also the perfusion solution, by lowering the temperature in the external circulating fluid in the organ bath. The temperature fell to 25°C over a period of about 10-15 minutes. After the temperature was stabilized the preparation was left for a further hour, to allow for equilibration at the lower temperature, before proceeding with the experiment. Arteries were warmed from 25°C to 37°C by allowing the external circulating fluid to heat over a period of about 15 minutes. After stabilization of the bath temperature at the new level one hour equilibration was allowed, as for cooled solutions, before proceeding with the experiment.

ISOTOPE.

1) Uptake and efflux of (±) ^{3}H NA.

Paired artery segments were tied at one end onto pieces of polythene cannula tubing. The free end was not occluded and the artery was not perfused. After nialamide treatment, as above, the arteries were incubated at 37°C or 25°C in purified (±) ^{3}H NA 0.5 µg/ml (Specific Activity 12 Ci/mM). The arteries were then washed for a total of 30 minutes in 6 successive 1 ml portions of Krebs bicarbonate solution maintained at 37°C or 25°C. After the wash period, all tissues were extracted at 37°C for 3 periods of 30 minutes each in a total of 2.0 ml 0.1 N HCl. The tissues were blotted, weighed and dissolved in 0.3 ml NCS reagent overnight at 37°C. 0.5 ml aliquots of the pooled acid extracts were counted in 20 ml Brays scintillant; and the NCS extracts counted in toluene scintillant (see Appendix for detail

of scintillant solutions). Quenching was corrected by internal standardization. All counting was done in a Packard Tricarb Liquid Scintillation Counter for 5 minutes in precounted pots.

2) Uptake of isotopic DA and formation of isotopic NA.

The methods used for the study of uptake of isotopic DA were essentially those described above for ^{3}H NA. However, there were some points of difference. In all experiments 4 segments of artery were used. These were pretreated as follows:

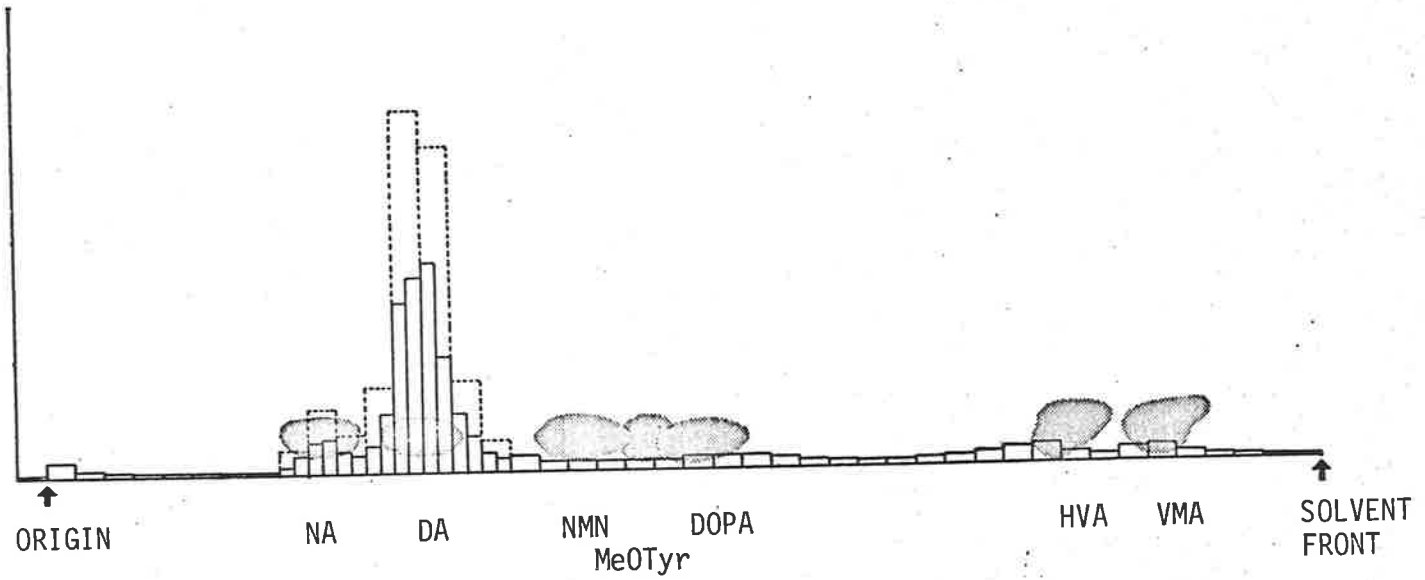
Segment	Pretreatment	^{14}C or ^{3}H DA Incubation Temperature	Wash Temperature
a	none	37°C	37°C
b	nialamide	37°C	37°C
c	nialamide	25°C	25°C
d	boiled	37°C	37°C

Segments were incubated with labelled DA 0.3 µg/ml (^{3}H DA Specific Activity 4.5 Ci/mM in 3 experiments and ^{14}C DA Specific Activity 56 mCi/mM in 2 experiments) for 30 minutes at the temperature indicated. After incubation the arteries were washed for a total of 12 minutes in 3 portions of Krebs bicarbonate solution (3 experiments with ^{3}H DA) and 30 minutes in 6 portions of Krebs bicarbonate solution (2 experiments with ^{14}C DA). The components of the acid (0.1 N HCl) tissue extracts were separated by the following methods. The acid tissue extracts were freeze dried, and the resultant residue extracted 3 times with a total of 1 ml ice cold acetone-ethanol mixture. The pooled extracts were evaporated under a stream of nitrogen gas to a small volume (approximately 0.02 ml). The concentrated extract, together with a cold carrier mixture containing NA, DA, and representative metabolites, was then applied to a cellulose phosphate ion exchange chromatography paper (see General Method, 2.11-2.13). The cold carrier mixture and appropriate standards were run concurrently

Figure 7.1

A typical chromatogram showing the positions of NA, DA and their major metabolites compared with the radioactive profile from a tissue extract.

Before liquid scintillation counting, the chromatogram was divided into 1 cm segments except in the region of NA and DA where 0.5 cm segments were cut. The dotted histogram shows the sums of pairs of 0.5 cm segments in the NA and DA region of the paper.



with the sample paper. A typical chromatogram showing the relative positions of metabolites compared with the radioactivity profile is shown in Fig. 1.

RESULTS

1. EFFECT OF LOWERED TEMPERATURE ON THE RESPONSE TO NA.

These experiments were carried out on both isolated perfused segments and helical strips.

In the perfused segments, dose response curves to both intra- and extraluminal NA were obtained on the same arteries at two bath temperatures, 37°C and 25°C, in 6 experiments. In 3 preparations, the first dose response curve was obtained at 37°C, then the bath solution cooled to 25°C and the dose response curve repeated. In the remaining 3 preparations, the first dose response curves were obtained at 25°C, then the bath solution warmed to 37°C, before repeating the dose response curves. In a further experiment, paired arteries, one maintained at 37°C, and the other at 25°C, were used. In all experiments there was no potentiation of NA at the lower temperature. The mean sensitivity ratio and standard error for extra- and intraluminal NA (37°C/25°C) were 1.2(0.95-1.5) and 0.87(0.55-1.4) respectively. These values were calculated from parallel dose response curves at a response of 60 mm Hg.

In the experiments using helical strips, 5 pairs of strips were used. One of the pair was maintained at 25°C, the other at 37°C throughout the experiment, and NA dose response curves were obtained on each of the strips. There was no significant difference between the sensitivities to NA; the mean ratio (37°C/25°C) at ED_{50} was 1.1(0.72-1.7).

The effect of reduced temperature on NA sensitivity in nialamide treated arteries was studied in both perfused segments

and helical strips.

In segments, there was little evidence of secondary sensitization at the lower temperature. Responses rose rapidly to the steady state level, and recovered quickly on washout of the drug.

In strips at 37°C, the effect of nialamide on the response to NA was as follows:

(a) There was no effect on the magnitude of the steady state response, or on the rate at which the steady state response was attained.

(b) There was a delay in recovery from the response, comprising mainly a slow further contraction after the strip had returned rapidly towards its resting length. This effect of nialamide on the recovery curve was less pronounced in strips than in segments.

Thus, the effect of nialamide on the strips was less dramatic than its effect on segments (as described by de la Lande and Jellett 1972). One phase of secondary sensitization, namely the slow attainment of the steady state, is largely absent, and the delay in recovery was less prominent.

Recovery curves after incubation at 25°C and 37°C with NA (0.5 μg/ml) for 30 minutes in paired artery segments and strips are shown in Figs. 2 and 3 respectively. It should be noted that in the experiments with segments, extraluminal NA only was tested, the intraluminal perfusion was stopped during the incubation period to avoid maintaining the artery at high perfusion pressures. Perfusion was resumed just prior to washout of the NA.

The only evidence of secondary sensitization at 25°C was observed in strips, where there was a slight tendency for recovery to be slower in the nialamide treated than in untreated arteries. Even so, in the nialamide treated arteries, the rates of recovery were not significantly different between innervated arteries at 25°C and a group of denervated arteries at 37°C.

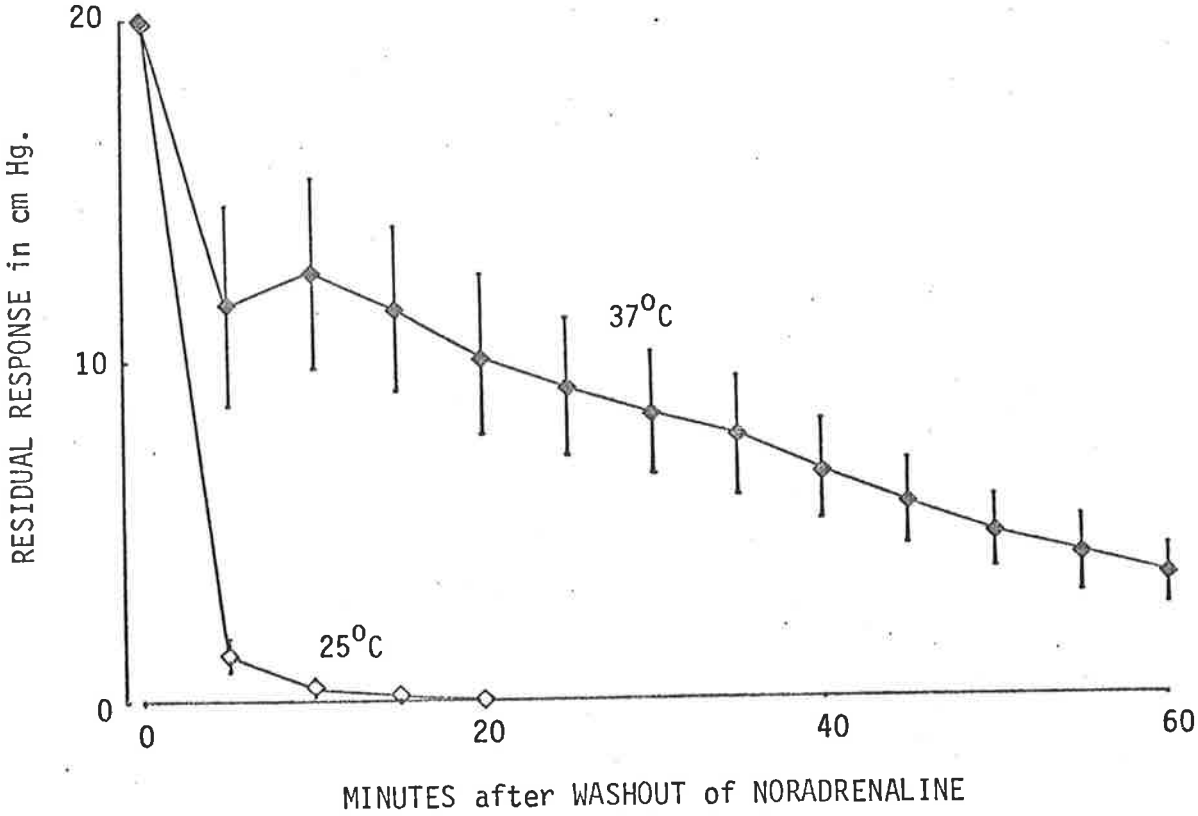


Figure 7.2

The effect of lowered temperature on recovery of nialamide treated artery perfused segments after incubation with NA.

Values shown are the means ± s.e. of 5 experiments in which paired segments were incubated with extraluminal NA 0.5 µg/ml for 30 minutes at 37°C or 25°C, the residual response being measured at regular time intervals after the washout of the NA. Perfusion was stopped during the incubation to avoid maintaining the artery at high perfusion pressures, and was resumed just prior to washout of the NA. Groups differ significantly at all points after zero time (t-test, $P < 0.01$).

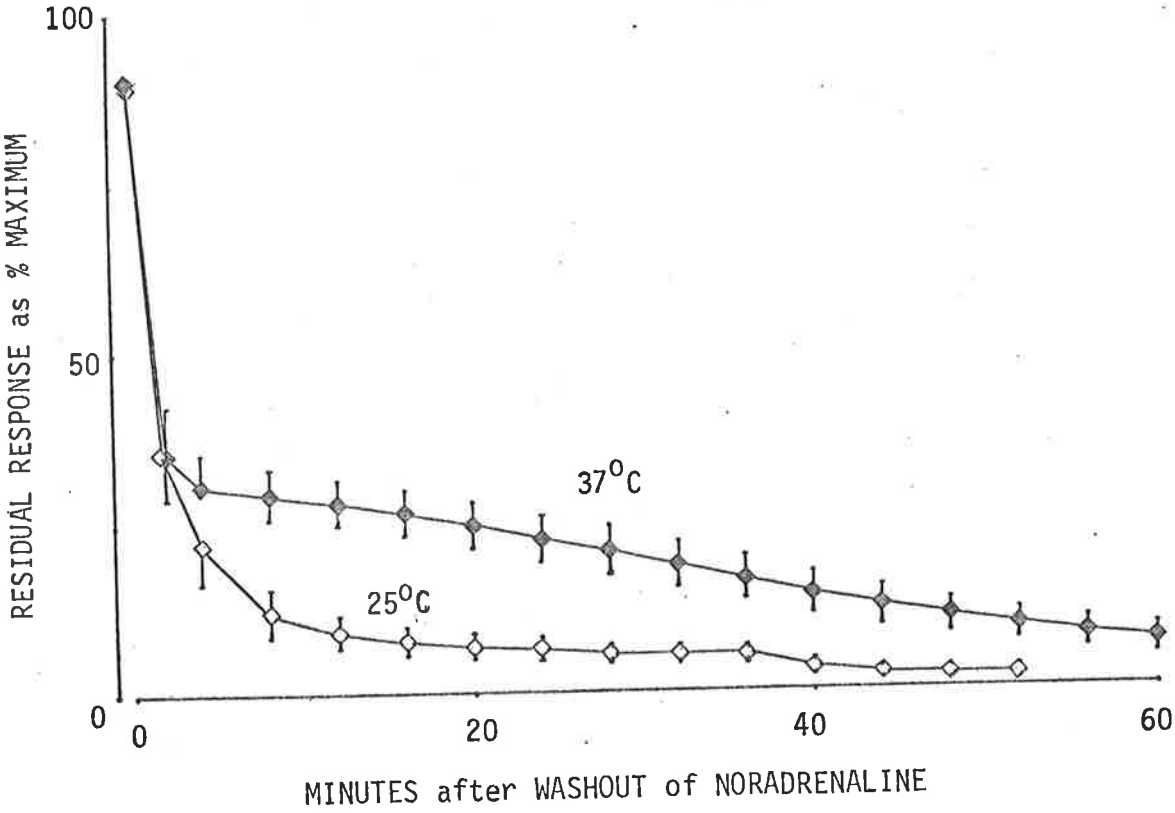


Figure 7.3

The effect of lowered temperature on the recovery from exposure to noradrenaline in artery helical strips pretreated with nialamide.

Values shown are the means ± s.e. from experiments in which strips were incubated with 0.5 µg/ml NA for 30 minutes at 37°C (12 experiments) or 25°C (9 experiments), after which the NA was washed out and the residual response measured at regular time intervals.

The curves differ significantly at every point after 4 minutes. (t-test, $P < 0.01$).

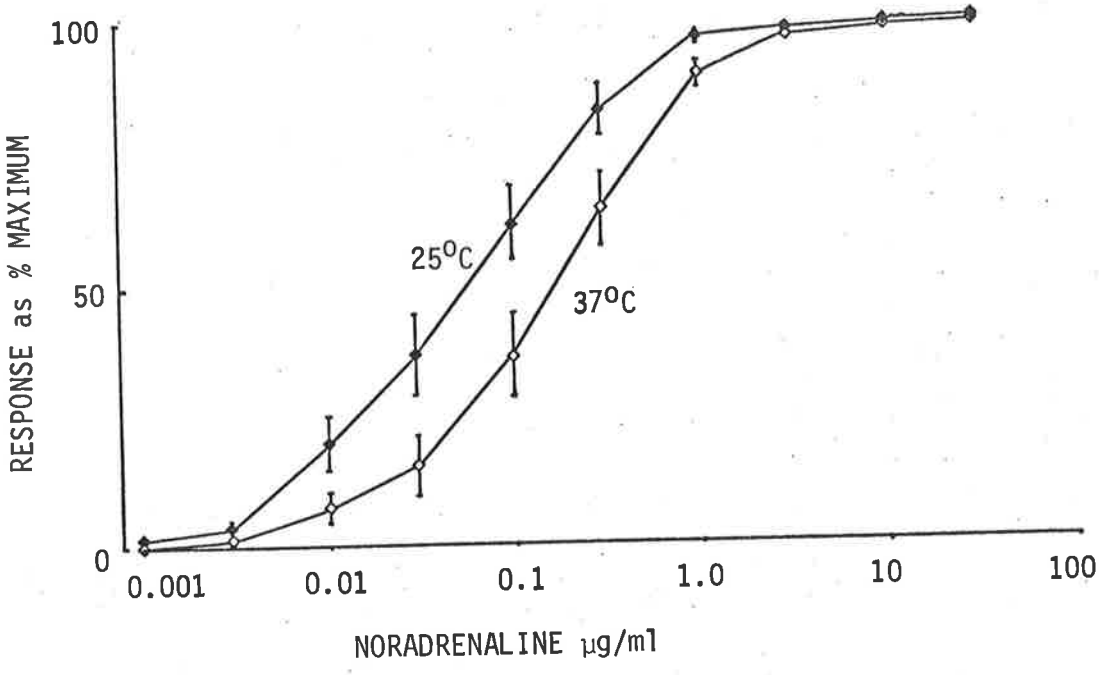


Figure 7.4

The effect of reduced temperature on the sensitivity of nialamide treated artery helical strips to noradrenaline.

Data shown is the means ± s.e. of responses to NA in 12 pairs of strips at 25°C and 37°C. The groups differ significantly at NA concentrations of 0.01 to 1.0 µg/ml (t-test, $P < 0.05$).

Despite the finding that secondary sensitization was absent at 25°C in treated arteries there was an increase in the magnitude of the response to NA at 25°C amounting to an increase in sensitivity to NA of 2.5-fold in 12 strips (Fig. 4). This increase in sensitivity was definite in strips but more variable in segments. The effects of lowered temperature in 9 segments ranged from 2.5-fold depression to 25-fold potentiation, the mean sensitivity ratio (37°C/25°C) being 2.9.

2. EFFECT OF LOWERED TEMPERATURE ON THE RESPONSE TO DA.

Dose response curves to DA and recovery curves from a submaximal (3 μg/ml) concentration of DA were obtained on paired untreated and paired nialamide treated strips at 37°C and 25°C. In the recovery studies, the artery remained in contact with the submaximal dose of DA for 15 minutes, and with the supramaximal dose of DA for varying periods of time (about 2-5 minutes); perfused segments were in contact with a submaximal dose of DA (0.3 μg/ml) for 30 minutes.

The main findings were that, at 25°C, both the strips and the segments exposed to submaximal doses of DA displayed rapid onset of the steady state response and rapid recovery from this response, i.e., secondary sensitization did not occur (Figs. 5 and 6). Recoveries of strips exposed to 300 μg/ml DA were also very much more rapid at 25°C than at 37°C, although slightly slower than the recoveries observed in untreated arteries at 37°C (Fig. 7). An interesting feature of the nialamide treated strips was that the recoveries from 300 μg/ml DA at 25°C were also very much more rapid than those of denervated arteries at 37°C. The same trend occurred after 3 μg/ml DA, but the difference was much less pronounced (Fig. 8). (The results of the denervated group are from experiments discussed in Chapter 4.7.)

With respect to the magnitude of the steady state response

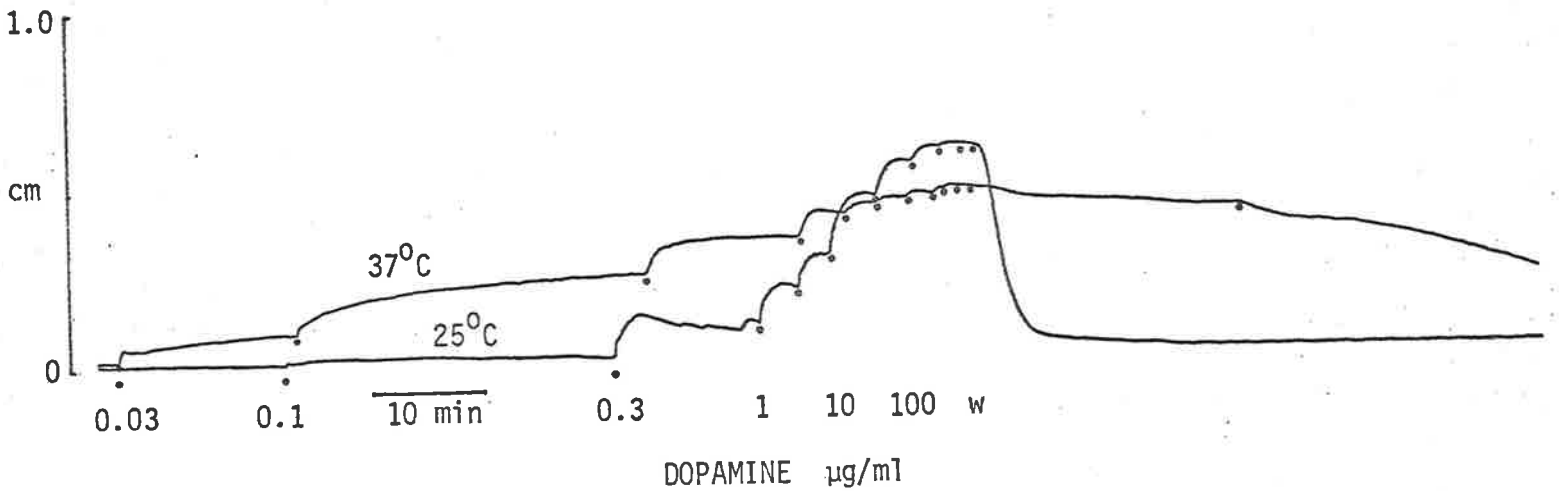


Figure 7.5

Responses to dopamine after nialamide treatment in paired artery helical strips at 37°C and 25°C, showing the lack of the features of secondary sensitization at the lower temperature.

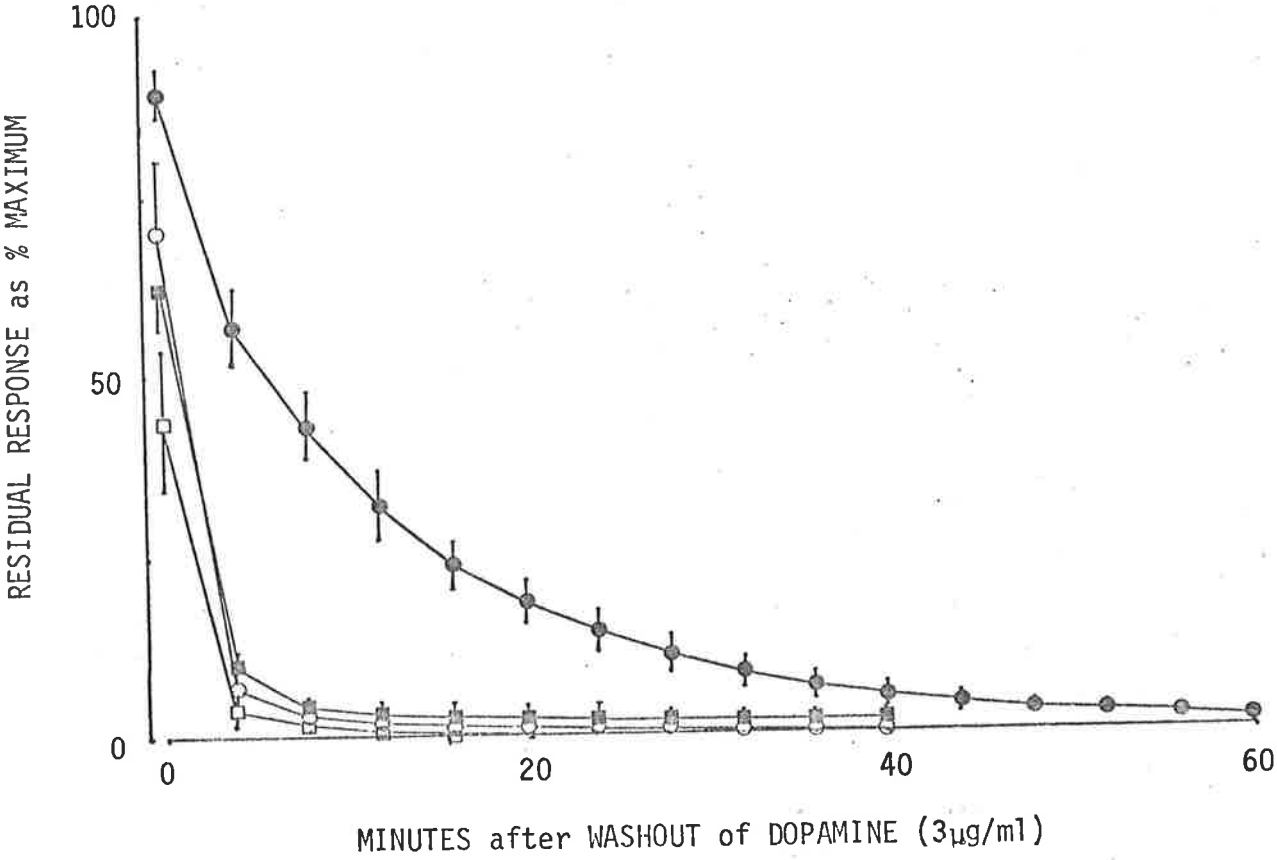


Figure 7.6

Recovery from a submaximal concentration of dopamine (3 μg/ml) in nialamide treated (closed symbols) and untreated (open symbols) artery helical strips at 37°C (circles) and 25°C (squares).

Data shown are the means ± s.e. of 6 experiments.

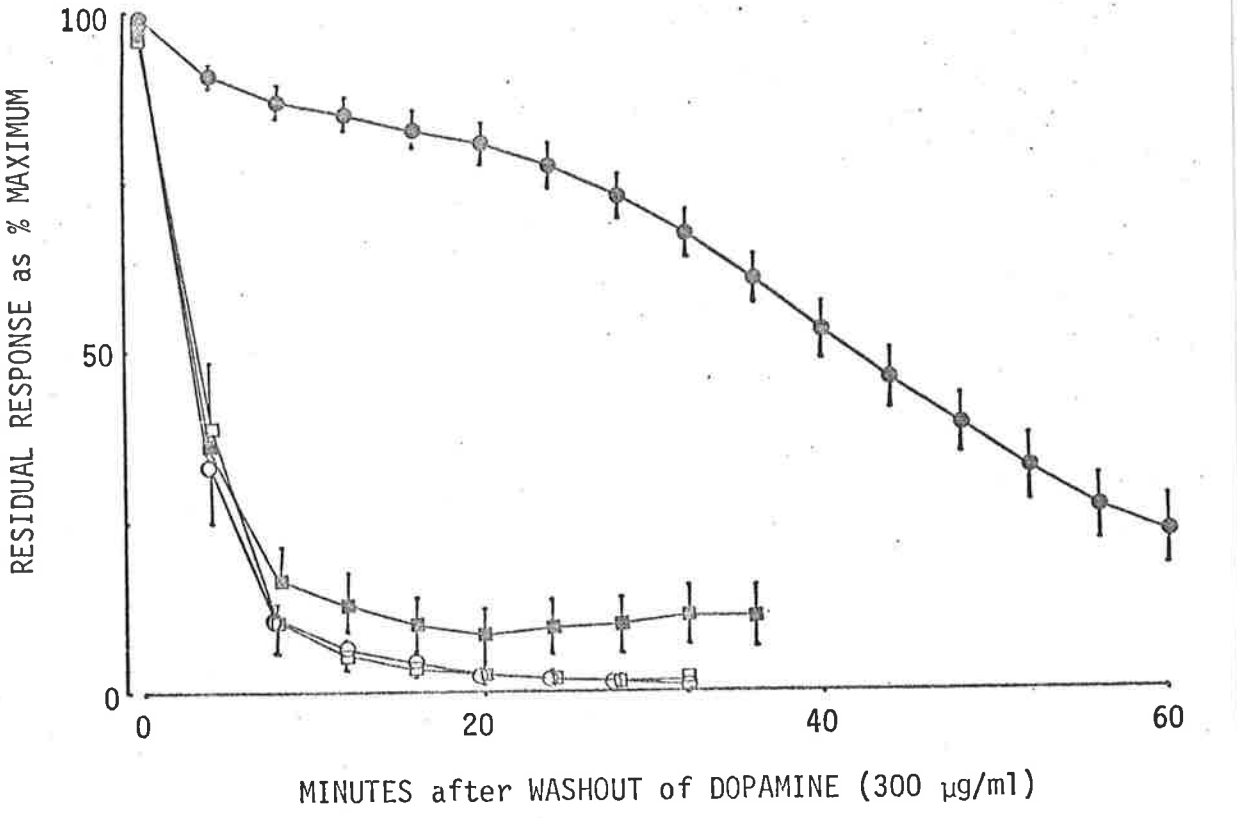


Figure 7.7

Recovery from a supramaximal concentration of dopamine (300 µg/ml) in nialamide treated (closed symbols) and untreated (open symbols) artery helical strips, at 37°C (circles) and 25°C (squares), showing the absence of prolonged recovery in the nialamide treated groups at 25°C. Values shown are the means ± s.e. of 6 experiments.

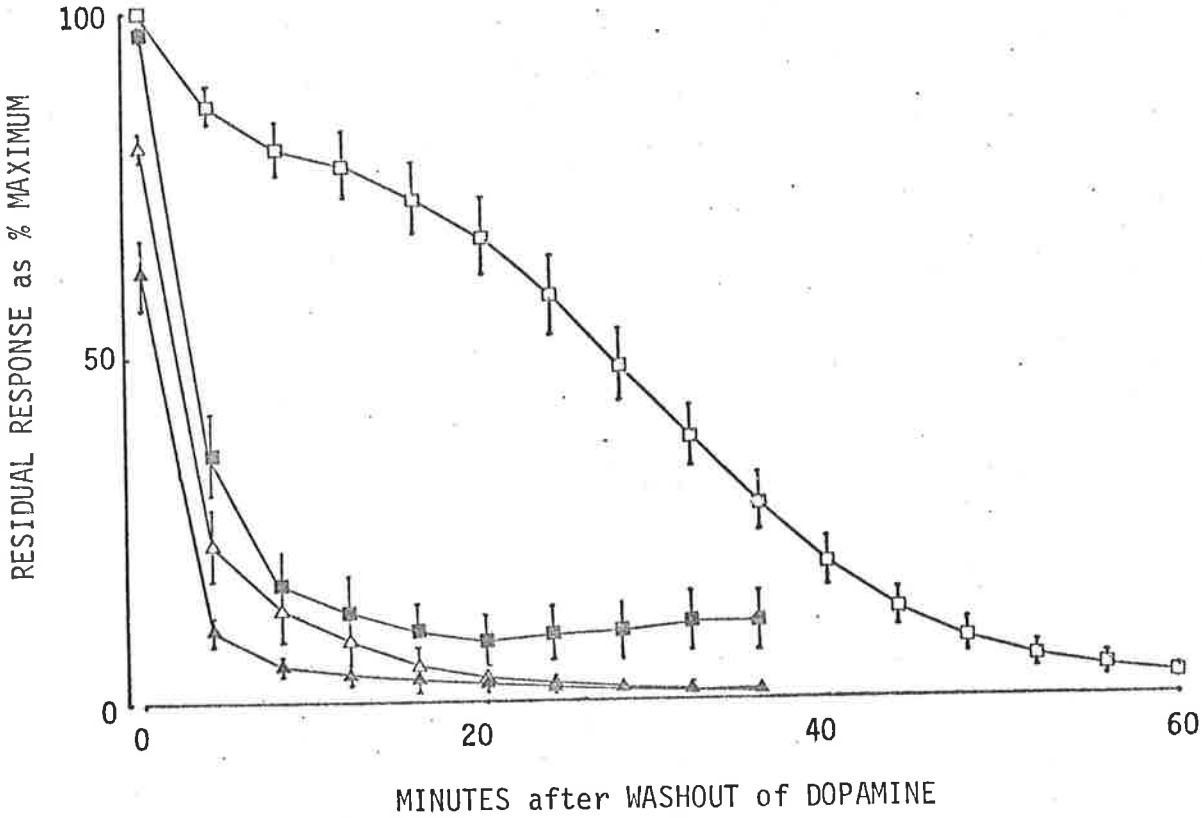


Figure 7.8

Recovery after dopamine 300 µg/ml and 3 µg/ml in innervated arterial strips at 25°C and in denervated arterial strips at 37°C, illustrating the relatively rapid recovery in the innervated nialamide treated groups at 25°C.

- denervated 300 µg/ml DA 37°C (5)
- denervated 3 µg/ml DA 37°C (5)
- innervated 300 µg/ml DA 25°C (6)
- innervated 3 µg/ml DA 25°C (6)

Figures in brackets refer to the number of experiments. The data for the denervated arteries is from experiments recorded in Chapter 4.7, and Figure 4.8.

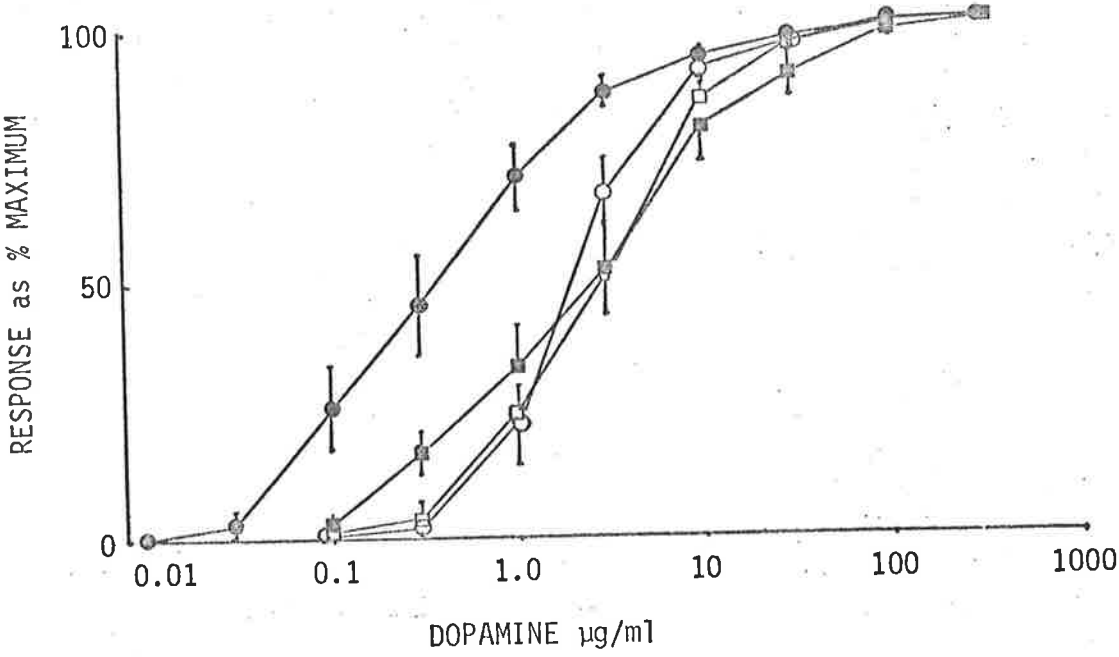


Figure 7.9

The effect of lowered temperature on the sensitivity to dopamine in nialamide treated and untreated artery helical strips.

Values shown are the means ± s.e. of responses in 5 experiments at 25°C (squares) and 37°C (circles), nialamide treated (filled symbols) and untreated (open symbols).

Analysis of variance showed that the 25°C nialamide group was not significantly different from the control groups at 37°C and 25°C except at one concentration level (0.3 µg/ml) ($P > 0.05$). However, all three of the above groups were significantly less sensitive than the 37°C nialamide treated group ($P < 0.05$).

to DA in untreated helical strips, there was no significant difference in the sensitivities at 25°C and 37°C. Nialamide treatment did not increase the sensitivity at 25°C but caused its anticipated increase in sensitivity (approximately 8-fold) in the arteries incubated at 37°C (Fig. 9).

3. EFFECT OF LOWERED TEMPERATURE ON THE RESPONSE TO L-DOPA AFTER NIALAMIDE.

Since untreated arteries did not respond to dopa, the following experiments were carried out only on nialamide treated artery strips. L-dopa was added to each preparation at cumulative concentrations of 10, 30, 100, and occasionally 200 µg/ml. If a response occurred in one of the paired strips, the concentration was not further increased until steady state was reached. If there was no response after 15 minutes, the concentration of dopa was increased. At the end of the experiment, a dose response curve to NA was obtained from each strip.

In each of 4 experiments the strips at 25°C did not respond to dopa at any of the above concentrations, although the contralateral arteries at 37°C showed their usual response (Fig. 10). The lack of response to dopa at 25°C was not due to deterioration of the preparations. This was indicated by the observation that the sensitivity to NA in these arteries was approximately equal to that of the contralateral arteries incubated at 37°C.

4. EFFECT OF LOWERED TEMPERATURE ON UPTAKE OF NA AND DA.

The effect of a reduction in bath temperature on the uptake of NA into nerve terminals was studied in two ways. First, indirectly, by assessing the effect of temperature on the potentiation of extraluminal NA by cocaine in the perfused ear artery segment, and second, in a more direct manner, by observing the net uptake of ^{3}H NA at 37°C and 25°C in nialamide treated arteries. An account of the methods used in the isotope

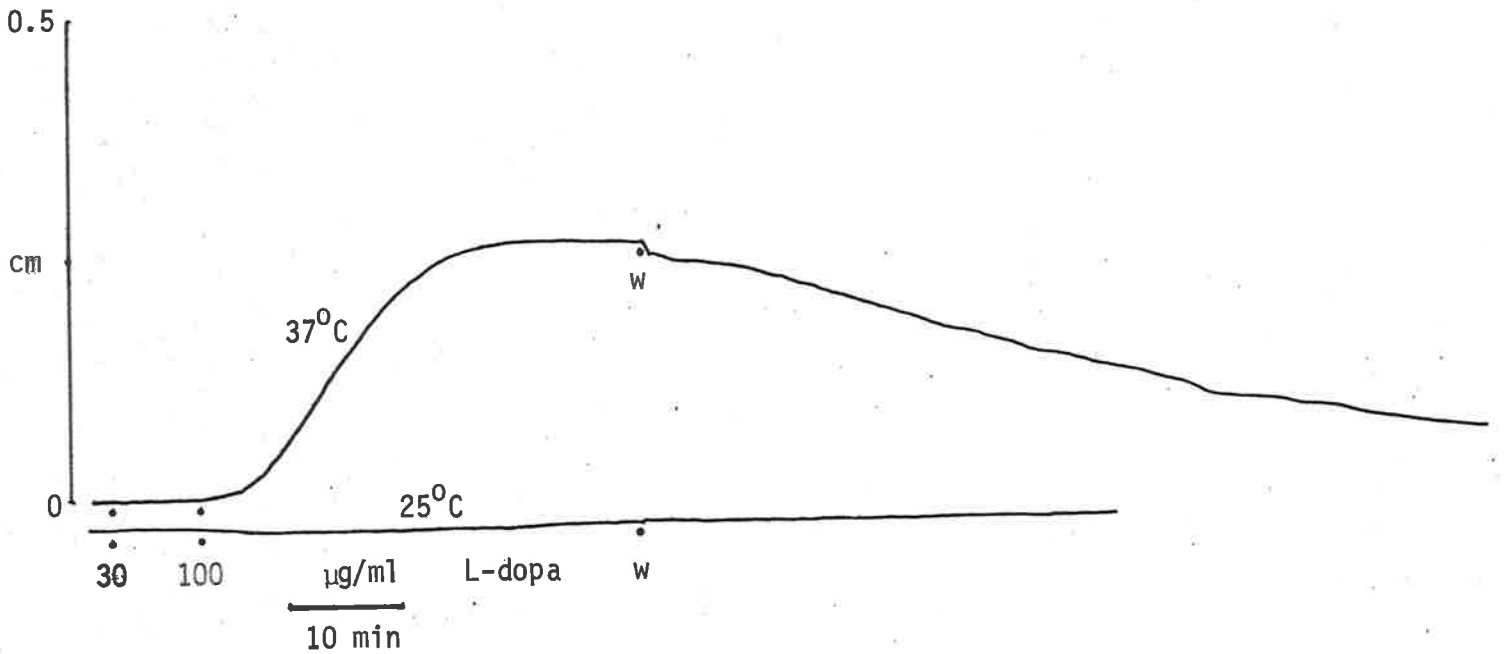


Figure 7.10

Responses to dopa in nialamide treated paired artery strips at 37°C and 25°C, showing the absence of the secondary response at 25°C.

experiments was presented on page 7.4.

(a) Potentiation of extraluminal NA by cocaine at 37°C and 25°C.

In 6 experiments, dose response curves to extraluminal NA were obtained at 25°C and 37°C in the presence and absence of cocaine. Those arteries which were initially at 25°C were then warmed to 37°C, and the arteries which initially were at 37°C were cooled to 25°C. The dose response curves to NA were then repeated in the presence and absence of cocaine. In 3 of the above experiments dose response curves were also obtained to intraluminal NA in the presence and absence of cocaine at 37°C and 25°C.

Table 1 shows that the potentiating effect of cocaine on extraluminal NA was significantly less (by 35%) at 25°C than at 37°C. On the other hand, the small degree of potentiation of intraluminal NA by cocaine was unaffected. These effects of temperature changes were unaffected by the sequence of the changes.

(b) Uptake and efflux of ^{3}H NA.

The amount of ^{3}H remaining in nialamide treated arteries at zero time and up to 30 minutes after incubation with ^{3}H NA at 37°C or 25°C for 30 minutes was measured as described on page 7.4. The total amount of label taken up during incubation with ^{3}H NA 0.5 μg/ml at 25°C did not differ significantly from that taken up at 37°C. However, significantly less label was retained in the tissue incubated at 25°C during the period 2 to 30 minutes after washout of the ^{3}H NA. Thus 30 minutes after washout, the mean concentration of ^{3}H in the 25°C arteries was 70% of that of the 37°C artery. Inspection of the efflux curve (Fig. 11) shows that the difference is in large part due to the much more rapid diffusion of label from the 25°C artery into the bathing solution during the first two minutes after washout. The data was analysed by the method of Paton (1973) to estimate the efflux rate constant. The results are summarised in Table 2.

TABLE 7:1

The effect of lowered temperature in the potentiation of extra- and intra- luminal NA by cocaine in isolated perfused segments.

	SENSITIVITY RATIO NA $\frac{\text{No Cocaine}}{\text{With Cocaine}}$	
	INTRALUMINAL	EXTRALUMINAL
25°C	1.4 1.0 - 1.9 (3)	5.8* 4.8 - 6.8 (6)
37°C	1.7 1.4 - 1.9 (3)	9.4* 8.2 - 10.9 (6)

Values shown are the geometric means of sensitivity ratios calculated from parallel dose response curves at a response of 60 mm Hg. The values below the mean are the mean ± s.e. The values in brackets refer to the number of experiments.

*Values significantly different (t-test, $P < 0.02$)

Figure 7.11

Residual ^{3}H content at time intervals after incubation with ^{3}H NA. ◆——◆ $37^{o}C$ ◇——◇ $25^{o}C$.

Data shown are the means ± s.e. of 8 experiments in which artery segments were incubated with (±) ^{3}H NA (0.5 µg/ml) for 30 minutes at $37^{o}C$ or $25^{o}C$. They were then removed from the incubation mixture into 6 successive 1 ml volumes of Krebs solution at $37^{o}C$ or $25^{o}C$ over a period of 30 minutes after which they were dissolved in NCS reagent and counted in a liquid scintillation counter. The total count is the corrected sum of wash fluids and tissue counts and was 36,400 ± 5,700 cpm/mg and 31,200 ± 4,000 cpm/mg for the $37^{o}C$ and $25^{o}C$ groups respectively (difference not significant, $P > 0.05$).

Mean residual counts are significantly different at each time interval after 0 time. ($P < 0.01$ at 2 minutes, $P < 0.001$ all other time intervals).

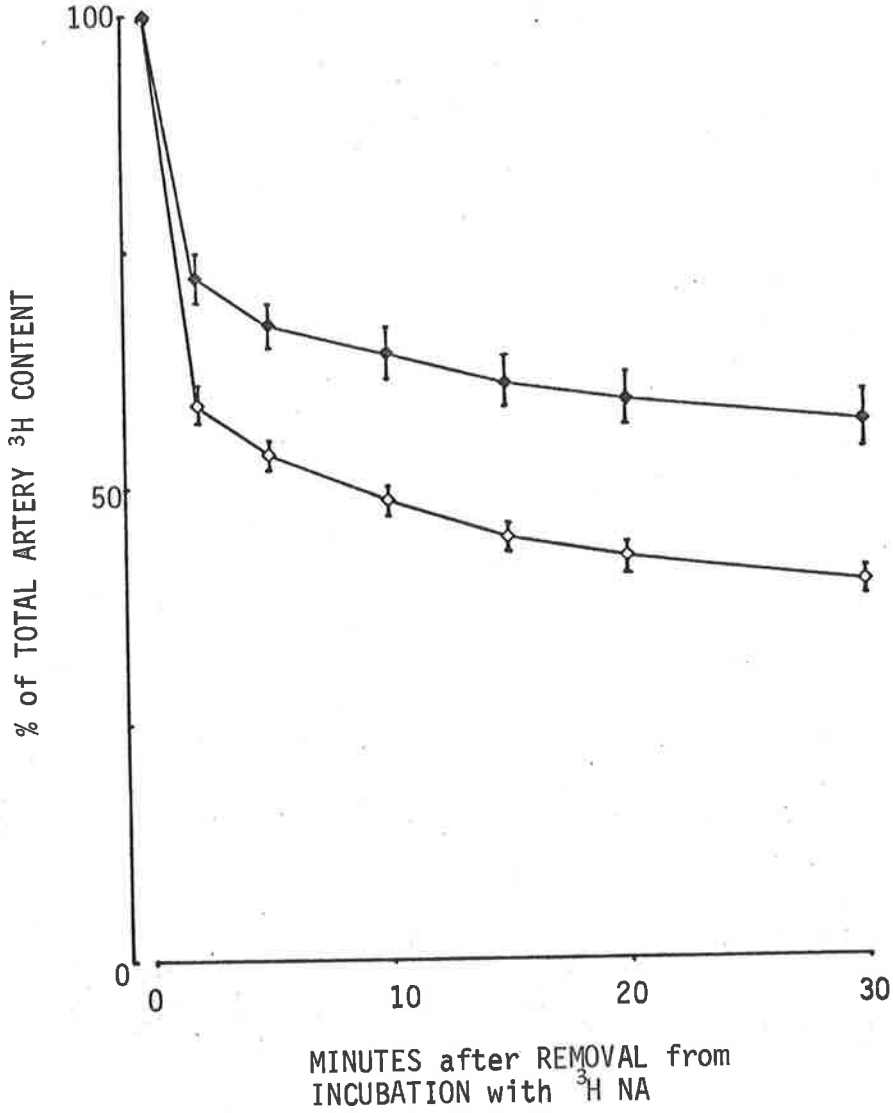

100
50
0
% of TOTAL ARTERY ^{3}H CONTENT
0
10
20
30
MINUTES after REMOVAL from
INCUBATION with ^{3}H NA

TABLE 7:2

Rate coefficients* of efflux of NA at 25°C and 37°C in nialamide treated segments.

TIME INTERVAL	Mean rate coefficient (min^{-1}) f^* ± s.e.	
	25°C	37°C
0 - 2	0.261† ±0.0173	0.162† ±0.0173
2 - 5	0.0304 ±0.0052	0.0243 ±0.0001
5 - 10	0.0197† ±0.0026	0.00972† ±0.0018
10 - 15	0.0169† ±0.0014	0.0101† ±0.00094
20 - 30	0.00743† ±0.00051	0.00448† ±0.00068

* Rate coefficients were calculated for each of 8 experiments according to the formula of Paton (1973a).

$$f = \frac{\Delta A}{\Delta t . At} \quad (min^{-1})$$

where ΔA = activity effluxed in time Δt.

At = amount of dl ^{3}H NA in the tissue at the midpoint of the time interval Δt.

† values differ significantly (t-test for paired observations $P < 0.01$).

They show that the efflux rate constant over the time 0 to 30 minutes after incubation was in fact greater at 25°C than at 37°C.

(c) Uptake of isotopic DA.

In 4 experiments the net uptake of isotopic DA and formation of isotopic NA was measured at 25°C and at 37°C. ^{3}H DA was used in 2 of these experiments, and ^{14}C DA in the remaining 2. In each experiment the quantity of label in the tissue was measured at a specified period of time after washout of the labelled DA from the bathing solution. The quantity of label accumulating in the bathing fluid during the foregoing specified period was also measured to provide an estimate of the total net uptake during the period of incubation, as well as an estimate of the rate of efflux of the label. The results are summarized in Table 3. It will be seen that the amount of label accumulating in the tissue during the 30-minute incubation with labelled DA, as well as the rates at which label accumulated in the bath solution following bath washout of the labelled DA were little different at 25°C and 37°C.

However, when the label retained in the tissue was analysed to assess the proportion of labelled NA to DA present, it was found that the proportion of labelled NA was significantly greater in the tissues incubated at 37°C than those incubated at 25°C. Since the net uptakes of label were similar at the two temperatures, the last result implies that more of the labelled DA was converted to NA at 37°C. However, these results must be interpreted with caution. The peaks of radioactivity, on which the estimates were based, co-chromatographed well with cold NA and DA in the chromatographic system used (see page 211 for details). However, about 50% of the label was also distributed over the chromatogram and did not display recognisable peaks for the expected labelled methylated metabolites of NA and DA (Fig. 7.1). In the light of subsequent experience of other

TABLE 7:3

^{3}H-DA net uptake and retention at 37°C and 25°C in Nialamide treated ^{14}C-Tissues.

EXPERIMENT	ISOTOPE	WASH TIME	TOTAL COUNT AT END OF INCUBATION cpm/mg tissue		RESIDUAL TISSUE ACTIVITY AFTER WASH % OF TOTAL COUNT	
			25°C	37°C	25°C	37°C
1	^{3}H	12 min	7581	7407	68.3	62.3
2	^{3}H	12 min	5264	8559	57.5	68.0
3	^{14}C	30 min	1141	1015	73.5	71.2
4	^{14}C	30 min	878	667	73.5	71.7

The values shown were obtained in 4 experiments in nialamide treated segments. In experiments 1 and 2 above the total count after incubation, and the residual activity after 12 minutes wash only were determined.

investigators in these laboratories (Head 1973), it is conceivable that oxidative degradation products of the catecholamines present in the original samples may have contributed to the diffuse activity spread over the chromatograms.

DISCUSSION

In order to interpret the results presented in this chapter it is first necessary to review the information already available on the effect of lowered temperature on the various mechanisms, both neuronal and extraneuronal, active in sympathetically innervated tissues. Most of this work has been done on tissues other than rabbit ear artery.

Many of the systems associated with the accumulation, release and metabolism of NA in adrenergic neurones has been shown to be inhibited by cooling. The uptake of NA into adrenergic neurones in cat heart was found to be temperature dependent (Gillis and Paton 1966), with a Q_{10} of approximately 2 (Iversen 1971). Giachetti and Shore (1966) reported that uptake of metaraminol by heart slices was greatly inhibited by incubation in the cold. Compared with the activity at 37°C, net uptake of ^{3}H NA was found to be reduced at 29°C in cat heart (Paton 1966), and a reduction of 30% in net uptake of ^{3}H NA was found in the rabbit aorta at 25°C (Nedergaard and Vagne 1969). The rate of uptake into intraneuronal vesicles was found to be 8 times faster at 37°C than at 27°C (Stjarne 1964). The rate of spontaneous release of NA from isolated bovine splenic nerve granules was also found to be reduced at lowered temperatures, the rate at 20°C being about 10% of that at 37°C (Euler and Lishajko 1963, 1967).

Lowered temperature also affects the release mechanisms in the sympathetic neurone. Kirpeker and others (1969) showed

that lowering the perfusion temperature to 15°C markedly reduced the amount of NA released in response to nerve stimulation or to infusion of potassium. Wennmalm and others (1970) found that reducing the temperature from 37°C to 8°C caused a depression of ^{3}H NA release from ^{3}H NA loaded cat spleen. The effect on the release by the indirectly acting amines tyramine (75% reduction at 25°C) was much more pronounced than the effect on release by stimulation (30% reduction at 25°C). However, this greater effect on release by tyramine may have been contributed to by the depression of the neuronal uptake mechanism, and a subsequent reduction in the amount of tyramine taken up by the neurone. Tsai and others (1967) suggested that the indirect effects of DA on the isolated guinea pig atria might be more prominent at higher temperatures.

The effect of lowered temperature on the activity of the enzymes of catecholamine synthetic chain is not well documented. However, Dr L. Austin (1972), in a personal communication, has stated that the reduction of the incubation temperature to 25°C from 37°C has little effect on the activity of the enzyme dopamine-β-hydroxylase.

The metabolism of catecholamines is known to be affected by reduced temperature. This could apply to both intra- and extraneuronal metabolism. At 25°C, in guinea pig atria, it was found that the MAO activity was reduced by 28% and COMT activity by 61% compared to 37°C (Opperman and others 1972).

Incubation at 15°C or less reduces the uptake of NA into smooth muscle of splenic arteries (Gillespie and Hamilton 1967) and cat spleen (Gillespie and others 1970). Foster (1967) found that cooling to 17.5°C potentiated the effect of NA and isoprenaline (an amine not taken up by adrenergic neurones) equally on guinea pig isolated tracheal chain, as did metanephrine and phenoxybenzamine, both of which are inhibitors of extraneuronal uptake. Clarke and others (1969) found that

perfusion with NA at 20°C reduced the extraneuronal uptake in the isolated perfused rat heart, but also caused myocardial swelling, as evidenced by an increase in the perfusion pressure and a decrease in ^{14}C sorbital space (intracellular space). They suggested that the reduction in extraneuronal uptake due to reduced temperature may be due to the hindrance of the passage of the NA from the coronary vessels to its final retention sites. However, Burnstock and others (1971) saw no effect of cold on the uptake of NA by cells of the thick muscle wall of the non-innervated umbilical artery at 2°C and suggested that the effect of cold is indeed on the membrane mechanism of extraneuronal uptake which appears to be absent in the non-innervated smooth muscle.

It is also a possibility that reducing the temperature has an effect on the effector cell. Glover and others (1968) showed that cooling reduced the response of the femoral artery to NA steadily from 37°C to 13°C, when it failed to respond. Herron and others (1971) reported a decrease in sensitivity to histamine and metaraminol in the rabbit ear artery with reduced temperature (3°C). Wennmalm and others (1970) found that in isolated perfused cat spleen the response to injected NA progressively decreased with the fall in temperature, to be almost abolished at 10°C. Smith (1952) noted changes in the nature of the response to adrenaline in the sheep artery strip at lowered temperature. The vessel contracted and relaxed more slowly at 17°C than at 37°C. Wennmalm and others (1970) also noted a change in the shape of the response to nerve stimulation at lowered temperature. Keatinge (1964) using sheep carotid artery spiral strips found that the contractile response to electrical stimulation was slower at 25°C than at 35°C. His evidence suggested that cold (down to 5°C) acts directly on the contractile mechanism, slowing it but only moderately affecting the total amount of shortening. He also found that the resting potentials

of arteries were significantly lower at 5°C than at 35°C.

Thus from the evidence it would appear that many systems in the adrenergic neurone and the effector cell may be affected by reduced temperature. Hence the change in the response of a tissue, produced by a decrease in temperature, is likely to reflect a complex interaction of many different factors.

In the work presented in this chapter it was found that a 12°C fall in temperature did not significantly alter the sensitivity to NA in the untreated rabbit ear artery, whether in the form of a perfused segment or strip. This is in contrast to the evidence of Glover and others (1968) using ear artery segments. However, the experimental conditions were not identical in the two studies. The rabbits used by Glover and others (1968) were cold acclimatised (personal communication from Prof. W. E. Glover). Later studies in the same laboratory (McClelland and others 1969) showed that the phenomenon of cold sensitization to NA only occurred in arteries removed from rabbits kept in a cold environment. The rabbits used in this study were kept at an ambient temperature of approximately 21°C, and thus were not cold acclimatised. This would account for the failure of reduced temperature to cause sensitivity to NA in these arteries.

A greater effect on the NA sensitivity was seen in arteries treated with nialamide to inhibit MAO. Here reduction of temperature potentiated the NA sensitivity 2-3 fold. In this case where the MAO is inhibited both intra- and extraneuronally, the ability of cooling to decrease $Uptake_1$ and $Uptake_2$ may come into play by reducing the loss of NA from the receptor area by the neuronal uptake system and the remaining metabolizing enzyme, COMT.

A puzzling feature of the data presented here on NA was the failure of the strips to reproduce all the features of secondary sensitization observed in segments (de la Lande and

Jellett 1972). A possible explanation is that the process of preparing the strips led to damage of the nerve terminals, so that the integrity of the neuronal uptake and binding system for NA was impaired in the strip compared with the segment. Sufficient NA may have accumulated during the period of incubation to permit the slow recovery phase of secondary sensitization to be manifest. The fact that secondary sensitization to DA was prominent in both strips and segments may reflect the fact that the concentrations of DA required to elicit responses were much higher than those required for NA and were probably supramaximal with respect to neuronal uptake (see Chapter 3).

Another possible explanation for the lack of secondary sensitization to NA during cumulative responses in the ear artery strip is that, in this preparation, neuronally mediated effects are not prominent, compared with the extraluminally dosed perfused segment (de la Lande and Urquilla 1969). The relative position of the nerve terminals and the smooth muscle cells in the artery wall may well be critical in determining the magnitude of neuronal effects. Thus the concentration of NA at the receptors in the media is governed by the concentration of NA in the fluid bathing the tissue, and the neuronally mediated effects of NA are masked by the direct effects.

However, the second phase of secondary sensitization, slow recovery after washout of the NA, is manifest in the nialamide treated strip. The concentration of NA at the receptors in the strip after washout may be then governed by the neuronal concentration of NA, since the bath concentration after washout should be very low. A similar dissociation of the first and second phases of secondary sensitization has been observed in the rabbit aortic strip (Trendelenburg 1974).

In the case of DA, the bathing solution contains only DA, to which the tissue is relatively insensitive. However, if DA is causing the release of endogenous NA from the nerve ending,

the tissue is presented with an amine to which it is much more sensitive, and so secondary sensitization occurs, the resultant response being not primarily due to the DA but to the released NA. It should be noted that secondary sensitization to DA occurs at dose levels of DA which are normally subthreshold in the untreated preparation (Chapter 4).

As with NA, in the untreated artery there was no effect of lowered temperature on the responses of strips to DA. Since neuronal uptake appears unimportant in the response to DA (Chapter 3), one might have expected a decrease in temperature to cause a decrease in sensitivity to DA. However, inhibition of other inactivating processes besides uptake may have compensated for a decrease in sensitivity of the smooth muscle. For example, the activity of MAO is decreased by 27% at 25°C (Opperman and others 1972).

The most significant of the findings presented in this chapter is the ability of cooling to largely abolish the secondary sensitization to dopa, DA and NA observed in nialamide treated arteries. In the case of NA, it has been shown previously that secondary sensitization in artery segments is also abolished by denervation and cocaine (de la Lande and Jellett 1972). It would appear that the occurrence of secondary sensitization to NA is, in fact, extremely sensitive to the activity of the neuronal uptake system, since the reduction of the temperature to 25°C reduced the uptake of NA only by about 30%. However, as already emphasised, it cannot be excluded that some other change induced by low temperature may also be involved. Another possibility which must be considered is that cooling may have also depressed the release of NA. The only evidence presented here on this point is that the amount of label released from the tissue within the first few minutes of washout of ^{3}H NA was in fact greater at 25°C than at 37°C. Although this label was probably from extraneuronal rather than neuronal sources,

the efflux rate over the ensuing 30 minutes, where the contribution of neuronal efflux would be greater, was also greater at 25°C than at 37°C. However, the rate of spontaneous efflux of ^{3}H NA from previously loaded rabbit atria, after 60 minutes of efflux, shows a Q_{10} of 2.5 between 27°C and 37°C, indicating a high degree of inhibition of neuronal efflux of ^{3}H NA by cooling (Paton 1973a). Release of NA by the indirect actions of sympathomimetic amines has also been shown to be decreased by cooling (Wennmalm and others 1970; Paton 1973b).

In the case of DA, a decrease in temperature, like chronic denervation, but unlike cocaine, abolished secondary sensitization. However, in the studies with labelled DA, it was found that the accumulation of label by the tissues was unaffected by cooling. This result implies that the inhibition of secondary sensitization was not due to a decrease in the amount of DA taken up by the nerve terminals, nor in the rate at which it was subsequently released. The studies on the formation of labelled NA from labelled DA, although limited and incomplete, however, suggest that less labelled NA was formed at the lower temperature. Hence this data does not conflict with one possible hypothesis advanced earlier (page 4.12), that secondary sensitization is in fact at least in part mediated by newly synthesized NA. Although consistent with the finding that DDC tends to prevent secondary sensitization in reserpine treated arteries, there still remains the difficulty (discussed on page 6.6) facing this hypothesis that DDC had little effect on secondary sensitization in arteries not pretreated with reserpine.

An interesting finding was that the decrease in temperature also abolished the delay in recovery of arteries exposed to an extremely high concentration of DA (300 µg/ml). This delay was reduced but not abolished by chronic denervation, and therefore was assumed to be only partly neuronal in origin. The more marked effect of cooling implies that the extraneuronal

accumulation and/or release of DA is also temperature-sensitive, as already proposed by Gillespie and Hamilton (1967) for NA.

The inhibition of the effects of cooling also extended to the constrictor response to L-dopa seen in arteries after treatment with nialamide. Evidence has already been presented that this response is neuronal in origin, since it does not occur in chronically denervated arteries. In this case, the mechanism by which the effects of cooling are mediated must also extend to possible inhibition of (a) uptake of dopa by the nerve terminal and (b) conversion to DA by l-aromatic amino acid decarboxylase. Both of these processes are presumably temperature-dependent. All that can be said at present is that the effects of cooling on the response to L-dopa are consistent with the hypothesis that secondary sensitization to this precursor is also mediated by NA, but that the effects may be augmented by inhibition of processes (a) and (b) above.

CHAPTER EIGHT

FATE OF INTRALUMINAL NORADRENALINE AND DOPAMINE IN THE PERFUSED RABBIT EAR ARTERY.

CHAPTER 8

INTRODUCTION

PRELIMINARY NOTE.

The study described in this chapter was one which, by its nature, required the co-operation of a number of individuals (I. S. de la Lande, D. A. S. Parker and the author). It is included in the thesis despite the fact that it concerns primarily some of the factors which influence the response to NA in the nialamide treated artery, the justification being the probability that the same factors influence the response to DA. This follows from the evidence in the preceding chapters that the response to DA in the nialamide treated artery is, in part at least, mediated by NA.

It has been shown previously in this laboratory that inhibition of MAO augmented the response to extraluminal NA in ear artery segments and delayed the recovery from this response (de la Lande and Jellett 1969, 1972). These secondary sensitization effects were found to be neuronal in origin, since they did not occur in chronically denervated arteries, or when cocaine was present throughout the exposure to NA.

These effects were not observed when the NA was applied intraluminally to the artery. The difference between the effects of extraluminal and intraluminal NA was explained in terms of the failure of the intraluminal NA to penetrate to the sympathetic nerve terminals located at the media-adventitia border.

The failure of intraluminal NA to reach the nerve terminals may be explained in at least two ways:

(a) there may be a diffusion barrier between the intimal surface and the media-adventitia border, or

(b) the NA applied to the intimal surface may be taken up and/or metabolised by the smooth muscle in the media.

The studies described in this chapter relate to the second possibility, and in particular, to the role of COMT in

influencing the concentration of NA at its receptors. Prior to commencing this study, it was known that COMT metabolises NA to normetanephrine, and that its distribution in other tissues appeared to be extraneuronal (Crout and Cooper 1962; Iversen and others 1966). Subsequently Jarrott and Iversen (1971) have shown that the distribution is, in part, neuronal. More was known of the role of MAO, since the pharmacological studies of de la Lande and co-workers provided indirect, but strong evidence that extraneuronal MAO did not exert a significant influence on the response to noradrenaline (de la Lande and Jellett 1969, 1972; de la Lande and Johnson 1972).

Evidence in Chapter 4 indicated that extraneuronal metabolism of DA by MAO occurred in the ear artery. It was of additional interest to determine whether metabolism by COMT influenced the response to DA, and whether DA was a substrate for extraneuronal uptake by the smooth muscle in the media of the artery. It has been shown that DA is a substrate for COMT *in vitro* (Axelrod and Tomchick 1958), but so far a role of COMT in the response to DA on other tissues has not been established (Trendelenburg and others 1971).

The present study was confined to:

(1) A histochemical analysis of the effect of inhibition of COMT and of $Uptake_2$ on the ability of intraluminally applied NA to penetrate across the artery wall to the sympathetic nerve terminals at the media-adventitia border;

(2) The effect of inhibition of COMT on the vasoconstrictor response to NA and DA;

(3) Histochemical analysis of the extraneuronal uptake of DA.

METHODS

HISTOCHEMICAL STUDIES WITH NA.

In these experiments, artery segments were taken from rabbits pretreated with reserpine to deplete endogenous NA (Methods 2.7). The artery segments were isolated and cannulated at both ends so that the intraluminal perfusion fluid did not mix with the extraluminal bathing fluid. MAO was inhibited by treatment with nialamide either *in vivo* or *in vitro* (see Methods 2.8) so that any NA taken up by the nerve terminals would be retained and hence observed histochemically. All segments were then exposed to either intraluminal or extraluminal NA (0.5 μg/ml) for 30 minutes. Some of the segments were exposed to either the COMT inhibitor, UO521 (3'4'dihydroxy-2-methyl-propiophenone) (10 μg/ml), or an $Uptake_2$ inhibitor, metanephrine (0.5 μg/ml), or combinations of these, for 15 minutes prior to and during the exposure to NA. At the end of 30 minutes the NA plus any other drug present was washed out, and the preparation further perfused intraluminally, and bathed extraluminally with drug-free Krebs bicarbonate solution for 10 minutes, after which the segment was removed for fluorescence histochemistry (Chapter 2.8). After histochemistry the intensity of fluorescence at the media-adventitia border was assessed on a scale ranging from + to +++ (see Chapter 2.10). It should be noted that in the above experiments, the intraluminal flow, normally 4-6 ml/min, was stopped entirely during exposure to extraluminal NA, and was decreased to approximately 1 ml/min when NA was perfused intraluminally. This was done in order to avoid possible leakage of intraluminal perfusion fluid into the extraluminal bathing medium as a result of the high perfusion pressures attained during the exposure to NA.

RESULTS

1. FATE OF INTRALUMINAL NA.

(a) Histochemistry.

Table 1 shows that, in 7 experiments after reserpine pre-treatment and MAO inhibition, arteries did not display evidence of monoamine fluorescence at the media-adventitia border. However, following exposure to extraluminal NA, there was typical green monoamine fluorescence at the media-adventitia border. In contrast, arteries exposed only to intraluminal NA failed to display similar monoamine fluorescence. However, arteries which had been treated with UO521 and/or metanephrine for 15 minutes prior to, and then simultaneously with, the intraluminal NA did show characteristic green monoamine fluorescence at the media-adventitia border. In the absence of previous exposure to NA, neither metanephrine nor UO521 alone caused the appearance of fluorescence.

There was no evidence in any of the above experiments of retention of NA in regions of the artery wall other than the adventitia. However, the fluorescence following exposure to extraluminal NA tended to be somewhat more widely dispersed in the adventitia than that seen in normal (untreated) rabbits.

In none of the arteries treated with intraluminal NA did the fluorescence appear of the same order of intensity as that seen in the arteries treated with extraluminal NA.

In summary, it was found that drugs which inhibit uptake into smooth muscle and extraluminal metabolism allowed intraluminal NA to accumulate in the nerve terminals to an extent which could be detected histochemically. Hence, these processes might be responsible for the failure of intraluminal NA to diffuse from the intimal surfaces of the artery to the nerve terminal.

(b) Recovery times.

In the course of the above experiments, rates of recovery

TABLE 8:1

The effect of a COMT inhibitor and an Uptake$_2$ inhibitor on the reappearance of catecholamine fluorescence after exposure to intraluminal NA in nialamide and reserpine pre-treated arteries.

EXP'T NO	NO TREATMENT	EXTRALUMINAL NA	INTRALUMINAL NA	U0521 + INTRALUMINAL NA	METANEPHRINE + INTRALUMINAL NA
1	0	+++	0	+	-
2	0	+++	0 - +	+ - ++	-
3	0	++ - +++	0	++	++
4	0	++ - +++	0 - +	+ - ++	++
5	0	++	0 - +	++	++
6	0	+	0	+	+
7	0	+++	0	++	+ - ++

KEY TO SYMBOLS USED:

0 no specific catecholamine fluorescence.

\+ catecholamine fluorescence present but not equally distributed around plexus.

++ catecholamine fluorescence in nerve terminals equivalent to that in normal arteries.

+++ catecholamine fluorescence visible in adventitia as well as in nerve terminals.

Data presented is from experiments in which segments of nialamide and reserpine pretreated artery were exposed to either intra- or extraluminal NA at a concentration of 0.5 µg/ml for 30 minutes. The arteries were then washed well and 10 minutes later were treated histochemically to display catecholamine fluorescence. Some segments were also exposed to U0521 (10 µg/ml) to exhibit COMT or metanephrine (0.5 µg/ml) to inhibit Uptake$_2$.

Assessment of the degree of fluorescence was carried out by two independent observers, both of whom were unaware of the experiment protocol.

from the vasoconstrictor response to NA were measured for the 10 minutes between washout of the NA and removal of the arteries for histochemistry. During this period the flow rates were at 5 ml/min. The time courses of the recoveries after the various treatments are shown in Fig. 1.

The main feature to note is that recoveries of arteries from intraluminal NA were extremely rapid compared with those of extraluminal NA. However, recoveries from intraluminal NA were significantly slower in the UO521 and the metanephrine treated arteries. The effect of UO521 was considerably greater than that of metanephrine, the latter becoming apparent only after 8 minutes.

(c) The effects of some COMT inhibitors on the sensitivity to NA.

The influence of catechol-O-methyl transferase on the concentration of NA at its receptors in the rabbit ear artery was studied by using enzyme inhibitors. The inhibitors tested were UO521, tropolone, and β-thujaplicin at the concentrations listed in Table 2.

Dose response curves to both extra- and intraluminal NA were obtained in isolated perfused artery segments, in the absence and presence of the inhibitor. The results were expressed as the ratio of equipotent doses which produced a response of 60 mm Hg.

The results are summarised in Table 2. The effects of the agents ranged from depression (tropolone) to slight potentiation (thujaplicin). UO521, which was more consistent in its effect than the above agents, nevertheless had little effect on the sensitivity to either intra- or extraluminal NA. However, in arteries previously perfused with nialamide to inhibit MAO, UO521 produced an unequivocal increase in sensitivity to intraluminal NA of the order of 2-3 fold. The increase was apparent both as a shift to the left of dose response curves to NA, and also as a further increase in the response when UO521 was added during the steady state constrictor response to NA. In some of these

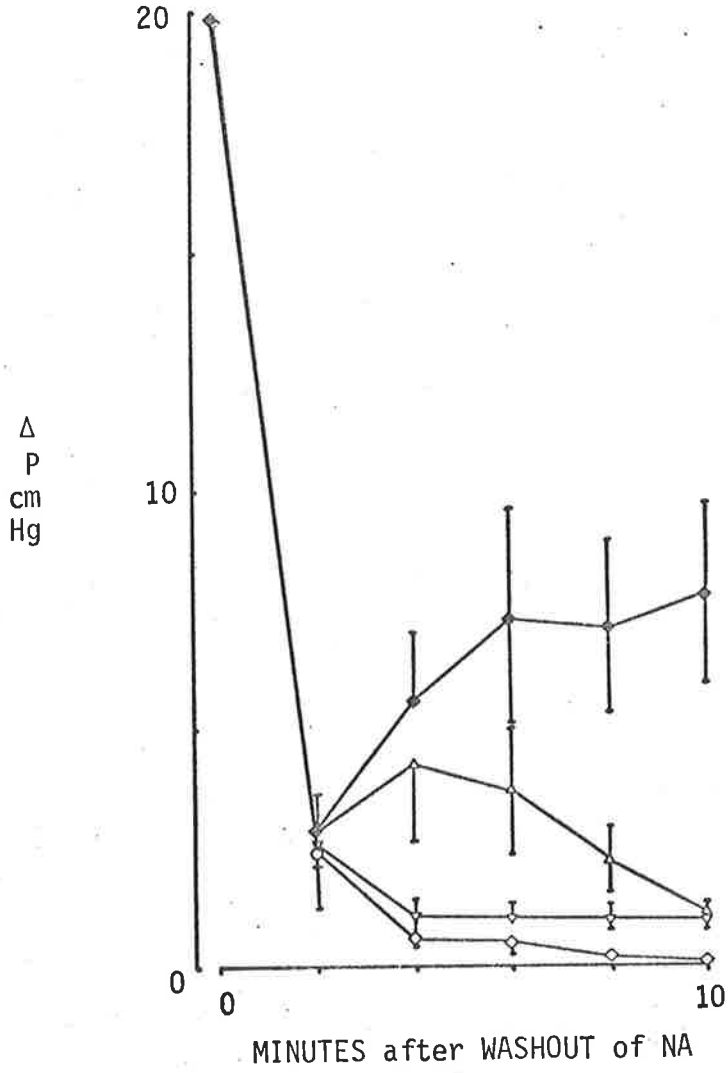


Figure 8.1

Recovery of perfused artery segments from noradrenaline 0.5 µg/ml.

Data shown is the means ± s.e. of 7 experiments in which the recoveries were recorded of segments treated with extra-luminal NA (◆——◆), intra-luminal NA (◇——◇), intra-luminal NA + U0521 △——△ , intra-luminal NA + metanephrine ▽——▽ (5 experiments only).

TABLE 8:2

The effect of various inhibitors of COMT on NA responses in the untreated, and nialamide treated ear artery.

	INHIBITOR	CONC'N	RATIO $\frac{\text{Conc. NA before inhibitor}}{\text{Conc. NA with inhibitor}}$	
			IL	EL
UNTREATED ARTERIES	TROPOLONE	2×10^{-4}M (2)	0.20 0.11-0.38	0.33 (0.14-0.81)
	U0521	5×10^{-5}M (6)	0.99 0.80-1.2	1.30 1.1-1.4
	β-THUJAPLICIN	1×10^{-5}M (2)	1.26 (0.53-3.0)	1.42 (0.9-2.2)
		5×10^{-5}M (3)	1.05 (0.64-1.7)	1.9 (1.1-3.1)
		1×10^{-4}M (2)	1.9 (0.7-5/.0)	1.5 (0.9-2.6)
NIALAMIDE TREATED ARTERIES	U0521 (Fast Perfusion)	1×10^{-5}M (11)	2.3* (2.1-2.5)	-
	U0521 (Slow Perfusion)	1×10^{-5}M (8)	3.2* (2.6-3.8)	-

*no significant difference between groups (t-test, $P > 0.05$)

Values shown are the geometric means of ratios of equipotent concentrations of NA producing a response of 60 mm Hg in the presence of inhibitor (calculated from parallel dose response curves).

The figures below the mean indicate mean ± s.e. where 5 or more observations were made, or where fewer observations were made, the figure refers to the upper and lower values found. The figure in brackets refers to the number of experiments.

In the nialamide + U0521 series, the artery was first treated with nialamide, then response to intraluminal NA measured. U0521 was then added, and the response to NA repeated.

experiments, in paired arteries, the perfusion rate of one of the pair was reduced to 0.9 to 1.2 ml/min to mimic the conditions of the histochemical experiments described above. At the slow rate of perfusion, the potentiating effects of UO521 appeared to be somewhat more prominent; however, the difference between the potentiations seen at the slow and fast rates of perfusion was not significant (paired t-test, $P>0.05$).

It was noted in these experiments that responses to NA were further enhanced immediately after washout of UO521. Furthermore, when UO521 was added during a steady state response, the perfusion pressure sometimes declined before further increasing to a new and elevated steady level. This behaviour of UO521 raised the possibility that the concentration used was excessive and was producing inhibitory as well as potentiating effects on NA. However, due to lack of time, further experiments to test this possibility were not carried out in this series.

2. EXTRANEURONAL FATE OF DA.

(a) Histochemistry.

In each of 4 experiments, five artery segments were removed from one ear of a rabbit, and allowed to remain in Krebs bicarbonate solution for 1 hour to equilibrate. Four of the sections were then treated with nialamide for 1 hour and rinsed for 15 minutes in Krebs bicarbonate solution. The segments were not perfused, but allowed to incubate for 15 minutes at room temperature according to the following plan:

Segment	Pretreatment	Treatment
a	nial	none
b	none	DA (20 µg/ml)
c	nial	DA (20 µg/ml)
d	nial	DA (20 µg/ml) + MN (20 µg/ml)
e	nial	MN (20 µg/ml)

The segments were then washed for 1 minute and immediately frozen

for fluorescence histochemistry (Chapter 2.8). Results, summarised in Table 3, were evaluated according to the following scheme:

0 No medial fluorescence,
Normal neuronal fluorescence observed,
+-++ Medial fluorescence apparent but slight or patchy,
+++ Bright fluorescence apparent in all parts of media,
++++ Very bright fluorescence apparent in all parts of media,

by two impartial observers.

Medial fluorescence was very marked in the nialamide and DA treated preparation, appearing to be brightest towards the outer areas of the media, grading inwards towards the lumen. After DA incubation in the non-nialamide treated preparations, the medial fluorescence was apparent but slight, as was the medial fluorescence in the nialamide and metanephrine treated segments. No fluorescence was seen in the media of the segments not incubated with DA, i.e. those treated with nialamide alone or nialamide with metanephrine. Normal neuronal fluorescence was apparent in all segments.

(b) Effect of a COMT inhibitor on the response to DA.

The effect of the COMT inhibitor UO521 (3'4'dihydroxy-2-methyl-propiophenone) on the sensitivity to DA was tested on four artery segments by comparing dose response curves to DA in the presence and absence of the drug. Neither the shapes of the responses to DA (Fig. 2) nor the sensitivity to extraluminal DA were affected by UO521. The sensitivity to intraluminal DA was decreased to a small extent. Thus the mean ratio ± se of the equipotent concentration of DA $\frac{\text{before UO521}}{\text{during UO521}}$ was 0.76 (0.62-0.92) and 0.98 (0.85-1.1) for intra- and extraluminal DA respectively (4 experiments). These experiments were not repeated in nialamide treated arteries due to the secondary sensitization to intraluminal DA (page 4.4) which made precise evaluation of small additional changes in sensitivity in perfused segments extremely difficult.

TABLE 8.3

The effect of metanephrine and nialamide on smooth muscle uptake of dopamine in the rabbit ear artery.

MEDIA FLUORESCENCE					
EXP'T	NIAL ONLY	DA 20 g/ml			
		NO NIAL	NIAL	NIAL + MN	MN + NIAL
1	0	++	++++	++	0
2	0	++	++++	++	0
3	0	++	+++-++++	++	0
4	0	+	+++-++++	+	0

For each experiment 5 artery segments from one rabbit ear artery were incubated for 1 hour in Krebs bicarbonate solution, then 4 of these pretreated with nialamide, and incubated with DA, DA + MN, MN, or Krebs bicarbonate solution containing no drug for 15 minutes, as indicated; washed for 1 minute, then frozen for fluorescence histochemistry.

Medial fluorescence was assessed in the arteries according to the following plan by two impartial observers:

0 No medial fluorescence
Normal neuronal fluorescence observed.

+-++ Medial fluorescence apparent but slight or patchy.

+++ Bright fluorescence apparent in all parts of media.

++++ Very bright fluorescence apparent in all parts of media.

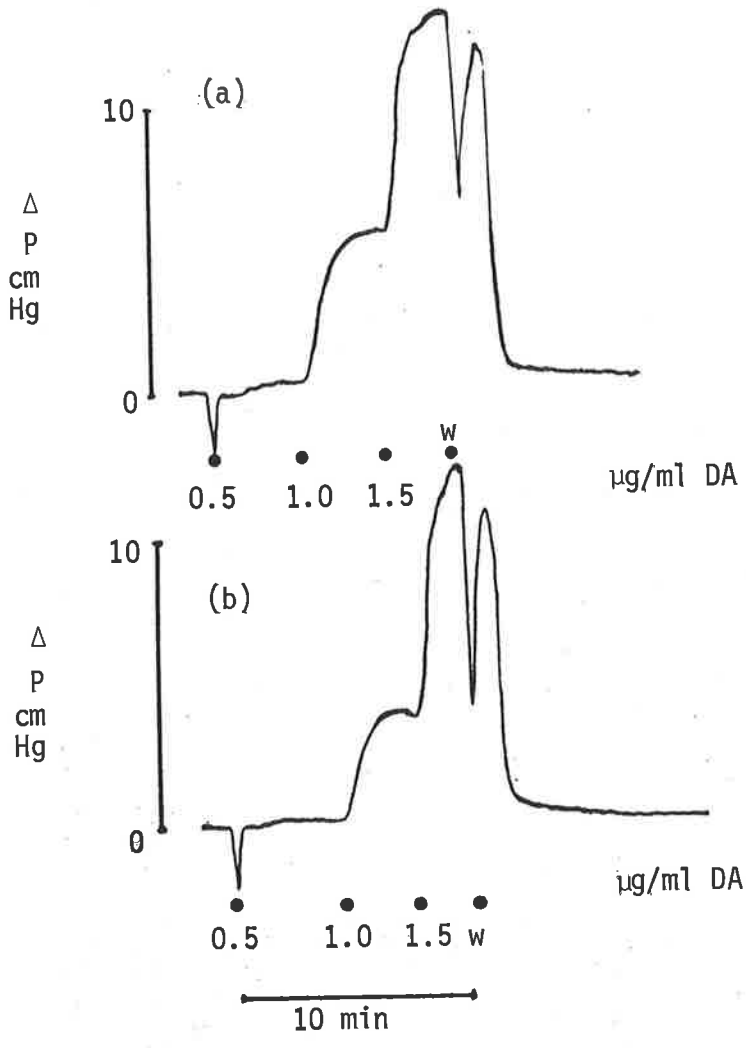


Figure 8.2

Responses to intraluminal dopamine in a perfused artery segment (a) before U0521 (b) in the presence of U0521 (10 µg/ml).

D I S C U S S I O N

The failure of intraluminal NA to produce neuronally mediated effects in the MAO inhibited rabbit ear artery, was attributed by de la Lande and Jellett (1969, 1972) to failure of the intraluminal NA to reach the nerve terminals.

Histochemical evidence for this explanation is provided by experiments which show that intraluminal NA failed to restore fluorescence to reserpine depleted nerve terminals after MAO inhibition except in the case when either COMT, or smooth muscle uptake, or both were inhibited. In this circumstance, the fluorescence, although not as marked as that produced by extraluminal NA, was nevertheless unequivocal. This may be interpreted to mean that intraluminal NA normally fails to achieve a sufficiently high concentration in the vicinity of the nerve terminals to enable its uptake by these terminals to be detected. Such a failure can be attributed to loss of NA as it diffuses across the media of the artery towards the nerve terminals either by uptake into smooth muscle or metabolism by MAO or COMT or both. Gillespie (1968) has shown that the smooth muscle cells of the media are a major site of extraneuronal uptake of NA in the rabbit ear artery, this uptake being selectively inhibited by metanephrine.

The results with UO521 indicate that the loss of NA is at least partly due to metabolism by COMT, which is situated largely extraneuronally in this tissue (Head and others 1974).

The histochemical results showed no difference in intensity of fluorescence between tissues treated with UO521 and intraluminal NA, and those treated with metanephrine and intraluminal NA. However, the recovery from the intraluminal NA in the UO521 treated group was retarded to a greater extent than the recovery of the metanephrine treated group. There are several possible explanations for this paradox. Firstly, the histochemical methods used are not finely quantitative, and depend on subjective

observation. Thus, within the rather broad framework of the system used, a large concentration difference could escape detection. Secondly, metanephrine may in some way be inhibiting the slow recovery characteristic of secondary sensitization, which has been shown to be dependent on neuronal mechanisms (de la Lande and Jellett 1969, 1972). Although metanephrine is known not to affect uptake into nerve terminals (Burgen and Iversen 1965), some other mechanism may be affected. Thirdly, UO521 in the concentration used here may inhibit $Uptake_2$ as well as COMT, and so increase the concentration of NA in the nerve terminal. Alternatively, if intraneuronal COMT is present in the nerve terminals of the rabbit ear artery, inhibition of this in addition to extraneuronal COMT and intra- and extraneuronal MAO may cause the intraneuronal NA concentration to increase to greater levels, thus producing the conditions necessary for the delayed recovery. Jarrott (1971) described a pre- and post-synaptic COMT with different substrate affinities. However, on the evidence at present available, it is not possible to distinguish between these possibilities.

In view of the histochemical evidence that COMT contributes to the inactivation of intraluminal NA as it diffuses across the media of the monoamine oxidase inhibited artery, it was considered that COMT may influence the concentration of NA at the receptors in the rabbit ear artery. However, in the otherwise untreated artery, the effects of UO521 and other COMT inhibitors on the response to intraluminal NA were slight and variable. After concomitant inhibition of MAO, however, a definite increase in sensitivity of the order of two-fold was seen.

It seems likely, therefore, that metabolism of low concentrations of NA in the media of the artery may be carried out by either COMT or MAO or both. The enzymes may act in parallel, such that if one of the two is inhibited, the other will function in its place. Thus the full effect of inhibition of one of the

enzymes is not seen while the other remains active. This effect has been shown to occur also in rabbit aorta (Kalsner and Nickerson 1969). Thus the relatively minor effects of procedures which influence neuronally mediated mechanisms, on the sensitivity to intraluminal NA, may well be a consequence of low neuronal uptake of intraluminal NA compared with that of extraluminal NA, rather than a simple reflection of the relative distribution of sympathetic nerve terminals and smooth muscle as was previously proposed.

The response to intraluminal DA in untreated arteries was not affected by inhibition of COMT. This study was not repeated in nialamide treated arteries due to the secondary sensitization effect seen with intraluminal DA (Chapter 4).

Trendelenburg and others (1971) have suggested that COMT affects the concentration at the receptors of only those substances to which the tissue is very sensitive. *In vitro*, the relative activities of DA and NA as substrates for COMT are approximately equal (Axelrod and Tomchick 1958), but DA is 100 to 150 times less potent than NA in producing a constrictor response in the rabbit ear artery. The lack of effect of COMT inhibition on the response to DA, together with the slight effect on the response to NA, would then be further corroborative evidence for this proposal.

The evidence presented here on the role of MAO, COMT and smooth muscle uptake in the response to intraluminal NA further suggests that the ability of intraluminal DA to cause secondary sensitization in nialamide treated arteries, in contrast to intraluminal NA, may also be due to the relatively high concentrations of DA which are required to produce a constrictor response in the artery. This suggests that these concentrations of DA may be sufficiently high to saturate the metabolizing enzymes and smooth muscle uptake mechanism in the artery wall, thus allowing relatively free access of intraluminal DA to the nerve terminals at the media-adventitia border of the artery. The histochemical

evidence, however, shows that even at the relatively high concentration of 20 μg/ml of DA, uptake into smooth muscle still occurs, since this may be reduced by equal concentrations of metanephrine.

CHAPTER NINE

GENERAL DISCUSSION

GENERAL DISCUSSION

Discussion of the results presented in this study fall naturally into two parts. Firstly, the factors controlling the response to DA in the untreated artery with normal MAO activity, and secondly, the factors which are operative in the DA response in the MAO inhibited artery.

In view of the fact that the response to DA, like NA, is mediated by the α-adrenergic receptors, it was of interest to determine whether the sympathetic nerves play a part in the response to DA in the ear artery, as has been shown for NA (de la Lande and Waterson 1967). Uptake of DA into the cytoplasm of sympathetic nerves was shown by histochemical methods to occur in this tissue at concentrations of DA which were sub-threshold for the constrictor. However, at threshold concentrations and above, the uptake appeared to be saturated. Langer and Trendelenburg (1969) have shown that saturation of neuronal uptake causes neuronal effects to become unimportant in the response to a drug, the concentration of that drug in the biophase then depending only on the concentration of drug in the tissue bathing fluid.

Indirect evidence showed that uptake was unimportant in the vasoconstrictor response to DA in the untreated artery, since (a) the intra- and extraluminal sensitivities to DA were identical, and (b) neither reserpine pretreatment, cocaine treatment, nor chronic denervation modified the sensitivity to intraluminal or extraluminal DA. It was therefore concluded that the vasoconstrictor response to DA in the rabbit ear artery is direct, and neuronal mechanisms play little or no part in the response.

It was also shown that DA does not act on dilator receptors in the ear artery under conditions where isoprenaline caused an unequivocal dilatation. Thus it would appear that DA is relatively inactive on the β-adrenergic receptors in the ear artery, and also that this artery contains relatively few of the DA

receptors which are belived to mediate dilatation in the dog renal and mesenteric arteries (McNay and others 1963, 1965).

In the case where the main metabolising enzyme for DA, MAO (Blaschko and others 1937),had been inhibited by nialamide, the features of the response to dopamine changed radically to those typical of secondary sensitization . The normally fast response and relaxation became very slow to reach steady state, and the relaxation after washout of the drug was prolonged. There was also an increase in the sensitivity. These effects were seen in both segments and strips, being most marked in segments after extraluminal DA and resembled the secondary sensitization to NA in the nialamide treated artery (de la Lande and Jellett 1969, 1972). In helical strips where complete dose response curves were obtained, it was shown that the sensitizing effects of DA are mostly present at levels where the direct effects are small, and taper off as the concentration of dopamine reaches ED 70 levels and above. A response similar in nature and time course to the response to DA was seen after L-dopa in nialamide treated arteries. L-dopa does not produce a constrictor response in untreated arteries. Denervation largely prevented secondary sensitization to both L-dopa and DA indicating that the secondary response depends on neuronal mechanisms.

Various treatments and pre-treatments were carried out to determine the conditions under which secondary sensitization to dopa, DA and NA occurred. This is summarized in Table 1. A scheme to explain the nature of secondary sensitization to DA is proposed in Fig. 1; a discussion of the evidence for this follows.

(1) DA is taken up into the nerve terminal.

Direct histochemical evidence for uptake of DA into nerve endings of the ear artery is presented in Chapter 3.9. Uptake was first visible at 0.01 µg/ml in nialamide and reserpine treated arteries, and further increases in brightness of fluorescence could not be detected by eye after 0.3 - 0.5 µg/ml

TABLE 9.1

Conditions for the occurrence of secondary sensitization to DA, L-dopa and NA in the rabbit ear artery.

DRUG	TREATMENT						
	NIALAMIDE UNTREATED	NIALAMIDE TREATMENT					
		DENERV--ATION	COCAINE	RESERPINE	DDC	RESERPINE + DDC	COOLING to 25°C
DA	-	-	+	+	+	-	-
L-DOPA	-	-	+	+	+ reduced	not tested	-
NA	- *	- *	- *	+ *	not tested	not tested	-

* Data from de la Lande and Jellett (1972).

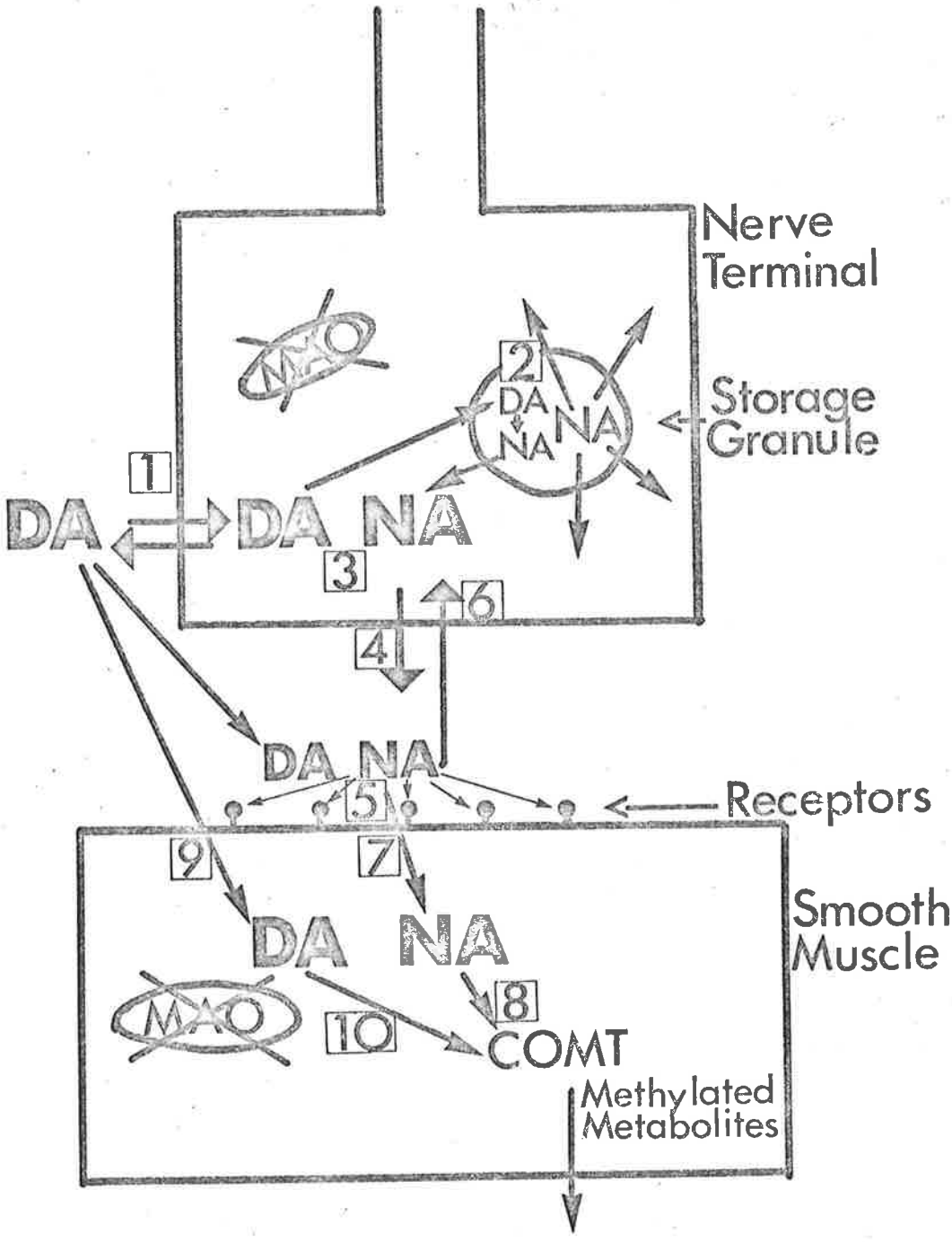


Fig. 9.1

Proposed mechanism for secondary sensitization to dopamine.

($\simeq 1.5 - 2.5 \times 10^{-6}$M). This evidence also showed that cocaine, which failed to modify secondary sensitization to DA, also failed to modify the neuronal uptake of DA at 1-3 µg/ml. However some modifying effect of cocaine was evident at 0.3-0.5 µg/ml.

(2) NA is formed from DA in nerve ending.

There is evidence for this step only in reserpine pretreated arteries where inhibition of dopamine-β-hydroxylase with diethyldithiocarbamate (DDC) largely prevented the manifestations of secondary sensitization (Chapter 6.3). In reserpine untreated arteries no effect of DDC on the secondary sensitization to DA was apparent. However, the fact that DDC partially reduced the secondary type response to L-dopa indicates that in a system where DA uptake is not involved, inhibition of synthesis of NA may have had some effect.

(3) In presence of MAO inhibition the intraneuronal accumulation of free amine increases.

No direct evidence of this step was obtained in this study. However, Furchgott and Sanchez Garcia (1968) postulated that the secondary sensitization to NA seen by them after MAO inhibition in guinea pig atria was due to the accumulation and release of NA from cytoplasmic binding sites. Direct evidence for such sites in sympathetic nerves after reserpine pretreatment and MAO inhibition has been presented by Taxi and Droz (1969) using autoradiographical techniques. Graefe and others (1971) suggested that any impairment of the intraneuronal mechanisms of inactivation (i.e. vesicular storage and MAO) leads to an increase in the axoplasmic concentration of free NA which seems to rise more after blockade of MAO than after pretreatment with reserpine and is most pronounced after both.

(4) NA is released from nerve endings.

An indication that secondary sensitization to DA is caused by release of NA was shown by the evidence (mentioned in *(2)* above)

that secondary sensitization to DA could be prevented by blockade of the synthesis of NA in reserpine and nialamide treated arteries. The released NA may be simply the result of a spontaneous efflux of free amine, such as occurs in NA treated tissues in which MAO has been inhibited (Lindmar and Loffelholz 1972); or it may be due to an indirect, or NA releasing effect, of DA. DA is known to have NA releasing effects in other tissues (Bulbring and Burn 1938; Stromblad 1960; Farmer 1965, 1966; Spiers and Calne 1969). Smith (1966) demonstrated that inhibition of MAO potentiates the effects of purely indirectly acting amines which are not good substrates of the enzyme. This potentiation was thought to be due to the decreased deamination of extra-granular NA in the absence of MAO activity. Also Luchelli-Fortis and Langer (1974) showed that in the case of phenylephrine, MAO inhibition unmasked an additional indirect effect due to the protection from deamination of the NA released by the phenylephrine; the NA being released in the untreated preparation in the form of deaminated metabolites only. Thus the indirect effects of DA may be unmasked in the presence of MAO inhibition by some or all of the following mechanisms:

(a) protection of the DA taken up by $Uptake_1$,

(b) protection of the intracellular NA released from the intragranular stores, and

(c) protection of released NA from prior deamination.

Cooling to $25^{o}C$ prevented the occurrence of secondary sensitization to L-dopa, DA and NA. However, since both the spontaneous efflux of free NA from MAO inhibited tissues, and the NA releasing effect of indirectly acting amines are reduced by cooling, (Wennmalm and others 1970; Lindmar and Loffelholz 1972; Paton 1973 a,b), it is not possible to distinguish between these mechanisms on the evidence presented here.

(5) Action of NA or DA/NA at receptors.

It is apparent from the experiments with DDC in reserpine and nialamide treated arteries (Chapter 6) that with concentrations

of DA which would be subconstrictor on the untreated artery, NA alone acts on the receptors to cause constriction. Thus, at low concentrations, the neuronal effect of DA, i.e. release of NA, is predominant in controlling the amount of active amine in the biophase. As the concentration of DA is raised, neuronal effects become increasingly less important, and the concentration of active amine in the biophase becomes more and more controlled by the concentration of DA in the bathing fluid.

The contrast between the ability of the artery helical strip to show secondary sensitization to DA and not to NA (Chapter 7) during cumulative responses is also of interest here. Neuronally mediated effects are not displayed prominently in the strip preparation, which, in this regard, approximates the intraluminally dosed ear artery segment (de la Lande and Urquilla 1969). Thus, in the case of NA, the concentration of NA in the biophase is regulated by the concentration of NA in the bathing fluid. However, for concentrations of DA which would be subthreshold in the untreated artery, the bathing fluid contains a concentration of DA to which the artery is relatively insensitive, but which may be acting on the neurones to release NA to which the artery is much more sensitive. Thus the concentration of active amine at the receptor is governed by the neurones, and the contraction seen at these low concentrations of DA is primarily due to the NA released.

It was of interest to observe that although nialamide treated artery helical strips did not show the first phase of secondary sensitization to NA, nevertheless the second phase of the response, that of slow recovery after washout of NA, was prominent, though quantitatively less than in the perfused segment.

(6) NA after its release undergoes reuptake by the nerve terminals.

The evidence for this step is shown in Chapter 4. Cocaine, which is without effect on the response to DA in the absence of

nialamide, potentiates the secondary response to dopa and DA when applied during the response, or during the recovery phase of the response. This action of cocaine is identical with its effect when applied during the secondary response to NA (de la Lande and Jellett 1972).

(7) NA which escapes reuptake by the neurones is taken up extra-neuronally by smooth muscle.

The evidence for this step is indirect. However, it was shown in Chapter 8 that in the reserpine and nialamide treated perfused artery segment, metanephrine, which is an inhibitor of smooth muscle uptake, permits intraluminally applied NA to achieve higher concentrations in the vicinity of the nerve terminals. Thus it is highly probable that NA released from the nerve terminals into the biophase is subject to the same process of extraneuronal uptake.

(8) NA which escapes reuptake into neurones is also inactivated by COMT.

Evidence for this step is also largely indirect, being based on inhibitor studies in the intraluminally dosed perfused segment, but nevertheless indicates the possibility that NA escaping from the biophase may be inactivated by COMT. Firstly, the COMT inhibitor,U0521, potentiates responses to intraluminal NA in nialamide treated perfused segments. It has been shown that in this preparation COMT is largely extraneuronal in distribution (Head and others 1974). Thus extraneuronal metabolism by COMT is a possible factor in governing the concentration of NA at the receptors in the MAO inhibited arteries. Histochemical evidence (Chapter 8) also showed that the use of U0521 permitted intraluminal NA to achieve high concentrations in the region of the nerve terminals, thus demonstrating that metabolism by COMT was a factor in preventing the diffusion of NA across the artery wall.

(9) DA undergoes extraneuronal uptake.

Some evidence for this step is shown in Chapter 8, where it was shown histochemically that in the nialamide treated artery, fluorescence appeared in the smooth muscle after incubation with DA (20 μg/ml). This fluorescence was reduced by the concomitant administration of metanephrine (20 μg/ml), indicating that DA is a substrate for smooth muscle uptake.

Gillespie and others (1970) showed in histochemical experiments that cooling to 15°C decreased smooth muscle uptake in arterial smooth muscle. In pharmacological experiments (Chapter 7), cooling to 25°C decreased the slow recovery phase of secondary sensitization to submaximal and supramaximal concentrations of DA to a much greater degree than denervation.

(10) DA is inactivated by COMT extraneuronally.

This step is assumed but was not tested directly. Axelrod and Tomchick (1958) showed that DA is a substrate of COMT *in vitro* with affinity equal to that of NA. COMT inhibition by U0521 had no effect on the magnitude of DA responses in untreated arteries and this work was not repeated in nialamide treated arteries. However, work by Trendelenburg and others (1971) has shown that inhibition of COMT appears to influence the concentration of an amine at the receptors only when the tissue is very sensitive to that amine (i.e. with ED50 approximately 10^{-6}M or below). The rabbit ear artery is relatively insensitive to DA (ED50 approximately 1.5×10^{-5}M), so the failure of COMT inhibition to influence the response to DA is not surprising. Thus this evidence does not rule out the possibility that DA may be metabolized extraneuronally by COMT.

Thus, the proposed mechanism of secondary sensitization to DA in the nialamide treated artery may be summarized in the following manner. The first phase of the secondary response to low doses of DA is an increase in sensitivity due to the continuous

slow release of intraneuronal NA, and reuptake of this NA. A progressive blockade of the $Uptake_1$ mechanism by the DA and NA in the presence of impaired intraneuronal inactivation of those amines probably also contributes to the supersensitivity which occurs, as suggested by Trendelenburg (1971).

As the concentration of DA is raised, the neuronal or indirect effect becomes less important in the response, and the manifestations of secondary sensitization become less prominent. This may be caused by the direct effect of the high concentrations of DA which then masks the relatively small indirect effect.

The prolonged recovery phase of the response is due to a slow release and reuptake of DA and/or NA from the nerve terminals, in the presence of the now slowly decreasing blockade of the $Uptake_1$ mechanism. This is augmented by a slow release of DA and possibly NA, from extraneuronal uptake and binding sites, since extraneuronal MAO (but not extraneuronal COMT) has been inhibited.

Reserpine pretreatment did not augment the degree of secondary sensitization when tested quantitatively on the nialamide treated artery strip, as might be suggested by the work of Trendelenburg (1971). However, it was noted that some aspects of secondary sensitization appeared to be enhanced in the reserpine and nialamide treated perfused segment (Chapter 4). Neuronal effects are more prominent in this preparation than in the helical strip (de la Lande and Urquilla 1969), so the effect may have been insignificant in the strip. Enhancement of the secondary sensitization to NA by reserpine pretreatment in the extraluminally dosed perfused segment was also noted by de la Lande and Jellett (1972).

Secondly, to consider the secondary response to L-dopa; this response does not occur in denervated arteries, or in the absence of MAO inhibition even though L-dopa is not a substrate

for MAO (Blaschko and others 1937). However, the synthetic products of L-dopa, DA and NA, are substrates for MAO. Thus the response of L-dopa, which occurs only in MAO inhibited arteries, must depend on the cytoplasmic accumulation of one or both of these amines, in the absence of intraneuronal MAO activity. Once an accumulation of DA is formed in the nerve ending, the mechanism of the secondary response to dopa may follow that of DA. The profound effect of cooling in the absence of any effect of cocaine pretreatment, however, suggests that cooling may affect the uptake of L-dopa and/or the synthesis of DA in addition to the other systems already mentioned.

The study of the mechanisms of the secondary response to DA and L-dopa may have thrown further light on the mechanism of the secondary sensitization phenomenon to NA seen in this and other tissues (Furchgott and Sanchez Garcia 1968; de la Lande and Jellett 1969, 1972). It was shown previously that, in contrast to L-dopa and DA, cocaine pretreatment could completely prevent the occurrence of secondary sensitization to NA. Thus uptake of NA into the neurone must be important in secondary sensitization to NA. An increased cytoplasmic concentration of NA has been shown to occur with NA (Furchgott and Sanchez Garcia 1968; Taxi and Droz 1969) after inhibition of MAO, and this may also be important in the mechanism. A continuous spontaneous efflux of NA from nerve terminals after MAO inhibition, which is unaffected by cocaine, but reduced by cooling, has been shown to occur in rabbit heart (Lindmar and Loffelholz 1972; Paton 1973 a,b). However Paton (1973 a,b) also showed that a significant amount of NA could be released by the addition of exogenous NA, or tyramine or metaraminol, and this indirect effect was also reduced by cooling. Thus an indirect effect of the exogenous NA may be factor in secondary sensitisation to NA. Reserpine pretreatment did not prevent secondary sensitization to NA, and in some ways even enhanced it (de la Lande and Jellett 1972). Pretreatment of tissues with reserpine has for many years

been a method used in the classification of indirect effects of sympathomimetic amines. The rationale for this method is that depletion of endogenous stores of NA, caused by reserpine, should prevent any release of NA by the indirectly acting amine under study. However, this method of classification of direct and indirect effects has come under some discussion in recent times (Trendelenburg 1972; Luchelli-Fortis and Langer 1974). The work with L-dopa and DA in this thesis also suggests that, in cases where replenishment of the intraneuronal NA stores is possible, such as when a precursor drug is used, or when NA itself is the amine under examination, this method cannot give a reliable answer. Another case is where the amine normally releases NA in the form of its deaminated metabolites. Inhibition of intraneuronal MAO allows indirect effects, previously masked, to become apparent (Luchelli-Fortis and Langer 1974).

Recent work has shown that there may be differences in type between MAO's in different locations (Goridis and Neff 1971), and that the enzyme may not be a single type but a series of isoenzymes with different substrate specificities. Differences in inhibitor sensitivity, substrate specificity and thermal inactivation have been found for MAO in normal and denervated vas deferens (Jarrott and Iversen 1971). Two forms of MAO, type A and B, have been located in rat mesenteric and femoral arteries with a selective loss of type A after chemical sympathectomy (Coquil and others 1973). Type A, which deaminated tyramine, 5-hydroxytryptamine and NA, was highly sensitive to inhibition by pargyline and clorgyline but resistant to carbonyl reagents. The second type, B, was resistant to chemical sympathectomy, did not deaminate NA or 5-hydroxytryptamine, but did deaminate tyramine. It was resistant to pargyline and clorgyline, but sensitive to carbonyl reagents. The type of MAO which is present intra- and extraneuronally in the rabbit ear artery has not been differentiated, and it is tempting to suggest that similar differences in the type of MAO in the two

situations exists in this artery also. However, the different pharmacological roles of the intra- and extraneuronal enzymes in the artery may simply reflect their different locations, inactivation by the intraneuronal enzyme being favoured by the higher intraneuronal concentrations resulting from $Uptake_1$.

Although all the work in this study has been carried out on a small muscular artery, it is probable that the relevance of the findings is not necessarily confined to that type of preparation. The similarity of the effects of DA after MAO inhibition noted on this preparation, and those noted by Helmer (1957) on the rabbit aorta (a large elastic conducting vessel) are very similar. Also the effects of L-dopa, DA and NA in the MAO inhibited artery resemble the effects seen in many other types of sympathetically innervated smooth muscle and it may be possible to extrapolate the results found in this study on the rabbit ear artery to other tissues. In particular, the mechanism of the increase in blood pressure seen after the concomitant administration of MAO inhibitors and L-dopa in man (Hunter and others 1970) and rabbits (Calne and Reid 1973) may have been at least partly elucidated by this study.

APPENDICES

APPENDIX 1

SOURCES OF DRUGS AND CHEMICALS

ascorbic acid	Koch Light Laboratories
ammonium acetate (Analar grade)	British Drug Houses
cocaine hydrochloride	MacFarlane-Smith
diethyldithiocarbamic acid sodium salt	May & Baker
diethyl ether (Anaesthetic ether B.P.)	Drug Houses of Australia
disulfiram	Ethnor
L-dopa	Koch Light Laboratories
dopamine hydrochloride	Koch Light Laboratories
ethanol absolute AR grade	Ajax Chemicals
halothane	ICI
isopropanol	BDH
lignocaine 2% + adrenaline 12.5 µg/ml	Astra Chemicals Pty Ltd
DL metanephrine hydrochloride	Sigma
NCS solubilizer	Nuclear Chicago
nialamide	Pfizer
L-noradrenaline bitartrate monohydrate	Koch Light Laboratories
paraformaldehyde	Merck
pentobarbital	(Sagatal) - May & Baker
phentolamine methane sulphonate	(Regitine) - Ciba
PPO {2,5-diphenyloxazole}	Koch Light Laboratories
POPOP {1,4-bis-2-(5-phenyloxazolyl)-benzene}	Koch Light Laboratories
reserpine	(Serpasil) - Ciba
β thujaplicin	Koch Light Laboratories
tropolone	K and K Laboratories
L-tyrosine	British Drug Houses
U0521 {3',4-dihydroxy-2-methyl-propiophenone}	Upjohn
urethane	Koch Light Laboratories
xylene (AR)	Ajax Chemicals

The dose of each was expressed as the weight of the salt except in the case of nialamide, noradrenaline, phentolamine and reserpine in which the weight of the base was used. Drugs used in tissue experiments were dissolved in 0.9% sodium chloride unless otherwise stated below.

Preparation of drugs

i. Dopamine hydrochloride was dissolved in cold ascorbic saline (Appendix 2). Further dopamine dilutions were also made in cold ascorbic saline. All dopamine solutions were kept in ice for the duration of the experiment.

ii. L-dopa was dissolved in warm (37°C) Krebs solution gassed with CO_2/O_2 containing ascorbic acid 50 µg/ml, immediately before use.

iii. Noradrenaline bitartrate was dissolved in ascorbic saline. Further dilutions were also made in ascorbic saline.

iv. Nialamide solution (a) for perfusion of artery segments. The required amount of nialamide was dissolved in approximately 20-30 ml of saline (0.9% NaCl) with the aid of gentle heat. This solution was then made to the required volume with warm gassed Krebs bicarbonate solution immediately before use.
(b) for pretreatment of animals. The required weight of nialamide was dissolved by the aid of gentle heat in saline (0.9% NaCl) and cooled to 37°C before injection.

v. Disulfiram. The required weight of disulfiram was dissolved in a few drops of absolute ethanol. This was then added to a large volume of saline (0.9% NaCl) with shaking until dissolved.

APPENDIX 2

Reagent Formulae

Krebs bicarbonate solution

NaCl	6.9 g
KCl	0.35 g
$CaCl_2$ (ml 10% solution)	2.8 g
$MgCl_2$ (ml 10% solution)	1.0 g
$NaHCO_3$	2.1 g
KH_2PO_4	0.16 g
Glucose	1.0 g
Glass distilled deionised water	to 1 litre

Gas - 95% O_2 5% CO_2

Reference: *Umbreit, W.W. and others. Manometric Techniques and Tissue Metabolism. Burgess Publication Co., Minneapolis, 1949.*

Ascorbic saline

Ascorbic acid 0.01% in normal (0.9%) saline adjusted to pH 5.5 with NaOH.

Bray's scintillant

PPO	4 g
POPOP	200 mg
naphthalene	60 g
methanol (absolute)	100 ml
ethylene glycol	20 ml
dioxane	to 1000 ml

Reference: *Bray, G.A. A simple efficient liquid scintillation method for counting aqueous solutions in a liquid scintillation counter. Analyt. Biochem., 1:279, 1960.*

Acetone - ethanol

acetone	1
ethanol absolute	1

Diazotised p-nitro-aniline reagent

		Proportions
Sol. (1) P-nitro aniline	0.2% in distilled water	1
Sol. (2) $NaNO_2$	2.5% in distilled water	1
Sol. (3) $NaCO_3$	5% in distilled water	2

Solutions (1) and (2) are mixed, then solution (3) added.
The reagent was freshly made prior to use.

Toluene scintillant

PPO	3 g
dimethyl POPOP	300 mg
toluene	to 1000 ml

APPENDIX 3

Apparatus and Techniques

A. Perfusion experiments.

1. Polythene cannulae were drawn from sterivac polythene tubing in sizes No. 3 (proximal cannula) and No. 2 (distal cannula). The distal cannula was U-shaped, and was supported by a metal hook.
2. The constant volume roller pump was designed by O. Saxby, Department of Pharmacology, Oxford University, and was manufactured in the Medical School workshop, University of Adelaide.
3. Pressure changes in the artery were measured with a Palmer mercury manometer (Condon model) recording on a Palmer Kymograph drum.
4. Field electrical stimulation was provided by a Grass Stimulator (model S4) via two platinum electrodes.

B. Helical strip experiments.

Length changes in the helical strips were recorded via a Harvard Heart/Smooth muscle transducer, through a Rikadenki double channel flat-bed chart recorder.

C. NA fluorescence histochemistry.

1. The freeze dryer used was a Thermovac model FD-3. The pump was not provided with a gas ballast.
2. Paraformaldehyde powder (Merck) was stored (in 5 g amounts) over 34% v/v H_2SO_4 at a relative humidity of 70%. The acid was changed every 2 weeks.
3. The apparatus used for vacuum infiltration was manufactured by the National Appliance Co. and was operated using a water vacuum.

4. Tissue sections were cut at 7 microns using a Leitz model 1212 microtome.
5. The tissue sections were examined by fluorescence microscopy using a Leitz microscope with a dry dark field condenser. Fluorescence was obtained with a HBO 200 W mercury vapour lamp using a 3 mm Schott B6 12 excitation filter and a 510 millimicron barrier filter. All artery sections were photographed on examination by a Leica Camera with microscope and exposure meter attachments. Photographic exposures varied between 10 and 30 seconds, using Kodak Photoflure film developed in Ilford ID2 developer.

BIBLIOGRAPHY

ALLWOOD MJ, and GINSBURG J: Peripheral vascular and other effects of dopamine infusions in man.
Clin. Sci. (London), 27:271-281, 1964.

AXELROD J, and TOMCHICK R: Enzymatic O-methylation of epinephrine and other catechols.
J. Biol. Chem., 233:702-705, 1958.

BALZER H, and HOLTZ P: Beeinflussung der wirkung biogener amine durch hemmung der aminoxydase.
Naunyn Schmiedebergs Arch. exp. Path. Pharmak., 227: 547-558, 1956.

BARGER G, and DALE HH: Chemical structure and sympathomimetic action of amines.
J. Physiol. (Lond.), 41:19-59, 1910.

BEJRABLAYA D, BURN JH, and WALKER JM: The action of sympathomimetic amines on heart rate in relation to the effect of reserpine.
Brit. J. Pharmacol., 13:461-466, 1958.

BIRMINGHAM AT, PATERSON G, and WOJCICKI J: A comparison of the sensitivities of innervated and denervated rat vas deferentia to agonist drugs.
Brit. J. Pharmacol., 39:748-754, 1970.

BLASCHKO H: The specific action of L-dopa decarboxylase.
J. Physiol. (Lond.), 96:50-51P, 1939.

BLASCHKO H, and CHRUSCIEL TL: The decarboxylation of amino acids related to tyrosine and their awakening action in reserpine treated mice.
J. Physiol. (Lond.), 151:272-284, 1960.

BLASCHKO H, RICHTER D, and SCHLOSSMANN H: The oxidation of adrenaline and other amines.
Biochem. J., 31:2187-2196, 1937.

BULBRING E, and BURN JH: The action of tyramine and adrenaline on the denervated nictitating membrane.
J. Physiol. (Lond.), 91:459-473, 1938.

BURGEN ASV, and IVERSEN LL: The inhibition of noradrenaline uptake by sympathomimetic amines in the rat isolated heart.
Brit. J. Pharmacol., 25:34-49, 1965.

BURN JH, and RAND MJ: The effect of precursors of noradrenaline on the response to tyramine and sympathetic stimulation.
Brit. J. Pharmacol., 15:47-55, 1960.

BURNSTOCK G, McLEAN JR, and WRIGHT M: Noradrenaline uptake by non-innervated smooth muscle.
Brit. J. Pharmacol., 43:180-189, 1971.

CALNE DB, and REID JL: Actions of levodopa on the blood pressure of conscious rabbits.
Brit. J. Pharmacol., 48:194-197, 1973.

CAMPBELL IK, and FARMER JB: The response of an isolated artery to sympathomimetic amines.
J. Pharm. Pharmacol., 20:19-25, 1968.

CLARKE DE, JONES CJ, and LINLEY PA: Histochemical fluorescence studies on noradrenaline accumulation by $Uptake_2$ in the isolated rat heart.
Brit. J. Pharmacol., 37:1-9, 1969.

COLLINS GGS, and WEST GB: Some pharmacological actions of diethyldithiocarbamate on rabbit and rat ileum.
Brit. J. Pharmacol., 32:402-409, 1968(a).

COLLINS GGS, and WEST GB: The release of ^{3}H-dopamine from the isolated rabbit ileum.
Brit. J. Pharmacol., 34:514-522, 1968(b).

COQUIL JF, GORIDIS C, MACK G, and NEFF NH: Monoamine oxidase in rat arteries: evidence for different forms and selective localization.
Brit. J. Pharmacol., 48:590-599, 1973.

CROUT JR, and COOPER T: Myocardial catechol-O-methyl transferase activity after chronic cardiac denervation.
Nature (Lond.), 194:387, 1962.

DE LA LANDE IS, CANNELL VA, and WATERSON JG: The interaction of serotonin and noradrenaline on the perfused artery.
Brit. J. Pharmacol., 28:255-272, 1966.

DE LA LANDE IS, and HARVEY JA: A new and sensitive bioassay for catecholamines.
J. Pharm. Pharmacol., 17:589-593, 1965.

DE LA LANDE IS, HILL BD, JELLETT LB, and McNEIL JM: The role of monoamine oxidase in the response of the isolated central artery of the rabbit ear to tyramine.
Brit. J. Pharmacol., 40:249-256, 1970.

DE LA LANDE IS, and JELLETT LB: 1969.
See Jellett LB, and de la Lande IS, 1969.

DE LA LANDE IS, and JELLETT LB: Relationship between the roles of monoamine oxidase and sympathetic nerves in the vaso-constrictor response of the rabbit ear artery to norepinephrine.
J. Pharmacol. exp. Ther., 180:47-55, 1972.

DE LA LANDE IS, and JOHNSON SM: Evidence for inactivation of noradrenaline by extraneuronal monoamine oxidase in the rabbit ear artery.
Aust. J. exp. Biol. med. Sci., 50:119-121, 1972.

DE LA LANDE IS, and RAND MJ: A simple isolated nerve-blood vessel preparation.
Aust. J. exp. Biol. med. Sci., 43:639-656, 1965.

DE LA LANDE IS, and URQUILLA PR: The effects of cocaine on a helical strip preparation of the rabbit ear artery.
Aust. J. exp. Biol. med. Sci., 47:P7, 1969.

DE LA LANDE IS, and WATERSON JG: Site of action of cocaine on the perfused artery.
Nature (Lond.), 214:313-314, 1967.

DE LA LANDE IS, and WATERSON JG: The action of tyramine on the rabbit ear artery.
Brit. J. Pharmacol., 34:8-18, 1968.

EBLE JN: A proposed mechanism for the depressor effect of dopamine in the anaesthetised dog.
J. Pharmacol. exp. Ther., 145:64-70, 1964.

EULER US von, and LISHAJKO F: Catecholamine release and uptake in isolated adrenergic nerve granules.
Acta physiol. scand., 57:468-480, 1963.

EULER US von, and LISHAJKO F: Reuptake and net uptake of noradrenaline in adrenergic nerve granules with a note on the affinity for l- and d-isomers.
Acta physiol. scand. 71:151-162, 1967.

FALCK B: Observations on the possibilities of the cellular localization of monoamines by a fluorescence method.
Acta physiol. scand., 56: Supp. 197, 1-25, 1962.

FARMER JB: Impairment of sympathetic nerve responses by dopa, dopamine and their alpha-methyl analogues.
J. Pharm. Pharmacol., 17:640-646, 1965.

FARMER JB: Indirect sympathomimetic actions of dopamine.
J. Pharm. Pharmacol., 18:261-262, 1966.

FELDBERG W: The peripheral innervation of the vessels of the external ear of the rabbit.
J. Physiol. (Lond.), 61:518-529, 1926.

FOSTER RW: The potentiation of the responses to noradrenaline and isoprenaline of the guinea pig isolated tracheal chain preparation by desipramine, cocaine, phentolamine, phenoxybenzamine, guanethidine, metanephrine, and cooling.
Brit. J. Pharmacol., 31:466-482, 1967.

FURCHGOTT RF, and SANCHEZ GARCIA P: Effects of inhibition of monoamine oxidase on the actions and interactions of norepinephrine tyramine and other drugs on guinea pig left atrium.
J. Pharmacol. exp. Ther., 163:98-122, 1966.

GATGOUNIS J: Structural activity relationships of a series of amines injected before and after monoamine oxidase inhibitor.
Arch. Int. Pharmacodyn., 154:412-420, 1965.

GIACHETTI A, and SHORE PA: Dual amine concentrating mechanisms in the adrenergic neurone.
Fed. Proc., 25:259, 1966.

GILLESPIE JS: The role of receptors in adrenergic uptake.
In: Adrenergic Neurotransmission.
Eds. Wolstenholme GEW, and O'Connor M. Ciba Foundation Study Group No. 33. Churchill (Lond.) 1968, p.61-72.

GILLESPIE JS, and HAMILTON DNH: A possible active transport of noradrenaline into arterial smooth muscle cells.
J. Physiol. (Lond.), 192:30P, 1967.

GILLESPIE JS, HAMILTON DN, and HOSIE RJA: The extraneuronal uptake and localization of noradrenaline in the cat spleen and the effect on this of some drugs, of cold and of denervation.
J. Physiol. (Lond.), 206:563-590, 1970.

GILLIS, CN, and PATON DM: Effects of hypothermia and anoxia on retention of noradrenaline by the cat perfused heart. *Brit. J. Pharmacol., 26:426-434, 1966.*

GLOVER WE, STRANGEWAYS DH, and WALLACE WFM: Responses of isolated ear and femoral arteries of the rabbit to cooling and to some vasoactive drugs. *J. Physiol. (Lond.), 194:78P-79P, 1968.*

GLOVER WE, and WALLACE WFM: Increased sensitivity of the rabbit ear artery produced by cooling. *J. Physiol. (Lond.), 201:95P-96P, 1969.*

GOLDBERG LI: Cardiovascular and renal actions of dopamine: potential clinical applications. *Pharmacol. Rev., 24:1-29, 1972.*

GOLDBERG LI, and SJOERDSMA A: Effects of several monoamine oxidase inhibitors on the cardiovascular actions of naturally occurring amines in the dog. *J. Pharmacol. exp. Ther., 127:212-218, 1959.*

GOLDBERG LI, and WHITSETT TL: Cardiovascular effects of levodopa. *Clin. Pharmacol. Ther., 12:376-382, 1971.*

GOLDBERG LI, and YEH BK: Attenuation of dopamine-induced renal vasodilation in the dog by phenothiazines. *Europ. J. Pharmacol., 15:36-40, 1971.*

GOLDSTEIN M, ANAGNOSTE B, LAUBER E, and McKEREGHAN MR: Inhibition of dopamine-β-hydroxylase by disulfiram. *Life Sci., 3:763-767, 1964.*

GOLDSTEIN M, LAUBER E, and McKEREGHAN MR: Studies on the purification and characterization of 3,4-dihydroxyphenylethylamine β-hydroxylase. *J. Biol. Chem., 240:2066-2072, 1965.*

GORIDIS G, and NEFF NH: Monoamine oxidase in sympathetic nerves: a transmitter specific type. *Brit. J. Pharmacol., 43:814-818, 1971.*

GRAEFE KH, BONISCH H, and TRENDELENBURG U: Time-dependent changes in neuronal net uptake of noradrenaline after pretreatment with pargyline and/or reserpine. *Naunyn-Schmiedebergs Arch. Pharmak., 271:1-28, 1971.*

GURD MR: The physiological action of dihydroxyphenylethylamine and sympathol.
Quart. J. Pharm., 10:188-211, 1937.

HAGGENDAL J, and MALMFORS T: The effect of nerve stimulation on catecholamines taken up in adrenergic nerves after reserpine pretreatment.
Acta physiol. scand., 75:33-38, 1969.

HAMET MR: Contribution a l'etude de la dihydroxy phenylethylamine.
Arch. int. Pharmacodyn., 40:427-443, 1931.

HAMILTON TC: Effects of dopamine on the conductance of perfused vascular beds of the chloralosed cat.
Brit. J. Pharmacol., 44:442-450, 1972.

HEAD RJ: Communication to Australian Society of Clinical and Experimental Pharmacologists, November 1973.

HEAD RJ, STITZEL RE, and DE LA LANDE IS: Changes in adenosine triphosphate, monoamine oxidase and catechol-O-methyl transferase accompanying chronic denervation of the rabbit ear artery.
Proc. Aust. Physiol. Pharmacol. Soc., 5:80, 1974.

HELMER OM: Estimation of urinary catechol amines by means of a strip of rabbit aorta as an aid in the diagnosis of pheochromocytoma.
J. Lab. clin. Med., 50:737-744, 1957.

HENNING M, and RUBENSON A: Evidence for a centrally mediated hypotensive effect of L-dopa in the rat.
J. Pharm. Pharmacol., 22:241-243, 1970.

HERRON A, PATTON JN, and WALLACE WFM: The effect of temperature on the responses of isolated rabbit ear arteries to externally administered L-noradrenaline, D-noradrenaline, L-adrenaline, histamine and metaraminol.
J. Physiol. (Lond.), 217:31-32P, 1971.

HILLARP N-A: Different pools of catecholamines stored in the adrenal medulla.
Acta physiol. scand., 50:8-22, 1960.

HOLTZ P, and CREDNER K: Die enzymatische Entstehung von Oxytyramine in Organismus und die physiologische Bedeutuny der Dopadecarboxylase.
Naunyn-Schmiedebergs Arch. Pharmak. exp. Path., 200: 356-388, 1942.

HORNYKIEWICZ O: Dopamine: its physiology, pharmacology and pathological neurochemistry.
In: Biogenic Amines and Physiological Membranes in Drug Therapy, Pt. B. Eds. Biel JH, and Abood LG. Dekker (NY) 1971, pp. 173-258.

HORWITZ D, GOLDBERG LI, and SJOERDSMA A: Increased blood pressure responses to dopamine and norepinephrine produced by monoamine oxidase inhibitors in man.
J. Lab. clin. Med., 56:747-753, 1960.

HUNTER KR, BOAKES AJ, LAURENCE DR, and STERN GM: Monoamine oxidase inhibitors and L-dopa.
Brit. med. J., 3:388, 1970.

IVERSEN LL: The uptake of noradrenaline by the isolated perfused rat heart.
Brit. J. Pharmacol., 21:523-537, 1963.

IVERSEN LL: The uptake of biogenic amines.
In: Biogenic Amines and Physiological Membranes in Drug Therapy, Pt. B. Eds. Biel JH, and Abood LG. Dekker (NY) 1971, pp. 259-327.

IVERSEN LL, GLOWINSKI J, and AXELROD J: The physiologic disposition and metabolism of norepinephrine in immunosympathectomised animals.
J. Pharmacol. exp. Ther., 151:273-284, 1966.

JARROTT B: Occurrence and properties of catechol-O-methyl transferase in adrenergic neurons.
J. Neurochem., 18:17-27, 1971.

JARROTT B, and IVERSEN LL: Noradrenaline metabolizing enzymes in normal and sympathetically denervated vas deferens.
J. Neurochem., 18:1-6, 1971.

JELLETT LB, and DE LA LANDE IS: The effect of nialamide on the constrictor response to noradrenaline in the isolated rabbit ear artery.
Aust. J. exp. Biol. med. Sci., 47:P15, 1969.

JONSSON G, and SACHS C: Synthesis of noradrenaline from 3,4-dihydroxyphenylalanine (DOPA) and dopamine in adrenergic nerves of mouse atrium - effect of reserpine, monoamine oxidase and tyrosine hydroxylase inhibition.
Acta physiol. scand., 80:307-322, 1970.

KALSNER S, and NICKERSON M: Disposition of norepinephrine and epinephrine in vascular tissue, determined by the technique of oil immersion.
J. Pharmacol. exp. Ther., 165:152-165, 1969.

KEATINGE WR: Mechanism of adrenergic stimulation of mammalian arteries and its failure at low temperatures.
J. Physiol. (Lond.), 174:184-205, 1964.

KIRPEKAR SM, PRAT JC, and YAMAMOTO H: Dependence of norepinephrine (NE) release from spleen on metabolic energy.
Fed. Proc., 28:415, 1969.

KOHLI JD: A comparative study of dopamine and noradrenaline on the rabbit aorta.
Canad. J. Physiol. Pharmacol., 47:171-174, 1969.

LANGER SZ, and TRENDELENBURG U: The effect of a saturable uptake mechanism on the slopes of dose response curves for sympathomimetic amines and on the shifts in dose response curves produced by a competitive antagonist.
J. Pharmacol. exp. Ther., 167:117-142, 1969.

LINDMAR R, and LOFFELHOLZ K: Differential effects of hypothermia on neuronal efflux, release and uptake of noradrenaline.
Naunyn-Schmiedebergs Arch. Pharmak., 274:410-414, 1972.

LIU PL, KRENIS LJ, and NGAI SH: The effect of levodopa on the norepinephrine stores in rat heart.
Anaesthesiology, 34:4-8, 1971.

LUCHELLI-FORTIS MA, and LANGER SZ: Reserpine-induced depletion of the norepinephrine stores: is it a reliable criteria for the classification of the mechanism of action of sympathomimetic amines?
J. Pharmacol. exp. Ther., 188:640-653, 1974.

McCLELLAND RJ, McGRANN S, and WALLACE WFM: Evidence that the responses of the perfused rabbit ear artery are influenced by the environmental conditions of the animal.
J. Physiol. (Lond.), 205:5P, 1969.

McDONALD RH Jr, and GOLDBERG LI: Analysis of the cardiovascular effects of dopamine in the dog.
J. Pharmacol. exp. Ther., 140:60-66, 1963.

McDONALD RH Jr, GOLDBERG LI, McNAY JL, and TUTTLE EP Jr: Augmentation of sodium excretion and blood flow by dopamine in man.
Clin. Res., II:248, 1963.

McDONALD RH Jr, GOLDBERG LI, McNAY JL, and TUTTLE EP Jr: Effect of dopamine in man: Augmentation of sodium excretion, glomerular filtration rate and renal plasma flow.
J. clin. Invest., 43:1116-1124, 1964.

McNAY JL, and GOLDBERG LI: Comparison of the effects of dopamine, isoproterenol, norepinephrine and bradykinin on canine renal and femoral blood flow.
J. Pharmacol. exp. Ther., 151:23-31, 1966.

McNAY JL, McDONALD RH Jr, and GOLDBERG LI: Comparative effects of dopamine on renal and femoral blood flows.
Pharmacologist, 5:269, 1963.

McNAY JL, McDONALD RH Jr, and GOLDBERG LI: Direct renal vasodilatation produced by dopamine in the dog.
Circulat. Res., 16:510-517, 1965.

MUSACCHIO JM, GOLDSTEIN M, ANAGNOSTE B, POCH G, and KOPIN IJ: Inhibition of dopamine β-hydroxylase by disulfiram *in vivo*.
J. Pharmacol. exp. Ther., 152:56-61, 1966.

MUSACCHIO JM, KOPIN IJ, and SNYDER S: Effects of disulfiram on tissue norepinephrine content and subcellular distribution of dopamine, tyramine and their β-hydroxylated metabolites.
Life Sci., 3:769-775, 1964.

NEDERGAARD OA, and VAGNE A: Uptake of norepinephrine by rabbit isolated aorta.
Proc. West Pharmacol. Soc., 12:98-102, 1969.

OPPERMANN JA, RYAN CF, and HAAVIK CO: The role of metabolism in temperature-dependent supersensitivity of guinea pig atria to sympathomimetic amines.
Europ. J. Pharmacol., 18:266-270, 1972.

PATON DM: Factors affecting the retention of norepinephrine-^{3}H by the perfused cat heart.
Fed. Proc., 25:260, 1966.

PATON DM: Evidence for carrier-mediated efflux of noradrenaline from the axoplasm of adrenergic nerves in rabbit atria.
J. Pharm. Pharmacol., 25:265-267, 1973(a).

PATON DM: Mechanism of efflux of noradrenaline from adrenergic nerves in rabbit atria.
Brit. J. Pharmacol., 49:614-627, 1973(b).

PESKAR B, HELLMAN G, and HERRTING G: Kinetik der Augnahme und der Transformation von 7-^{3}H-dopamin im isoliert perfundierten Rattenherzen.
Naunyn-Schmiedebergs Arch. exp. Path. Pharmak., 260: 186-187, 1968.

POLLIN W, CARDON PV, and KETY SS: Effects of amino acid feedings in schizophrenic patients treated with iproniazid.
Science, 133:104-105, 1961.

ROBERTS M: A note on the use of cellulose phosphate cation-exchange paper for the separation of catecholamines, and some other biogenic amines.
J. Pharm. Pharmacol., 14:746-749, 1962.

RUBENSON A: The action of dopamine on arterial blood pressure in the rat.
Acta Pharmacol. Toxicol., 29:135-144, 1971.

SCHUELKE DM, MARK AL, SCHMID PG, and ECKSTEIN JW: Coronary vasodilatation produced by dopamine after adrenergic blockade.
J. Pharmacol. exp. Ther., 176:320-327, 1971.

SMITH CB: The role of monoaminoxidase in the intraneuronal metabolism of norepinephrine released by indirectly-acting sympathomimetic amines or by adrenergic nerve stimulation.
J. Pharmacol. exp. Ther., 151:207-220, 1966.

SMITH DJ: Constriction of isolated arteries and their vasa vasorum produced by low temperatures.
Amer. J. Physiol., 171:528-537, 1952.

SPECTOR S, GORDON R, SJOERDSMA A, and UDENFRIEND S: End product inhibition of tyrosine hydroxylase as a possible mechanism for regulation of norepinephrine synthesis.
Molec. Pharmacol., 3:549-555, 1967.

SPIERS ASD, and CALNE DB: Action of dopamine on the human iris.
Brit. med. J., 4:333-335, 1969.

STJARNE L: Studies of catecholamine uptake, storage and release mechanisms.
Acta physiol. scand., 62: Supp. 228, 1964.

STJARNE L: Studies of noradrenaline biosynthesis in nerve tissue.
Acta physiol. scand., 67:441-454, 1966.

STROMBLAD BLR: Effect of denervation and of cocaine on the action of sympathomimetic amines.
Brit. J. Pharmacol., 15:328-332, 1960.

TAINTER ML: Comparative actions of sympathomimetic compounds: the influence of cocaine and certain related compounds upon the actions of a group of sympathomimetic amines.
Quart. J. Pharm., 3:584-598, 1930.

TAXI J, and DROZ B: Radioautographic study of the accumulation of some biogenic amines in the autonomic nervous system.
Int. Soc. Cell. Biol. Symp., 8:175-190, 1969.

TODA N, and GOLDBERG LI: Dopamine induced relaxation of isolated arterial strips.
J. Pharm. Pharmacol., 25:587-589, 1973.

TRENDELENBURG U: Supersensitivity of isolated nictitating membrane of the cat to sympathomimetic amines after impairment of the intraneuronal mechanisms of inactivation.
Naunyn-Schmiedebergs Arch. Pharmak., 271:29-58, 1971.

TRENDELENBURG U: Classification of sympathomimetic amines.
In: Handbook of Experimental Pharmacology, Vol. 33: Catecholamines. Eds. Blaschko H, and Muscholl E. Springer Verlag, Berlin, 1972, pp. 336-362.

TRENDELENBURG U: The relaxation of rabbit aortic strips after a preceding exposure to sympathomimetic amines.
Naunyn-Schmiedebergs Arch. Pharmak., 281:13-46, 1974.

TRENDELENBURG U, HOHN D, GRAEFE KH, and PLUCHINO S: The influence of block of catechol-O-methyl transferase in the sensitivity of isolated organs to catecholamines.
Naunyn-Schmiedebergs Arch. Pharmak., 271:59-92, 1971.

TSAI TH: Time course of contraction of the isolated nictitating membrane of the cat in response to l-norepinephrine.
Fed. Proc., 27:709, 1968.

TSAI TH, LANGER SZ, and TRENDELENBURG U: Effects of dopamine and α-methyldopamine in smooth muscle and on the cardiac pacemaker.
J. Pharmacol. exp. Ther., 156:310-324.

TSENG LF, and WALASZEK EJ: Blockade of the dopamine depressor response by bulbocapnine.
Fed. Proc., 29:741, 1970.

VAN ORDEN LS III, BENSCH KG, LANGER SZ, and TRENDELENBURG U: Histochemical and fine structural aspects of the onset of denervation supersensitivity in the nictitating membrane of the spinal cat.
J. Pharmacol. exp. Ther., 157:274-283, 1967.

VANOV S: Effect of pronethalol on inhibitory actions of catecholamines.
J. Pharm. Pharmacol., 15:723-730, 1963.

WATERSON JG, and HUME WR: A simple tissue holder for formaldehyde-vapor induced fluorescence.
J. Appl. Physiol., 34:116, 1973.

WATERSON JG, and SMALE DE: Location of noradrenergic structures in the central artery of the rabbit ear.
Aust. J. exp. Biol. med. Sci., 45:301-308, 1967.

WENNMALM A, HEDQVIST P, and STJARNE L: Effects of hypothermia on norepinephrine release and effector response in isolated perfused cat spleen.
Circulat. Res., 27:39-44, 1970.

WHITSETT TL, HALUSHKA PV, and GOLDBERG LI: Attenuation of postganglionic sympathetic nerve activity by L-dopa.
Circulat. Res., 27:561-570, 1970.

YEH BK, McNAY JL, and GOLDBERG LI: Attenuation of dopamine renal and mesenteric vasodilation by haloperidol evidence for a specific dopamine receptor.
J. Pharmacol. exp. Ther., 168:303-309, 1969.