FACTORS INFLUENCING THE DIFFUSION OF NORADRENALINE ACROSS THE WALL OF AN ARTERY

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SUMMARY

1. The present study was undertaken to define some of the factors which influence the diffusion of noradrenaline (NA) in the wall of the rabbit ear artery. It was prompted by earlier observations in this laboratory that the artery wall constituted a barrier to the unrestricted diffusion of exogenous NA. When the work for this thesis commenced it had been shown that the uptake of NA in the neuronal plexus limited the diffusion of the amine, and it appeared that the diffusion of NA might be restricted in the tunica media as well.

2. The study commenced with a histochemical investigation of the diffusion of exogenous NA. The results confirmed that neuronal uptake of NA occurred in the rabbit ear artery, and indicated that the uptake of NA by the smooth muscle cells and O-methylation of the amine following its uptake by these cells restricted the diffusion of NA from the lumen to the nerve plexus surrounding the tunica media. Isotopic techniques were used mainly to investigate these and other factors influencing the diffusion of NA in the artery wall.

3. The neuronal uptake of NA was found to be the single most important factor limiting the diffusion of transmitter away from its site of release, and in limiting the diffusion of exogenous NA from the adventitial surface of the artery to the lumen. The results of these studies implied that nearly 80% of the released transmitter was taken up by the nerves; approximately two-thirds of it were

retained, and one-third was deaminated by intraneuronal monoamine oxidase (MAO) and sequestered, mainly as 3,4-dihydroxyphenylglycol (DOPEG). O-methylation did not appear to occur intraneuronally.

4. At least 90% of the NA and its metabolites which effluxed from the neuronal plexus diffused through the tunica adventitia and only 10% through the tunica media.

5. Approximately 40% of the NA which diffused through the tunica media was taken up by the smooth muscle cells, where it was O-methylated and sequestered as normetanephrine (NMN). This study did not indicate whether some of the NA which diffused across the tunica media was also taken up by the smooth muscle cells, but effluxed from them unchanged. It was concluded that the retention of NA in these cells did not restrict the diffusion of NA across the artery wall.

6. The main metabolic pathways for NA in the rabbit ear artery were found to be deamination by intraneuronal monoamine oxidase (MAO), and O-methylation by extraneuronal catechol-O-methyl transferase (COMT). COMT did not appear to be present in the nerves, and although MAO is known to exist in the smooth muscle cells extraneuronal MAO did not appear to deaminate NA under the conditions of the present study.

7. At the frequency (5 Hz) most commonly used to stimulate the sympathetic nerves in the experiments reported, modulation of the release of NA by presynaptic alpha receptors appeared to influence the magnitude of the overflow of NA and its metabolites less than the effects of vasoconstriction.

8. As the release studies were complicated by the postulated presynaptic alpha adrenoreceptor inhibition of transmitter release, the diffusion of exogenous NA in the artery wall was examined under conditions which mimicked the release studies. These experiments confirmed that neuronal uptake was the single most important factor influencing the diffusion of NA, and that approximately one-quarter of the NA which was taken up by the nerves was deaminated. The significance of the extraneuronal uptake of NA and of O-methylation were also confirmed in these studies.

9. In ear arteries in which the neuronal uptake of NA and the smooth muscle uptake of NA were inhibited it was apparent that the diffusion of exogenous NA from the adventitial surface to the lumen was restricted by vasoconstriction. This diffusion was enhanced when the post-synaptic alpha receptors were inhibited by phentolamine, or when the concentration of NA applied to the artery did not elicit a marked constrictor response. Thus, it is suggested that vasoconstriction is a further factor limiting the diffusion of NA in the rabbit ear artery.

10. Included in this thesis are studies relating to:

    a. the purity of tritiated NA,

    b. some attempts to visualize, by fluorescent means, the diffusion of endogenous NA following its release by nerve stimulation,

c. the effects of some inhibitors of the smooth muscle uptake and the metabolism of NA on the vasoconstrictor responses to sympathetic nerve stimulation, and on the overflow of NA from the artery following stimulation,

d. the inhibitory actions of cocaine on the neuronal uptake of NA and on the release of transmitter.

DECLARATION

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

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PUBLICATIONS

Part of the material in this thesis has been published in the following journals:

Aust. J. exp. Biol. med. Sci.	52: 193-200	(1974)
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CHAPTER 1

GENERAL INTRODUCTION

FACTORS INFLUENCING THE RELEASE, DIFFUSION AND OVERFLOW OF NORADRENALINE IN THE RABBIT EAR ARTERY

CHAPTER 1

During the course of the twentieth century, man's understanding of the mechanisms of neurohumoral transmission in the sympathetic nervous system has advanced considerably. In the early part of this century physiologists, like Barger and Dale, suggested that chemical substances, such as amines, might be involved in the transmission of excitation from nerve terminals to effector cells. In the middle of the century von Euler proposed that the transmitter involved in the post-ganglionic sympathetic nervous system was predominantly noradrenaline (NA), and later demonstrated that this NA was firmly bound to small subcellular particles or granules in the sympathetic neurones (Euler, 1954; Euler and Hillarp, 1956; Euler, 1958). These storage granules, although present in other regions of the nerves, are concentrated in swellings or nodes in the terminal portion of the axons. Hence, the nodes are sometimes referred to as nerve terminals even though they do not constitute the ends of the axons. In the last two decades many of the mechanisms involved in the formation of the transmitter and in its storage, mode of release, interaction with effector cells and inactivation have been investigated. Several of these mechanisms influence both the quantity and the nature of the transmitter as it diffuses away from its site of release.

It is the purpose of this introduction to outline the major features of the release process, and to summarize some properties of the mechanisms responsible for the termination of the response

of the effector cells. Subsequently, some properties of the rabbit ear artery, which was used almost exclusively in the experiments for this thesis, will be discussed. Finally, the aims of the study undertaken will be enunciated.

1. Release of noradrenaline

Historically, several methods have been used to investigate the release of NA in sympathetically innervated tissues and the factors which terminate the action of the released transmitter. The two most commonly used methods involved either direct assay of the amount of transmitter which overflowed from the tissue concerned, or quantitation of the nature and magnitude of the responses of the effector cells during and following the release of transmitter by nerve stimulation. Although the latter technique does not permit direct quantitation of the amount of NA released, it has been used extensively to study the release mechanisms. Hence, it is known that the responses of sympathetically innervated tissues are maximal at relatively low rates of stimulation, usually between 10 and 20 Hz (Folkow, 1952), and that the rate of change in response is maximal with frequencies between 1 and 10 Hz (Häggendal et al, 1970). By comparing the magnitudes of the responses of rabbit ear arteries to exogenous NA and to NA released by nerve stimulation, Waterson (1973) was able to estimate the concentration of endogenous NA at the receptors on the smooth muscle cells closest to the nerve terminals. Similarly, by comparing the mechanical responses of rabbit ear arteries to nerve stimulation and exogenous NA, Bevan and Waterson (1971) postulated that the contractile process involved two mechanisms, namely direct excitation and myogenic propagation of

excitation (page 1.30). Other pharmacological studies, which have provided considerable information about the fate of NA following its release, will be discussed later in this chapter.

Several reports have appeared in the literature, in which the amounts of endogenous NA which overflowed* from sympathetically innervated tissues in response to nerve stimulation were assayed. In some of these the magnitudes of the responses of the effector cells were also related to the amount of transmitter which overflowed.

In their classical study, Brown and Gillespie (1957) demonstrated that a substance was released from the cat spleen following nerve stimulation which, when injected into a pithed rat, caused its blood pressure to rise. Chromatographic analysis indicated that the substance was probably NA. In these experiments, as the frequency of stimulation increased above 10 Hz the output of NA per stimulus increased to reach a maximum at about 30 Hz. At higher frequencies this output declined.

Vogt (1973) summarized the results of a number of studies in which the amount of endogenous NA released from a variety of phenoxybenzamine-treated tissues was measured. Phenoxybenzamine (PBZ) was used to inhibit the uptake of NA by neuronal and extraneuronal tissues and its subsequent metabolism (page 1.12). Vogt compared the amount of NA released relative to the total tissue content, and

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* *It is important to distinguish between the overflow of NA, that is the amount of transmitter which effluxed from the tissue into the bathing or perfusion medium, and the amount of NA released from the sympathetic nerves. As discussed later (page 1.8), a number of factors are thought to reduce the amount of NA in the extracellular space following its release and, hence, the amount of NA which overflows from a tissue can only represent the amount released when those factors have been eliminated.*

demonstrated that for small isolated tissues the fractions released per impulse were comparable. She suggested that the apparently lower yields of NA from larger organs might be caused by the slower diffusion of the transmitter through the bulky tissues.

As described in Chapter 6, attempts were made in the present study to assay the overflow of endogenous NA from isolated rabbit ear arteries. In most of these experiments the amount of NA which reached the medium bathing the artery was too small to permit detection by biological assay.

Subsequent to the findings that tritiated NA (^{3}HNA) was taken up by sympathetic nerves (page 1.9) and released by nerve stimulation (Hertting and Axelrod, 1961), numerous studies have been reported in which this isotopic technique was used to study the release and overflow of transmitter. In many of these studies the nature of the tritiated material in the overflow was characterized by separation into NA and its various metabolites (for example, Su and Bevan, 1970; Langer, 1970; Tarlov and Langer, 1971).

Häggendal et al (1970) used an isotopic technique to examine transmitter release in the isolated portal vein as a function of stimulation frequency, and found that the amount of NA released per impulse did not change markedly when the nerves were stimulated at frequencies between 2 and 16 Hz. In accord with the earlier findings of Brown and Gillespie (1957), the output of NA per pulse declined at higher frequencies.

Since the work for this thesis commenced, a number of substances including NA, dopamine, some prostaglandins and acetyl choline, have

been shown to inhibit the release of transmitter by mechanisms which are believed to be presynaptic. Of these the interaction of NA with presynaptic alpha adrenoreceptors appears to have the greatest physiological significance (Langer, 1974). This inhibitory feedback mechanism was postulated to explain the actions of alpha adrenoreceptor antagonists on the overflow of transmitter from tissues following nerve stimulation. For example, Farnebo and Hamberger (1971) demonstrated that PBZ enhanced the stimulation-induced overflow of transmitter in isolated rat irides more than another β-haloalkylamine (GD 131) which, like PBZ, inhibited neuronal and extraneuronal uptake of NA but which, unlike PBZ, was only a weak inhibitor of the alpha adrenoreceptors. PBZ and another alpha adrenoreceptor antagonist, phentolamine, increased the overflow of transmitter at concentrations which did not block neuronal and extraneuronal uptake of NA (Starke et al, 1971; Farnebo and Hamberger, 1971) and, furthermore, enhanced the overflow in tissues in which the post-junctional receptors were predominantly of the beta type (Starke et al, 1971; McCulloch et al, 1972).

This inhibitory feedback hypothesis was strengthened by the findings that alpha receptor agonists, including NA, inhibited transmitter release (Starke, 1972; McCulloch et al, 1973, 1974, 1975), and that the effectiveness of PBZ in increasing the overflow of NA during nerve stimulation was almost completely lost when endogenous NA stores were depleted by reserpine (Enero and Langer, 1973).

These findings of Enero and co-workers were compatible with the hypothesis that a threshold concentration was required to trigger the inhibitory mechanism. Also in support of this threshold concept were

the findings of Rand et al (1973) who showed that, during stimulation of guinea pig atria at a frequency of 1 Hz, the efflux of NA per impulse declined with increasing numbers of pulses, so that the efflux following 16 pulses was 14% of the efflux with 1 pulse. This decline was largely prevented by PBZ. However, these workers were unable to demonstrate a similar decline in the efflux per pulse in the rabbit ear artery which suggested that, in this tissue and at a frequency of 1 Hz, the transmitter did not accumulate in sufficient concentration to exert an inhibitory effect on release (Rand et al, 1975).

Dopamine also inhibited the release of transmitter during nerve stimulation in a number of tissues, including the rabbit ear artery, but appeared to act on different presynaptic inhibitory receptors to NA (McCulloch et al, 1973; Langer, 1974). It is not clear yet whether the concept of a dopaminergic inhibitory mechanism has a physiological function in the regulation of endogenous transmitter release.

Several studies have indicated that acetylcholine inhibited the stimulation-induced release of NA in the rabbit heart (Löffelholz and Muscholl, 1969) and in the rabbit ear artery (Rand and Varma, 1970; Hume et al, 1972; Steinsland et al, 1973a). Although it was concluded that this action of acetylcholine was mediated by muscarinic receptors at or near the adrenergic nerve terminals, the influence of this mechanism on the release of transmitter has not be defined.

Hedqvist (1970) proposed that locally formed prostaglandins (PGs) of the E series modulated the release of NA. The evidence for this was firstly, that PGs were produced in tissues following sympathetic nerve stimulation or the application of exogenous NA, and secondly,

that exposure of these tissues to exogenous PGE depressed the release of NA, whereas drugs which inhibited PGE synthesis enhanced the neuronally evoked release of NA. This mechanism, which appeared to be independent of the regulatory mechanism mediated through the presynaptic alpha receptors (Stjärne, 1973, Starke and Montel, 1973; Hedqvist, 1974), produced only a very small increase in transmitter release relative to the increase observed when these presynaptic receptors were inhibited (Starke and Montel, 1973). In fact, Stjärne (1973) found that the PGE-mediated system appeared to be operative only at low frequencies in the guinea pig vas deferens, and de la Lande et al (1975) demonstrated that at least part of the inhibitory action of PGE_2 on the vasoconstriction produced by sympathetic nerve stimulation in the rabbit ear artery was caused by depressing the response to NA. De la Lande and co-workers concluded that, although there was evidence for an active prostaglandin-synthesising system in the ear artery, there was no evidence of a functional role for this system. It seems likely that the putative inhibitory feedback mechanism mediated by endogenous PGs does not have an important function in the modulation of neuro-transmission (Langer, 1974, 1975).

In summary, it has been demonstrated that NA is the principal transmitter at the sympathetic neuro-effector junction in peripheral tissues and that it can be released by nerve stimulation. The amount of endogenous NA which overflows from the sympathetic tissues is usually very small and isotopic techniques have been developed which are generally accepted as being valid indicators of transmitter release (Langer, 1970; Langer and Vogt, 1971). Four mechanisms have

been discussed which play a greater or lesser role in modulating the release of transmitter. Of these, the interaction of NA with the presynaptic alpha adrenoreceptor inhibitory mechanism appears to have greatest physiological significance.

2. Factors influencing the concentration of released noradrenaline

A number of mechanisms are believed to reduce the concentration of NA in the extracellular space following its release from the nerve terminals and, hence, terminate the response of the effector cells. These mechanisms are:

i. uptake into the sympathetic nerve terminals — neuronal uptake,

ii. uptake into the effector cells — extraneuronal uptake,

iii. intracellular metabolism by monoamine oxidase (MAO) and/or catechol-O-methyl transferase (COMT),

iv. diffusion of NA through the surrounding tissues,

v. non-specific binding to extraneuronal structures other than the effector cells.

i. Neuronal uptake

The active transport of NA across the neuronal membrane from the extracellular fluid to the axoplasm of the sympathetic nerves - referred to as neuronal uptake - has been investigated extensively in the past two decades.

According to Iversen (1973) the idea that catecholamines might be bound in tissues was suggested by Burn in 1932, although it was

another thirty years before the significance of the uptake mechanisms was appreciated.

Whitby et al (1961) demonstrated that intravenously administered tritium-labelled noradrenaline (^{3}HNA) was removed from the circulation and accumulated mainly in tissues containing a rich sympathetic innervation. Histochemical and autoradiographic techniques have confirmed that this accumulation occurred in the sympathetic nerves (Gillespie and Kirpekar, 1966; Malmfors, 1965), and that the subcellular distribution of ^{3}HNA closely paralleled that of endogenous NA. That is, the catecholamine was stored in the dense-cored vesicles or granules within the nerves (Wolfe et al, 1962; Potter and Axelrod, 1963).

Iversen (1963, 1965) demonstrated that this neuronal uptake process was saturable and obeyed Michaelis-Menten kinetics. Burgen and Iversen (1965) demonstrated that the high affinity of this uptake for NA was decreased by O-methylation, so that normetanephrine (NMN) had a low affinity for the uptake site. In some tissues at least, the uptake process is stereochemically selective. For example, in rat heart the affinity of the naturally occurring laevo (l) isomer of NA was five times greater than the dextro (d) isomer (Iversen, 1967). In the isolated rabbit ear artery Allen et al (1972) demonstrated that both the l- and d-isomers of NA accumulated following incubation of the tissues in l-^{3}HNA and d-^{14}CNA respectively. However, it was not possible from these experiments to determine whether the affinity of the neuronal uptake system for d-NA and l-NA in the rabbit ear artery resembled that in the rat heart.

Trendelenburg and Draskóczy demonstrated that the net neuronal uptake of NA depended not only on the extracellular concentration of NA, but also on its axoplasmic concentration. The axoplasmic concentration was influenced by the ability of the granular vesicles to store NA and of the enzyme, monamine oxidase (MAO), to deaminate it (Draskóczy and Trendelenburg, 1968, Trendelenburg and Draskóczy, 1970). The evidence for this was derived from experiments in which they perfused isolated rabbit hearts with d-NA or l-NA and noted that equal amounts of the two isomers were removed from the perfusion media when the concentration of NA was low (0.12 µmol l^{-1}), but that significantly more l-NA than d-NA was removed when the concentration was increased to 1.2 µmol l^{-1}. At this higher concentration, inhibition of MAO did not alter the amounts of the two isomers which were removed from the perfusion media, but when the binding of NA to the granular vesicles was impaired, the net removal of l-NA, but not d-NA, was decreased. When MAO and vesicular binding were inhibited simultaneously, the net uptake of both the laevo and dextro isomers was very small. These results can be explained by the evidence of Stjärne and Euler (1965) that the vesicular retention of NA was stereospecific for l-NA, and that of Giachetti and Shore (1966) that the stereospecificity of MAO was not very pronounced. Hence, although at the lower concentration (0.12 µmol l^{-1}) the intraneuronal mechanisms were able to prevent the axoplasmic accumulation of either isomer of NA from reaching a concentration which impaired the net neuronal influx, at the higher concentration, these mechanisms were unable to prevent the accumulation of the dextro isomer and the resultant decrease in the net influx of d-NA.

The importance of vesicular binding to the process of uptake and accumulation of NA in neuronal tissues has been demonstrated by other workers (for example, Iversen, 1965).

In the rabbit ear artery, de la Lande and Jellett (1972) found that inhibition of MAO produced a secondary constrictor response to extraluminal NA (that is, NA applied to the adventitial surface of the artery) and delayed the recovery from this response. This phenomenon was termed "secondary sensitization" by Furchgott and Sanchez Garcia (1968). Pre-treatment of these MAO-inhibited arteries with reserpine, to impair the binding of NA to the granular vesicles, accentuated the secondary sensitization. Such results are in accord with the above evidence of Trendelenburg and co-workers that NA which accumulates in the axoplasm is normally deaminated by MAO or bound in storage granules.

In the studies undertaken for this thesis, cocaine and PBZ were used to inhibit the neuronal uptake of NA. Iversen (1967) reviewed the actions of cocaine in sympathetically innervated tissues and concluded that it acted "fairly selectively on adrenergic transmission". Although Kalsner and Nickerson (1969) and Bevan and Verity (1967) suggested that cocaine had a post-synaptic action in aortic strips, de la Lande et al (1967a and b) demonstrated pharmacologically that the major effect of cocaine on the response of the rabbit ear artery to exogenous NA was the result of inhibition of neuronal uptake. Another action of cocaine, namely its local anaesthetic effect, produced some complications during the work for this thesis and are reported in Chapter 10.

PBZ has been used extensively to inhibit the neuronal uptake of exogenous NA (Iversen and Langer, 1969) and to inhibit re-uptake of NA following its release by sympathetic nerve stimulation (for example, Su and Bevan, 1970; Langer, 1970). However, it is well recognized that PBZ also inhibits the post-synaptic alpha adrenoreceptors, extraneuronal uptake of NA and more recently it has been shown to inhibit the presynaptic inhibitory feedback mechanism (Brown and Gillespie, 1957; Iversen and Langer, 1969; Lightman and Iversen, 1969; Starke et al, 1971). Presumably because of its action on the uptake processes, PBZ also inhibits the metabolism of released transmitter in a variety of tissues (Langer, 1970; Su and Bevan, 1970; Tarlov and Langer, 1971).

Although cocaine is known to inhibit the neuronal uptake of exogenous NA and potentiate the responses of various tissues to exogenous NA and sympathetic nerve stimulation (de la Lande and Waterson, 1967; Nedergaard and Bevan, 1971; Bevan and Verity, 1967), its effect on the stimulation-induced overflow of NA has been variable. Hughes (1972) demonstrated that cocaine increased the amount of NA which overflowed from the vas deferens and portal vein of the rabbit following nerve stimulation, and Langer (1970) found that cocaine increased the efflux of transmitter from the cat nictitating membrane at low frequencies of stimulation (4 Hz) but not at higher frequencies (25 Hz). Farnebo and Malmfors (1971) were unable to demonstrate a greater overflow of transmitter as a result of stimulation in the mouse vas deferens, when neuronal uptake was blocked by desipramine, although the contractile response was potentiated.

The effect of neuronal uptake on the diffusion and overflow of transmitter in the rabbit ear artery will be reported in this thesis.

ii. Extraneuronal uptake

The process of transfer of NA from the extracellular medium onto or into a cell, other than a neurone, is known as extraneuronal uptake. Gillespie (1973) distinguished between this process of transfer and the accumulation of NA in a tissue; accumulation only occurred when the rate of inward transport (uptake) exceeded the rate of loss by metabolism and sequestration.

Anden et al (1963) and later Fischer et al (1965) noted that extraneuronal binding of catecholamines occurred in the salivary gland of the rat after intravenous administration of ^{3}HNA. Subsequently it was shown that a variety of non-neuronal cells accumulated NA, for example, cardiac muscle (Clarke et al, 1969), smooth muscle (Doležel, 1966; Gillespie and Hamilton, 1966), collagen and elastin (Avakian and Gillespie, 1968).

Avakian and Gillespie (1968) described the binding of NA to smooth muscle cells in the rabbit ear artery and found that, although some of the amine accumulated on the membranes of the cells, most was distributed throughout the cytoplasm. As this intracellular NA concentrated preferentially on the nucleus (Gillespie et al, 1970; Burnstock et al, 1971), Gillespie (1973) suggested that at least part of the NA in the smooth muscle cells was bound. Considerable differences in the ability of smooth muscle cells in various organs and species to retain NA were described by Gillespie and Muir (1970).

In studies in which he perfused the isolated rat heart with NA or adrenaline (A), Iversen (1965) noted a dramatic increase in the uptake of the amines at concentrations which had previously been shown to saturate neuronal uptake. Iversen showed that this process, which he termed uptake$_2$, operated at catecholamine concentrations of 5 µmol l^{-1} and above, exhibited a low affinity but high capacity for binding of the amine, was not stereoselective or sensitive to cocaine and was inhibited by the O-methylated metabolites of NA and A, normetanephrine (NMN) and metanephrine (MN). Although Iversen proposed that this second uptake process was mediated neuronally, histochemical studies have since demonstrated that the uptake occurred into extraneuronal tissue, principally into cardiac muscle cells (Clarke et al, 1969). Subsequently, Lightman and Iversen (1969) provided evidence which indicated that extraneuronal uptake occurred at very much lower concentrations than previously described by Iversen and that it might occur at all concentrations. Their findings indicated that the failure of low concentrations of NA to accumulate extraneuronally was the result of extraneuronal metabolism by MAO and COMT and was not related to a threshold phenomenon.

This relationship between the metabolizing enzymes and extraneuronal uptake has been investigated using specific uptake inhibitors. Kalsner (1969a and b) demonstrated that steroids inhibited the extraneuronal uptake of NA and postulated that they exerted their influence by inhibiting extraneuronal COMT. Subsequently, Iversen and Salt (1970) found that, although steroids depressed the extraneuronal formation of metabolites, the accumulation of unchanged

amine decreased to the same extent. As these steroids also prevented NA accumulation, even when metabolism was prevented by inhibition of MAO and COMT, they concluded that the steroids functioned by inhibiting the access of the substrate to the enzymes.

One of these steroids, deoxycorticosterone acetate (DOCA) has been used extensively in this present study to inhibit selectively the uptake of NA by smooth muscle cells.

Avakian and Gillespie (1968) described the binding of NA to collagen, elastin and smooth muscle cells in the rabbit ear artery and suggested that the concentration of amine required for uptake to occur was high (60 μmol l^{-1}). Since then the findings reported in this thesis and elsewhere (de la Lande et al, 1974), and those of Burnstock et al (1971), indicate that the apparent failure of NA to accumulate in smooth muscle cells (at low concentrations) is related to extraneuronal metabolism and not to a threshold phenomenon. Although the affinity of the smooth muscle uptake system for NA is much less than that of the neuronal uptake system, the quantity of amine which can be retained is high because of the amount of tissue available (Gillespie and Towart, 1973).

The movement of NA across the membranes of smooth muscle cells is not unidirectional (Avakian and Gillespie, 1968), however the efflux of the amine following uptake can be inhibited by PBZ and NMN (Gillespie et al, 1970). It was suggested that the efflux of unchanged NA from extraneuronal stores enhanced the concentration of NA in the synaptic cleft and created a shift in NA storage from an extraneuronal to an intraneuronal site (Draskóczy and Trendelenburg,

1970; Trendelenburg, 1972). Trendelenburg (1974) examined the effect of extraneuronal efflux on the relaxation of rabbit aortic strips following exposure to exogenous NA, and found that inhibition of COMT delayed the recovery from the contractile response. He concluded that this effect was the result of prolonged efflux of NA from the extraneuronal stores consequent upon an enhanced accumulation of unchanged NA. This study also indicated that, despite the presence of MAO in the smooth muscle cells, inhibition of MAO did not alter the efflux, and therefore presumably the accumulation, of NA. Trendelenburg suggested that this was due to COMT activity masking the effect of MAO, or alternatively, that NA was a poor substrate for extraneuronal MAO.

Recently evidence has been presented which suggested that, in the cat nictitating membrane, two extraneuronal O-methylating systems existed, only one of which was sensitive to hydrocortisone (Graefe and Trendelenburg, 1974). The hydrocortisone-resistant compartment had a low affinity, but high capacity, for catecholamines, whereas the hydrocortisone sensitive compartment had a high affinity, but low capacity. Whether this also occurs in the rabbit ear artery has not been determined, although Johnson (1975) demonstrated that the artery possessed a readily saturable O-methylating extraneuronal mechanism in addition to the low affinity, high capacity accumulation described by Avakian and Gillespie (1968). De la Lande and Johnson (1972) also found that extraneuronal MAO played an important role in the inactivation of high concentrations of NA, but not low concentrations (Johnson, 1975), following uptake into smooth muscle cells.

The significance of extraneuronal uptake to the inactivation of endogenous NA has been investigated directly by estimating the overflow of NA and its metabolites from tissues following sympathetic nerve stimulation. Hughes (1972) found that inhibition of extraneuronal uptake with corticosterone caused a 1.4-fold increase in the overflow of NA from rabbit vas deferens and that this was increased considerably when neuronal uptake was inhibited as well. Hughes suggested that slightly more than 90% of the transmitter which was released by nerve stimulation was inactivated by these two uptake processes. On the other hand, Bell and Vogt (1971) reported that NMN (which was used to inhibit extraneuronal uptake of NA) did not increase the overflow of endogenous NA from the uterine artery of the guinea pig, in which neuronal uptake had been inhibited, and Farnebo and Hamberger (1971) found that inhibition of extraneuronal uptake in the rat iris did not increase the overflow of tritiated products following field stimulation of irides which had been previously loaded with ^{3}HNA. In fact, at a concentration of 10 µmol l^{-1}, NMN caused a slight decrease in the overflow.

The influence of extraneuronal uptake on the diffusion of NA across the wall of the rabbit ear artery was investigated in this study.

iii. Metabolism

a. Monoamine oxidase

The monoamine oxidases are a group of enzymes which catalyse the oxidative deamination of catecholamines to their corresponding aldehyde derivatives. Subsequently, these aldehydes are either oxidized to their acid derivatives or reduced to the corresponding

glycol. It is believed that this oxidation is catalysed by an aldehyde dehydrogenase (Erwin and Deitrich, 1966) and the reduction by an aldehyde reductase (Tabakoff and Erwin, 1970). The metabolites so formed include:

3,4-dihydroxyphenylglycol (DOPEG), and

3,4-dihydroxymandelic acid (DOMA).

DOPEG and DOMA are substrates for catechol-O-methyl transferase (COMT) and can be O-methylated to form:

4-hydroxy,3-methoxyphenylglycol (MOPEG), and

4-hydroxy,3-methoxymandelic acid (VMA).

Similarly, NMN, the O-methylated metabolite of NA is a substrate for MAO and can be metabolized to MOPEG or VMA. The metabolic pathways for NA are outlined in Figure 1.1.

There is now evidence that different forms of MAO exist in some tissues and that the different forms might be associated with specific cell types. In a number of studies it has been shown that the MAO associated with sympathetic nerves is different to that associated with the extraneuronal tissues, (for example, pineal gland, Goridis and Neff, 1971; rat vas deferens, Jarrott, 1971a; mesenteric artery, Goridis and Neff, 1973). NA was found to be a substrate for neuronal MAO (Type A), but not for extraneuronal (Type B) (Neff et al, 1973).

In the rabbit ear artery, de la Lande et al (1970) demonstrated extraneuronal MAO by histochemical techniques, but could not identify neuronal MAO. However, subsequent studies by de la Lande and Jellett

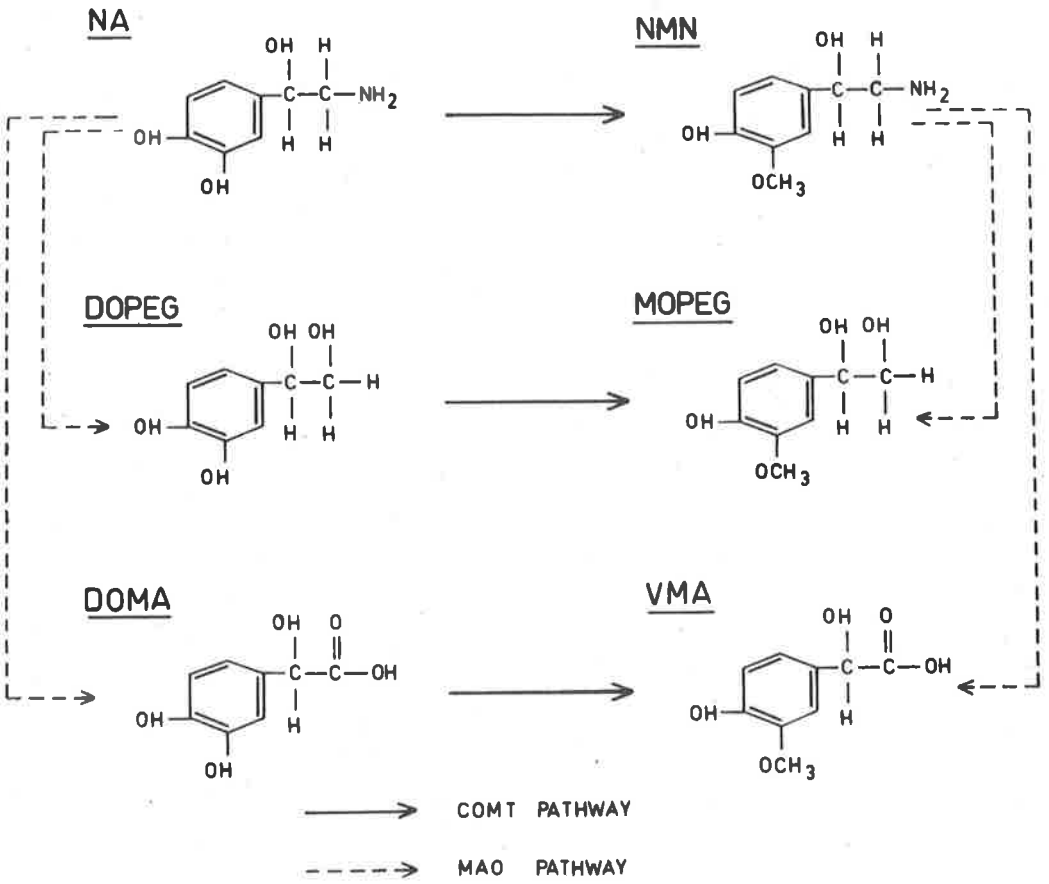

FIGURE 1.1 Metabolic pathways for NA.

The diagram indicates the structure of NA and the structures of its deaminated and O-methylated metabolites. The symbols used represent:

NA - noradrenaline
NMN - normetanephrine
DOPEG - 3,4-dihydroxyphenylglycol
MOPEG - 4-hydroxy,3-methoxyphenylglycol
DOMA - 3,4-dihydroxymandelic acid
VMA - 4-hydroxy,3-methoxymandelic acid.

(1972) indicated that not only was MAO present intraneuronally but that it was physiologically more important that that sited extraneuronally. This evidence was derived from pharmacological studies in which exogenous NA was applied to the adventitial surface of the artery. Inhibition of MAO caused a slow secondary response and a marked delay in recovery from vasoconstriction following washout of the NA. As these changes were prevented by cocaine or chronic denervation, and only occurred when NA was administered extraluminally, it appeared that they were neuronal in origin. Further evidence that MAO was present intraneuronally in the rabbit ear artery was provided by Head et al (1974), who found that the MAO content of these arteries fell by 9% following chronic denervation. The importance of intraneuronal MAO to the neuronal uptake system and to the efflux of NA from the nerves following uptake has been discussed previously (page 1.10).

De la Lande and Johnson (1972) demonstrated that extraneuronal monoamine oxidase played an important role in the inactivation of high concentrations of exogenous NA (118 μmol l^{-1}) in the rabbit ear artery. Whether this finding (and that of de la Lande and Jellett discussed above) indicates the presence of two types of MAO in the ear artery is not clear. It might, in fact, simply reflect the different locations of the same form of MAO; inactivation by the neuronal enzyme being apparent because of the higher concentration of NA normally achieved in the neurone subsequent to uptake (de la Lande, 1975).

In the rabbit aorta, the neuronal tissues (situated in the tunica adventitia) can be separated from the smooth muscle cells

(forming the bulk of the tunica media) and, hence, it is possible to examine uptake and metabolism in nerve-free and nerve-rich tissues. Levin (1974) used this procedure and found that DOPEG was the major metabolite formed in the adventitia during incubation in NA, whereas NMN was the major metabolite arising from the medial tissues. When intact aortic strips were incubated in NA, approximately equal amounts of O-methylated and deaminated metabolites were formed. Levin suggested that the O-methylation which occurred in the adventitial strips was probably extraneuronal in origin and resulted from the uptake of NA into the few fibroblasts and smooth muscle cells which were found attached to the adventitial layer. This suggestion was supported by the finding that the level of O-methylation was not decreased by cocaine.

Recently, Head (1976) investigated the metabolism of exogenous NA in the rabbit ear artery and concluded that there were striking resemblances in the metabolism of NA in this artery and in the rabbit aorta. Head's findings will be discussed further in Chapter 8.

Several workers have examined the effects of metabolism on the overflow of NA from tissues during and prior to sympathetic nerve stimulation. Bell and Vogt (1971) found that inhibition of MAO had no consistent effect on the overflow of endogenous NA in the guinea pig uterine artery; they did not assay the overflow of metabolites. Other studies revealed that the deaminated metabolites of NA were present in the overflow prior to and following sympathetic nerve stimulation in a variety of tissues, and that

inhibition of MAO usually produced a shift in the metabolism from deamination to O-methylation (Langer, 1970; Tarlov and Langer, 1971; Langer et al, 1972).

Although pharmacological evidence pointed to the influence of intraneuronal MAO in the termination of the response of the rabbit ear artery to sympathetic nerve stimulation and exogenous NA (de la Lande and Jellett, 1972), biochemical evidence was absent. While it was not the intention of the present study to investigate this specifically, some evidence accumulated which supported the concept that deamination of NA occurred mainly, if not entirely, within the nerves and O-methylation within the smooth muscle cells.

b. Catechol-O-methyl transferase

Catechol-O-methyl transferase (COMT) is an intracellular enzyme which 3-O-methylates catecholamines in the presence of the methyl donor S-adenosyl methionine (S-AMe). Following its discovery by Axelrod (1957), COMT has been investigated extensively and its properties and functions have been reviewed recently by Guldberg and Marsden (1975).

The influence of COMT on the metabolism of NA is summarized in Figure 1.1. It can be seen that COMT O-methylates NA to NMN and the deaminated catechols, DOPEG and DOMA, to MOPEG and VMA respectively.

The cellular distribution of COMT appears to be both tissue and species dependent, although the lack of an adequate histochemical procedure has so far prevented visualization of the enzyme. Only a small fraction of COMT appears to be firmly bound to membranes

within the cell, however, it is not clear whether the remainder is free in the cytoplasm or loosely bound to membranes. COMT does not appear to be stereochemically specific and possesses similar affinities for the l- and d-isomers of NA (Axelrod and Tomchick, 1958). Although there is evidence that multiple forms of the enzyme might exist, the biological function and significance of this multiplicity is not certain (Guldberg and Marsden, 1975).

Several inhibitors of COMT have been used in biochemical and pharmacological studies and included 3,4-dihydroxy-2-methyl propiophenone (UO521), pyrogallol and tropolone. UO521 has been used exclusively in this present study in inhibit COMT. Although PBZ, cocaine and several steroids are apparently without direct effect on COMT activity (Eisenfeld et al, 1967; Holtz et al, 1960; Hapke and Green, 1970), they possess an indirect effect by preventing access of NA to the cytoplasm of neuronal and extraneuronal cells.

The distribution of COMT in sympathetically innervated tissues has not been completely defined, despite an earlier assumption that it was predominantly extraneuronal. The evidence in support of this assumption included the findings that:

a. COMT activity persisted following chronic denervation of tissues (Verity et al, 1972; Head et al, 1974),

b. O-methylation of exogenous NA was increased, not decreased, by inhibition of neuronal uptake or denervation in the rabbit ear artery (Head et al, 1975),

c. little or no O-methylation occurred in strips of adventitial tissue from the rabbit aorta following incubation in NA, whereas O-methylated metabolites were formed in segments of nerve-free medial tissue (Levin, 1974),

d. O-methylation of NA and its deaminated metabolites was prevented in the isolated, resting cat spleen following inhibition of extraneuronal uptake (Cubeddu et al, 1974).

However, other studies have indicated that in some tissues COMT activity appeared to be intraneuronal as well. Crout and Cooper (1962) demonstrated that COMT activity in the cat heart decreased after chronic denervation, and Jarrott (1971b) showed a similar decline in the cat nictitating membrane and in the vas deferens of the rat and rabbit. The studies of Langer (1970) and Langer et al (1972), in which O-methylation of ^{3}HNA occurred extraneuronally, as well as intraneuronally, in the cat nictitating membrane are in accord with the observations of Jarrott and Langer (1971) that COMT was present in both sites in homogenates of these membranes. In their experiments Langer et al (1972) demonstrated that the NMN which effluxed from the resting nictitating membrane was formed intraneuronally and that which overflowed as a result of stimulation was formed extraneuronally. They also suggested that O-methylation of the deaminated metabolites probably occurred intraneuronally following nerve stimulation.

In a comprehensive study of the metabolism of ^{3}HNA released by nerve stimulation Langer (1970) found that inhibition of COMT had little effect on the magnitude of the overflow of tritium-labelled material in the isolated medial muscle of the cat nictitating

membrane, but eliminated the overflow of NMN in both the resting and stimulated preparation. Concurrently, the overflow of NA and its deaminated catechol metabolites was increased.

iv. Diffusion

In the preceding sections of this introduction it was suggested that the concentration of NA in the synaptic cleft, following its release from sympathetically innervated tissues, was influenced by a number of factors including neuronal and extraneuronal uptake, metabolism subsequent to uptake by either of these processes and the putative inhibitory feedback mechanisms. Similarly, the diffusion of transmitter away from its site of release might influence the concentrations of NA in the synapse and have, in addition, important implications for the release, uptake and metabolic processes. Conversely, these processes are likely to influence the characteristics of the diffusion of NA through a tissue. In fact, it has been suggested that the diffusion of transmitter through the tunica media determined part of the vasoconstrictor response of the rabbit ear artery to exogenous NA and to sympathetic nerve stimulation (Bevan and Waterson, 1971). These workers proposed that the second more slowly developing phase of the constrictor response was the result of penetration of NA through the tunica media, and its contact with smooth muscle cells remote from its site of release (page 1.30).

Gerová et al (1967) attempted to visualize the diffusion of NA in the wall of the dog dorsal pedal artery using a fluorescent technique similar to that described by Falck (1962). They noted that, following sympathetic nerve stimulation of COMT and MAO-inhibited

arteries, the tunica media was infiltrated with fluorescent material which they attributed to NA. These results support the hypothesis that NA released by nerve stimulation diffuses from the nerve endings to smooth muscle layers remote from the nerve terminals. Subsequently, this study was extended to include the rat femoral and rabbit saphenous arteries and the histochemical observations were confirmed by autoradiography. In this thesis, the fluorescent histochemical technique was used to visualize the diffusion of NA across the tunica media following its release, but did not provide a sensitive or reliable indication of diffusion (Chapter 5).

In a number of studies, Bevan and co-workers have investigated several aspects of neuromuscular transmission in vascular tissues. One aspect of these studies related to the concentration of transmitter in and around the synaptic cleft following its release. Bevan and Su (1973) calculated the peak concentration of NA within the synapse (intrasynaptic) and the mean concentration in the extracellular space within the media-adventitia border (extrasynaptic) during sustained nerve stimulation in three vessels of known cleft width, namely, the pulmonary artery, the ear artery and the portal vein of the rabbit. They demonstrated that, as the width of the cleft decreased, the transmitter tended to be confined to the synapse, so that the intrasynaptic concentration increased and the concentration gradient between intra- and extrasynaptic NA was greater. The density and thickness of the neuronal plexus also appeared to restrict the diffusion of NA away from its site of release. Thus, in the rabbit ear artery, although the thickness of the plexus and the number of nodes per unit of surface area were considerably greater than in the rabbit

aorta, the net release of transmitter was not increased proportionally because of this node crowding effect (Bevan et al, 1972).

Bevan and Su (1973) proposed that once the NA emerged from the confines of the synaptic cleft, its concentration was influenced by the diffusion coefficient of the tunica involved. In the rabbit aorta, where the diffusion coefficient in the tunica media (7.3×10^{-7} cm^2 sec^{-1}) was much lower than that in the tunica adventitia (4×10^{-6} cm^2 sec^{-1}), it was suggested that the lower medial coefficient probably reflected a smaller extracellular space and the presence of extracellular diffusion barriers. Török et al (1971) used ^{14}C-inulin to investigate the extracellular space in the rabbit aorta, and found that the inulin space occupied 40% of the media and 60% of the adventitia. The greater coefficient of diffusion in the adventitia partly explained the findings of Su and Bevan (1971) that considerably more NA overflowed from the adventitial than the medial surface of the rabbit pulmonary artery following nerve stimulation.

Other studies by Bevan and co-workers indicated that the diffusion of NA across the wall of elastic arteries was not uniform. In one investigation, Bevan and Osher (1970) examined the distribution of tritium-labelled material in the wall of the rabbit aorta following stimulation of the nerves which had been pre-loaded with ^{3}HNA. When compared with paired non-stimulated arteries, the amount of tritiated material associated with the nerve plexus was reduced and the amount of tritium in the media was increased. The distribution of tritiated material in the media was not uniform; the greatest increase occurred

close to the media-adventitia border, and the lowest at the intimal surface. Further evidence that the diffusion of NA across the wall of an artery was not uniform was provided by the studies of Török and Bevan (1971), in which they examined the diffusion characteristics of tritiated material across strips of rabbit aorta following exposure of the adventitial or intimal surface to ^{3}HNA. When the NA was applied to the adventitial surface, they found a rapid and uniform diffusion of tritium-labelled material across the tunica adventitia which was consistent with the large inulin space described previously. As only 50% of this space was saturated after 60 seconds of exposure, they suggested that the large communicating channels were filled rapidly and that the slower saturation of the rest might reflect the saturation of small interstices. In cocaine-treated arteries, tritium-labelled materials were concentrated in the outer layers of the tunica media when the ^{3}HNA was applied to the adventitial surface, and in the inner layers of the media when the NA was applied to the intima.

v. Non-specific binding

The binding of NA to connective tissue elements in the rabbit ear artery was described by Avakian and Gillespie (1968) and Powis (1973). Avakian and Gillespie used a fluorescent technique to examine the binding of NA to collagen and elastin and found that both tissues showed a concentration-dependent binding of NA with a threshold of approximately 60 µmol l^{-1}. The binding of NA to these tissues differed from that in smooth muscle cells, in that the amine was less firmly bound and was PBZ-insensitive.

Powis (1973) investigated the effects of changes in the ionic composition and pH of the incubation medium on the binding of NA to bovine collagen and elastin. As this binding was relatively resistant to changes in the composition and pH of the bathing medium, Powis suggested that it was largely hydrophobic in nature. On the basis of kinetic analyses, it was suggested that both collagen and elastin possessed high affinity-low capacity and low affinity-high capacity binding sites. Binding of NA to these connective tissues was insensitive to PBZ, phentolamine, NMN and cocaine, but was inhibited by oxytetracycline. At a concentration of 100 μmol l^{-1}, oxytetracycline inhibited the binding of l-NA (0.6 μmol l^{-1}) to bovine collagen by 68%. Although collagen showed no specificity in the binding of l- and d-NA (0.6 μmol l^{-1}), in elastin, the binding of l-NA was two-fold greater than d-NA.

The physiological importance of the binding of NA to these connective tissues was investigated by examining the influence of oxytetracycline on the responses of the isolated rabbit ear artery to exogenous NA and sympathetic nerve stimulation. Powis (1973) found that oxytetracycline potentiated the responses of the artery to NA and nerve stimulation. As these effects were not further potentiated by inhibition of the neuronal and smooth muscle uptake systems and were greater than those produced in the presence of neuronal and smooth muscle uptake inhibitors, Powis proposed that the removal of NA by connective tissue binding might be the major factor responsible for terminating the immediate constrictor response of the tissue. In support of this hypothesis, Powis found that MAO and COMT activity

in the rabbit liver was not suppressed by oxytetracycline and that the neuronal and smooth muscle uptake of NA in rabbit ear artery strips was not inhibited by the drug.

3. Structure and properties of the rabbit ear artery

The rabbit ear artery, which was used almost exclusively for the experiments described in this thesis, is a small muscular artery which has been used extensively for *in vitro* and, to a lesser extent, for *in situ* studies. Since de la Lande and Rand (1965) described the use of this artery *in vitro*, it has been used for pharmacological and histological studies, for biological assay (as either a donor or a test artery) and, more recently, for isotopic and biochemical studies.

The structure of the artery has been described and conforms to the morphology of other small arteries in that the intimal lining is surrounded by 6-10 layers of smooth muscle cells, which in turn are enveloped by a dense plexus of sympathetic nerves situated in the inner aspect of the tunica adventitia (Waterson and Smale, 1967; Waterson and de la Lande, 1967). The plexus at the base of the ear artery, and in that portion used for the studies reported in this thesis, comprised the terminal axons of post-ganglionic nerves which arose in the superior cervical ganglion (de la Lande et al, 1967b). Bevan et al (1972) reported that the nerve plexus was 12 microns thick and contained approximately 21×10^7 nodes.

The fluorescence characteristics of the rabbit ear artery were demonstrated by Waterson and Smale (1967), (Figure 1.2). They suggested that the fluorescence adjacent to the lumen was non-specific and corresponded to the autofluorescence of the intima

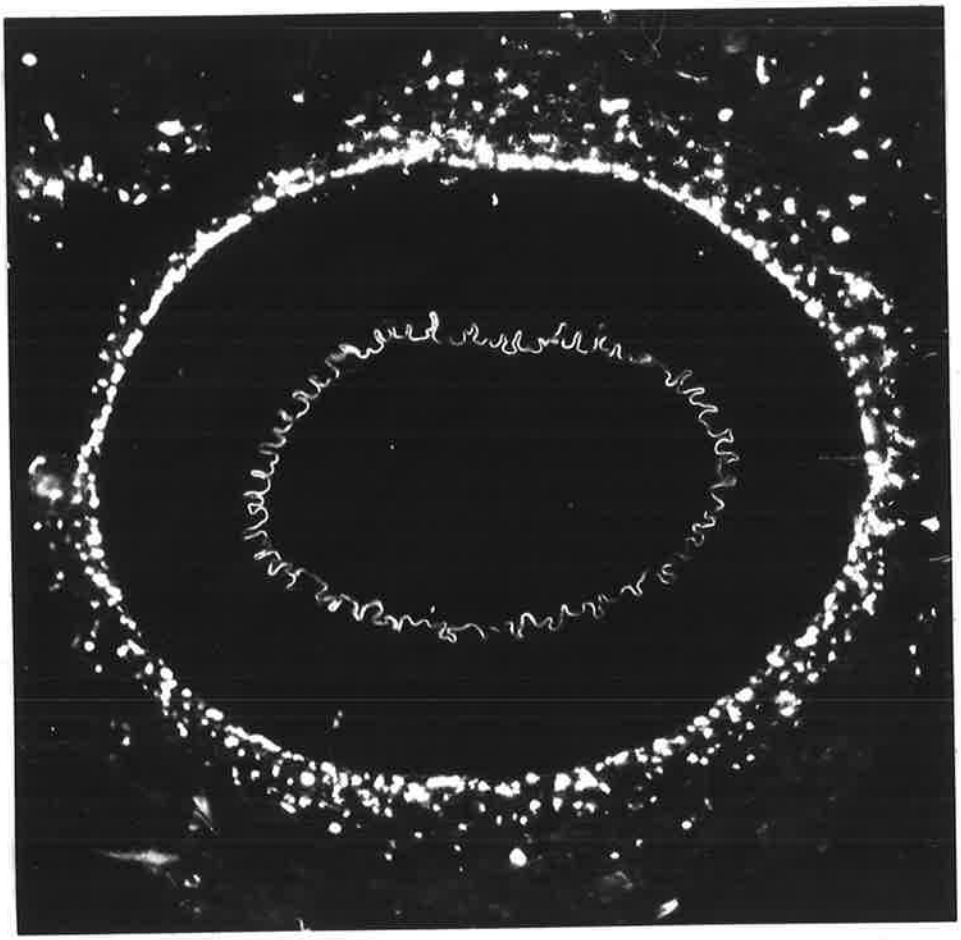

FIGURE 1.2 Transverse section of the rabbit ear artery, treated by the fluorescent histochemical method for demonstration of noradrenergic structures.

The location of the sympathetic nerve terminals at the media-adventitia border is indicated by the dense noradrenergic fluorescence in this region. Non-specific autofluorescence is also seen at the intima.

previously described by Fuxe and Sedvall (1965) and Norberg and Hamberger (1964) in other blood vessels. The intense green fluorescence at the media-adventitia border corresponded to the position of the sympathetic nerve plexus, and provided evidence that the fluorescence was produced by NA containing nodes. Previously, de la Lande and Head (1967) had demonstrated that the predominant, if not the only, catecholamine in the rabbit ear artery was NA. In accord with evidence in many other small arteries, Waterson and Smale (1967) were unable to demonstrate fluorescent structures in the smooth muscle layers of the rabbit ear artery, which suggested that NA was liberated only at the media-adventitia junction. Hence, although the outermost smooth muscle cells came into close contact with the nerves (0.5 microns; Hume, 1973) the inner cells were somewhat removed. It was assumed by Waterson and de la Lande (1967) that excitation of the inner cells resulted either by diffusion of the transmitter across the artery wall or by propagation of excitation from the outer smooth muscle cells. Support for the latter suggestion was provided by the electron microscopic analysis of small arteries by Rhodin (1967), in which he demonstrated that intimate membrane contacts were found between some of the smooth muscle cells. Bevan and Waterson (1971) investigated the biphasic responses of arteries to NA and sympathetic nerve stimulation and inferred that the two phases of constriction were associated with the two mechanisms of excitation described above. Bevan et al (1973) provided further evidence to support the hypothesis that the initial phase of vasoconstriction (Phase A) was caused by excitation of the outer layers of smooth muscle cells and myogenic propogation of this

excitation to deeper layers, and that the second phase (Phase B) resulted from the penetration of NA through the vessel wall. Steinsland et al (1973b) proposed that the fast Phase A constriction depended on the release of calcium from an intracellular pool, and that the slow Phase B depended on the influx of extracellular or membrane-bound calcium. Bevan et al (1973) also provided evidence that was compatible with the concept that two pools of calcium were involved in the biphasic response.

Much of the information about the rabbit ear artery has been derived from pharmacological studies of isolated arteries. De la Lande and Rand (1965) demonstrated that the artery could be readily cannulated and kept in physiological solution in an organ bath for several hours without apparent loss of sensitivity to sympathetic nerve stimulation or to vasoactive drugs. In this preparation solutions were perfused through the lumen, and the responses of the tissue to various procedures were monitored by change in the perfusion pressure. Other techniques have been used to measure the responses of artery segments to various procedures. For example, Kalsner (1972) maintained the perfusion pressure at a constant level and noted the changes in the flow rate through the artery, while Gillespie and Rae (1972) measured the stiffness or compliance of the artery wall.

The constrictor response to NA is mediated by alpha receptors (de la Lande and Rand, 1965) and, as noted previously, the response to sustained applications of NA or to sympathetic nerve stimulation is usually biphasic. Hence, as some procedures which evoke a response in the artery are of short duration it is probable that they reflect only the transient component of the biphasic response (de la Lande, 1975).

Response studies have provided useful information regarding the role of the artery wall in terminating the action of NA. For example, de la Lande et al (1966) found that the ear artery was less sensitive to extraluminal than to intraluminal NA. As cocaine and denervation reduced or abolished this difference in sensitivity, it was proposed that neuronal uptake prevented much of the extraluminally applied NA from reaching the alpha receptors on the smooth muscle cells, whereas intraluminally applied NA had uninhibited diffusion across the tunica media to the nerve terminals (de la Lande et al, 1967a and b) (Figure 1.3). At the time that the work for this thesis commenced, this simplistic model was being questioned as a result of the findings of de la Lande and Jellett (1972). These workers demonstrated that inhibiting intraneuronal MAO caused secondary sensitization to extraluminal NA and delayed the recovery of the artery from vasoconstriction following the withdrawal of the amine (page 1.19). As these effects were not apparent when NA was applied intraluminally, it was suggested that the access of NA to the nerve terminals was limited. The histochemical studies described in Chapter 5 were undertaken to clarify this problem and indicated that the diffusion of NA from the lumen to the nerve terminals was impeded by the smooth muscle cells.

The aim of the project described in this thesis was to examine some of the factors which influence the diffusion of NA across the wall of the rabbit ear artery. The importance of the neuronal and extraneuronal uptake processes, the metabolizing enzymes and vasoconstriction on the overflow of transmitter released by

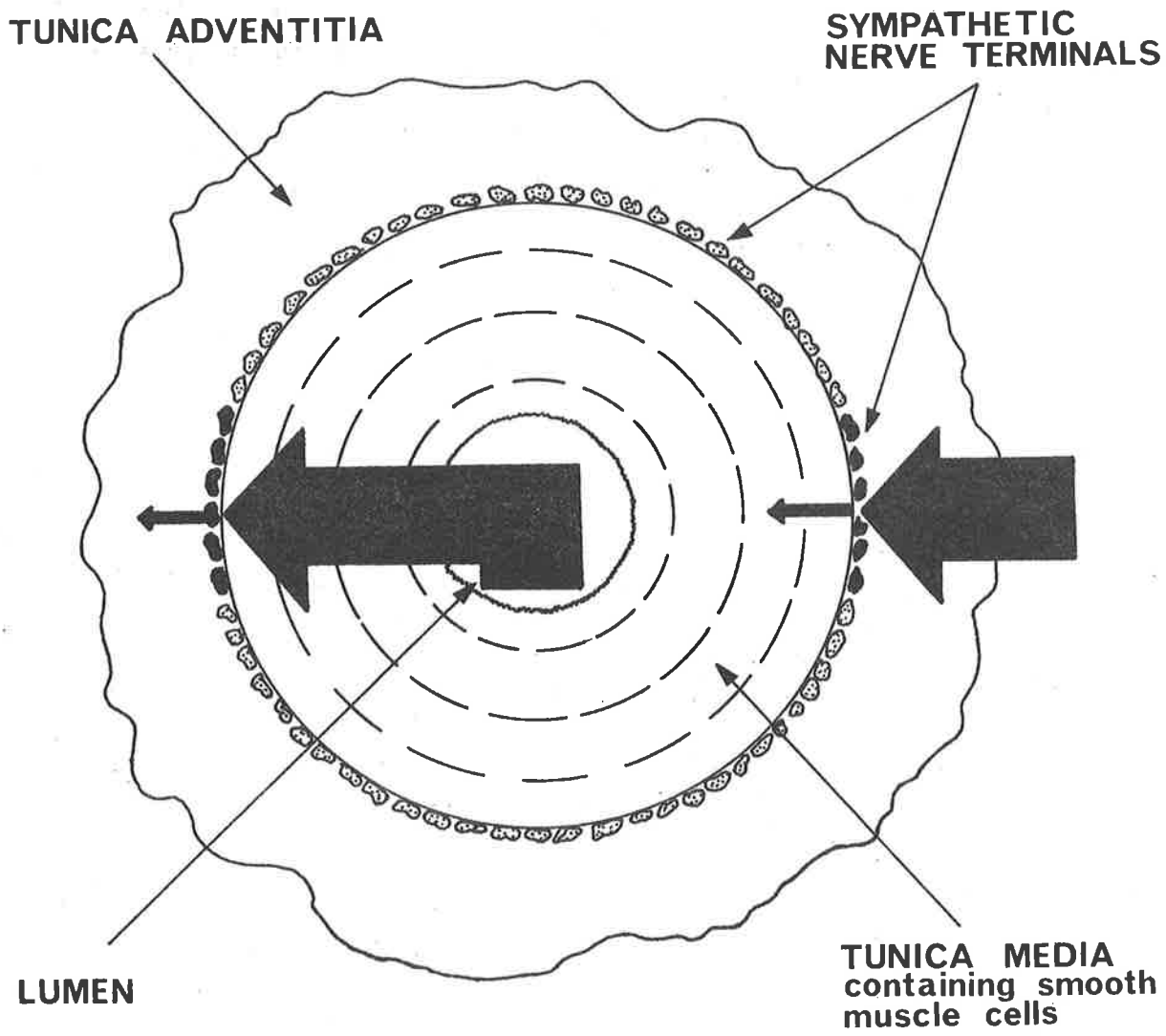

FIGURE 1.3 Diagrammatic representation of the influence of uptake by the sympathetic nerves on the diffusion of NA across the wall of the rabbit ear artery.

The direction of the arrows indicates the direction of diffusion of exogenous NA and the thickness represents the concentration of NA. The model was proposed by de la Lande et al (1967a), and illustrates free penetration of extraluminal and intraluminal NA through the adventitia and media respectively to the nerve terminals.

sympathetic nerve stimulation, and on the diffusion of exogenous NA across the artery wall, have been investigated.

Initially, the diffusion of exogenous NA was studied using a histochemical approach, but as this did not permit quantitation isotopic techniques were developed. The histochemical technique also proved to be unsuccessful when used to visualize the movement of released transmitter through the artery wall. Similarly, the overflow of NA following sympathetic nerve stimulation could not be detected by biological assay and, hence, more sensitive isotopic techniques were used in most experiments.

CHAPTER 2

GENERAL METHODS: NON-ISOTOPIC

CHAPTER 2

Two methodology chapters are included in this thesis. In this chapter, the commonly used non-isotopic techniques are described, and in the second, those methods in which radio-isotopes were used are outlined. Less commonly used techniques or modifications to the methods described here are indicated in the relevant chapters under the heading "Methods".

A list of the drugs used in this study and their method of preparation is included in Appendix 2.

1. Preparation of arteries for *in vitro* experimentation

Semi-lop-eared rabbits, bred in the Central Animal House of the University of Adelaide, were used almost exclusively in this study. For one series of experiments (Chapter 10), it was necessary to use short-eared rabbits, bred in the Animal House of the Institute of Medical and Veterinary Science, Adelaide because of a severe shortage of the semi-lop variety. Animals of both sexes, ranging in weight from 1.5 - 2.5 kg, were used.

Unless otherwise stated, rabbits were stunned by a blow to the cervical vertebrae and bled from the right carotid artery. One ear was moistened with Krebs' solution (see Appendix 2), and the cartilaginous notch at the base of the superior surface of the ear was palpated. The skin was removed from the area over the central ear artery, from below the cartilaginous notch to above the

bifurcation of the central vein, and the artery was exposed by careful excision of fascial layers, adipose tissue, muscle bundles and excess supporting connective tissue (Figure 2.1). The preparation was kept moist with Krebs' solution which had been gassed with a mixture of 95% O_2:5% CO_2 and warmed to 37°C.

A cannula, drawn from No. 1 Sterivac tubing, was inserted into the proximal end of the exposed artery and ligated. Usually a second cannula with a slightly finer tip was inserted and tied into the distal portion of the artery, approximately 3 cm above the first tie (for some experiments No. 2 Sterivac tubing was used). The distance between the two ties was measured before the artery was dissected from the ear. The paired artery, that is the artery from the opposite ear, was cannulated and removed in a similar manner.

These arteries were placed in organ baths or in superfusion holders and were equilibrated for a minimum of 60 minutes prior to experimentation.

Organ bath

Those arteries which were placed in small organ baths (approximately 1.5 cm^3 capacity) were held at their *in situ* length with their distal ends towards the bases of the organ baths as shown in Figure 2.2. The arteries were bathed in Krebs' solution (37 ± 0.5°C), gassed with a 95% O_2:5% CO_2 mixture and perfused with Krebs' solution at a rate of 4.5 ± 0.5 cm^3 min^{-1}. The perfusion system, as shown in Figure 2.3, was similar to that described by de la Lande et al (1966). Note that the artery was clamped at a fixed length

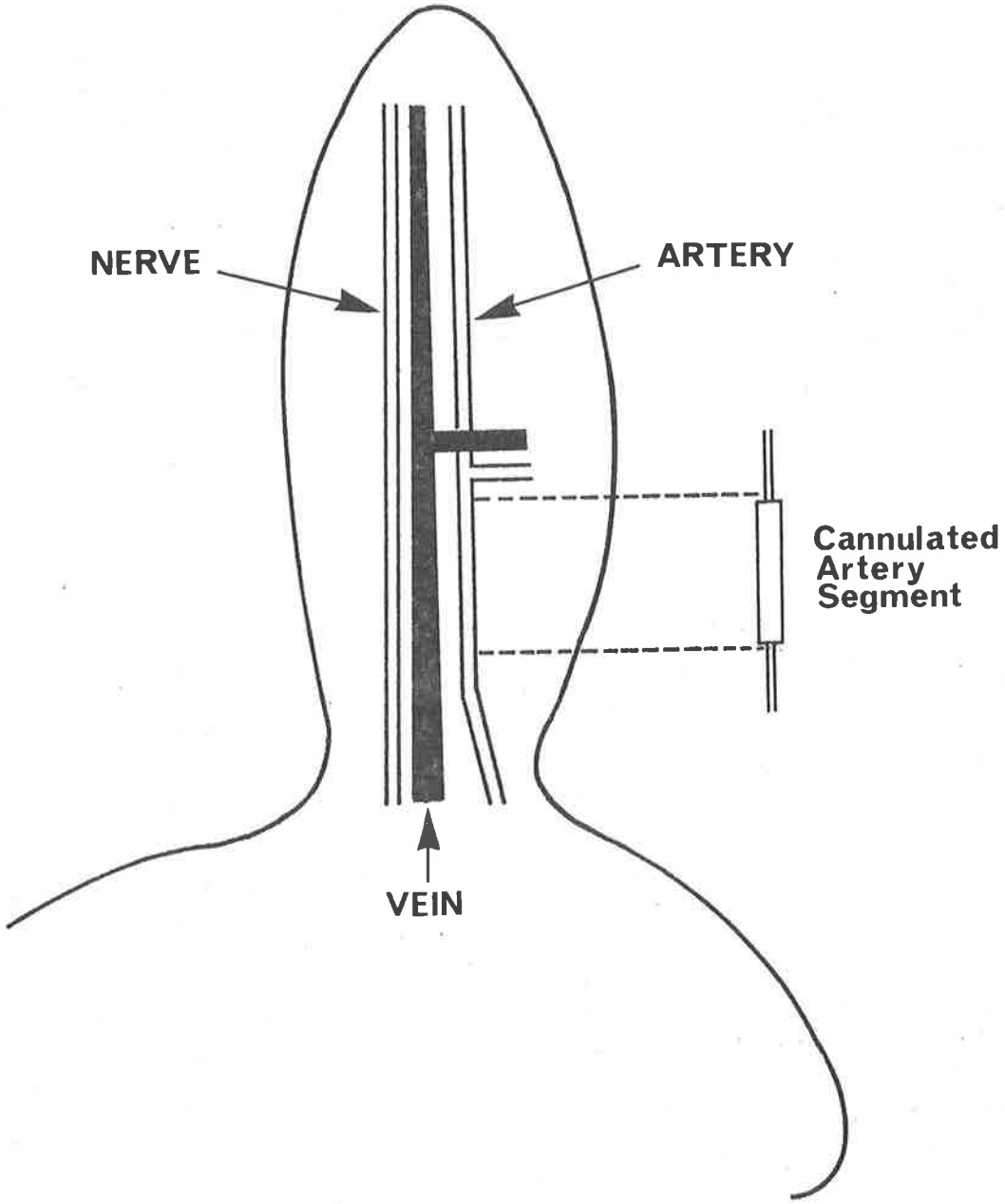

FIGURE 2.1 Diagram of the rabbit ear showing the great auricular nerve, the central vein and the central artery. The diagram represents the left ear held erect and viewed from the right side of the animal. The section of the artery which was cannulated for use in the experiments in this study is indicated.

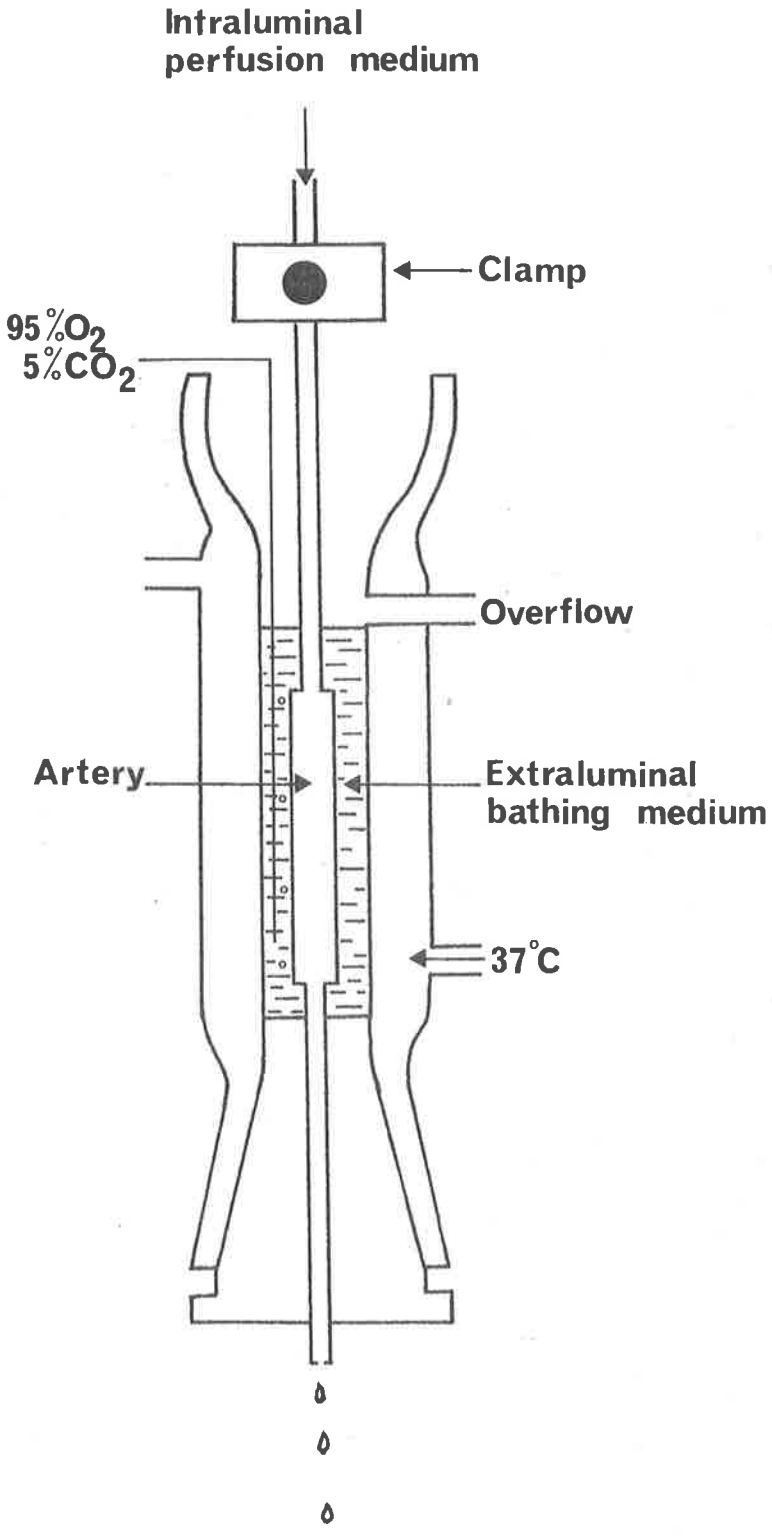

FIGURE 2.2 Diagram of a small, double-jacketed organ bath (1.5 cm^3) in which the cannulated ear artery segment was held at its *in situ* length.

The artery was perfused intraluminally, and bathed extraluminally, with Krebs' solution. The organ bath and perfusion medium were maintained at 37°C, and the Krebs' solution was gassed with 95% O_2:5% CO_2.

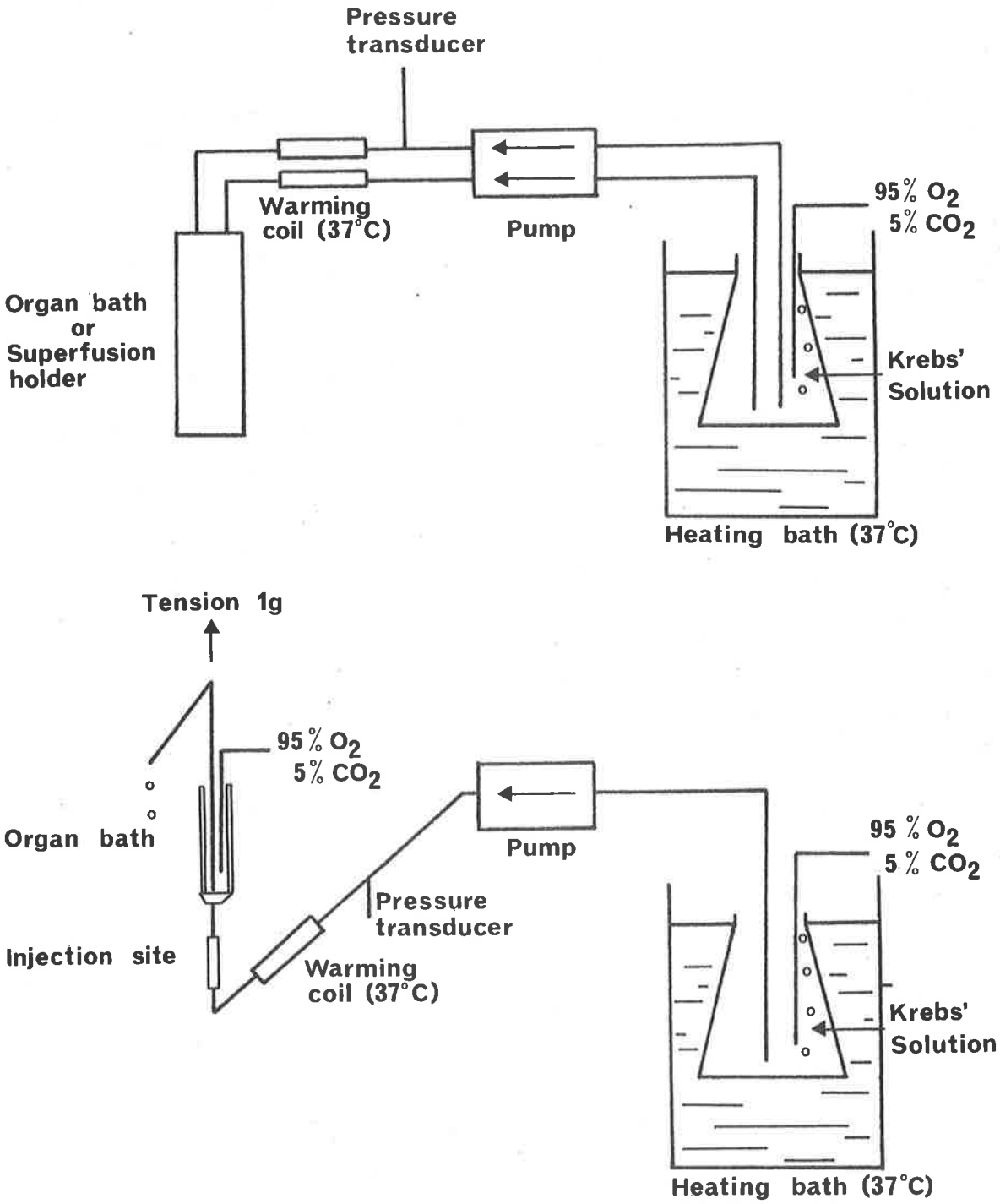

FIGURE 2.3 Diagrams of the apparatus used to perfuse the isolated rabbit ear artery. The perfusion system in the upper diagram was used in conjunction with a small organ bath or a superfusion holder (Figures 2.2 and 2.4). When the small organ bath was used only one perfusion line was required.

The perfusion system in the lower diagram was used in conjunction with a large organ bath, in which a double-cannulated artery, held at a tension of 1 g, was perfused from below.

and not held at 1 g tension, and was perfused from above to facilitate collection of the perfusate. Changes in vascular resistance were monitored on a paper chart recorder* via a pressure transducer.†

In other experiments, single or double-cannulated arteries were placed in larger organ baths (12 - 20 cm^3 capacity) according to the method of de la Lande and Rand (1965) and de la Lande et al (1966) and were perfused from below (Figure 2.3). Those arteries which were double cannulated were held at a constant tension of 1 g and not at their *in situ* length. Pressure changes in the perfusion system were monitored on a smoked kymograph paper by means of a mercury manometer with a floating pointer, or on a paper chart recorder via a pressure transducer as described above. Completed kymograph traces were preserved with shellac.

Superfusion apparatus

In some experiments, the double-cannulated arteries were clamped at their *in situ* length in polystyrene holders between parallel platinum electrodes (Figure 2.4). The outside of each artery was superfused at a flow rate of 3.5 ± 0.5 $cm^3\ min^{-1}$ so that a continuous film of warmed, gassed Krebs' solution existed between the transmural electrodes and the artery. The intraluminal flow rate was 3.5 ± 0.5 $cm^3\ min^{-1}$ and the perfusion

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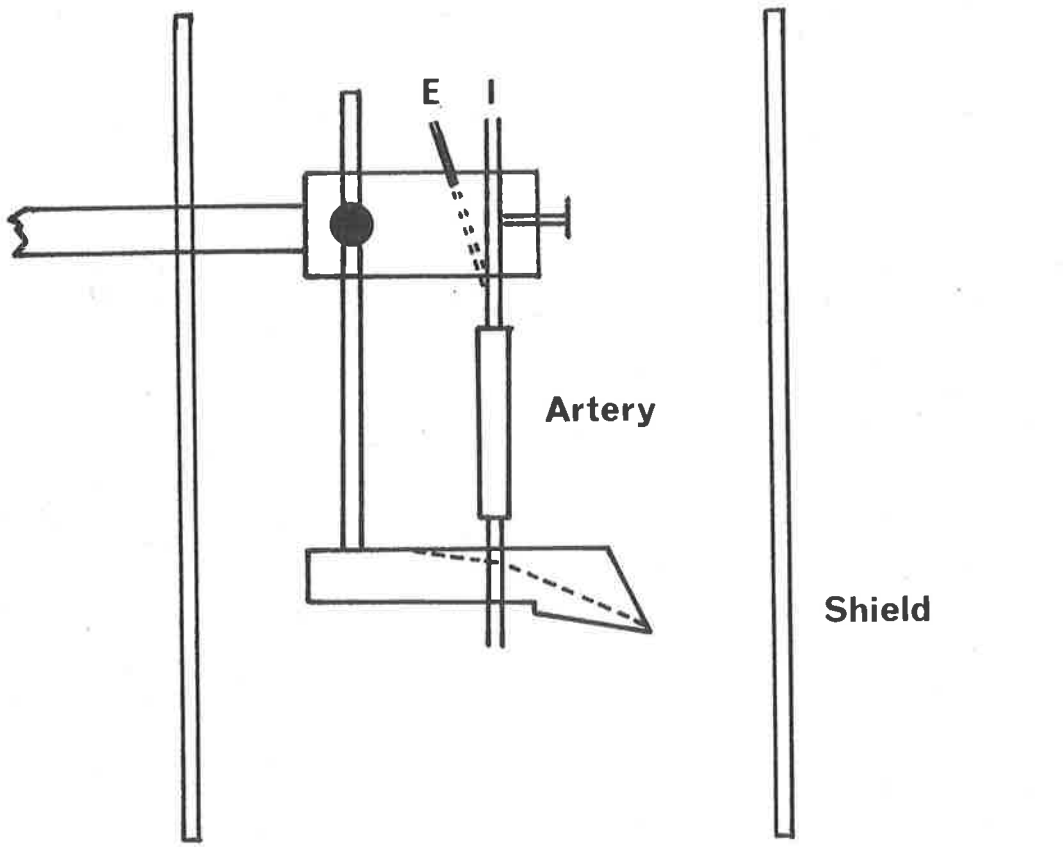
* *B-240 dual pen recorder, Rikadenki Kogyo Company, Tokyo, Japan, or Polygraph Model 5D pen recorder, Grass Instrument Company, Quincy, Mass., U.S.A.*

† *Statham P23 AC, Hato Rey, Puerto Rico.*



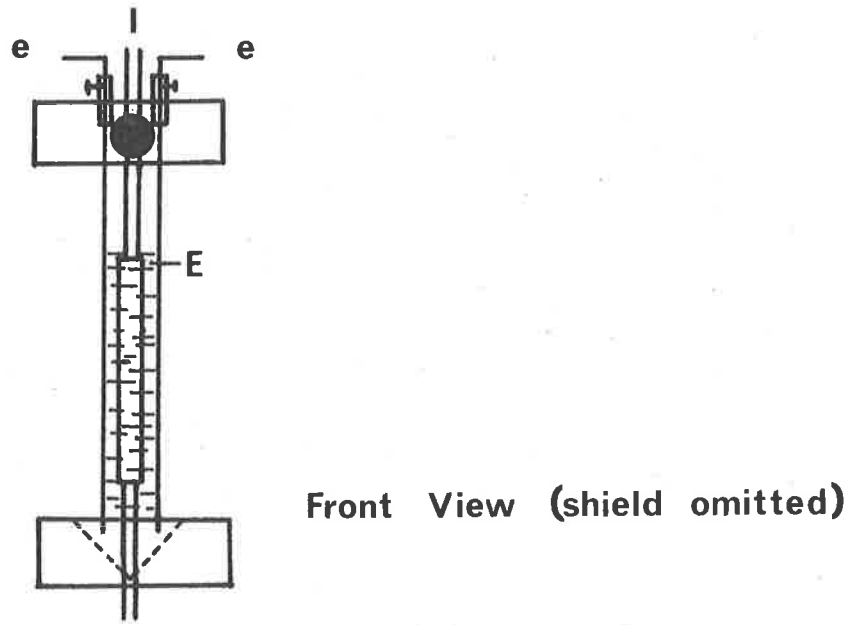


FIGURE 2.4 Diagram of the holder and shield used for the superfusion experiments. The artery, which was held at its *in situ* length, was superfused extraluminally (E) and perfused intraluminally (I) with warmed, gassed Krebs' solution, and was stimulated through parallel platinum electrodes (e) placed as shown in the lower diagram.

pressure was monitored as described above. Superfusion and perfusion fluids could be collected separately.

In the double-cannulated preparations described, drugs could be applied intraluminally (that is, to the luminal surface of the artery) by infusion or injection, or extraluminally (that is, to the adventitial surface) by addition to the bath or perfusate. Details of drug application and modifications to the perfusion rates will be given in the relevant chapters.

Changes in tone in the arteries were recorded as changes in perfusion pressure, so that contraction of smooth muscle cells produced an increased resistance in the vascular segment and a corresponding rise in the perfusion pressure.

2. Stimulation of sympathetic nerves

The sympathetic nerves in the artery segments were stimulated by square wave pulses delivered through 25 gauge platinum wire electrodes from a stimulator.* The platinum electrodes were arranged in various ways as indicated diagrammatically in Figures 2.4 and 2.5.

Field electrodes

All but the terminal 2.5 mm of each platinum electrode was shielded by a polypropylene cover. The electrodes were positioned on either side of the artery with the top electrode near the proximal end of the artery and the second electrode near its distal end.

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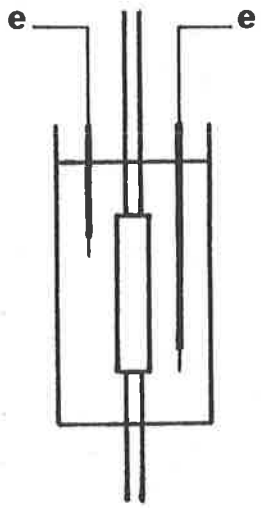
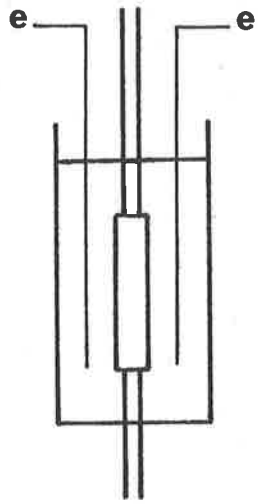
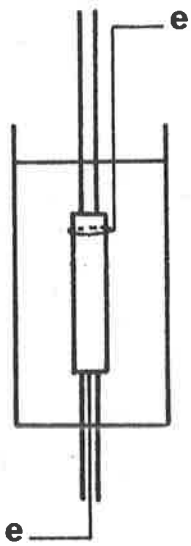
* *Models S4 or S44, Grass Instrument Company, Quincy, Mass., U.S.A.*



Field electrodes



Transmural electrodes (unshielded)



Internal-external electrodes

FIGURE 2.5 Diagrammatic representation of the three electrode arrangements used for stimulating the sympathetic nerves in the ear arteries. Only the inner chamber of each organ bath is represented.

Transmural electrodes

Unshielded, parallel electrodes were placed in the same plane as the artery and approximately 1 mm away from it.

Internal-external electrodes

This arrangement of the electrodes was devised to permit electrical stimulation of arteries bathed in liquid paraffin. As the upper periarterial electrode was contacting the moist artery current flow between this and the second electrode was possible. The latter electrode was inserted into the cannula to a level approximately 1 mm below its tip. Such an arrangement was suited also to those experiments in which the overflow of NA and its metabolites was to be chromatographed, as it minimized the risk of electrolytic destruction of NA and its O-methylated metabolites (see Chapter 7).

For each of the electrode arrangements described, the appropriate stimulation parameters were determined experimentally. To ensure selective stimulation of the maximum number of neurones in the artery wall, a voltage was chosen which produced maximum constriction, and a pulse duration was selected which did not stimulate the smooth muscle cells directly. The voltage required to produce this maximal response in the artery was determined by increasing the applied voltage during repetitive stimulation with low frequency, short duration pulses. When possible, the voltage selected for experimentation was approximately twice that which evoked this maximal response. Using this supramaximal voltage and low frequency pulses, the pulse duration was increased until the constrictor effects of nerve stimulation were

not blocked by tetrodotoxin or guanethidine. The pulse duration selected was below that which evoked direct muscle stimulation.

The frequency of stimulation most commonly used with all electrode types was 5 Hz, as this was within the physiological range and produced a marked constrictor response in the arteries tested.

The most commonly used stimulation parameters were:

Electrode type	Frequency (Hz)	Duration (msec)	Voltage (mV)
Field	5	0.5 - 1.0	80 - 120
Transmural			
Organ Bath	5	0.5	40
Superfusion	5	0.3 - 0.5	12 - 20
Internal-external	5	0.5	70

3. Fluorescent histochemistry

The fluorescent technique used for the demonstration of NA in arteries was based on the modification of the classical Falck technique (Falck, 1962) described by Waterson and Smale (1967).

Artery segments were frozen in an acetone-dry ice mixture and transferred to a chilled aluminium holder in a vacuum flask containing dry ice (Waterson and Hume, 1973). When all segments had been frozen, the holder was placed in a vacuum freeze-drying chamber and maintained at -45 ± 5°C at a pressure of 2 - 6.5 Nm^{-2} for 16-20 hours. Subsequently, the specimens were transferred to a one litre glass jar

containing 5 g of paraformaldehyde powder which had been stored over sulphuric acid (34% V/V) for at least 7 days. The sealed jar was placed in an oven at 80°C for 60 minutes. The formaldehyde-treated tissues were vacuum infiltrated with paraffin wax at a temperature of 60°C for 30 minutes and embedded in paraffin wax.

Tissue sections were cut at 7 microns, mounted on glass slides in an Entellan (Merck) and xylol mixture and examined using a Leitz microscope with a dark field condenser.

Fluorescence was produced by an HBO 200 W mercury vapour lamp using a 1.5 mm Schott BG 12 excitation filter and a 530 nm barrier filter. Photographs were taken using a (Leitz) orthomat camera back with microscope attachment and Kodak photoflure negative film. The film was developed in ID 2 (Ilford) and prints were produced on Ilford Grade 5, single weight, glossy paper (developer: Ilford ID 20).

4. Descending paper chromatography

Chromatography of NA and its metabolites was carried out using strips of Whatman P81 cellulose phosphate ion exchange paper (approximately 3 x 57 cm) according to the method of Roberts (1962). Usually 10 - 20 µl of the sample and 0.1 µmol of pure compound were applied at the origin. The pure compound was added to act as a marker and to minimize variations due to the concentration of salt in the sample. Papers were air dried and equilibrated in an atmosphere of the developing solvent, isopropanol:ammonium acetate ($200\ \mu mol\ l^{-1}$; pH 6.5) (2:1) before being developed for a distance of approximately 46 cm (12 - 16 hours; ambient temperature). Papers were allowed to dry in the air at room temperature before

visualization of the spots in ultraviolet light or after spraying with diazo-p-nitroaniline reagent. A pencil tracing was taken of the chromatogram, and rf values were calculated when required.

Most papers were cut into 1 cm lengths and were agitated at room temperature for a minimum of 16 hours in either:

a. 1 cm^3 of saline (0.16 mol l^{-1}, pH 5.5) containing ascorbic acid (0.6 mmol l^{-1}), or

b. 1 cm^3 of HCl (300 - 500 mmol l^{-1}).

The NA content of the eluates obtained was assayed biologically or fluorometrically (page 2.9).

5. Assay of catecholamines

i. Biological

Bioassay of catecholamines (CAs) was performed using a technique similar to that described by de la Lande and Harvey (1965). A double-cannulated artery was perfused with Krebs' solution containing 5-hydroxytryptamine (5HT) (12 - 85 x 10^{-3} µmol l^{-1}) and cocaine (3 µmol l^{-1}). 5HT and cocaine were also added to the extraluminal Krebs' solution, and increased the sensitivity of the artery to injections of NA. Injections (0.05 - 0.3 cm^3) of the solutions to be assayed were made into the perfusate stream at a uniform distance from the artery (Figure 2.3). The rises in perfusion pressure produced by injection of the unknown solutions were matched against the rises produced by known CA standards.

ii. Fluorometric

The trihydroxyindole (THI) method, first described by Ehrlen in 1948 and later adapted for automatic analysis (Merrills, 1963) was modified by Head (1976) and used in this form to assay the catecholamine (CA) content of various samples. This procedure involved:

a. the automated sampling of the test solution in HCl (500 mmol l^{-1}) or standard solutions of CAs in HCl (500 mmol l^{-1}) at a sample rate of 20 hr^{-1},*

b. neutralization of the solution to pH 5.8 by NaOH (1 mol l^{-1}) and phosphate buffer (1 mol l^{-1}),

c. oxidation of the CA with potassium ferricyanide (300 mmol l^{-1}) buffered with sodium phosphate (200 mmol l^{-1}), and

d. lutine formation with alkaline ascorbate [NaOH (2.5 mol l^{-1}); ascorbic acid (17 mmol l^{-1})].

The lutines were estimated fluorometrically at the following wavelengths:

activation: 395 nm (interference filter)

emission: 495 nm (Wratten No. 8 sharp cut filter).

The output of the fluorometer was monitored continuously on a paper chart recorder.†

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* *A Technicon Model 11 Autoanalyser train (Technicon Australia Pty. Ltd.) was used for this assay. The manifold arrangement is represented diagrammatically in Figure 2.6.*

† *Model B-161, Rikadenki Kogyo Company, Tokyo, Japan.*

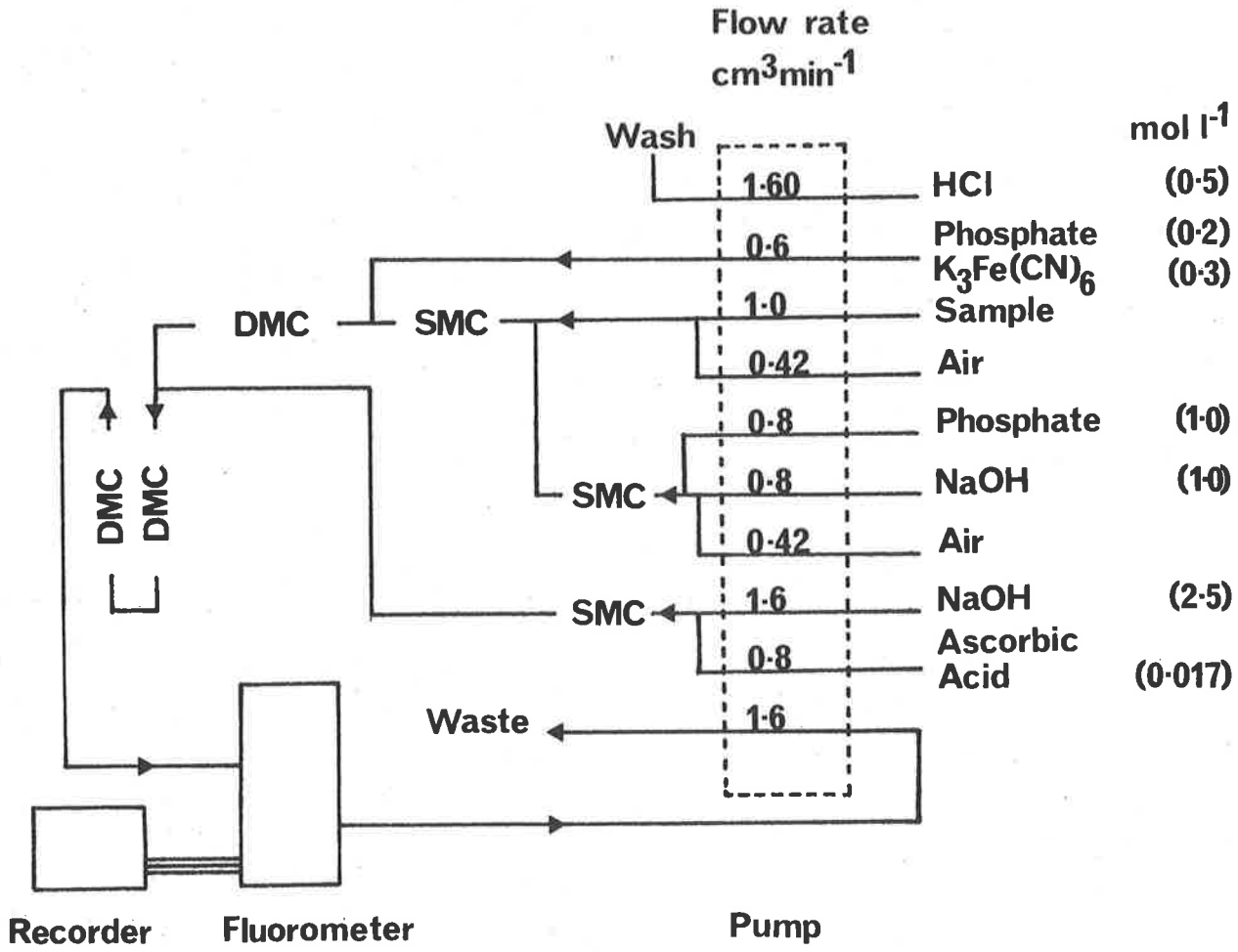

FIGURE 2.6 Flow diagram for the automatic analysis of catecholamines using the Technicon Autoanalyser. The reagents used, their concentrations and flow rates are indicated. Samples in HCl (500 mmol l^{-1}) were processed at a rate of 20 per hour.

SMC and DMC refer to single and double mixing coils respectively.

The CA content of the test solutions was determined by reference to the level of fluorescence produced by the standard solutions following lutine formation.

6. Determination of artery weights

At the conclusion of each experiment, arteries were removed from the organ baths or superfusion holders and severed at the tip of each cannula. The segment obtained was blotted on moist filter paper and weighed, usually in a sealed, disposable tube.

7. Tests of significance

Paired or unpaired students' t-tests were used to indicate the significance of groups of data. Differences between these groups were considered to be significant when the probability was below the 5% level. For convenience, the differences between observations are described in the text as either "significant" (that is, $p < 0.05$) or "not significant" (that is, $p > 0.05$). The tests used and the levels of significance for the data appear in the relevant tables or figures.

CHAPTER 3

THE PURITY OF TRITIATED-NORADRENALINE

CHAPTER 3

In view of the failure of a number of early experiments to produce an overflow of tritium following stimulation of sympathetic nerves which had been pre-loaded with tritium-labelled noradrenaline (^{3}HNA), the question was raised as to whether the incubate medium contained NA or a proportion of impurities. Biological and fluorometric assay revealed that the concentration of NA in these samples of ^{3}HNA was less than that quoted by the manufacturer. Subsequently, all batches of ^{3}HNA were assayed on arrival, usually by the fluorometric method, but occasionally by bioassay as well (see page 2.9). The purity of each sample of NA was calculated as the ratio of the concentration of NA in the sample (determined by assay) relative to the concentration quoted by the manufacturer. This ratio was expressed as a percentage.

As indicated in Table 3.1, the purity of those samples, which arrived as aqueous solutions at ambient temperature in glass vials, ranged between 5% and 68%. This discrepancy was investigated by Head (1976), who showed that:

a. the tritium contents of all samples analysed approximated those quoted by the manufacturer,

b. there was no obvious correlation between the quoted specific activities of either dl-^{3}HNA or l-^{3}HNA and the purity of the solution,

c. when the stock solutions were analysed chromatographically, two tritiated fractions were found in addition to ^{3}HNA;

Sample	Specific Activity* (Ci $mmol^{-1}$)	^3H content* (mCi cm^{-3})	Purity (%)† Fluorometric Assay	Purity (%)† Biological Assay
dl-^{3}HNA				
1	12.0	1.0	12	9
2	7.7	1.0	11	9
3	13.0	1.0	42	36
4	12.0	1.0	15	8
5	6.1	2.0	53	-
6	11.6	1.0	20	-
7	9.6	1.0	55	-
8	12.2	1.0	40	-
l-^{3}HNA				
1	4.1	1.0	5	6
2	8.1	1.0	65	-
3	10.3	1.0	65	-
4	8.8	0.9	68	-
5	8.9	1.0	60	-

* *Values quoted by manufacturer.*

† *Amount of NA in the stock solutions as determined by assay relative to that quoted by the manufacturer and expressed as a percentage.*

TABLE 3.1 Purity of aqueous stock solutions of ^{3}HNA which arrived from the manufacturer in glass vials and at ambient temperatures.

nevertheless, the amount of tritium associated with these two fractions was insufficient to account for the discrepancy in purity,

d. there was evidence of a tritiated impurity which co-chromatographed with NA in paper systems, but which did not exhibit the THI fluorescence characteristics of authentic NA,

e. the impurities formed more slowly in polypropylene than in glass containers.

In an attempt to improve the purity of the NA samples when they arrived in these laboratories, the manufacturer was requested to despatch the isotopes in polypropylene containers, acidified with hydrocholic acid (100 mmol l^{-1}), and either frozen or at ambient temperature. Although delivery under these conditions appeared to increase the purity of the samples on arrival, there was still a substantial discrepancy between the stated concentrations of NA and that determined by fluorometric or biological assay.

As the ^{3}HNA stock solutions contained impurities, it was important to determine whether such impurities might influence the isotopic experiments envisaged in this study. Consequently, it was decided to investigate the possible accumulation of these impurities in sympathetically innervated tissues. Experiments were carried out, in conjunction with R.J. Head, in which the neuronal uptake of NA was studied in rabbit ear arteries and in rat vas deferens. These preparations were treated with nialamide and UO521 to inhibit MAO and COMT, and with DOCA to inhibit the smooth muscle uptake of NA.

METHODS

Semi-lop-eared rabbits and albino Wistar rats were injected with reserpine (1.64 μmol kg^{-1}; IP) 16 hours prior to the removal of the central ear arteries (rabbits) or the vas deferens (rats) (Waterson and Smale, 1967). The effectiveness of the reserpine pre-treatment in depleting endogenous NA in these tissues was assessed by:

a. processing a small segment of the appropriate tissue by the fluorescent histochemical method described previously (page 2.6), or

b. extracting the remaining endogenous NA from tissue segments in hydrochloric acid (page 4.15), and estimating the NA content by fluorometric assay (page 2.9).

Segments of ear artery and vas deferens from non-reserpinized control animals were included for comparison.

The remaining segments from the reserpinized animals were equilibrated in Krebs' solution and then treated with nialamide (340 μmol l^{-1}) for 45 minutes, followed by UO521 (55 μmol l^{-1}) for 15 minutes. In most experiments, DOCA (27 μmol l^{-1}) was added to the UO521 for a further 15 minutes prior to incubation. Subsequently,

a. each artery segment was incubated in 1 cm^3 of Krebs' solution containing UO521 and DOCA (above concentrations), ascorbic acid (300 μmol l^{-1}) and dl-^{3}HNA (0.6 μmol l^{-1})* for 60 minutes; at the end of the incubation period each

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* *For these experiments l-NA was not added to the incubate medium.*

artery was washed in Krebs' solution for 60 seconds, blotted, weighed and the catecholamine content extracted in HCl,

b. four vas deferens were incubated in 10 cm^3 of the dl-^{3}HNA incubate described above. After 45 minutes these tissues were removed and a second group of 4 vas deferens was placed in the incubate for a further 45 minutes. Both groups of vas deferens were washed in Krebs' solution for 60 seconds, blotted and weighed and the catecholamines were extracted in HCl.

Samples of the incubate solutions collected after 0, 45 and 90 minutes of tissue incubation and the acid extracts of the tissues were:

a. chromatographed by descending paper chromatography (page 2.7),

b. assayed for NA content by the fluorometric method (page 2.9),

c. counted for radioactivity in Bray's scintillant (page 4.12).

RESULTS

The effectiveness of the reserpine pre-treatment procedure in depleting the stores of endogenous NA was confirmed histochemically in the arteries by the absence of specific noradrenergic fluorescence, and in the vas deferens by the reduced NA content of the tissues (7.1 nmol g^{-1} cf. 44.4 nmol g^{-1} for non-reserpinized control segments).

1. Rabbit ear artery

Chromatographic separation of the incubate solutions (at 0, 45 and 90 minutes of incubation), indicated that the tritiated material separated into three peaks with approximate rf values of 0.2, 0.5 and 0.8. One of these peaks co-chromatographed with non-labelled NA. Separation of the artery extracts produced a single peak which also co-chromatographed with non-labelled NA.

The chromatographic profiles obtained from a typical experiment are illustrated in Figure 3.1. The specific activity of the ^{3}HNA extracted from these arteries (25.5 Ci mmol^{-1}) was much closer to the specific activity of the stock solution of ^{3}HNA quoted by the manufacturer (12.2 Ci mmol^{-1}) than that determined experimentally (55.5 Ci mmol l^{-1}) (that is, on the basis of the ^{3}HNA content determined by fluorometric assay and the ^{3}H content determined by scintillation spectrometry).

2. Rat vas deferens

The results of the chromatographic analyses were similar to those for the ear artery, in that the tritiated material in the incubate solutions separated into three peaks, one of which co-chromatographed with non-labelled NA (Figure 3.2). However, the tritiated material from the tissue extracts now separated into two distinct peaks, one of which co-chromatographed with authentic NA and the other (rf 0.8) with one of the peaks produced by the incubation solutions. The tritium content of this second peak was equivalent to approximately 5% of the tritium content of the NA peak.

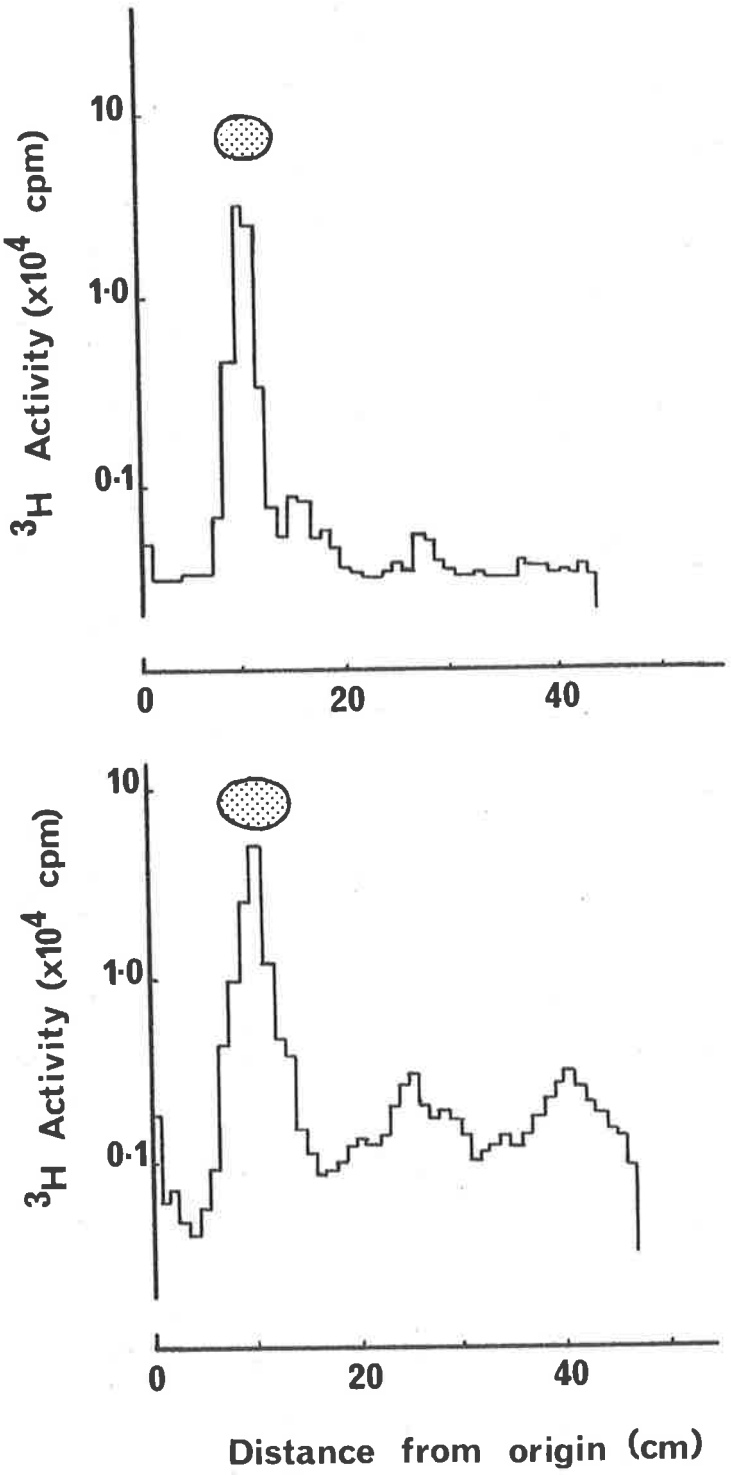

FIGURE 3.1 The distribution of tritium-labelled material on ion-exchange paper chromatograms.

Upper diagram: acid extract from ear artery.

Lower diagram: incubate used for same artery - sampled at T=0

The location of pure NA on the same chromatographic system is represented by the hatched area.

Ordinate: Tritium contents (cpm) of chromatogram eluates.

Abscissa: Distance (cm) from origin of chromatogram.

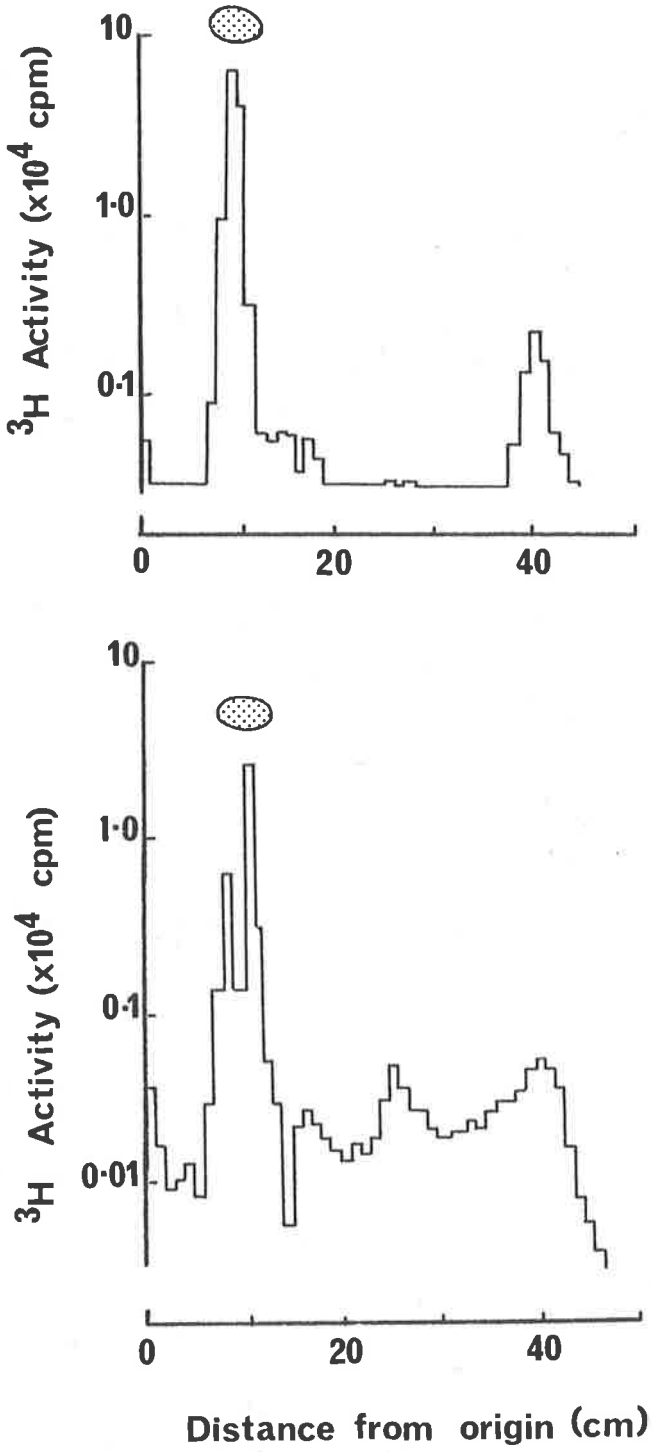

FIGURE 3.2 The distribution of tritium-labelled material on ion-exchange paper chromatograms.

Upper diagram: acid extract from vas deferens

Lower diagram: incubate used for same vas deferens - sampled at T=0.

The location of pure NA on the same chromatographic system is represented by the hatched area.

Ordinates and Abscissae as for Figure 3.1.

The acid extracts from the two groups of vas deferens were pooled and the specific activity was found to be 5.8 Ci $mmol^{-1}$. This figure was less than that quoted by the manufacturer (20 Ci $mmol^{-1}$) and that determined experimentally (86 Ci $mmol^{-1}$).

DISCUSSION

The results of these experiments indicated that little or none of the impurity present in the incubation medium accumulated in either the ear arteries or the vas deferens. The small amount of tritium-labelled material not associated with NA which was detected in the vas deferens, but not in the rabbit ear arteries, might indicate a tissue or species difference, but could also be a manifestation of the larger mass of tissue used (190 mg of vas deferens cf. 7 mg of ear artery).

The possibility that these tissues accumulated the tritiated "impurity" that seemingly co-chromatographed with ^{3}HNA cannot be excluded, although the finding that the specific activity of the ^{3}HNA in the tissue extracts was less than that in the stock solution might indicate that such an impurity was not taken up by neuronal tissues.

Although the data presented here showed that the tritiated impurities present in incubate solutions were probably not retained in the neuronal tissues, it must be remembered that smooth muscle uptake of NA was blocked and the metabolizing enzymes were inhibited. The results of other experiments, in which it was shown that cocaine inhibited neuronal uptake of tritiated material from "impure" incubate solutions, and that, following this incubation period, tritiated-material was not released from these arteries as a result

of sympathetic nerve stimulation (Figure 8.5), provided further evidence that the impurities present in the stock solutions were probably not interfering with the uptake of ^{3}HNA from the incubate.

CHAPTER 4

GENERAL METHODS: ISOTOPIC

CHAPTER 4

Isotopic techniques were used extensively in this study of the factors influencing the release of NA and its diffusion across the artery wall. The purpose of this chapter is to describe these isotopic techniques and to provide some experimental evidence supporting their use.

The radio-isotopes used throughout this study were:

(dl)-noradrenaline-7-^{3}H-hydrochloride (dl-^{3}HNA) and

(l)-noradrenaline-7-^{3}H-acetate (l-^{3}HNA)

and were purchased from:

The Radiochemical Centre, Amersham, United Kingdom.

1. Purity and storage of ^{3}HNA

Experimental evidence, discussed in the preceding chapter, indicated that the concentration of NA in the ^{3}HNA stock solutions was less than the figure quoted by the manufacturer, and that impurities could be detected by paper chromatography. It appeared that these impurities were not taken up by the neuronal stores in the rabbit ear artery, and hence, in most of the experiments which were designed to evaluate the overflow of tritium-labelled material following the release of ^{3}HNA by sympathetic nerve stimulation, the incubation medium was not purified prior to use. However, for the exogenous diffusion studies (Chapter 11) the stock solution was usually purified by Dowex 50 (Na^{+}) chromatography as described

later (page 4.11). Stock solutions of ^{3}HNA were stored at 4°C in polypropylene containers to which HCl was added; 100 mmol l^{-1} before despatch, or 10 mmol l^{-1} on arrival in these laboratories.

Samples which had been purified on Dowex 50 ion-exchange resin were held in HCl (500 mmol l^{-1}) at 4°C until required for use, when the appropriate amount was transferred to a freeze-drying tube, frozen in a mixture of alcohol or acetone and dry-ice and lyopholysed to dryness. The ^{3}HNA was reconstituted in Krebs' solution containing ascorbic acid.

2. Preparation of ^{3}HNA

i. Overflow studies

Incubation solutions were prepared by adding the requisite volume of ^{3}HNA* and non-labelled 1-NA to warmed, gassed Krebs' solution† which contained ascorbic acid (300 µmol l^{-1}).

The concentrations used for these experiments were:

dl-^{3}HNA incubates

dl-^{3}HNA (0.6 µmol l^{-1})
plus
1-NA (0.3 µmol l^{-1})

to provide a final 1-NA concentration of 0.6 µmol l^{-1}

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* *The concentration of ^{3}HNA in the stock solution was determined by fluorometric assay and not from the manufacturer's quoted value.*

† *The phrase "warmed, gassed Krebs' solution" is used to denote Krebs' solution which was warmed to 37°C and gassed with 95% O_2:5% CO_2.*

l-^{3}HNA incubates

l-^{3}HNA (0.3 µmol l^{-1})
plus
l-NA (0.3 µmol l^{-1})

ii. Exogenous diffusion studies

In most of the diffusion experiments, purified stock solutions were lyopholysed and reconstituted in warmed, gassed Krebs' solution containing ascorbic acid (300 µmol l^{-1}). Non-labelled l-NA was added as indicated.

The concentrations used for the diffusion experiments were:

dl-^{3}HNA diffusion

dl-^{3}HNA (0.18 µmol l^{-1})
plus
l-NA (0.09 µmol l^{-1})

l diffusion

l-^{3}HNA (0.09 µmol l^{-1})
plus
l-NA (0.09 µmol l^{-1})

Occasionally other concentrations of NA were used, but they will be referred to in the appropriate chapters.

3. Duration of incubation

In the isotopic overflow studies undertaken in this thesis, the sympathetic nerves in the artery segments were pre-loaded with ^{3}HNA for 60 minutes. This parameter was selected on the basis of experiments undertaken to determine the effect of the duration of

incubation on the neuronal accumulation of ^{3}HNA, and on the magnitude of the overflow of tritium-labelled material from artery segments prior to and as a result of nerve stimulation.

i. Neuronal accumulation

Seven ear artery segments, tied at one end with pieces of cotton, were removed from each rabbit and equilibrated in glass vials containing warmed, gassed Krebs' solution. Subsequently, six of the seven segments were immersed in Krebs' solution which contained dl-^{3}HNA (page 4.2) and ascorbic acid (300 µmol l^{-1}) for periods of 1 to 120 minutes. One segment was placed in NA-free Krebs' solution for 120 minutes. After incubation, each segment was washed in three changes of Krebs' solution for a total of 60 minutes. Arteries were blotted, weighed and digested in solubilizer (page 4.15). 10 cm^{3} of toluene scintillant was added to each vial and the tritium content of the digested arteries was determined by liquid scintillation spectrometry (page 4.12).

As indicated in Figure 4.1, the amounts of tritium retained by these artery segments after incubation in dl-^{3}HNA increased linearly with incubation periods of up to 60 minutes. There was no significant difference in the amount of tritium retained in those arteries which were incubated for 60, 90 or 120 minutes.

Although the ratio of ^{3}HNA to endogenous NA was not analysed in the present experiments, it was shown subsequently that approximately 12% of the total NA in the artery was ^{3}HNA following 60 minutes incubation in dl-^{3}HNA and 60 minutes washout in Krebs' solution.

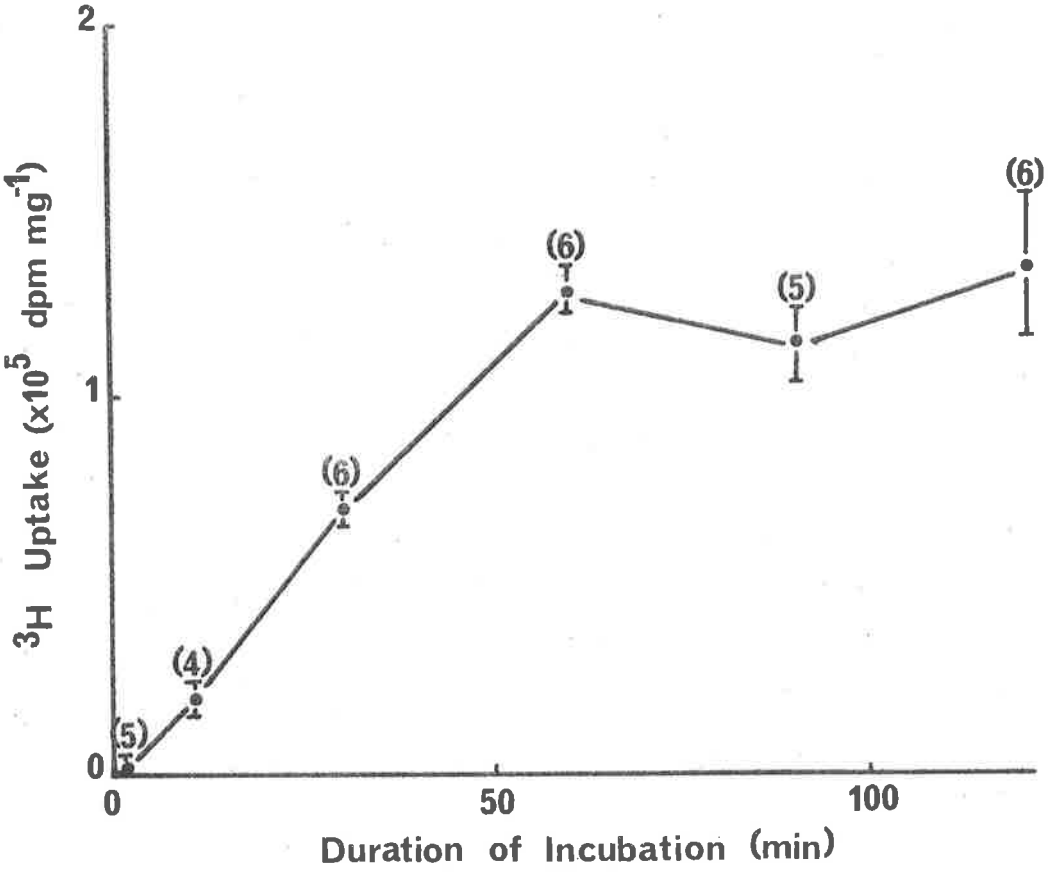

FIGURE 4.1 The effect of the duration of incubation on the mean uptake of ^{3}HNA (±S.E.M.) by rabbit ear artery segments.

Numbers in parentheses refer to the number of artery segments incubated in dl-^{3}HNA (0.9 µmol l^{-1}).

The five arteries which were incubated in ^{3}HNA-free Krebs' solution for 120 min contained negligible amounts of tritium (58 ± 26 dpm mg^{-1}), and are not included in the figure.

Ordinate: mean uptake of ^{3}HNA (dpm mg^{-1})
Abscissa: duration (min) of the incubation period

ii. Overflow studies

Double-cannulated artery segments were placed in small organ baths (page 2.2) and after equilibration were incubated in dl-^{3}HNA (0.9 μmol l^{-1}); one of each pair of arteries was incubated for 30 minutes and the other for 60 minutes. The flow of Krebs' solution through the lumen was stopped during the incubation period and was restarted at the beginning of the washing period. Washing continued for 60 minutes during which the extraluminal bathing medium was replaced every 10 minutes. At the end of this washing period, the extraluminal Krebs' solution was replaced with liquid paraffin and the intraluminal flow rate was reduced to 0.23 cm^{3} min^{-1} (for discussion of the paraffin technique see Chapter 9).

Ten minutes after the application of liquid paraffin, the intraluminal perfusate was collected in vials containing Bray's scintillant (page 4.12) at intervals of 2 or 4 minutes. The sympathetic nerves in each artery were stimulated three times by square wave pulses delivered through the internal-external electrode arrangement (page 2.5). The collection and stimulation times are indicated in Figure 4.2. At the end of the experiment both arteries were weighed, and the tritium content of each aliquot of perfusate collected was determined by liquid scintillation spectrometry (page 4.12).

The mean overflow of tritium-labelled material into the luminal perfusate for each collection period is shown in Figure 4.2. A more detailed discussion of the magnitude and kinetics of this

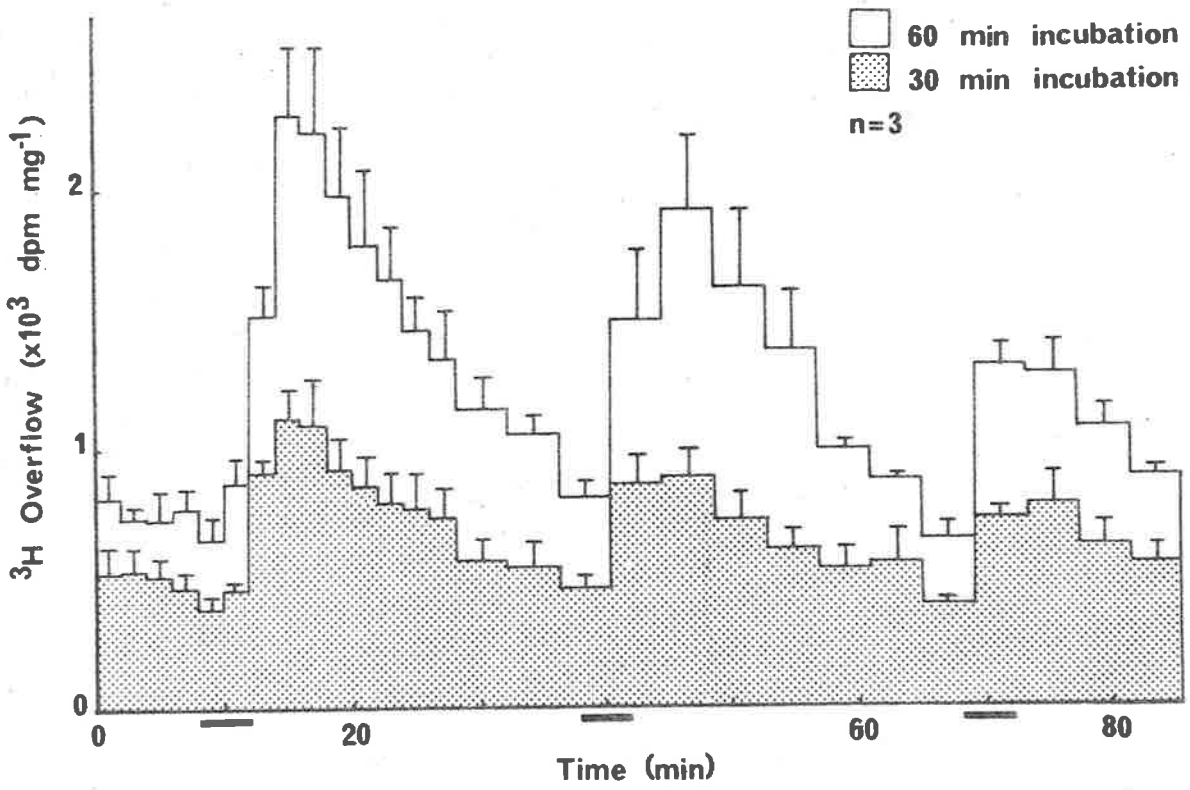

FIGURE 4.2 The intraluminal overflow of tritium from paraffin-bathed arteries incubated in dl-^{3}HNA for 30 or 60 minutes (mean +S.E.M.).

Arteries were stimulated (▬) at a frequency of 5 Hz for 4 minutes.

Ordinate: Tritium overflow per 2 minutes of collection (dpm mg^{-1})
Abscissa: Duration of experiment (min)

overflow will be given in Chapter 9. However, it was apparent from this figure that for the first stimulation period:

a. the prestimulation overflow of tritiated material was greater (50%) from those arteries incubated for 60 minutes than those incubated for 30 minutes,

b. the overflow of tritium increased and reached a peak within 8 minutes of the onset of nerve stimulation and subsequently declined to the prestimulation level,

c. the stimulation-induced overflow (SI-overflow)* was 170% greater in those arteries incubated for 60 minutes than in those incubated for 30 minutes.

During the subsequent two stimulation periods the overflow of tritium was qualitatively similar to that described above, although the magnitudes of the overflows subsequent to the onset of stimulation declined.

iii. Comments on the uptake and overflow studies

It was evident from these studies that the uptake and overflow of tritiated material was greater in those arteries incubated in NA for 60 minutes than in those incubated for 30 minutes. Incubation of the arteries for longer periods than 60 minutes did not further increase the amount of tritiated material retained by the arteries.

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* *Stimulation-induced overflow represents the difference in the overflow of tritium which occurred as a result of nerve stimulation, and the overflow which would have resulted from the resting artery during the same period. This resting overflow was calculated by extrapolation of the prestimulation overflow.*

The present uptake studies are in accord with the findings of Nedergaard and Bevan (1971) who showed that the amount of ^{3}HNA retained in aortic rings increased rapidly for the first 45 minutes of incubation in ^{3}HNA (1.0 µmol l^{-1}) and subsequently remained constant. Nedergaard and Bevan also showed that when the NA concentration was decreased to 10 nmol l^{-1}, the amount of ^{3}H taken up by the aortic rings was greater, but that the accumulation of tritium continued to increase for the duration of the incubation period (180 minutes).

On the basis of the experiments described above it was decided that, for the concentration of NA selected, an incubation time of 60 minutes followed by a washing period of 60 minutes was suitable for the present study.

4. Separation of NA and its metabolites

i. Dowex 50 chromatography

The technique of Taylor and Laverty (1969), as modified by Head (1976), was adapted for use in those experiments in which NA, NMN and the deaminated metabolites (DOPEG, DOMA, MOPEG, VMA) were separated. In this fractionation, the amines, but not the deaminated metabolites, were adsorbed onto a column of cation exchange resin [Dowex 50 (Na^{+})]. The adsorbed NA and NMN were eluted differentially from the column with HCl to complete the separation.

In those experiments in which the tritium overflow was fractionated, a portion of the Krebs' solution containing the tritiated materials was added to standard amounts of NA, NMN and each

of the deaminated metabolites*. Following acidification, the pH was adjusted to approximately 6.4 and the solution was loaded onto a Dowex column (60 mm x 4 mm) by means of a continuous flow pump† at a rate of 0.5 cm^3 min^{-1}. NA and its metabolites were added in order that the recovery of the ^3H compounds could be estimated colourometrically (page 4.10).

Each column was perfused with distilled water and the effluent was collected in two 10 cm^3 fractions; the first fraction contained the deaminated metabolites. The NA fraction was eluted in HCl (500 mmol l^{-1}) and the NMN in HCl (1.5 mol l^{-1}). The usual collection periods were:

Eluent	Fraction Number	Volume (cm^3)	Eluate
Distilled water	1	10	Deaminated metabolites
	2	10	
HCl	3	13	
(500 mmol l^{-1})	4	10	NA
	5	5	
HCl	6	3	
(1.5 mol l^{-1})	7	8	NMN
	8	3	

* *Typical volumes used were:*
Krebs' solution containing ascorbic acid (300 µmol l^{-1}) - 1.5 cm^3
NA, NMN (12 mmol l^{-1}) - 100 µl of each
DOPEG, DOMA, MOPEG, VMA (12 mmol l^{-1}) - 50 µl of each
HCl (100 mmol l^{-1}) - 270 µl
phosphate buffer (200 mmol l^{-1}; pH 6.4) - 450 µl.

† *Technicon peristaltic pump; Technicon Australia Pty. Ltd.*

When the deaminated metabolites were to be further separated by adsorption onto alumina (see below), sodium metabisulphite (630 mmol), EDTA (320 mmol) and glacial acetic acid (100 µl) were added to the collection vials to minimize loss by oxidation.

Usually 1 cm^3 of each of the eluate fractions was transferred to 15 cm^3 of toluene-triton scintillant (Appendix 2) and the tritium activity was determined as described elsewhere (page 4.12). The fractions were assayed by the colourometric procedure (page 4.10). By this means, it was possible to compensate for the loss of tritiated material during the fractionation procedure.

Prior to re-use the Dowex 50 was regenerated to the Na^+ form by perfusing each column with the following solutions for 60 minutes at a rate of 0.5 cm^3 min^{-1}:

NaOH (2 mol l^{-1})

distilled water [containing EDTA (26 mmol l^{-1})]

HCl (2 mol l^{-1})

distilled water (EDTA)

phosphate buffer (200 mmol l^{-1}; pH 6.4).

ii. Batch alumina chromatography

In some experiments the fraction which contained the deaminated metabolites following Dowex 50 chromatography was further fractionated by adsorption of the two catechol metabolites onto Al_2O_3, and their differential elution. The O-methylated metabolites (MOPEG and VMA) were not adsorbed and were removed in the effluent.

The procedure used was as follows:

a. 5 cm^3 of the effluent from the Dowex column were added to a tube containing Al_2O_3 (4 mol), EDTA (3.2 mol) and sodium metabisulphite (525 mmol),

b. the pH was adjusted to 8.4 with Na_2CO_3 (2.0 mol l^{-1}) and maintained at this level for 4 minutes,

c. the effluent (which contained MOPEG and VMA) was removed,

d. the alumina was washed in two volumes (20 cm^3) of distilled water, one volume (5 cm^3) of acetic acid (100 mmol l^{-1}) and two volumes of acetic acid (300 mmol l^{-1}). The first volume of acetic acid (100 mmol l^{-1}) contained DOPEG,

e. DOMA was eluted in 6 cm^3 of HCl (500 mmol l^{-1}).

Each of the solutions obtained was mass assayed by the colourometric procedure, and its tritium activity determined in toluene-trition scintillant.

5. Colourometric assay

The para-nitroaniline method used for the visualization of catechols and 3-0-methylated catechols on chromatography media was adapted by Head (1976) for use on the Technicon Autoanalyser.

Samples of catecholamines or their metabolites prepared in HCl (300 - 500 mmol l^{-1}) were introduced into the manifold of the Autoanalyser and mixed with K_2CO_3 (1.45 mol l^{-1}), para-nitroaniline (7.2 mmol l^{-1}) and $NaNO_2$ (290 mmol l^{-1}). The coloured derivate formed was passed through the flow cuvette of the colourometer and

changes in optical density (O.D. 505) were recorded on a paper chart recorder. The manifold design and the flow rates of the various reagents are shown in Figure 4.3 The optical density changes produced by the test solutions were equated to the changes produced by known concentrations of NA and its metabolites.

6. Purification of ^{3}HNA

Stock solutions of ^{3}HNA which required purification prior to use were added to phosphate buffer (200 mmol l^{-1}; pH 6.4) and ascorbic acid (600 µmol l^{-1})* and loaded onto Dowex 50 (Na^{+}) columns. The columns were washed with water (4 collections of 5 cm^{3}) and the NA eluted in HCl (500 mmol l^{-1}). Approximately twenty-five aliquots of HCl (3 cm^{3}) were collected. 0.01 or 0.02 cm^{3} of each collection was added to toluene-triton scintillant and the tritium activity determined. The NA content of each aliquot was determined by fluorometric assay (page 2.9). Figure 4.4, which is a profile of the NA contents and tritium activities of these aliquots, indicates that most of the tritium activity occurred in those aliquots containing NA.

Usually the three aliquots containing the greatest concentration of NA were combined and assayed by the fluorometric method. The tritium activity was determined by liquid scintillation spectrometry. The resultant purified stock solution was held at 4°C until required for use.

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* *Typical volumes used were:*

^{3}*HNA* - *0.5* cm^{3}

phosphate buffer - *2.0* cm^{3}

ascorbic acid - *0.1* cm^{3}

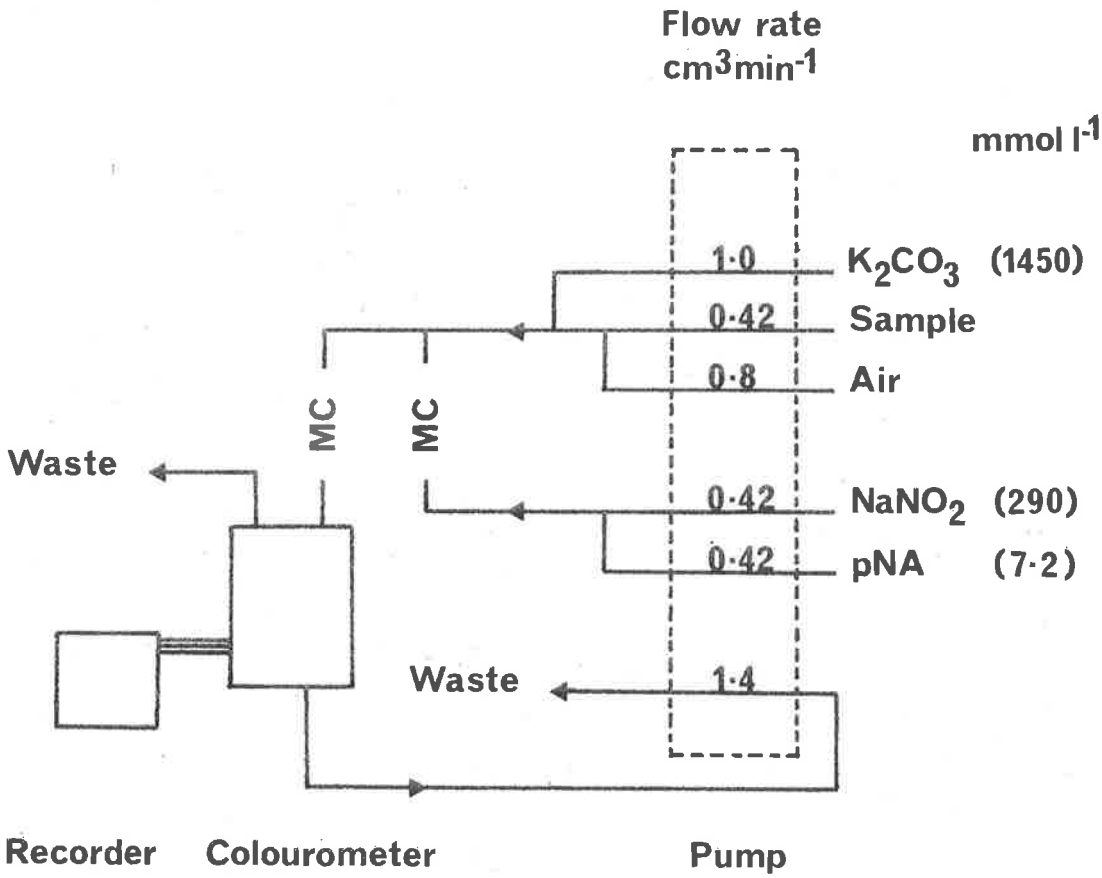

FIGURE 4.3 Schematic representation of the colourometric assay of noradrenaline and its 3-methoxy metabolites.

The reagents used, their concentrations and flow rates are indicated. Samples, in HCl (300 or 500 mmol l^{-1}), were introduced into the manifold of the Autoanalyser.

MC refers to the mixing coils and
pNA to para-nitroaniline.

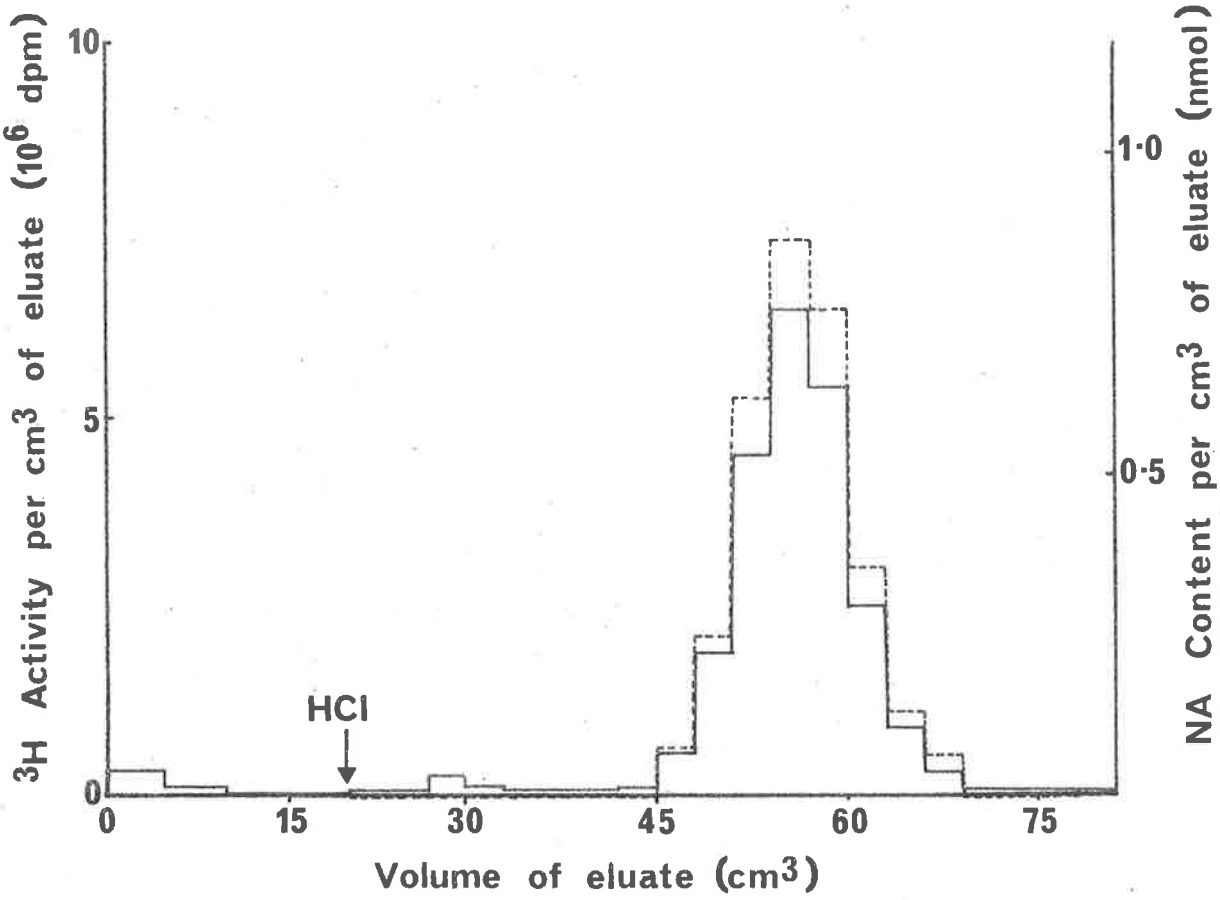

FIGURE 4.4 Typical profiles of tritium activity and NA content obtained from Dowex 50 chromatographic purification of l-NA. The elution and collection procedures are outlined in the text.

NA was eluted from the column with HCl (500 mmol l^{-1}) as indicated (arrow).

The broken lines represent the NA content per cm^3, and the continuous lines the tritium activity per cm^3, in each aliquot of eluate collected.

7. Liquid scintillation spectrometry

i. Scintillants

The dioxane-based cocktail described by Bray (1960) was used in most experiments. 15 cm^3 of the scintillator, described as Bray's scintillant, provided satisfactory counting efficiencies for samples of tritium-labelled materials contained in up to 1.5 cm^3 of Krebs' solution. This scintillant was prepared in 10 l batches according to the formulation and method outlined in Appendix 2.

In some experiments, the radioactivity in samples was determined using 10 - 15 cm^3 of toluene-based scintillants. For descriptive purposes, these cocktails have been designated as toluene or toluene-triton scintillants (see Appendix 2).

ii. Counting procedures

The radioactivity present in solutions containing tritium-labelled compounds was measured by liquid scintillation spectrometry using a Packard Model 3310 Liquid Scintillation Spectrometer. Count rates were determined for periods of 5 or 10 minutes (usually 10 minutes), and the background count rates were subtracted prior to quench and counting efficiency corrections. In early experiments, the background count rate for each vial containing scintillant was determined prior to the addition of the unknown tritiated sample, but this became impractical and mean background rates were determined and used.

As the channels ratio method of quench correction was used for samples counted in Bray's scintillant, standard curves, relating

counting efficiency to the ratio of the net count rates in two different channels of the spectrometer, were prepared for each batch of scintillator. Duplicate curves were determined according to the following procedure:

a. tritiated toluene of known activity (a_K) was added to 8 vials containing 15 cm^3 of scintillant, and the counting rate (r_1) in channel 1* was determined for each,

b. the mean counting rate (MCR) was calculated and vials with counting rates of more than ± 10% were replaced,

c. 7 of the 8 vials were quenched with different volumes of Krebs' solution and the counting rates (r_2) in all 8 vials were re-determined using channels 1 and 2*,

d. the counting efficiency, expressed as a percentage (E), and the channels ratio (R) were determined according to the following formulae:

$$E = \frac{MCR}{r_1} \times \frac{r2}{a_K} \times 100$$

$$R = \frac{r_2 \text{ (channel 1)}}{r_2 \text{ (channel 2)}}$$

e. standard curves were determined by plotting E against $\frac{1}{R}$.

Hence, for each unknown sample, E was determined and the counting rate corrected for efficiency and quenching. Because of

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* *The parameters for the two channels were:*

channel 1 - 50% gain
50 - 1000

channel 2 - 50% gain
50 - 250

the large number of samples involved, a programme was derived for use with an Olivetti Programma 101, which calculated E for each sample, corrected the counting rate for quenching and efficiency of counting and divided by the mass of the artery segment used for the particular experiment. In some experiments, other correction factors were added to the programme (for example, in Chapter 10, the tritium activity which effluxed into the lumen was standardized against a constant activity in the extraluminal bathing medium).

In this thesis, tritium activity, rather than the amount of NA and its metabolites which this represented, has been used as the standard unit, as there was doubt about the specific activity of the stock solutions provided by the manufacturer (see Chapter 3).

The internal standardization procedure used to correct for quenching and efficiency of counting with the toluene-based scintillants involved determination of the counting rate (r_1) for the unknown sample, followed by a second determination (r_2) after the addition of ^3H toluene of known activity (a_K).

Hence, the counting efficiency (E) was derived from:

$$E = \frac{r_2 - r_1}{a_K}$$

and the activity (A) in the test sample from:

$$A = \frac{r_1}{E}$$

8. Extraction of tritium-labelled material from tissues

In those experiments in which the amount of NA and its metabolites remaining within the tissues was determined, the tissues were:

a. agitated in 0.5 cm^3 of NCS solubilizer (Nuclear Chicago) at 36°C for at least 16 hours, or

b. immersed in 1.0 cm^3 of HCl (100 mmol l^{-1}) containing EDTA (22 µmol l^{-1}) at 4°C for a minimum of 16 hours (Head, 1976).

The latter method was preferred as it enabled the NA to be extracted into a supernatent relatively free from protein. When required, the tritium activity of the supernatent was determined in the toluene-triton scintillant, and the amount of NA was assayed fluorometrically or by bioassay.

CHAPTER 5

HISTOCHEMICAL STUDIES

Part A: DIFFUSION OF EXOGENOUS NORADRENALINE ACROSS THE ARTERY WALL

Part B: LOCALIZATION OF ENDOGENOUS NORADRENALINE FOLLOWING SYMPATHETIC NERVE STIMULATION

CHAPTER 5

The studies described in this thesis commenced in 1970 with an analysis of some of the factors which influence the diffusion of exogenous NA across the artery wall, and continued with a histochemical investigation of the diffusion of endogenous NA following its release by sympathetic nerve stimulation. The chapter is divided into two parts; part A relates to the exogenous studies and part B to the endogenous studies.

PART A DIFFUSION OF EXOGENOUS NORADRENALINE ACROSS THE ARTERY WALL

This study was prompted by the findings of de la Lande and Jellett (1972), who showed that although inhibition of MAO augmented the constrictor response of the rabbit ear artery to extraluminal NA and prolonged the relaxation of the artery from this response, these effects were not observed when the NA was applied intraluminally. This difference was explained in terms of the failure of the intraluminal NA to diffuse across the artery wall to the sympathetic nerve terminals.

The work described in this part of Chapter 5 was undertaken, in collaboration with Mrs. M.A. Lazner, to determine whether smooth muscle uptake and metabolism by COMT influenced the diffusion of NA across the artery wall, and hence its concentration in the region of the sympathetic nerves. An indirect technique, namely the ability

of NA to restore monoamine fluorescence in nerve terminals which had been depleted of NA by reserpine, was used to assess the influence of these factors.

Metanephrine (MN), an inhibitor of smooth muscle uptake, and 3,4-dihydroxy-2-methyl propiophenone (UO521), an inhibitor of the enzyme COMT, were employed to examine the influence of smooth muscle uptake and metabolism by COMT on the diffusion of intraluminal NA across the artery wall.

METHODS

Semi-lop-eared rabbits were injected with reserpine (1.64 μmol kg^{-1}; IP) (Waterson and Smale, 1967) 24 hours before being anaesthetized with urethane (19.7 mol kg^{-1}; IP). Artery segments, cannulated at the proximal end or at the distal end as well, were placed in organ baths and equilibrated for 60 minutes as described previously (page 2.3). The perfusion pressure was monitored on smoked kymograph papers. In addition, at least one segment was removed from each animal and immediately frozen in an acetone/dry ice mixture. Such sections were included to verify the effectiveness of the reserpine treatment in depleting the nerves of their specific monoamine fluorescence.

At the end of the equilibration period nialamide (340 μmol l^{-1}) was added to the intraluminal and extraluminal Krebs' solution for 60 minutes, after which the arteries were washed in nialamide-free Krebs' solution for a further 10 minutes. Evidence that this procedure inhibited MAO was presented by de la Lande et al (1970).

In each experiment Krebs' solution containing NA (3.0 µmol l^{-1}) and ascorbic acid (300 µmol l^{-1}) was perfused through one artery segment (intraluminal) and bathed the outside of a second segment (extraluminal) for 30 minutes.* Other segments were treated with either UO521 (55 µmol l^{-1}) or MN (2.5 - 5.0 µmol l^{-1}) for 15 minutes prior to and for the duration of the application of intraluminal NA.

At the end of the period of exposure to NA the arteries were washed in drug-free Krebs' solution for 10 minutes. For the duration of this washing period the intraluminal flow rate was 5 cm^3 min^{-1}. Subsequently, each segment was rapidly frozen in an acetone/dry ice mixture and processed according to the fluorescent histochemical technique described earlier (page 2.6).

Control segments, treated only with nialamide, UO521, MN or ascorbic acid, were included to determine whether these agents restored fluorescence in the absence of added NA.

In a subsequent series of experiments the artery segments were not treated with nialamide prior to the administration of UO521, MN and NA (Table 5.2). Note that in two experiments segments of nialamide-treated arteries, which had been exposed to extraluminal NA, were included to confirm that NA restored fluorescence in MAO-inhibited arteries.

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* *In those experiments in which NA was added intraluminally the artery segments were cannulated at both ends to prevent the NA from mixing with the external bathing medium. Further, since an elevated perfusion pressure might have caused the intraluminal fluid to leak into this bathing medium, the flow rate was reduced to 1.0 cm^3 min^{-1} during the application of intraluminal NA, and was stopped during exposure to extraluminal NA.*

Sections from each artery segment were examined independently by at least two observers who were unaware of the particular treatment given to the segments.

The intensity of specific fluorescence at the media-adventitia border was classified on a scale ranging from:

0 - absent,

+ - present but sparse compared with that found in a "normal" artery (i.e. freshly excised artery from an untreated rabbit),

++ - comparable with that observed in a "normal" artery,

+++ - greater than that in a "normal" artery.

RESULTS

Those segments of freshly excised arteries included to verify the effectiveness of reserpine pre-treatment showed that the drug had depleted monoamine fluorescence in the region of the media-adventitia border in all arteries.

1. Restoration of monoamine fluorescence

i. Nialamide-treated arteries

Those nialamide-treated arteries in which NA (3 μmol l^{-1}) was applied extraluminally displayed intense monoamine fluorescence at the media-adventitia border. However, this fluorescence was not restored when NA was perfused through the lumen of the arteries, that is applied intraluminally, unless the arteries were treated

with MN or UO521 as well (Table 5.1). In one artery segment, not included in Table 5.1, extraluminal NA (0.3 μmol l^{-1}) restored intense fluorescence (rated as +++).

ii. Non-nialamide-treated arteries

In the three experiments in which nialamide treatment was omitted, monoamine fluorescence was not observed at the media-adventitia border in arteries following exposure to either extraluminal or intraluminal NA. Furthermore, fluorescence was not restored in UO521 or MN-treated arteries following the application of intraluminal NA (Table 5.2).

iii. Control arteries

Fluorescence was not restored in artery segments treated with nialamide alone, nor in nialamide-treated segments which were exposed to either MN or UO521 as well. Similarly, arteries treated with MN alone or UO521 alone did not show evidence of monoamine fluorescence at the media-adventitia border.

The precaution was taken of testing whether ascorbic acid itself caused restoration of fluorescence, since ascorbic acid was always present in solutions containing NA. There was no evidence that ascorbic acid alone restored fluorescence.

2. Relaxation of artery segments following washout of NA

The rates of relaxation of the artery segments from the constriction caused by NA were recorded during the 10 minutes' washout period. The results are summarized in Figure 5.1. It will be noted that in nialamide-treated arteries this rate of recovery from

Experiment Number	Ext. NA	Int. NA	Int. NA + UO521	Int. NA + MN
1	+++	0	+	
2	+++(++)	+?	++	
3	+++(++)	0	++(+)	++
4	+++	+?	++	++
5	++	+?	++(+)	++(+)
6	+	0	++	++(+)
7	+++	+?	++	++
8	+++	0		

TABLE 5.1 Restoration of monoamine fluorescence by NA at the media-adventitia border of ear artery segments following depletion of the NA stores by reserpine. All arteries were perfused *in vitro* with nialamide to inhibit MAO activity. MN (2.5 - 5.0 µmol l^{-1}) and UO521 (55 µmol l^{-1}) were present throughout the application of NA. NA (3 µmol l^{-1}) was applied intraluminally (Int. NA) to some segments and extraluminally (Ext. NA) to others.

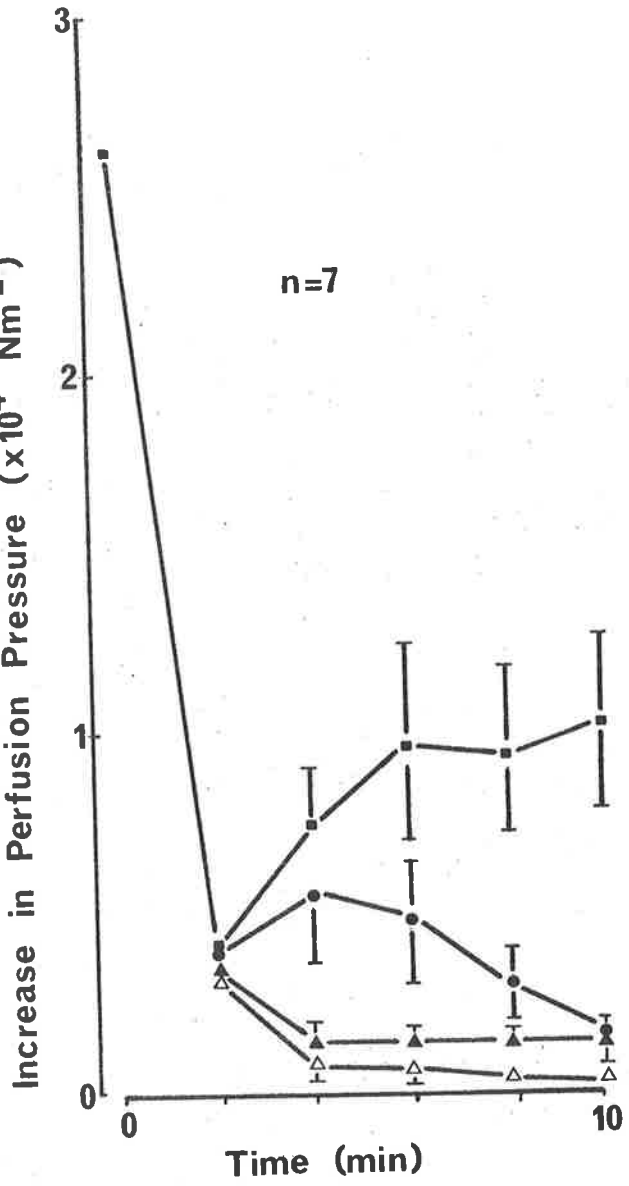
Fluorescence was rated 0 to +++ (see text). +? refers to equivocal fluorescence, rated at < + by both observers and not readily distinguishable from non-specific fluorescence. Where estimates of fluorescence differed between observers, the lower value is shown in brackets.

Experiment Number	Ext. NA	Int. NA	Int. NA + UO521	Int. NA + MN	Ext. NA + Nialamide
1	0	0	0	0	
2	0	0	0	0	+++(++)
3	0	0	0	0	+++

TABLE 5.2 Failure of NA to restore monoamine fluorescence at the media-adventitia border of ear artery segments following depletion of the NA stores by reserpine.

MN (2.5 - 5.0 µmol l^{-1}) and UO521 (55 µmol l^{-1}) were present throughout the application of NA.

NA (3.0 µmol l^{-1}) was applied intraluminally (Int. NA) or extraluminally (Ext. NA). Fluorescence was restored by extraluminal NA only in the two segments pre-treated with nialamide. Where estimates of fluorescence differed between observers, the lower value is shown in brackets.



n - represents the number of arteries used

FIGURE 5.1 Relaxation of nialamide-treated and reserpine pre-treated artery segments from vasoconstrictor responses to NA, following the washout of the amine.

NA was applied intraluminally to untreated (△ —— △), UO521 (● —— ●) and MN (▲ —— ▲) treated arteries, and extraluminally to untreated (■ —— ■) arteries.

Ordinate: increase in perfusion pressure above that in the resting artery

extraluminal NA was very much slower than that following intraluminal NA. Furthermore, the rate of recovery following intraluminal NA in those arteries treated with nialamide and UO521 was slower than in nialamide and MN-treated arteries. Both MN and UO521 reduced the rate of recovery in nialamide-treated arteries.

DISCUSSION

The reappearance of fluorescence at the media-adventitia border of reserpine and nialamide-treated arteries following exposure to extraluminal NA was considered to be the result of accumulation of NA in sympathetic nerves. The evidence for this included the findings of:

a. other workers that NA was taken up by, and stored in, sympathetic nerves (page 1.9),

b. this study, in which the fluorescence reappeared following the application of NA in the same location as, and with a similar appearance to, that produced by endogenous NA,

c. de la Lande et al (1974) who showed that cocaine, which is a potent inhibitor of neuronal uptake prevented the reappearance of fluorescence in reserpine-depleted nerve terminals when extraluminal NA was applied to nialamide-treated arteries.

Hence, the reappearance of fluorescence at the media-adventitia border in the present studies appeared to be a valid indication that NA had accumulated in the sympathetic nerves in sufficient quantity for it to be detected histochemically.

The failure of intraluminal NA to restore fluorescence in nialamide-treated arteries suggested that, at the concentration used, NA did not reach a sufficiently high concentration in the vicinity of the nerve terminals for its uptake to be demonstrated histochemically. This failure could be attributed to:

a. a physical barrier in the artery wall between the lumen and the nerve terminals, or

b. loss of NA as it diffused across the artery wall.

That loss of NA as it diffused across the artery wall was the more likely explanation for the failure of intraluminal NA to restore fluorescence in nialamide-treated arteries was supported by the findings of the present study, in which fluroescence was restored when smooth muscle uptake of NA was prevented by MN, or when O-methylation of NA was inhibited by UO521.

The latter argument assumed that either COMT was not present intraneuronally or that the contribution of intraneuronal COMT to O-methylation was negligible in the present experiments. Recently, Head (1976) investigated the distribution of COMT in the rabbit ear artery and concluded that it was entirely extraneuronal. On the assumption that COMT is not sited intraneuronally in the rabbit ear artery, it is suggested that the slower rate of recovery from the vasoconstrictor response to intraluminal NA in UO521-treated arteries, relative to MN-treated arteries, might be a manifestation of the continuing efflux of NA from the smooth muscle cells in the UO521-treated artery segments. As MN prevented the uptake of NA into smooth muscle cells this efflux would not occur. As discussed previously (page 1.16), Trendelenburg (1974) found that inhibition

of COMT in the rabbit aorta prolonged the efflux of NA from the extraneuronal stores and delayed the recovery from vasoconstriction.

The inability of extraluminal NA to restore fluorescence, when nialamide pre-treatment was omitted in the second series of experiments, provided further evidence for the presence of intraneuronal MAO in this artery. Previously de la Lande and Jellett (1972) had produced pharmacological data which indicated the presence of intraneuronal MAO, although histochemical techniques had revealed only extraneuronal MAO (de la Lande et al, 1970).

In summary, this fluorescent study indicated that, at a concentration of 3.0 μmol l^{-1}, exogenous NA was taken up by smooth muscle cells and inactivated by COMT as it diffused across the artery wall. The evidence indicated that extraneuronal uptake of NA occurred at much lower concentrations than proposed by Avakian and Gillespie (1968).

PART B LOCALIZATION OF ENDOGENOUS NORADRENALINE FOLLOWING SYMPATHETIC NERVE STIMULATION

In the preceding section of the chapter, evidence was presented which indicated that diffusion of intraluminal NA (3 μmol l^{-1}) across the artery wall was restricted by uptake into the smooth muscle cells in the media and possibly by extraneuronal metabolism. This work also supported the concept that metabolism by intraneuronal MAO occurred following neuronal uptake of NA. As described in Appendix 1, higher concentrations of NA (600 μmol l^{-1}) produced an intense and even distribution of fluorescence across the wall of untreated arteries,

some of which appeared to be bound to connective tissue and some taken up by the smooth muscle cells. Clearly, the evidence which has accumulated from these and other studies indicates that the diffusion of NA across the wall of the rabbit ear artery is influenced by several factors which might include neuronal and extraneuronal uptake, binding to connective tissue elements and metabolism by MAO and COMT.

An important question is whether these factors limit the diffusion of endogenous NA from the nerve terminals following its release. The present study describes an attempt to investigate this question histochemically and was prompted by the work of Gerová et al (1967) who used the fluorescent histochemical technique to demonstrate diffusion of NA within the tunica media of the dog dorsal pedal artery, following electrical stimulation of the appropriate sympathetic supply. In addition to a diffuse fluorescence in the media, they noted "fluorophore cloudlets" around the nerve endings in the stimulated, but not in the unstimulated, arteries.

In an initial series of experiments, 19 artery segments were stimulated for up to 60 seconds by square wave pulses delivered through platinum field electrodes at frequencies of up to 30 Hz. These segments, and 22 non-stimulated segments, were either untreated or treated with nialamide (340 µmol l^{-1}), UO521 (55 µmol l^{-1}), cocaine (3 µmol l^{-1}), MN (2.5 µmol l^{-1}) or PBZ (33 µmol l^{-1}). At the end of the period of stimulation the arteries were removed from the organ baths as rapidly as possible, frozen in an acetone/dry ice mixture and prepared for fluorescence microscopy (page 2.6).

Identification and interpretation of changes in these arteries was difficult because of the problems encountered in preparing adequate sections from the constricted arteries, and because the morphology of the smooth muscle cells and surrounding connective tissue was altered relative to the unstimulated sections. Although it was not possible to demonstrate monoamine fluorescence outside the nerve terminals, in some sections prepared from stimulated arteries these terminals appeared to lack definition. However, it was not clear whether this represented a "halo" of fluorescence outside the neuronal membrane or not. The presence of this "halo" could not be related to any of the drug treatments used.

It seemed possible that the failure of the histochemical technique to demonstrate extraneuronal NA reflected the delay in removing the arteries from the organ baths and freezing them. Consequently, similar experiments were conducted in which the techniques of superfusion and transmural stimulation were combined, as this permitted the arteries to be frozen more rapidly.

METHODS

Usually four single cannulated segments of artery were removed from each animal, mounted in superfusion holders between parallel platinum electrodes and held at their *in situ* length by a piece of cotton attached to their distal ends (page 2.3). A small cut was made in each artery above the cotton tie to permit the escape of the intraluminal perfusate.

Drugs were added to both the intraluminal and extraluminal Krebs' solution and included nialamide (340 μmol l^{-1}), cocaine (3 μmol l^{-1}) and PBZ (33 μmol l^{-1}). Nialamide was added for 40 minutes and was washed out of the artery segment for 10 minutes prior to sympathetic nerve stimulation. Cocaine and PBZ were added to the arteries for 20 minutes prior to and for the duration of the stimulation period.

Control segments were bathed in Krebs' solution or Krebs' solution containing the particular drug, but were not stimulated.

Usually, arteries were stimulated for 4 minutes at 5 Hz and were frozen in an acetone/dry ice mixture either during stimulation or at the instant stimulation stopped. The distribution and intensity of monoamine fluorescence was assessed visually by the fluorescent histochemical technique.

RESULTS

In the absence of stimulation, the drug-treated arteries were indistinguishable histochemically from the untreated arteries in that they both showed strongly fluorescing, discrete structures at the media-adventitia border, and neither displayed evidence of fluorescence in the media or in the connective tissue of the adventitia. A photomicrograph of an untreated, unstimulated artery is shown in Figure 5.2.

The appearance of the stimulated arteries differed from these control segments in that the majority showed:

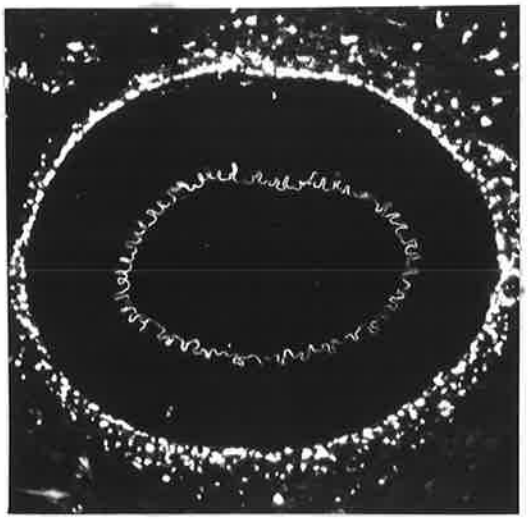
FIGURE 5.2 Transverse sections of formaldehyde-treated rabbit ear arteries showing the effects of sympathetic nerve stimulation on the distribution and intensity of monoamine fluorescence.

A. Untreated, non-stimulated artery which displayed discrete noradrenergic fluorescence at the media-adventitia border. Fluorescence was not observed in the media or the connective tissue of the adventitia.

B and C. Cocaine-treated (B) and nialamide-treated (C) segments following nerve stimulation. In both arteries the fluorescence in parts of the neuronal plexus was absent or less intense than in non-stimulated control arteries. The individual nodes were less discrete and the fluorescence tended to coalesce into bands. The bluish fluorescence surrounding parts of the lumen in the cocaine-treated artery was thought to be an increased level of background fluorescence as a result of the constricted nature of the media.

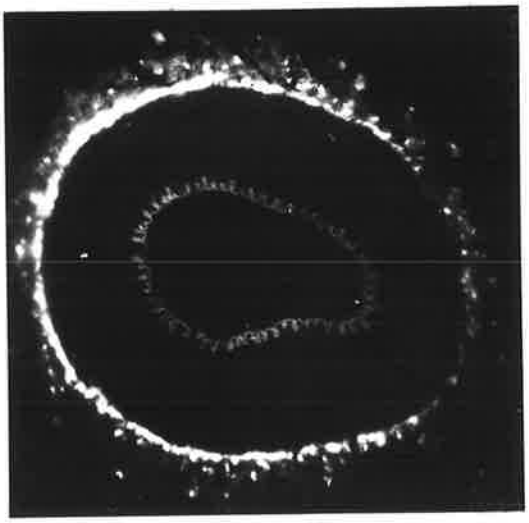
D. PBZ-treated artery following nerve stimulation. Monoamine fluorescence in a portion of the neuronal plexus was absent, and in other regions was less intense and the nodes appeared to coalesce.

Scale represents 100 µm

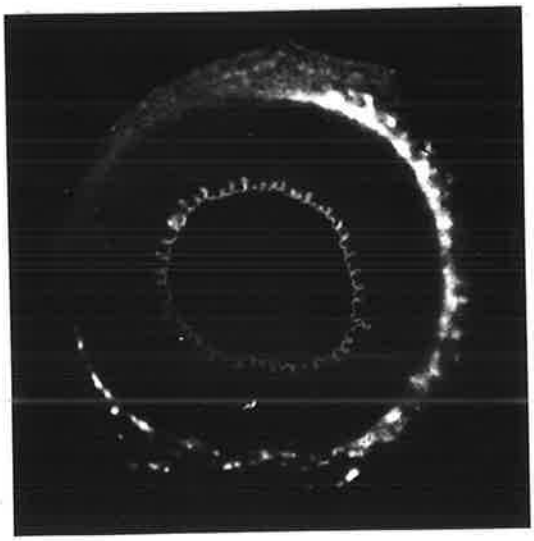


A

B



C



D

a. decreased intensity or absence of fluorescence in some regions of the neuronal plexus,

b. coalescence of nerve terminal fluorescence; that is, the discrete nature of the fluorescence described in the non-stimulated arteries at the media-adventitia border was lost,

c. some suggestion of monoamine fluorescence in the adventitial connective tissue close to the nerve terminals.

Occasionally, patches of fluorescence appeared to exist in the outer regions of the tunica media, but this was not a consistent finding.

The characteristics described above are shown at a higher magnification in the photomicrographs which constitute Figure 5.3.

All arteries, whether they were untreated or treated with nialamide, cocaine or PBZ, displayed some evidence of altered nerve terminal fluorescence as a result of sympathetic nerve stimulation. Of the 31 arteries which were stimulated, connective tissue elements close to the nerve terminals appeared to fluoresce in 19, while in 7 there was some evidence that fluorescence might have been present in the media. These findings did not appear to be dependent on the drug regime used.

DISCUSSION

The findings of the present study are in agreement with observations of Gerová et al (1967) and Doležel et al (1975) that

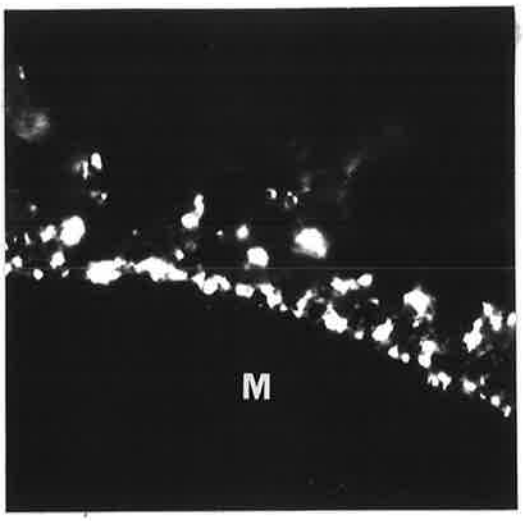
FIGURE 5.3 Transverse sections of formaldehyde-treated rabbit ear arteries showing a portion of the tunica adventitia, the neuronal plexus and the tunica media (M).

A. Non stimulated, PBZ-treated artery. Note the absence of fluorescence in the media and the discrete nature of the nerve terminals in the neuronal plexus.

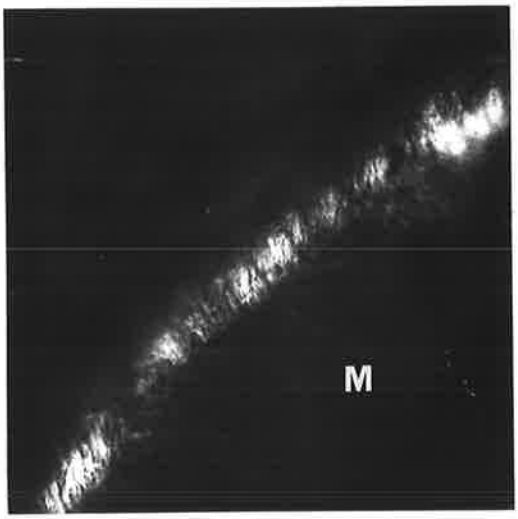
B and C. PBZ-treated arteries following nerve stimulation. The discrete nature of the fluorescing nodes was lost, and the fluorescence tended to coalesce. Fluorescence was apparent in the adventitia adjacent to the neuronal plexus, but was not positively identified in the media.

D. PBZ-treated artery following nerve stimulation. Nerve terminal fluorescence was absent, and diffuse monoamine fluorescence was noted in the media and adventitia. One portion of the media displayed intense fluorescence (arrow).

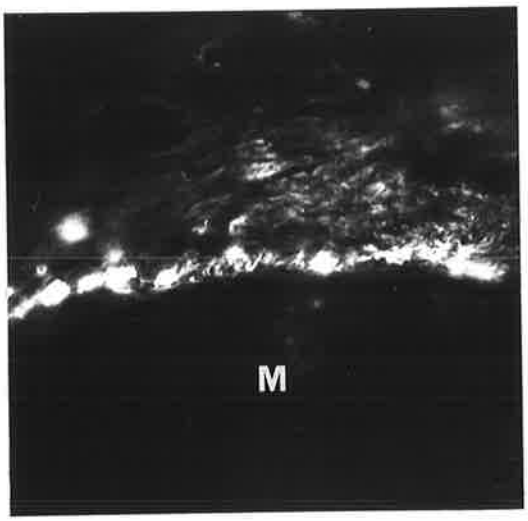
Scale represents 20 µm



A



B



C

D

"fluorophore cloudlets" surrounded the nerve terminals in the artery wall following stimulation of the sympathetic nerves. However, in the experiments reported in this chapter, the halo was usually accompanied by a partial or complete loss of fluorescence in some areas of the neuronal plexus. Furthermore, the finding of Gerová and co-workers that monoamine fluorescence appeared throughout the tunica media of the dog dorsal pedal artery, the rat femoral artery and the rabbit saphenous artery as a consequence of sympathetic nerve stimulation was not demonstrated in the present study, in which medial fluorescence, if present at all, was limited to isolated patches in a few arteries.

Although nerve terminals have been described in the outer half of the tunica media in the dorsal pedal and saphenous arteries used by Gerová and co-workers, the plexus in the femoral artery, like that in the ear artery, is apparently confined to the media-adventitia border. Hence it is unlikely that variations in the position of the nerve plexus account for the differences between the two studies. However, a number of differences in the experimental procedures used might explain the paucity of medial fluorescence in the rabbit ear artery:

> Firstly, Gerová and co-workers (Gerová et al, 1967; Doležel et al, 1975) stimulated the sympathetic nerves of the artery segments *in situ* and, although they did not indicate whether the arteries used in their experiments were bathed in a physiological solution or not, it is likely that the volume of fluid in contact with the outside of these arteries was considerably less than that which superfused the ear arteries

(3.5 ± 0.5 cm^3 min^{-1}). In view of findings presented elsewhere, that most of the NA released by nerve stimulation in the isolated ear artery overflowed from the adventitial surface (Chapter 8), and that the luminal overflow increased when extraluminal diffusion was limited (Chapter 9), it is possible that this discrepancy in the volume of extraluminal fluid in the two experiments altered the diffusion pattern. That is, the diffusion of NA into the bathing medium in those arteries "stimulated *in situ*" might have been restricted by the small volume of extraluminal fluid.

Secondly, whereas Gerová and co-workers demonstrated medial fluorescence in MAO and COMT inhibited arteries, they did not indicate whether this fluorescence was apparent when neither, or only one, enzyme was inhibited. Hence, in the present experiments, the absence of fluorescence in the media of most arteries might be a result of NA metabolism by COMT or MAO. Such an explanation, however, does not account for the lack of medial fluorescence in the PBZ-treated arteries, in which uptake into the neuronal and smooth muscle tissues was inhibited and NA was unable to reach the sites of metabolism. In this instance, the non-development of fluorescence in the media might be related to the failure of NA to enter the smooth muscle cells.

Finally, the ability of Gerová and co-workers to detect fluorescence throughout the media might have resulted from an increased intensity of fluorescence in the prepared microscopic sections as a consequence of:

a. the longer period of freeze-drying used by them prior to formaldehyde treatment of the artery segments (7 - 10 days cf. 16 hours in this study), and

b. the preparation of thicker sections (30 - 60 μm cf. 7 μm in this study).

In conclusion, although the findings of this study indicated that some changes occurred in the distribution of fluorescence in the neuronal plexus following sympathetic nerve stimulation, it was apparent that the fluorescent histochemical technique used did not provide a suitable method for studying the factors which influence the release and diffusion of endogenous NA in the isolated rabbit ear artery. Although more sensitive isotopic methods were developed to investigate the release and diffusion of NA some changes in the technique used in the above study might facilitate the histochemical localization of NA. Of these, bathing the external surface of the isolated artery with liquid paraffin might be the most significant as it would prevent the rapid diffusion of NA through the tunica adventitia.

CHAPTER 6

THE EFFECTS OF SOME INHIBITORS OF SMOOTH MUSCLE UPTAKE AND METABOLISM OF NORADRENALINE ON THE CONSTRICTOR RESPONSES TO AND THE OVERFLOW OF NORADRENALINE FOLLOWING SYMPATHETIC NERVE STIMULATION

CHAPTER 6

In this Chapter, two series of experiments are described which were intended to further define the influence of metabolism and smooth muscle uptake on the concentration and diffusion of NA in the artery wall following its release by nerve stimulation. In the first series the influence of the smooth muscle uptake of NA, MAO and COMT on the constrictor responses of isolated arteries to nerve stimulation were examined. Paired artery segments, one of which was treated with nialamide to inhibit MAO, were used to investigate the influence of neuronal and extraneuronal MAO in untreated arteries and in those treated with MN and COMT.

In the second group of experiments, the overflow of NA from isolated arteries following sympathetic nerve stimulation was measured by biological assay. Previously, de la Lande et al (1968) reported that the stimulation-induced overflow of NA in the rabbit ear artery was too small to quantitate by bioassay. However, the present study was undertaken in view of the finding of de la Lande and Harvey (1965) that 5-hydroxytryptamine (5HT) increased the sensitivity of the assay artery to NA about six-fold.

In exploratory experiments the sympathetic nerves in the donor arteries were stimulated, and the amount of NA which overflowed into the extraluminal and intraluminal Krebs' solution bathing each artery was assayed on a 5HT-sensitized artery segment. Several electrode arrangements were used and some donor arteries were treated with PBZ,

to inhibit the uptake of released NA by neuronal tissues and smooth muscle cells. The frequency of stimulation ranged from 2 Hz to 10 Hz. In most experiments, the amount of NA which overflowed into the extraluminal Krebs' solution could not be assayed unequivocally, and NA was never demonstrated in the intraluminal perfusate. In the few experiments where extraluminal NA was detected, the maximum overflow was 0.4 pmol $pulse^{-1}$ g^{-1}. As these results indicated that the amount of NA collected from the donor artery was invariably less than the threshold of the assay artery, further development of a bioassay system depended on maximizing the concentration of NA collected from the donor artery and increasing the sensitivity of the assay artery.

Consequently, in later experiments the techniques of transmural stimulation and superfusion were combined, since this permitted the use of smaller volumes of Krebs' solution and a more convenient electrode arrangement. Su and Bevan (1970) claimed that such a technique stimulated "all nervous elements", and allowed collection of the released NA "with minimum delay and dilution". It was also found that the concentration of NA which induced a constrictor response in the assay artery was lower when the artery was superfused than when it was bathed in an organ bath ($16 \pm 2 \times 10^{-7}$ µmol cf. $5 \pm 1 \times 10^{-6}$ µmol). In the experiments described here, the assay arteries were superfused and the donor arteries were either superfused or placed in small organ baths. In either event the sympathetic nerves were stimulated transmurally.

METHODS

1. Response and recovery studies

Double cannulated artery segments were superfused as described previously (page 2.3). The perfusion pressure was monitored on a Grass Polygraph Model 5D pen recorder via a Statham pressure transducer. The sympathetic nerves were stimulated until a steady-state response was attained (50 to 150 pulses). Arteries were stimulated at intervals of 10 to 15 minutes except during the application of nialamide (Figure 6.1). MN (0.8 - 1.0 μmol l^{-1}) and UO521 (55 μmol l^{-1}) were added to the Krebs' solution perfusing the adventitial and luminal surfaces of the arteries for at least 10 minutes prior to the first of the two stimulations. Nialamide (340 μmol l^{-1}) was added to artery B for 60 minutes and was washed out for 10 minutes prior to stimulation.

The responses of the arteries to nerve stimulation were estimated during steady-state vasoconstriction. Recovery times were calculated as the time taken for the perfusion pressure to return to its prestimulation level following stimulation.

2. Overflow of endogenous NA

The overflow of transmitter following sympathetic nerve stimulation was measured in separate experiments, in which artery segments were held either in a superfusion holder or in a small organ bath (page 2.2). Platinum electrodes were placed in the transmural position and the sympathetic nerves in the arteries were stimulated at supramaximal voltages, with a pulse duration of 0.5 msec, and at the frequencies and train lengths listed in Tables 6.1 and 6.2. The

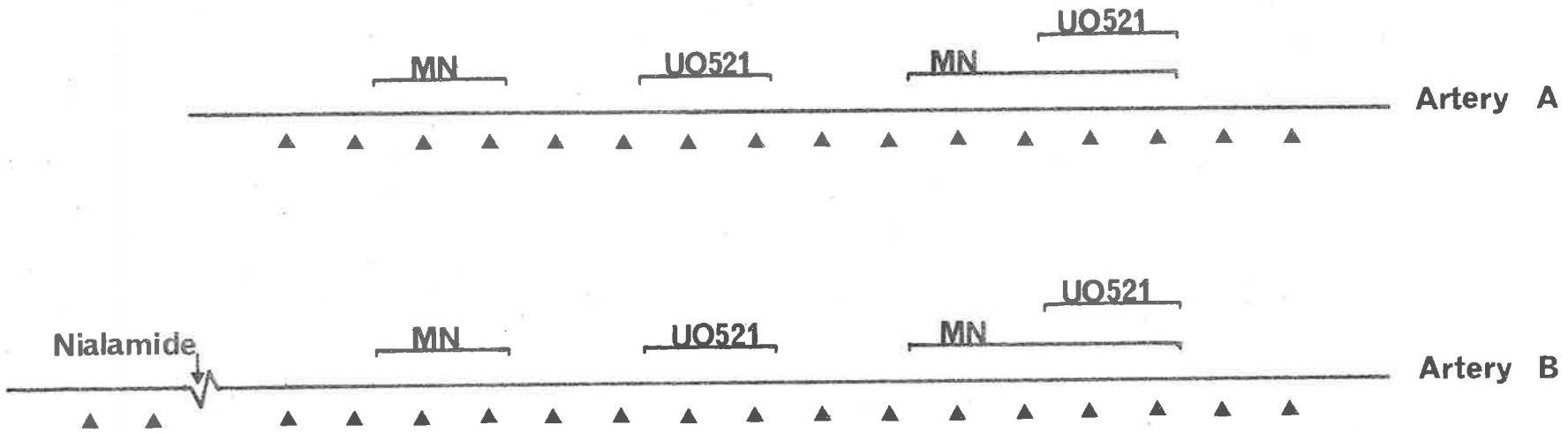


FIGURE 6.1 Diagrammatic representation of the experimental regime used in the response studies.

Paired arteries were stimulated (▲) at intervals of 10 - 15 minutes, except during the application (60 minutes) of nialamide (340 µmol l^{-1}) to artery B. MN (0.8 - 1.0 µmol l^{-1}) and UO521 (55 µmol l^{-1}) were added to the arteries for 10 minutes prior to, and for the duration of, the stimulation period, and were washed out of the arteries for 10 minutes prior to the subsequent train of stimuli.

Stimulation parameters: supra maximal voltage - 15 V

pulse duration - 0.5 msec

frequency - 2 Hz usually

various drug regimes used are shown in these tables. Nialamide was added to the Krebs' solution contacting the arteries for 60 minutes, and was washed out for 10 minutes prior to stimulation. All other drugs were added to the Krebs' solution at least 10 minutes prior to the period of stimulation, and remained in contact with the artery for the duration of the stimulus. Ascorbic acid (300 or 600 $\mu mol\ l^{-1}$) was always present in the Krebs' solution.

A second artery was removed from each rabbit and placed in a superfusion holder. These assay arteries were perfused with Krebs' solution containing 5HT [$(12 - 85) \times 10^{-3}\ \mu mol\ l^{-1}$] and cocaine ($3\ \mu mol\ l^{-1}$). Dose-response curves to intraluminal injections of NA were determined according to the technique of de la Lande et al (1967c). Standard injections of NA were given at intervals of approximately 2 minutes, and were interspersed with 0.3 cm^3 injections of Krebs' solution collected from the donor artery prior to, during or following sympathetic nerve stimulation.

RESULTS

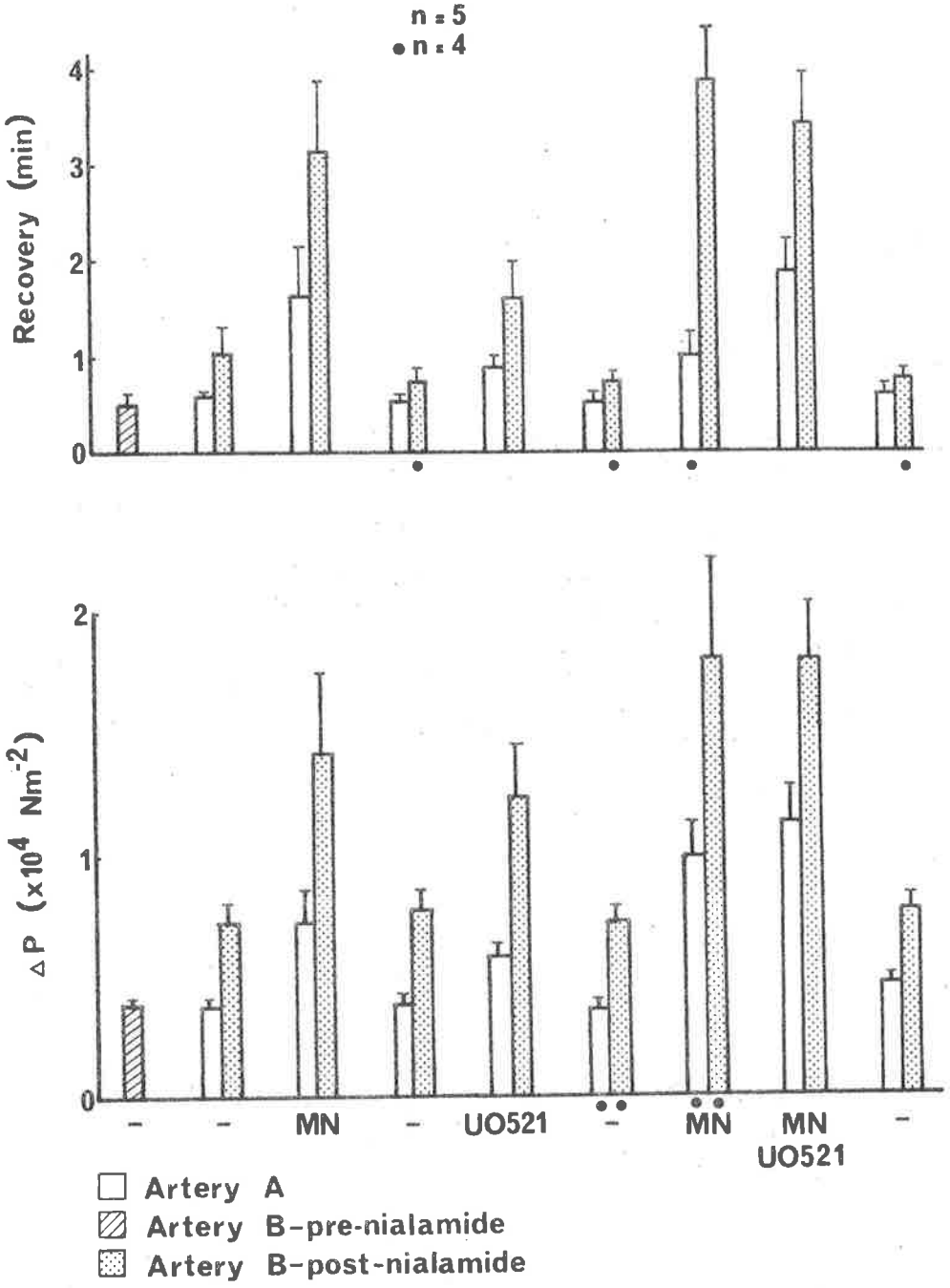
1. Response and recovery studies

The magnitudes of the steady-state constrictor responses of the arteries to sympathetic nerve stimulation, and the recovery times are shown in Figure 6.2. In the non-nialamide-treated arteries (artery A), MN and UO521 increased the magnitude of the constrictor response to nerve stimulation by 86% and 51% respectively. The effects of UO521 and MN were not additive, that is, UO521 failed to further increase the response when this had been increased by MN. It should be noted

FIGURE 6.2 The effects of MN and UO521 on the steady-state constrictor responses of untreated and nialamide-treated ear arteries to sympathetic nerve stimulation, and on the time taken for the arteries to recover from this response.

Tests of significance: paired t-test

1. Nialamide treatment significantly increased the constrictor responses of untreated, MN-treated, UO521-treated and MN plus UO521-treated arteries.
2. Nialamide treatment did not significantly alter the rate of recovery of untreated arteries, but significantly increased the recovery time in MN, UO521 and MN plus UO521 treated arteries.
3. MN significantly increased the constrictor responses and the recovery times of untreated and nialamide-treated arteries.
4. UO521 significantly increased the constrictor responses and the recovery times of untreated and nialamide-treated arteries, but not MN-treated arteries (whether nialamide-treated or not).
5. MN potentiated the responses of untreated arteries, but not nialamide-treated arteries, significantly more than UO521, and delayed the recoveries in both untreated and nialamide-treated arteries more than UO521. Note: the second application of MN was used for these tests.



n - represents the number of arteries used
Δ P - rise in perfusion pressure during nerve stimulation

that MN itself, in the concentration range employed (0.8 - 2.0 μmol l^{-1}), caused a small increase (6 x 10^{2} Nm^{-2}) in the perfusion pressure in the resting artery.

The nialamide-treated segments (artery B) consistently displayed an augmented response to stimulation, which was also apparent during the application of the various drugs. However, when the responses of these nialamide-treated arteries were calculated as ratios, $\frac{\text{drug present}}{\text{drug absent}}$, the effects of MN and UO521 were of the same order as in the untreated arteries. There was no significant difference between the potentiation produced by MN in nialamide and non-nialamide-treated arteries, and by COMT in nialamide and non-nialamide-treated arteries.

In untreated and nialamide-treated arteries the effects of MN and UO521 were completely reversible on drug washout.

In untreated and nialamide-treated arteries the recovery times from the responses were significantly slowed by MN and to a lesser extent by UO521, but UO521 did not further delay the recoveries produced by MN. In the presence of MN and/or UO521, nialamide treatment significantly delayed the recoveries from nerve stimulation, and although nialamide treatment alone delayed the recoveries this was not significant at the 0.05 level.

2. Overflow of endogenous NA

The overflow of NA in a number of experiments, in which the donor arteries were stimulated in superfusion holders or small organ baths, is shown in Tables 6.1 and 6.2 respectively. These tables do not include those experiments in which an overflow was not detected.

ARTERY	STIMULATION TRAIN No.	Freq (Hz)	Duration (Pulses)	DRUGS (μmol l^{-1}) Nial* (340)	Coc (3)	UO521 (55)	MN (0.8-1.0)	NA OVERFLOW (pmol p^{-1} g^{-1})
A	1	10	600					0.24
		10	600					0.30
B	1	10	600					†
		10	600					0.12
	2	5	600					0.24
		5	600					0.18
	3	5	600					0.36
		5	600					0.30
C	1	2	120					†
	2	2	120					0.30
	3	5	300					0.12
	4	10	600					0.12
D	1	5	300	+		+	+	1.66
		5	300	+		+	+	1.48
	2	5	300	+		+	+	1.78
		5	300	+		+	+	1.30
E	1	5	300	+	+	+	+	†
	2	5	300	+		+	+	2.96
	3	5	300	+	+	+	+	2.90
	4	5	300	+	+			2.31
	5	5	300	+	+	+	+	2.31
F	1	5	300	+				2.96
	2	5	300	+	+	+	+	†
	3	5	300	+		+	+	3.37

+ - *drug present*
* - *arteries were pretreated with nialamide*
† - *overflow was not assayed*

TABLE 6.1 Overflow of NA (pmol $pulse^{-1}$ gm^{-1}) from the adventitial surface of 6 rabbit ear arteries following sympathetic nerve stimulation. The donor arteries were held in superfusion holders, and the extraluminal superfusate was collected and assayed as indicated. For arteries A, B and D two collections of the superfusate were made during continuous stimulation. The concentrations of the drugs used, and the frequency and number of stimuli in each stimulation period are indicated.

ARTERY	STIMULATION TRAIN No.	Freq (Hz)	Duration (Pulses)	DRUGS (μmol l^{-1}) Nial* (340)	Coc (3)	UO521 (55)	MN (0.8-1.0)	NA OVERFLOW (pmol p^{-1} g^{-1})
G	1	10	2400	+	+	+		0.41
	2	10	2400	+	+	+		0.60
	3	10	2400	+	+	+		0.41
	4	10	2400	+	+	+		0.24
	5	10	2400	+	+	+		0.00†
H	1	10	2400	+	+	+		0.48
	2	10	2400	+	+	+		0.40
	3	10	2400	+	+	+		0.48
I	1	10	2400	+	+	+		0.18
	2	10	2400	+	+	+		0.18
J	1	10	2400	+	+	+		2.25
	2	10	2400	+	+	+	+	1.90
	3	10	2400	+	+	+	+	2.55†
	4	10	1200	+	+	+	+	1.30†
	5	10	1200	+	+	+	+	2.13‡
	6	10	2400		+	+	+	0.83§

+ - *drug present*
* - *arteries were pretreated with nialamide*
† - *TTX (0.3 μmol l^{-1})*
‡ - *TTX (1.6 μmol l^{-1})*
§ - *artery was removed from the organ bath prior to "stimulation"*

TABLE 6.2 Overflow of NA (pmol $pulse^{-1}$ g^{-1}) from the adventitial surface of 4 rabbit ear arteries following nerve stimulation. The donor arteries were stimulated in small organ baths for 4 minutes (except artery J which was stimulated for 2 minutes for periods 4 and 5), and the extraluminal fluid was removed for assay 6 minutes after stimulation ceased. The concentrations of the drugs used, and the frequency and number of stimuli in each period are indicated.

It will be noted that in all experiments, except those in which the donor arteries were treated with MN, the overflow of NA was very small. In fact, in most experiments the responses of the assay preparations to injections of the test or donor solutions were too close to the threshold to permit an unequivocal assertion that they reflected an overflow of transmitter. Despite the presence of unequivocal vasoactivity in the bathing-medium of MN-treated arteries following nerve stimulation, it was concluded that at least part of this activity was unrelated to the overflow of transmitter. The evidence for this was a) that tetrodotoxin (TTX; 0.3 - 1.6 μmol l^{-1}), a drug which inhibits the release of NA, did not prevent the formation of vasoactivity, even though it inhibited the constrictor response of the donor artery to nerve stimulation, and b) that stimulation of Krebs' solution containing MN and ascorbic acid, (that is, without an artery), yielded vasoconstrictor activity. The cause of this is discussed in Chapter 7.

DISCUSSION

The results described in this chapter showed that inhibition of MAO by nialamide in the rabbit ear artery increased the magnitude of the constrictor response to sympathetic nerve stimulation. This would be the expected result if the net reuptake of NA after its release was decreased in MAO-inhibited arteries, as described by de la Lande and Jellett (1972). An alternate explanation for this enhanced response was that inhibition of MAO increased the output of transmitter. In experiments in which the isolated nictitating membrane of the cat was pre-loaded with ^{3}HNA, it was demonstrated

that the overflow of tritium-labelled material (that is, NA and its metabolites) following nerve stimulation, was not significantly increased by MAO inhibition (Langer, 1970), although the amount of unmetabolized NA which overflowed was increased greatly. Hence, a likely explanation for the present findings was that the concentration of NA in the region of the adrenoreceptors was increased as a result of the enhanced efflux of NA from the nerve terminals following re-uptake of the transmitter. This relationship between the uptake of NA by neuronal tissues, its metabolism by MAO and the efflux of NA from the axoplasm was discussed previously for exogenous NA (page 1.10).

The effects of MN, namely, to increase the magnitude of the response of the artery to nerve stimulation and to delay the recovery from this response, were consistent with its activity as an inhibitor of smooth muscle uptake of NA. Moreover, the results suggested that part of the NA released from neuronal tissues is normally taken up by the smooth muscle cells. The effect of UO521 was also consistent with the suggestion, from the histochemical data (Chapter 5), that COMT contributes to the inactivation of NA as it crosses the artery wall.

There are two qualifications to the proposed mechanism of action of MN. One is the finding that "stimulation" of Krebs' solution containing ascorbic acid and MN produced vasoconstrictor activity. This point is clarified in Chapter 7. The second qualification is that MN, at the concentration used, invariably caused a small increase in perfusion pressure; that is, the effects of stimulation were studied in partly constricted arteries in which, for the same degree of muscle shortening as in unconstricted arteries, the increase in

perfusion pressure would be greater. However, these artefacts would not account for the failure of UO521 in MN-treated arteries to augment the responses produced by MN alone. This interaction resembled that between steroid inhibitors of smooth muscle uptake and UO521, described by Kalsner (1969a,b), in which it was demonstrated that inhibition of smooth muscle uptake prevented the substrate (NA) from gaining access to the enzyme (COMT).

The results of the present study support the postulate that the concentration of NA in the synapse, following its release by nerve stimulation, is in part determined by the net uptake of NA into neuronal tissues and smooth muscle cells and by the intracellular mechanisms of binding and metabolism. Hence, inhibiting the smooth muscle uptake system increased the extracellular concentration of NA and potentiated the magnitude of the response of the ear artery to nerve stimulation. It probably also delayed the inactivation of NA by preventing access of the transmitter to the metabolites, and by preventing intracellular binding. Simultaneous inhibition of smooth muscle uptake and MAO further increased the vasosocontrictor response and delayed the recovery, whereas the simultaneous inhibition of smooth muscle uptake and COMT did not. These results provided further evidence for the presence of active intraneuronal MAO and extraneuronal COMT. Presumably, COMT was either not present intraneuronally, or if it was present, it was physiologically insignificant.

The data on output contributed little to this study other than to highlight the extremely small quantity of transmitter released by nerve stimulation, a fact already apparent from the earlier study of de la Lande et al (1968). Because of this, the biological assay

technique was not pursued further; instead, more sensitive isotopic methods were introduced to study the overflow of transmitter. However, this study was included for a second reason, as it drew attention to some possible artefacts induced by field stimulation.

CHAPTER 7

ELECTROLYTIC O-DEMETHYLATION OF METHOXYCATECHOLAMINES

CHAPTER 7

During the course of the experiments undertaken in the preceding chapter it was noted that the passage of electrical pulses through Krebs' solution which contained MN and ascorbic acid produced vasoactivity.

The purpose of this chapter was to define the nature of the vasoconstrictor substance produced.

METHODS

Catecholamines (dl-noradrenaline, NA; dl-adrenaline, A) or their 3-methoxy analogues (dl-normetanephrine, NMN; dl-metanephrine, MN) were dissolved in Krebs' solution or saline (155 x 10^3 µmol l^{-1}) which was gassed with 95% oxygen and 5% carbon dioxide and maintained at 37°C.

Current was passed through the solutions by means of two platinum electrodes approximately 8 cm in length and positioned 1 cm apart. In most experiments the current source was a Grass model S-44 stimulator. Voltage and frequency were standardised at 70 V and 5 Hz except where otherwise indicated. Solutions treated in this way will be described as stimulated.

1. Biological assay

A double-cannulated artery was used as described in Chapter 2 (page 2.8), and cocaine (3 µmol l^{-1}) and 5-hydroxtryptamine

(13×10^{-3} µmol l^{-1}) were added to the Krebs' solution to enhance sensitivity. For assay, solutions were injected intraluminally in a volume not exceeding 0.3 cm^3.

2. Fluorometric analysis

The catecholamine (NA or A) contents of samples were assayed by a trihydroxyindole method similar to that described previously (page 2.9). Briefly, this procedure involved the automated sampling of catecholamines into acetic acid (300 mmol l^{-1}), oxidation at pH 5.8 with potassium ferricyanide (300 µmol l^{-1}) buffered with sodium acetate (1.5 mol l^{-1}) and lutine formation with alkaline-ascorbate [NaOH (2.5 mol l^{-1}), ascorbic acid (17 mmol l^{-1})]. The lutines were estimated fluorometrically at the wavelengths described in Chapter 2. Under these conditions A and NA, but not MN and NMN, contributed to the fluorescence estimated. Standard solutions of A and NA were dissolved in Krebs' solution or saline, depending on the nature of the test solution, and made 300 mmol l^{-1} with respect to acetic acid.

In other experiments, so that the catecholamine contents of stimulated solutions could be monitored continuously, a small portion of the sample was removed from the organ bath (0.1 cm^3 min^{-1}), mixed with ten times its own volume of acetic acid (300 mmol l^{-1}) and added to the oxidant. The 3-methoxycatecholamines were dissolved in Krebs' solution and assayed for non-THI fluorescence. Subsequently, current was passed through each solution and the THI fluorescence was measured. Known concentrations of catecholamines in Krebs' solution were used as standards.

3. Spectrophotometric analysis

The visible absorption spectra of the catecholamines and their 3-methoxy derivatives were examined photometrically using a Unicam SP 1800 spectrophotometer. Where such an analysis indicated the existence of an aminochrome,* the absorption maxima (λ max) of the solution was determined and the rate of increase of aminochrome during the passage of current was estimated continuously at that wavelength. In these experiments, the amine was omitted from the physiological solution in the reference cuvette.

4. Paper chromatography

Catecholamines were separated from their 3-methoxy analogues by descending chromatography on cellulose phosphate paper (Whatman P81) according to the procedure described elsewhere (page 2.7). Briefly, 20 µl aliquots of stimulated saline solution were applied to the paper, air-dried and chromatographed in isopropanol:ammonium acetate. For comparison, 20 µl aliquots of unstimulated saline solution containing the individual catecholamines and their 3-methoxy derivatives were run in parallel. After the solvent had migrated approximately 40 cm, the papers were air-dried and either sprayed with freshly prepared diazotized para-nitroaniline or cut into 1 cm sections. Sections were eluted overnight in 2 cm^3 of saline (pH 5.5) containing ascorbic acid (600 µmol l^{-1}). The saline eluates were assayed either fluorometrically or by bioassay.

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* *Aminochrome refers to the highly coloured cyclic oxidation products of β-(3,4 dihydroxyphenyl)-ethylamines and related products (Heacock et al, 1958).*

RESULTS

The vasoconstrictor activities of unstimulated and stimulated saline solutions containing MN (2.3 µmol l^{-1}) and ascorbic acid (600 µmol l^{-1}) are illustrated in Figure 7.1. It will be noted that only the stimulated solution elicited a response and that this response and that to NA were largely abolished by phentolamine (2 µmol l^{-1}). The results were identical when Krebs' solution was used instead of saline.

By placing the electrodes in separate solutions of MN and ascorbic acid connected with a moist filter-paper bridge, it was established that the vasoconstrictor activity appeared in the anodic compartment. Omission of ascorbic acid, or the use of distilled water instead of saline or Krebs' solution, led to loss of constrictor activity.

It was concluded from the preceding experiments that the appearance of constrictor activity depended on the presence of electrolyte and a reducing agent, and involved oxidation of metanephrine since the reaction occurred at the anode. The following experiments indicated that the constrictor material produced from MN or NMN was the corresponding catecholamine.

a. Fluorometric assay of MN (500 µmol l^{-1}) or NMN (500 µmol l^{-1}) in Krebs' solution containing ascorbic acid (600 µmol l^{-1}) showed that stimulation of these solutions was accompanied by an increase in fluorescence (Figure 7.2). The increase was linear over the period of stimulation (15 minutes). That the fluorescence possessed identical characteristics

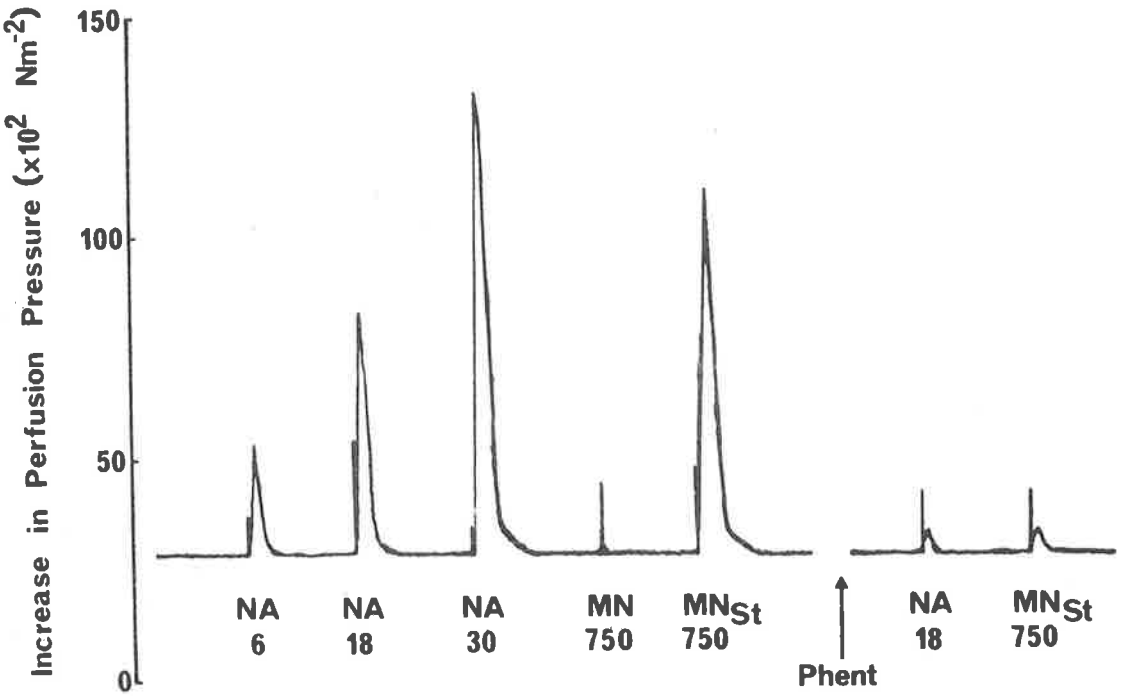

FIGURE 7.1 Responses to noradrenaline (NA), and unstimulated (MN) and stimulated (MN_{St}) solutions of metanephrine. All doses (µmol x 10^{-6}) were injected intraluminally. At the arrow, phentolamine was perfused intraluminally at a concentration of 2 µmol l^{-1}. Ordinate: increase in perfusion pressure (x10^2 Nm^{-2}).

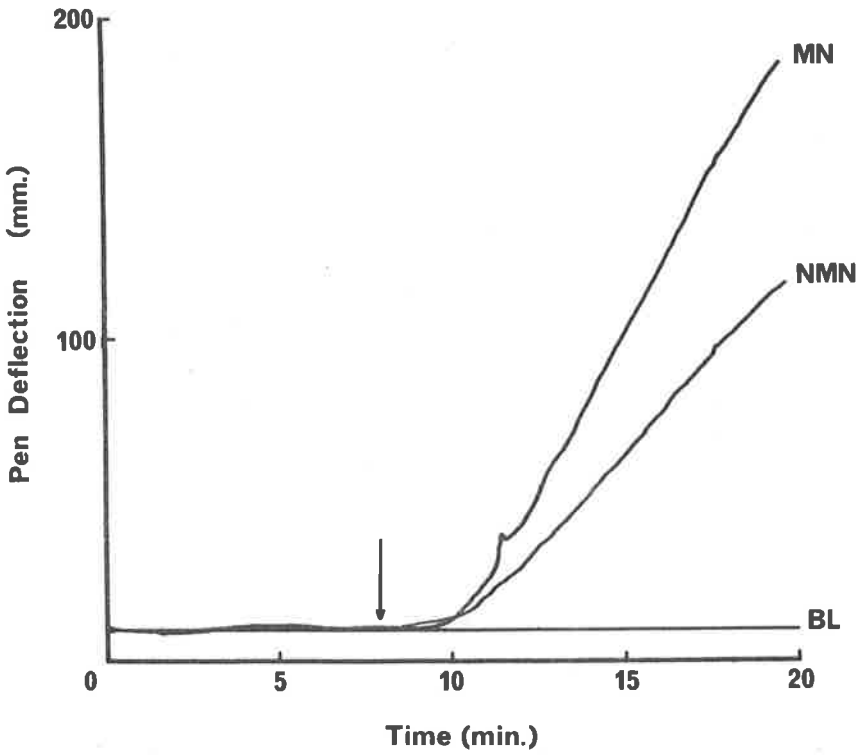

FIGURE 7.2 The trihydroxyindole fluorescence of solutions of MN (500 µmol l^{-1}) and NMN (500 µmol l^{-1}) continuously assayed before and during stimulation. The line (BL) indicated that, when the order of adding the reagents was reversed to prevent fluorescence of adrenaline, the fluorescence of the MN solution did not increase during the stimulation.

Ordinate: fluorescence measured in terms of pen deflection (mm)

to the corresponding catecholamine (A or NA) was indicated by its failure to appear when the order of adding potassium ferricyanide and the alkaline-ascorbate solution in the THI assay procedure was reversed. In this respect, pure solutions of A and NA behaved in an identical manner to the above stimulated solutions.

b. In the course of the preceding experiments it was shown that the fluorescence produced by stimulating solutions of MN was specific for A. This was achieved by replacing the ascorbic acid in the THI assay with thioglycollic acid, since the latter permitted the formation of the fluorescent lutine of NA but not A (Merrills, 1963). Under these conditions fluorescence developed when solutions of NMN were stimulated.

c. Diazonium-sprayed ion exchange paper chromatograms of stimulated solutions of MN and of NMN indicated the presence of spots which co-chromatographed with pure A and NA respectively (Figure 7.3). These spots were not detected in unstimulated solutions. Eluates of the same regions from unsprayed papers displayed vasoconstrictor activity on the rabbit ear artery and catecholamine-like fluorescence when analysed by the THI procedure. This is illustrated for NMN in Figure 7.4. In contrast, the chromatograms of stimulated and unstimulated solutions of Krebs' solution or saline containing NA and A were identical. (Note: all solutions described above contained ascorbic acid; 600 μmol l^{-1}).

FIGURE 7.3 Chromatogram of Krebs' solution containing ascorbic acid (600 μmol l^{-1}) and one or other of NA, NMN, A or MN (all 5 mmol l^{-1}), in the absence of stimulation (RHS) and following stimulation (LHS). The stimulated samples of NMN and MN show the presence of the catecholamines NA and A, respectively. The compounds were visualized after spraying with diazotized para-nitroaniline. Stimulation parameters: 70 V, 5 Hz, 30 min.

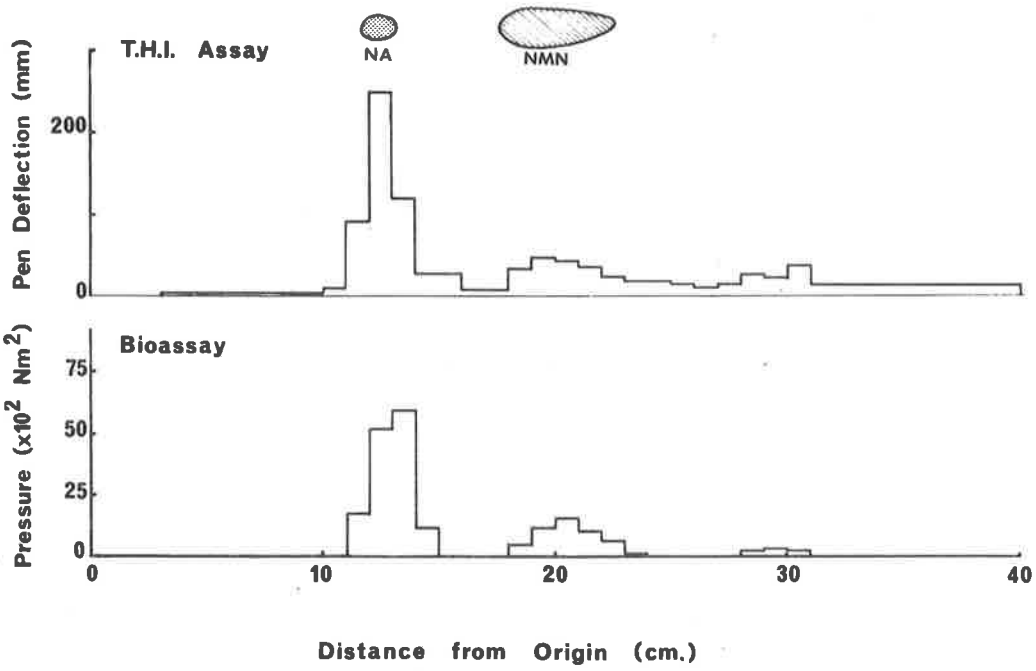

FIGURE 7.4 Illustrates the coincidence of fluorescence (determined by THI assay) and vasoconstrictor activity (measured by bioassay) in eluates of 1 cm sections of a chromatogram of a stimulated solution of NMN in saline containing ascorbic acid (600 µmol l^{-1}). The location of pure NA and NMN after visualization on the same chromatographic system is shown.

d. In the absence of ascorbic acid a red colour developed during stimulation of solutions containing NMN or MN. Colour formation was restricted to the anodic compartment. Paper chromatography of these solutions indicated that the spots which co-chromatographed with the catecholamines, as described in c. above, were now greatly decreased in intensity, both when tested by diazonium spraying and by fluorometric assay of the paper eluates. These eluates also failed to display vasoconstrictor activity.

Ascorbic acid-free solutions of NA or A also became coloured during stimulation. When analysed spectrophotometrically, and λ max values of the coloured solutions of NMN, MN, NA and A so produced were identical (Figure 7.5). These maxima were the same as those reported for adrenochrome (487 nm) and noradrenochrome (484 nm) by Heacock and Mattock (1963). Hence, the term aminochrome will be used subsequently to describe the coloured materials. It was of interest that the absorption spectrum of the aminochrome found in the stimulated MN (and also A) solution was qualitatively similar to that reported for adrenochrome by Beaudet (1951), and by Stock and Hinson (1955).

The rate of formation of aminochrome during stimulation was measured by the change in OD at 480 nm and was found to be linear with time. In ascorbic acid-free Krebs' solution containing A, NA, MN or NMN (all 500 μmol l^{-1}) the rate of aminochrome formation (OD 480 min^{-1}) was greater for A (0.062) than NA (0.054), and greater for MN (0.022) than NMN (0.014) (Figure 7.6). It will be noted that some aminochrome was formed in the solutions of A and NA prior to stimulation. This did not occur when ascorbic acid was present.

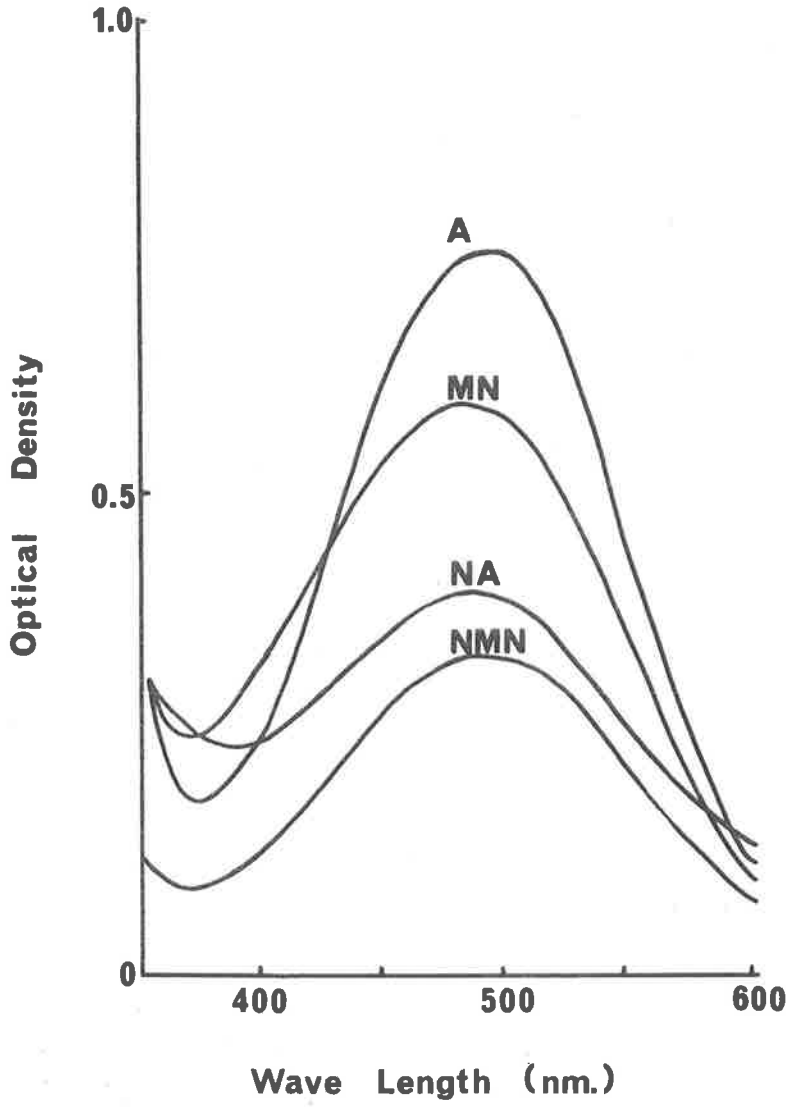

FIGURE 7.5 The visible absorption spectra of A, MN, NA, NMN (all 5 mmol l^{-1}) following stimulation in ascorbic acid-free Krebs' solution.

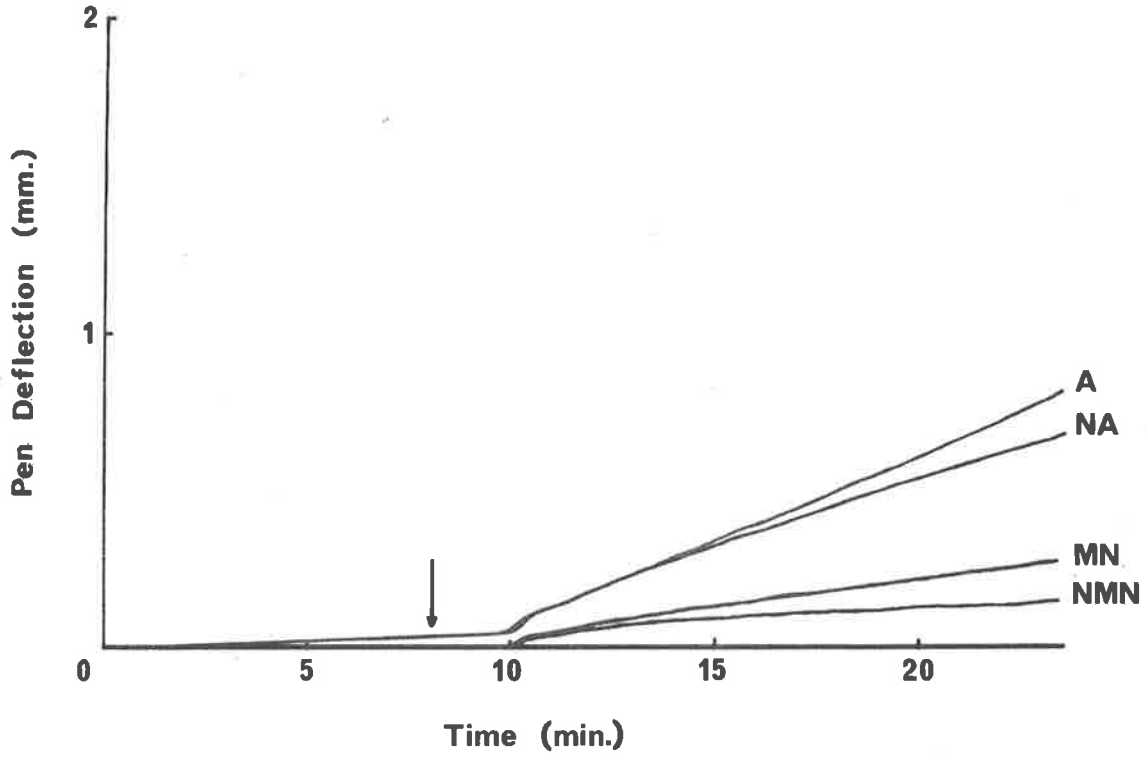

FIGURE 7.6 Increase in optical density at 480 nm of A, NA, MN and NMN (all 5 mmol l^{-1}) in Krebs' solution before and during stimulation. Stimulation was commenced at 8 minutes (shown by arrow). Note the small increase in optical density which occurred in the solutions of A and NA before stimulation.

DISCUSSION

The results indicated that the vasoactive substances which were produced when current was passed through solutions of MN or NMN resembled the corresponding catecholamines in the following respects:

a. vasoconstriction was mediated by alpha receptors,

b. the materials co-chromatographed with authentic catecholamines,

c. the materials possessed the same THI fluorescence as the catecholamines.

A scheme which accounts for the formation of a catecholamine (II) from its parent 3-methoxycatecholamine (I) is outlined in Figure 7.7. The scheme is based on the mechanism for the oxidation of guaiacol by periodate described by Adler and Magnusson (1959). Their mechanism involved oxidative demethoxylation of the methoxy group, as well as the oxidation of the phenolic hydroxyl group, to form the corresponding O-benzoquinone. The scheme shown in Figure 7.7 involves the oxidation of the 4-hydroxy,3-methoxy amine to the corresponding O-benzoquinone and the subsequent reduction of the latter to the corresponding catecholamine in the presence of ascorbic acid. In the absence of ascorbic acid cyclisation of the O-benzoquinone to the corresponding indole occurs (adrenochrome or noradrenochrome) (III), which accounted for the colour formed in the ascorbic acid-free solutions after the methoxy amines were stimulated.

The significance of the electrolytic O-demethylation of NMN or MN to the normal activity of the sympathetic neuroeffector systems

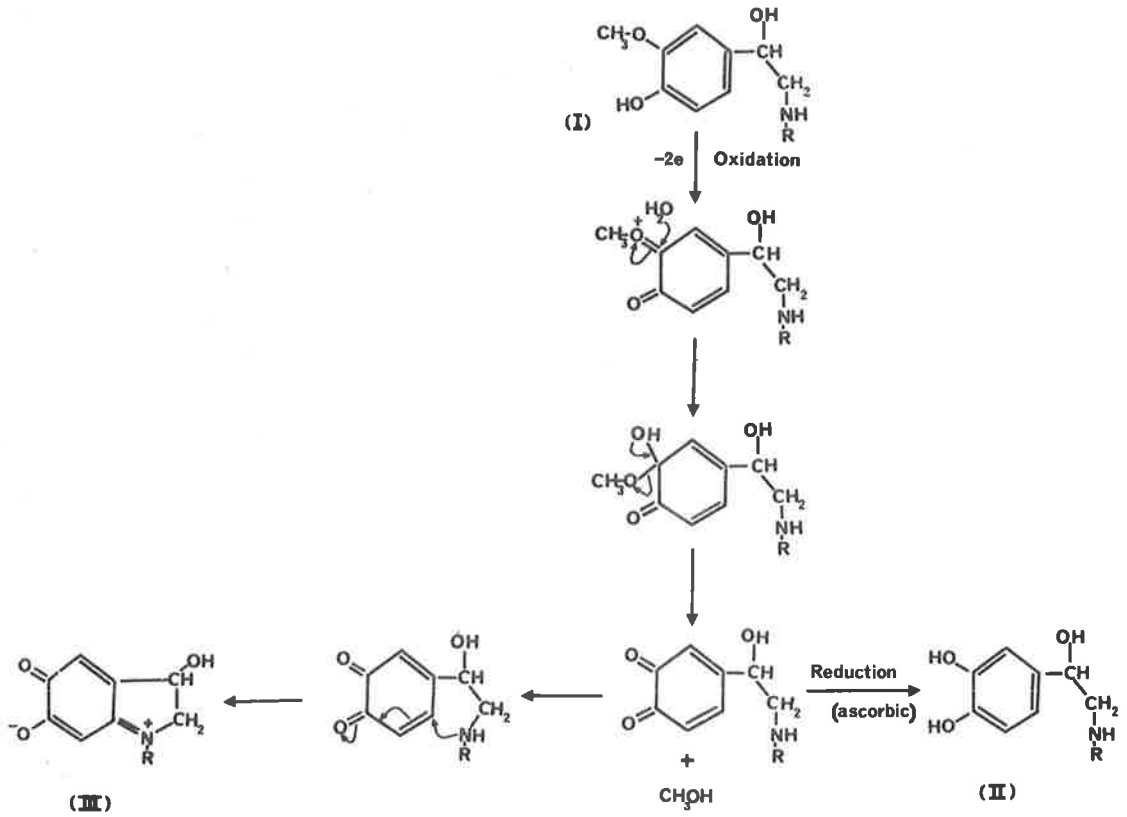

FIGURE 7.7 The proposed mechanisms involved in the oxidative demethylation of the 3-methoxy catecholamines (I) (normetanephrine R=H, metanephrine R=CH_3). This scheme accounts for the formation of the catecholamines (II) (noradrenaline R=H, adrenaline R=CH_3) in the presence of ascorbic acid and the aminochromes (III) (noradrenochrome R=H, adrenochrome R=CH_3) in its absence.

in vivo is not known. However, it has important implications for the use of the methoxy derivatives of catecholamines as pharmacological agents for inhibiting extraneuronal uptake of the latter amines. Obviously, in studies dealing with the relationships between stimulation of the nerves and the responses (functional or metabolic) of the tissues, the technique of field stimulation as a method of stimulating the intramural nerves is contraindicated where the methoxy analogues are added in high concentrations to the solutions bathing the tissue. Even in the absence of added NMN electrolytic O-demethylation may represent a significant artefact in such studies, since NMN is a major metabolite of the NA released from sympathetic nerves (Langer, 1970).

Consequently, in experiments undertaken in this thesis, when the internal-external electrode arrangement (page 2.5) was used, care was taken in selecting the position for the positive electrode. Therefore, in those experiments in which the arteries were bathed in liquid paraffin, the positive electrode was usually placed around the upper cannula. On the other hand, when the arteries were bathed in Krebs' solution and intraluminal and extraluminal collections were made, the anode was placed in the perfusate, as it was felt that the rapid passage of catecholamines or their methoxy analogues over the internal electrode was less likely to result in their destruction, than prolonged contact with the external electrode.

CHAPTER 8

FACTORS INFLUENCING THE OVERFLOW OF ENDOGENOUS NORADRENALINE AND ITS METABOLITES IN THE AQUEOUS-BATHED RABBIT EAR ARTERY

CHAPTER 8

In view of the small amount of endogenous NA released by nerve stimulation in the rabbit ear artery, isotopic techniques were developed to examine some features of transmitter release, diffusion and overflow in this tissue. Although these techniques were highly sensitive they suffered from the disadvantage that the tritium-labelled material consisted of metabolites as well as the physiologically active amine.

The purpose of the work to be described in this chapter was:

a. to examine the influence of sympathetic nerve stimulation in the rabbit ear artery on the relative overflows of tritium-labelled material into the intraluminal perfusate and into the extraluminal bathing medium,

b. to examine the influence of PBZ on the overflow of transmitter,

c. to examine the relative proportions of NA and its metabolites which effluxed from the artery, and the effect of PBZ on these proportions.

PBZ was used in view of the evidence that it inhibited neuronal and extraneuronal uptake of NA (page 1.12). Hence its use in this study provided a method of analysing the combined roles of the two uptake processes on the diffusion of transmitter across the artery wall.

Evidence will be presented which indicated that the uptake processes exerted a considerable influence on the diffusion of the transmitter in the rabbit ear artery. The results also have implications to the concept that the sympathetic nerve terminals contain presynaptic adrenoreceptors which modulate the release of NA.

METHODS

Pairs of double-cannulated arteries were mounted in small organ baths, and were incubated in either dl- or l-^{3}HNA (for details see Chapters 2 and 4).* During the post-incubation washing period (approximately 60 minutes) the outside of the artery was washed in four changes of Krebs' solution (containing the appropriate drugs), and the luminal surface was perfused with the same solution at a rate of 4.5 ± 0.5 cm^3 min^{-1}. At the end of the washing period the intraluminal flow rate was reduced to 0.23 cm^3 min^{-1} and was maintained at this rate for the remainder of the experiment.

The first collection period began 10 minutes after the flow rate was reduced. Prior to each collection the outside of the artery was washed with 20 cm^3 of Krebs' solution, and a standard volume of Krebs' solution (usually 1.5 cm^3) was added to the bath. At the conclusion of each collection period the fluid in the bath was withdrawn, its volume was determined, and a portion was added to Bray's scintillant for determination of the tritium activity. The intraluminal perfusate was collected throughout and was counted for tritium activity.

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* *The intraluminal perfusate was stopped for the duration of the incubation period (60 minutes).*

The sympathetic nerves in these arteries were stimulated at a frequency of 5 Hz for 4 minutes.

At the end of each experiment the arteries were weighed and the residual tritium content was determined after digestion of the tissues in NCS solubilizer or extraction of the catecholamines in HCl (page 4.15).

As three series of experiments will be described in this chapter, details of the electrode arrangement, the duration of the collection periods, the drug regimes used and the procedures required to separate NA and its metabolites will be given separately.

Series 1. Overflow of tritium-labelled material

Forty minutes after the incubate (dl-^{3}HNA) was removed, PBZ (33 μmol l^{-1}) was added to the extraluminal Krebs' solution bathing one artery in each pair for at least 25 minutes prior to the first collection period, but was not maintained throughout the experiment. Five coincident collections of the perfusate and bath fluid were made (Figure 8.2).

The transmural electrode arrangement described on page 2.5 was used to stimulate the sympathetic nerves.

Series 2. Overflow of tritium-labelled material, ^{3}HNA and ^{3}H metabolites

In these experiments the Krebs' solution contained ascorbic acid (300 μmol l^{-1}) to minimize the risk of oxidation of the catecholamines, and the possible electrolytic demethylation of the O-methyl derivatives as described in Chapter 7.

Based on the experience gained in the first series of experiments, the sequence and duration of the extraluminal and intraluminal collection periods were modified as illustrated in Figure 8.3 and Table 8.1. The aliquots of Krebs' solution collected were divided; one portion was added to Bray's scintillant for determination of the total tritium content, and the second was loaded onto a Dowex 50 (Na^+) ion exchange resin column for the separation of NA and its metabolites (for details, see Chapter 4).

PBZ (33 µmol l^{-1}) was added to the Krebs' solution bathing one of each pair of arteries at least 30 minutes before the first collection. Sympathetic nerves in the arteries were stimulated through electrodes arranged in the internal-external position described earlier (page 2.5). The internal electrode formed the anode.

Series 3. Overflow of NA and its metabolites

One artery in each pair was treated with cocaine (usually 90 µmol l^{-1}) 20 minutes prior to and during incubation in dl-^{3}HNA. Following the removal of the incubate PBZ (33 µmol l^{-1}) was added to the Krebs' solution washing the arteries (that is, intraluminally and extraluminally), and remained in it for the duration of the experiments.

The experimental design was similar to that described above (Series 2), except that:

a. the intraluminal collections were not fractionated,

	COLLECTION SITE					
	Extraluminal			Intraluminal		
COLLECTION	Time (min)	Volume (cm^3) *Scintillant*	*Dowex 50*	Time (min)	Volume (cm^3) *Scintillant*	*Dowex 50*
Prestimulation	8	0.3-0.5	1.0	4	1.0	-
				4	1.0	-
	Wash					
Prestimulation	8	0.3-0.5	1.0	8	0.3	1.0
	Wash					
Stimulation	8	0.3-0.5	1.0	4	0.3	1.0
Post-stimulation				4	0.3	
Post-stimulation	8	1.0	-	4	1.0	-
				4	1.0	-

TABLE 8.1 Diagrammatic representation of the collection periods (extraluminal and intraluminal) in Series 2, before, during, and after stimulation. The volumes of Krebs' solution added to Bray's scintillant and applied to the Dowex 50 columns are indicated for each collection period. The artery and organ bath were washed extraluminally between collections as indicated.

b. three extraluminal prestimulation collections were made, and for those arteries not treated with cocaine two were fractionated,

c. following separation of the NA and its metabolites by Dowex 50 chromatography, that portion which contained the deaminated metabolites was further fractionated by adsorption onto alumina (for details, see Chapter 4).

RESULTS

1. Responses to nerve stimulation

The mean increases in perfusion pressure elicited by stimulation of the sympathetic nerves in the three series of experiments are shown in Table 8.2.

It was noted that the response usually comprised an initial spike followed by a fairly well sustained increase in perfusion pressure. This response was virtually eliminated by PBZ in all arteries tested (Figure 8.1).

2. Series 1 - Overflow of tritium-labelled material

i. Untreated arteries

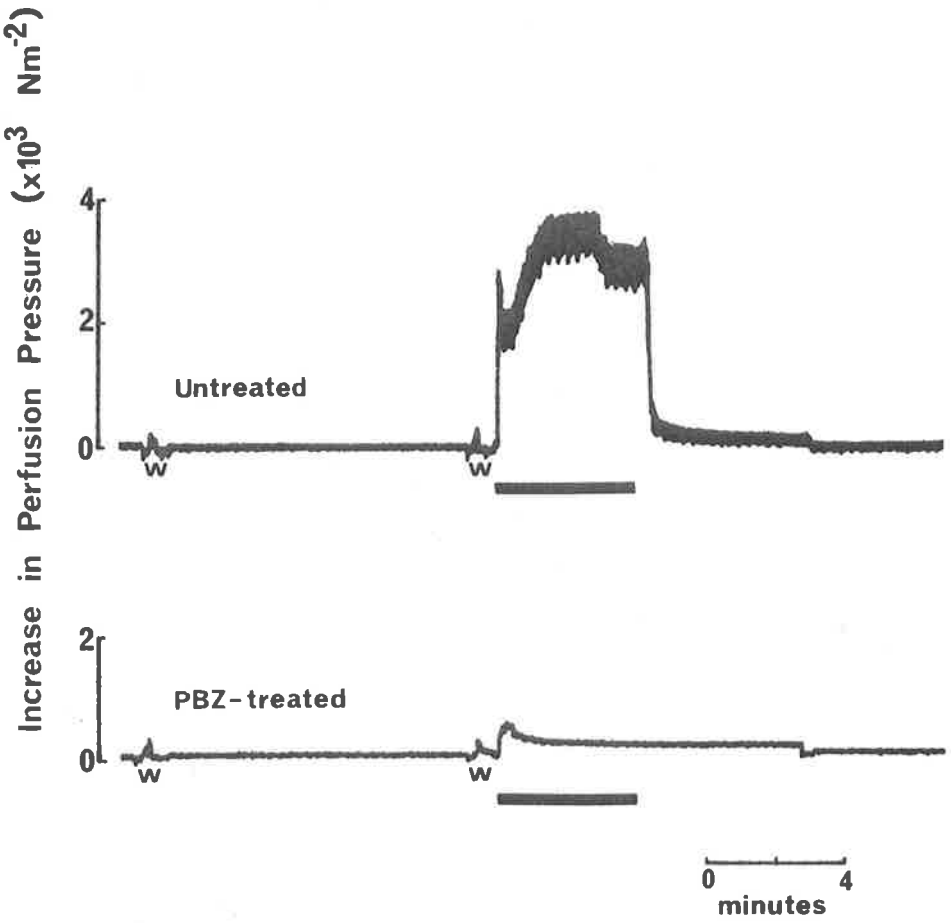
In untreated arteries, the amount of tritiated-material which overflowed into the extraluminal solution prior to stimulation was approximately twelve-fold greater than that which reached the intraluminal perfusion medium. Stimulation of the sympathetic nerves in these arteries increased the mean overflow of tritium-labelled

	SERIES 1	SERIES 2		SERIES 3
	(dl-^{3}HNA)	(dl-^{3}HNA)	(l-^{3}HNA)	(dl-^{3}HNA)
Untreated artery	28 (97-13)	49 (60-40)	75 (125-45)	-
PBZ-treated artery	4 (5-0)	3 (5-1)	2 (2-2)	*{ 1 (3-0) } 2 (4-0)
n	7	3	4	6

* *Arteries incubated in ^{3}HNA containing cocaine (90 µmol^{-1}) and then washed in PBZ prior to stimulation.*

n - represents the number of pairs of arteries used in each series.

TABLE 8.2 Mean rise (range) in perfusion pressure (x10^{2} Nm^{-2}) elicited by stimulation of the sympathetic nerves in untreated and PBZ-treated (33 µmol l^{-1}) arteries. When a biphasic response was produced the rise in perfusion pressure was calculated from the plateau of the more slowly developing second phase (Figure 8.1).



W - indicates the extraluminal washing period

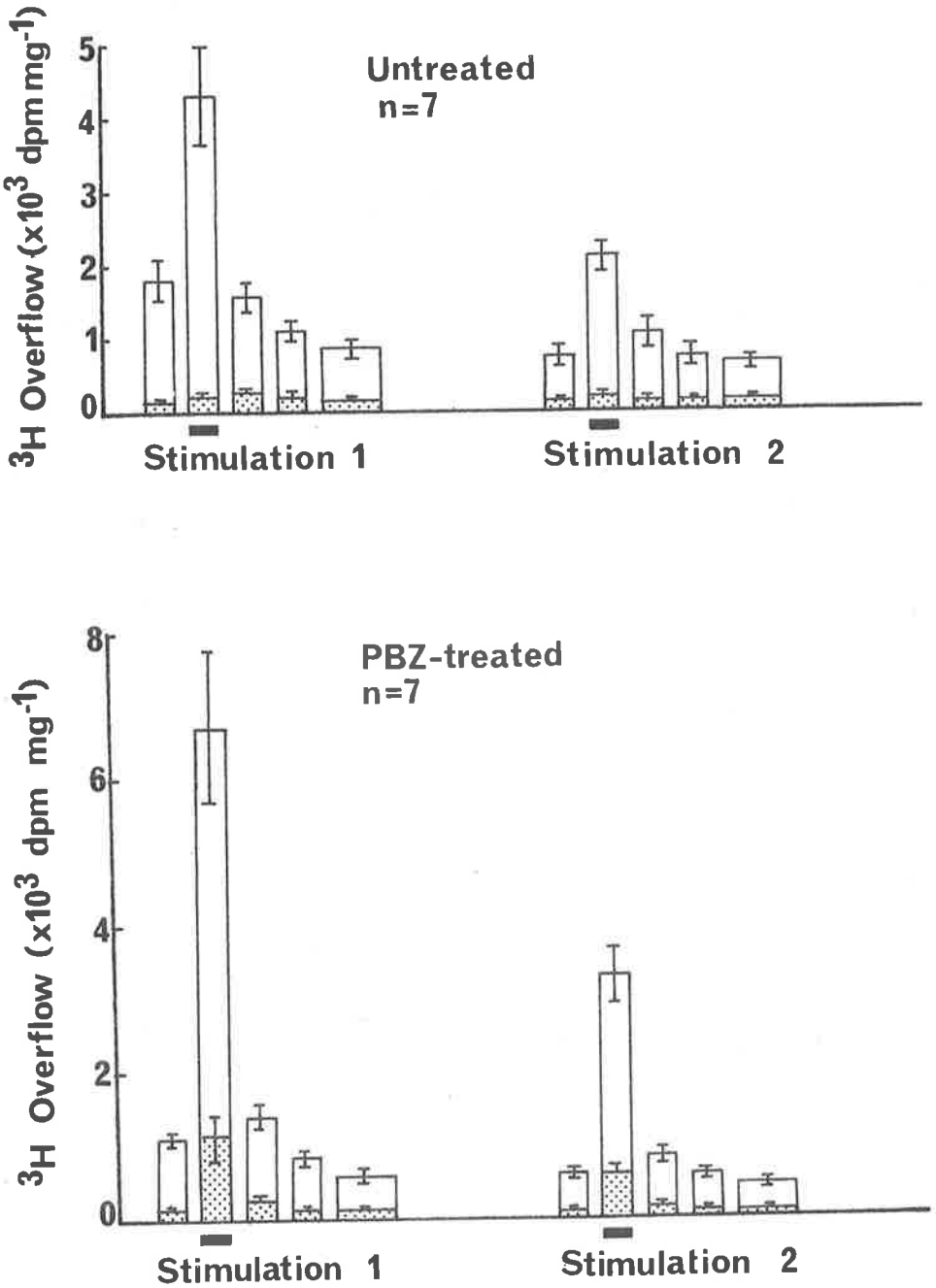
FIGURE 8.1 Typical constrictor responses of untreated and PBZ-treated rabbit ear arteries to sympathetic nerve stimulation. The response of the untreated artery usually comprised an initial spike followed by a more slowly developing second phase. PBZ (33 μmol l^{-1}) either greatly reduced or eliminated this response.

Ordinate: increase in perfusion pressure ($x10^3$ x Nm^{-2})

material into the extraluminal bathing medium by 140%, and into the intraluminal perfusate by 100%. A consistent feature of all experiments was that the extraluminal overflow reached its peak during stimulation, whereas the peak overflow into the perfusate occurred after stimulation stopped (Figure 8.2).

It will be noted that during stimulation the ratio of tritium-labelled material which overflowed extraluminally relative to that which overflowed intraluminally increased from the prestimulation value of 12.5 to 19.7, and that in the collections following stimulation it declined to approximately 6 (Table 8.3). This ratio will be referred to as the overflow ratio $\frac{\text{extraluminal}}{\text{intraluminal}}$. The mean stimulation-induced (SI) overflow ratio $\frac{\text{extraluminal}}{\text{intraluminal}}$ was 11.3 (that is, less than the ratio during the stimulation period). It should be recalled that the SI-overflow represents the difference between the overflow of tritium which occurred as a result of stimulation and the overflow which would have resulted from the resting artery during the same period. However, in these experiments, because of the possible loss of tritium during the washing period following stimulation, the ratio might be overestimated; this point will be considered later (page 8.9).

The changes which occurred during the second period of stimulation are summarized in Figure 8.2 and Table 8.3. It can be seen that the overflow of tritium prior to and during stimulation was less than that for the first stimulation period. As this decrease was more marked in the extraluminal bathing medium, the overflow ratios $\frac{\text{extraluminal}}{\text{intraluminal}}$ were consistently lower.



n - represents the number of paired arteries used
▬ - stimulation period (4 min, 5 Hz)

FIGURE 8.2 The overflow of tritium labelled material from untreated and PBZ-treated rabbit ear arteries (Series 1). The heights of the open and hatched columns represent the mean overflows into the extraluminal bathing medium and intraluminal perfusate respectively. The vertical bars represent + or ±S.E.M.

Ordinates: tritium activity (dpm mg^{-1}) in the overflow per 4 minutes of collection.

	DRUG TREATMENT			
	Untreated		PBZ-treated	
COLLECTION	Stim 1	Stim 2	Stim 1	Stim 2
Prestimulation	12.5 (14.8-10.6)	6.0 (6.1-5.9)	8.5 (9.7-7.4)	6.3 (6.8-5.9)
Stimulation	19.7 (23.2-16.7)	12.5 (14.4-10.8)	6.8 (7.9-5.8)	5.9 (6.6-5.4)
Post-stimulation 1	6.0 (6.6-5.5)	5.1 (5.7-4.6)	5.8 (6.9-4.8)	5.5 (6.1-5.0)
2	6.1 (6.5-5.7)	5.5 (5.9-5.2)	7.0 (8.8-5.7)	7.4 (7.9-6.9)
3	5.7 (5.8-5.5)	4.7 (5.0-4.5)	5.6 (6.3-4.9)	5.5 (6.1-5.0)
SI-overflow*	11.3 (14.8-8.7)	12.1 (14.0-10.4)	5.9 (6.9-5.0)	6.3 (7.8-5.1)

* *The term SI-overflow was used to denote the difference between the overflow of tritium-labelled material which occurred as a result of stimulation and the overflow which would have resulted from the resting artery during the same period.*

TABLE 8.3 The geometric means (+S.E.M. -S.E.M.) of the tritium overflow ratios $\frac{\text{extraluminal}}{\text{intraluminal}}$ prior to, during and following nerve stimulation in untreated and PBZ-treated arteries (Series 1). The SI-overflow ratios are indicated for each stimulation. Seven pairs of arteries were used in these experiments.

ii. PBZ-treated artery

In the resting artery PBZ significantly reduced the overflow of tritium into the extraluminal bathing medium, but did not alter the overflow into the lumen. Stimulation of these PBZ-treated arteries increased the mean overflow of tritium into the bathing medium by 520% (cf. 140% for untreated arteries), and into the lumen by 820% (cf. 100% in untreated arteries). The combined extraluminal and intraluminal increase was 660% (cf. 130% in untreated arteries). The above effects of PBZ can be seen also from Figure 8.2, and from the data in Table 8.3 in which the tritium overflow ratios $\frac{\text{extraluminal}}{\text{intraluminal}}$ are summarized for each collection period.

The kinetics of tritium overflow into the lumen were also affected by PBZ; the peak overflow occurred during stimulation and not during the first recovery collection as in the untreated arteries. On the other hand the kinetics of overflow into the bathing medium did not appear to be altered (Figure 8.2).

Apart from the reduced level of tritium overflow during all stages of the repeat stimulation, the effects of PBZ were similar (qualitatively and quantitatively) to those described for the first stimulation period.

3. Series 2 - Overflow of tritium-labelled material

For this series the experimental conditions were varied to avoid the loss of tritium-labelled material subsequent to the onset of nerve stimulation. Hence the collection periods were longer, and the artery and organ bath were not rinsed with Krebs' solution following stimulation.

The results with respect to total tritium overflow are presented in Figure 8.3 and in Tables 8.4 and 8.5. Some comparative figures from the first series of experiments are included in the tables.

The overflow of tritium-labelled material from the arteries incubated in dl-^{3}HNA resembled that obtained in the first series of experiments, in that:

a. the major proportion of the overflow effluxed from the tunica adventitia,

b. stimulation increased both the extraluminal and intraluminal overflows,

c. PBZ further increased the stimulation-induced overflows; the luminal overflow was enhanced proportionally more than the extraluminal,

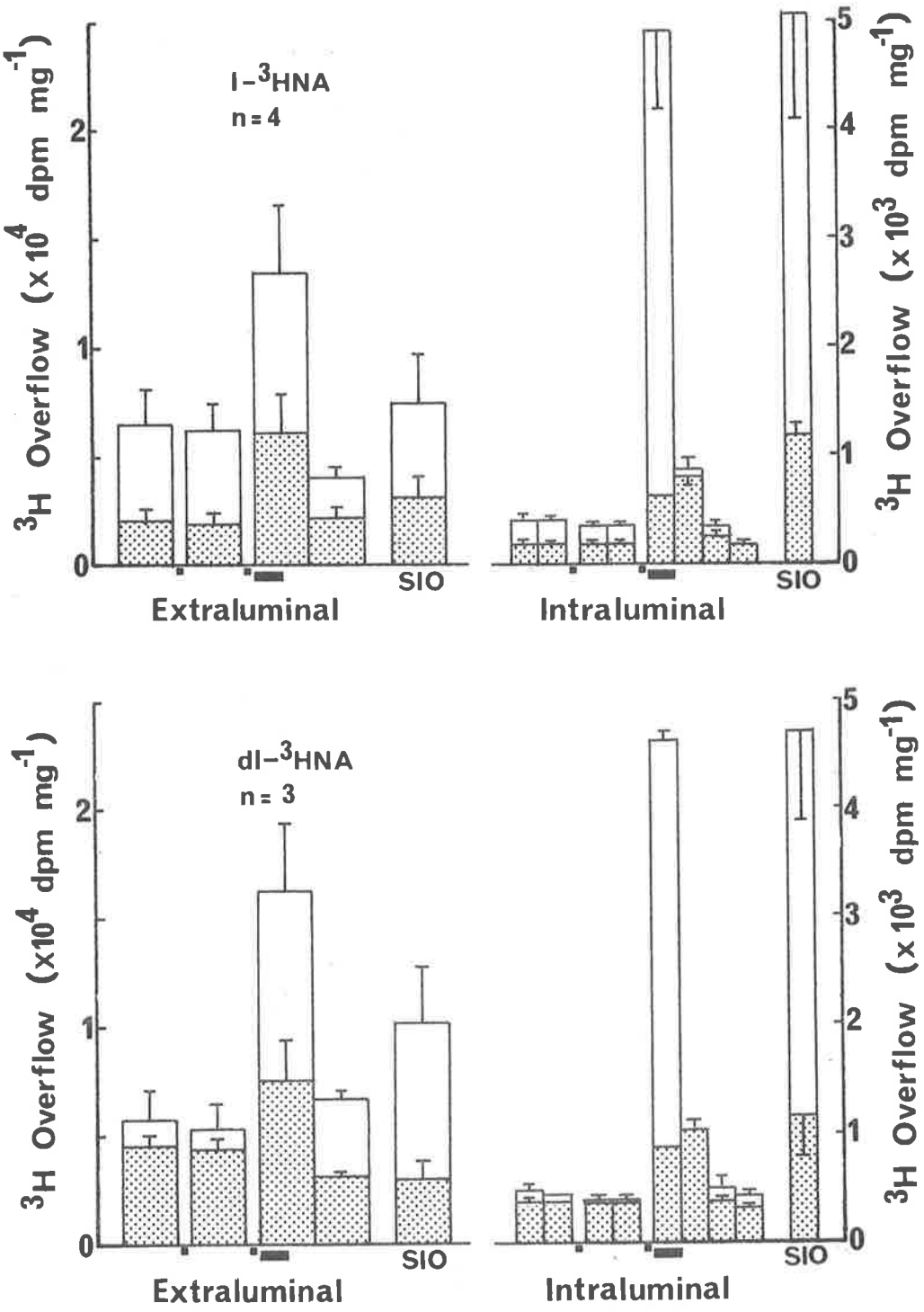
d. the maximum intraluminal overflow following the onset of nerve stimulation was reached earlier in the PBZ-treated arteries.

However, a number of differences between the first and second series of experiments were also noted. In the first series:

a. PBZ significantly reduced the efflux of tritiated material into the extraluminal bathing medium and tended to decrease the intraluminal overflow prior to nerve stimulation, whereas in the second it tended to increase the extraluminal overflow and had little or no effect on the intraluminal efflux (Table 8.5),

FIGURE 8.3 The overflow of tritium-labelled material from untreated (hatched) and PBZ-treated (open) arteries (Series 2). The extraluminal and intraluminal overflows from arteries incubated in l-^{3}HNA and dl-^{3}HNA are indicated. Two extraluminal collections of 8 minutes each were made prior to nerve stimulation and two following the onset of stimulation. Intraluminal collections were made each 4 minutes.

Ordinates: tritium activity (dpm mg^{-1}) in the extraluminal and intraluminal overflows per 4 minutes of collection.



n - represents the number of pairs of arteries used

SIO - stimulation-induced overflow (see Table 8.3)

▬ - *stimulation period (4 min, 5 Hz)*

• - *extraluminal washout*

	SERIES 1	SERIES 2	
	(dl-^{3}HNA)	(dl-^{3}HNA)	(l-^{3}HNA)
Untreated Artery			
Prestimulation	12.5 (14.8-10.6)	9.4 (10.6-8.3)	10.4 (11.2-9.7)
SI-overflow	11.3 (14.8-8.7)	5.2 (6.5-4.2)	6.1 (7.1-5.2)
PBZ-treated Artery			
Prestimulation	8.5 (9.7-7.4)	13.5 (16.4-11.2)	15.2 (20.5-11.3)
SI-overflow	5.9 (6.9-5.0)	4.4 (4.7-4.1)	3.0 (3.5-2.6)
n	7	3	4

n - represents the number of pairs of arteries used

TABLE 8.4 The geometric means (+S.E.M. -S.E.M.) of the tritium overflow ratios $\frac{\text{extraluminal}}{\text{intraluminal}}$ for the first stimulation period in untreated and PBZ-treated arteries. Prestimulation and SI-overflow ratios are included for experiments undertaken in Series 1 and Series 2. For Series 2 the ratios are indicated separately for the two incubates used.

	SERIES 1	SERIES 2	
	(dl-^{3}HNA)	(dl-^{3}HNA)	(l-^{3}HNA)
Prestimulation			
Extraluminal	0.5 (0.6-0.5)	1.4 (1.9-1.0)	3.3 (3.7-2.9)
Intraluminal	0.8 (0.9-0.7)	1.0 (1.2-0.9)	2.2 (2.8-1.8)
Extraluminal + Intraluminal	0.6 (0.7-0.5)	1.4 (1.8-1.0)	3.2 (3.5-3.0)
SI-overflow			
Extraluminal	2.2 (3.2-1.5)	3.8 (5.3-2.7)	1.9 (2.6-1.4)
Intraluminal	7.4 (10.8-5.1)	4.5 (5.6-3.7)	3.8 (4.8-3.0)
Extraluminal + Intraluminal	3.5 (5.4-2.3)	3.9 (5.3-2.9)	2.2 (2.8-1.7)
n	7	3	4

n - represents the number of pairs of arteries used

TABLE 8.5 The effects of PBZ (33 μmol l^{-1}) on the extraluminal, intraluminal and combined extraluminal and intraluminal overflow of tritium in the rabbit ear artery. The geometric means (+S.E.M. -S.E.M.) of the ratios of the overflows in $\frac{\text{PBZ-treated arteries}}{\text{untreated arteries}}$ are indicated for the prestimulation and SI-overflows.

Tests of significance: unpaired t-test

There was no significant difference between the geometric means of the SI-overflow ratios for the dl- and l-incubates in Series 2, but the extraluminal, intraluminal and combined prestimulation overflow ratios were significantly higher for the l- than the dl-incubates.

b. in both untreated and PBZ-treated arteries stimulation enhanced the extraluminal overflow relative to the intraluminal more than in Series 2 (Table 8.4),

c. PBZ tended to increase the stimulation-induced intraluminal overflow of tritium more than in the second (Table 8.5).

These differences might have been caused by the altered experimental conditions, at least for the SI-overflows, since in the first series there would have been some loss of tritium from both the adventitial and intimal surfaces during the wash period. The differences in the effect of PBZ on the efflux from the resting arteries cannot be explained in this manner, and in fact there does not appear to be a satisfactory explanation for them.

The data derived from the arteries incubated in l-^{3}HNA are summarised also in Figure 8.3 and Tables 8.4 and 8.5. It will be seen that, with one qualification, there was little difference between the characteristics of the tritium overflow from these arteries and those incubated in dl-^{3}HNA. The qualification related to the effect of PBZ on the resting overflow, which was now increased approximately three-fold above that prevailing in the untreated arteries.

4. Series 2 - Chromatographic separation of the tritium overflow

The mean tritium activity in each of the fractions separated by Dowex 50 chromatography are shown for those arteries incubated in l-^{3}HNA and dl-^{3}HNA in Tables 8.6 and 8.7. The tritium activity in each fraction was calculated as a percentage of the total tritium

	l-^{3}HNA (n=4)			dl-^{3}HNA (n=3)		
	DEAM	NA	NMN	DEAM	NA	NMN
Extraluminal Overflow						
Prestimulation	2352 (±824)	957 (±419)	*	3330 (±438)	1682 (±485)	*
Stimulation	6535 (±1538)	3823 (±1099)	850† (±236)	6040 (±961)	4956 (±1437)	1211 (±357)
Intraluminal Overflow						
Prestimulation	590† (±223)	*	*	1043 (±127)	*	*
Stimulation	618 (±52)	*	*	1078 (±219)	*	*

n - *represents the number of arteries used.*

* - *insufficient tritium activity for positive identification.*

† - *positively detected in only 3 of the 4 arteries.*

Note: Arteries used in these experiments and those in Table 8.7 were paired. That is, each pair was incubated in either dl- or l-^{3}HNA and one of each pair was treated subsequently with PBZ (33 μmol l^{-1}) while the other remained untreated.

TABLE 8.6 Dowex 50 chromatography of the extraluminal and intraluminal overflows of tritium-labelled material from untreated arteries, which had been incubated in either l- or dl-^{3}HNA, prior to and following nerve stimulation. The tritium activities (dpm mg^{-1}) ±S.E.M.s associated with the fractions containing the deaminated metabolites, NA and NMN are indicated.

	l-^{3}HNA (n=4)			dl-^{3}HNA (n=3)		
	DEAM	NA	NMN	DEAM	NA	NMN
Extraluminal Overflow						
Prestimulation	5578 (±847)	2253 (±1387)	*	5470 (±1200)	3447 (±1209)	*
Stimulation	5518 (±1076)	18125 (±3164)	*	5520 (±1044)	19833 (±5088)	*
Intraluminal Overflow						
Prestimulation	865 (±202)	*	*	581 (±60)	*	*
Stimulation	744 (±145)	4468 (±894)	*	639 (±134)	4513 (±916)	*

n - represents the number of arteries used.

* - *insufficient tritium activity for positive identification.*

Note: Arteries used in these experiments and those in Table 8.6 were paired. That is, each pair was incubated in either dl- or l-^{3}HNA and one of each pair was treated subsequently with PBZ (33 µmol l^{-1}) while the other remained untreated.

TABLE 8.7 Dowex 50 chromatography of the extraluminal and intraluminal overflow of tritium-labelled material from PBZ-treated arteries, which had been incubated in either l- or dl-^{3}HNA, prior to and following nerve stimulation. The tritium activities (dpm mg^{-1}) ±S.E.M.s associated with the fractions containing the deaminated metabolites, NA and NMN are indicated.

activity in the three fractions, and the results from both series are shown in Figure 8.4. As there was no significant difference between the distribution of NA and its metabolites for those arteries incubated in dl-^{3}HNA when compared with those incubated in l-^{3}HNA, the results will be considered together.

i. Untreated arteries

Prior to the onset of stimulation 70% of the tritiated material which overflowed into the bathing medium surrounding the untreated arteries consisted of the deaminated metabolites (DEAM) of NA (page 1.17). The remainder of the tritiated material was unchanged NA. Normetanephrine (NMN) was not detected.

Only deaminated metabolites could be detected in the small amount of tritium-labelled material which overflowed into the luminal perfusate prior to stimulation.

The composition of the extraluminal overflow following the onset of stimulation differed from the prestimulation overflow in the following respects:

a. NMN was detected and represented a small, but consistent, proportion of the overflow (11% and 9% for the dl- and l-incubates respectively),

b. the percentage of NA increased from 30% to 39% (dl) and from 22% to 33% (l),

c. the absolute amount of the deaminated metabolites increased by factors of 2.8 (dl) and 1.8 (l), although

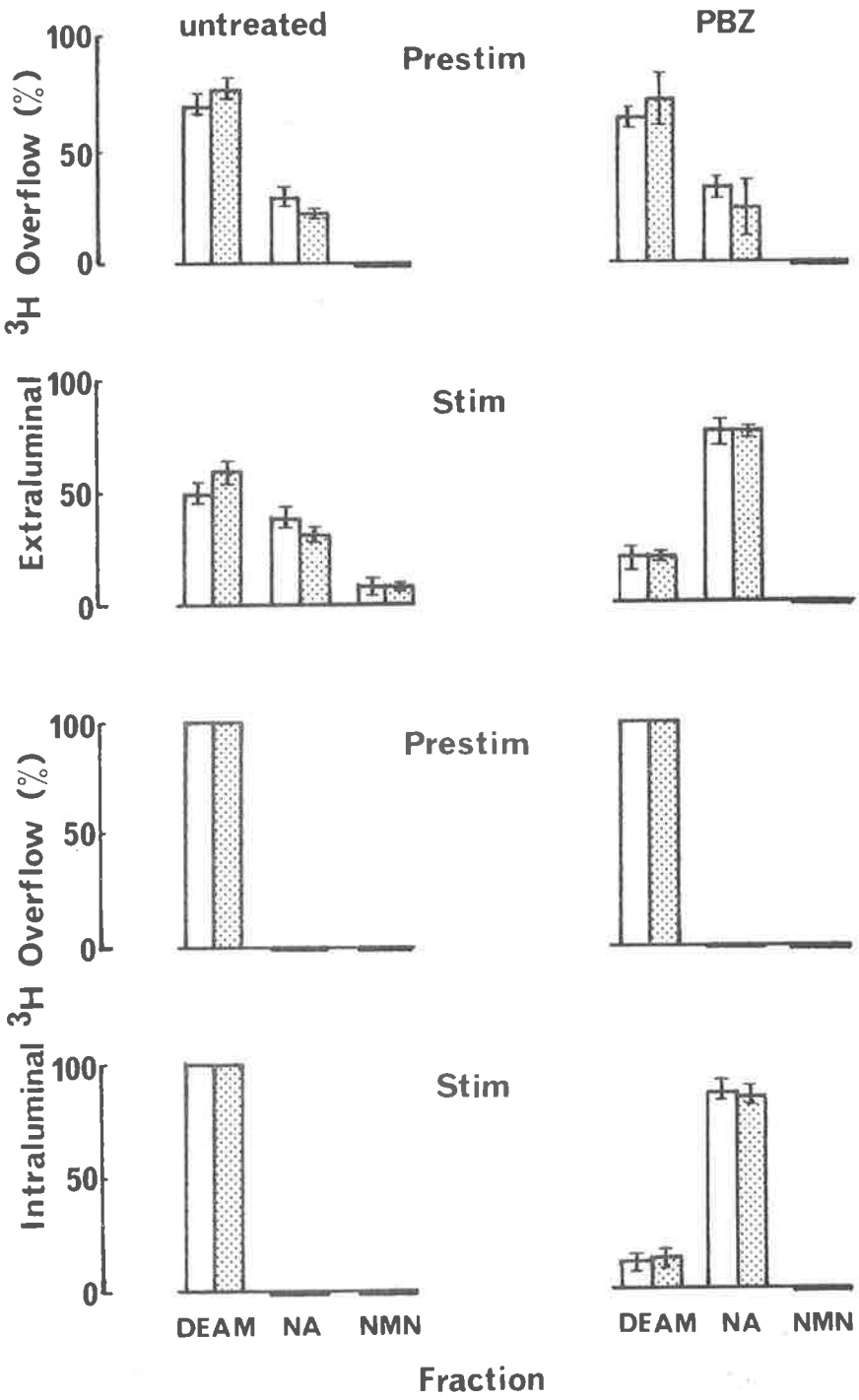


FIGURE 8.4 The percentage of deaminated metabolites, NA and NMN in the extraluminal and intraluminal overflows of tritium-labelled material prior to and as a result of nerve stimulation in untreated and PBZ-treated rabbit ear arteries. 3 pairs of arteries were incubated in dl-^{3}HNA (open columns) and 4 in l-^{3}HNA (hatched columns).

Tests of significance: unpaired t-test

There was no significant difference between the overflows from arteries incubated in dl-^{3}HNA compared with l-^{3}HNA, for any of the collections in either untreated or PBZ-treated arteries ($p > 0.05$).

the proportion of these metabolites in the total tritium overflow was less.

Analysis of the corresponding intraluminal overflow showed that, although the absolute amount of tritiated material was greater than that prior to stimulation, still only that fraction containing the deaminated metabolites could be positively identified.

ii. PBZ-treated arteries

PBZ had little effect on the composition of the extraluminal and intraluminal overflows prior to stimulation compared with untreated arteries, in the sense that DEAM formed the only fraction which could be unequivocally detected in the lumen, whereas NA and DEAM were present in the extraluminal overflow in similar proportions to those which overflowed from untreated arteries.

Stimulation had little effect on the absolute overflow of DEAM compared with the resting arteries, but greatly increased the overflow of NA so that the major fraction in both the extraluminal and intraluminal overflows was unchanged NA. NMN was not detected in either overflow.

5. Series 3 - Overflow of NA and its metabolites

In the final series of experiments, undertaken in collaboration with R.J. Head, the deaminated metabolites which overflowed from four pairs of PBZ-treated arteries were fractionated by adsorption onto Dowex 50 and then alumina. This procedure permitted the separation of NA, NMN, DOPEG, DOMA and a combined MOPEG/VMA fraction. One of each pair of arteries was treated with cocaine (90 μmol, l^{-1}) prior

to and during incubation in dl-^{3}HNA. This was done to provide a measure of the tritium-labelled materials which might have effluxed from non-neuronal structures.

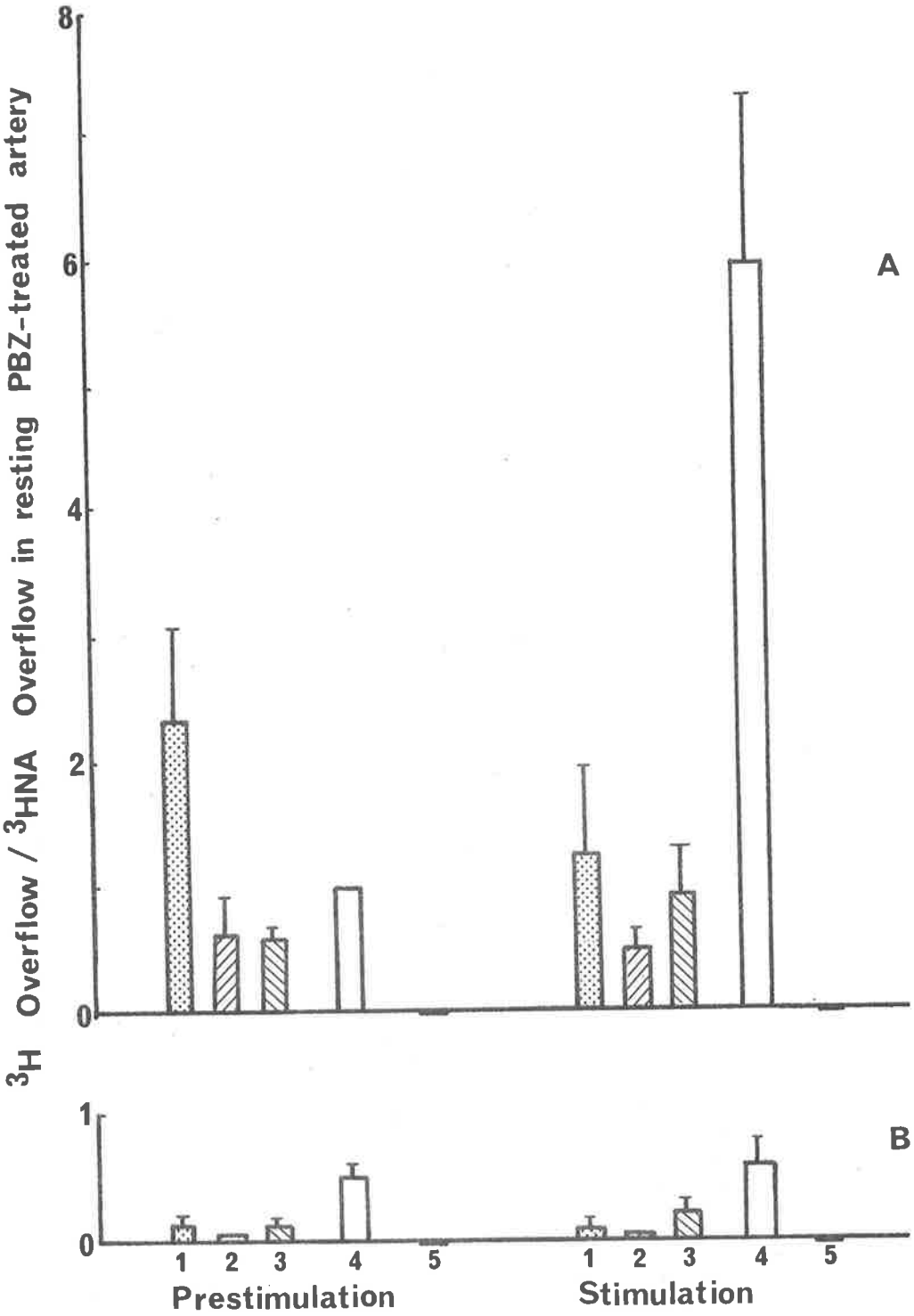
The distribution of NA and its metabolites in the extraluminal overflow from these cocaine and non-cocaine-treated arteries prior to and following the onset of stimulation is indicated in Figure 8.5.

Dowex 50 chromatography of the overflows from the non-cocaine-treated arteries indicated that the relative proportions of NA, NMN and DEAM were similar to those described previously (Figure 8.4). That is, in the resting artery the extraluminal overflow consisted of NA (22%) and DEAM (78%), while the proportion of NA in the overflow following stimulation was increased to 70% and the DEAM fraction decreased to 30%. NMN was not detected in either overflow.

The total overflow of tritium prior to nerve stimulation was reduced by over 80% in those arteries which were treated with cocaine during the period of incubation. Surprisingly, there was a measurable overflow of NA which amounted to approximately 50% of the NA overflow in the non-cocaine-treated arteries. NMN was not detected. Stimulation slightly increased the overflow of both DEAM and NA, but neither trend was significant.

Batch alumina chromatography of the fractions which contained the deaminated metabolites indicated that:

a. the resting overflow from the non-cocaine-treated arteries contained DOPEG (51%), DOMA (14%) and a residual fraction (13%) consisting of MOPEG and VMA and probably most of the impurities (page 8.19),



1 - DOPEG, 2 - DOMA, 3 - MOPEG + VMA, 4 - NA, 5 - NMN

FIGURE 8.5 The extraluminal overflow of NA and its metabolites in PBZ-treated arteries (A), and in cocaine and PBZ-treated arteries (B), relative to the overflow of NA in resting PBZ-treated arteries. The overflows were fractionated prior to (prestimulation), and following (stimulation) nerve stimulation.

b. cocaine-treatment reduced the overflow of DOPEG and DOMA in the resting arteries by over 94% and the overflow of MOPEG and VMA by about 75%,

c. in the non-cocaine-treated arteries the absolute overflows of DOPEG and DOMA were reduced by sympathetic nerve stimulation; the difference was significant for DOPEG but not for DOMA. There was also a slight increase in the amount of MOPEG and VMA which overflowed,

d. only that fraction of the overflow from the cocaine-treated arteries which contained MOPEG and VMA was increased by stimulation; DOPEG and DOMA were unaffected.

6. Summary of results

The results presented in this chapter showed that the major portion of the tritiated material which overflowed from both the resting and stimulated rabbit ear artery effluxed from the adventitial surface. PBZ, at a concentration which inhibited neuronal and extraneuronal uptake of NA, increased the overflow from both surfaces following sympathetic nerve stimulation; the effect on the intraluminal overflow was greater.

In untreated arteries the tritiated material which overflowed prior to nerve stimulation consisted mainly of the deaminated metabolites, although some NA was detected. Stimulation increased the proportion of NA in the efflux and a small amount of NMN also appeared. Only deaminated metabolites were detected in the intraluminal overflow from both the resting and stimulated arteries.

PBZ slightly increased the proportion of NA in the resting overflow and greatly increased the proportion in the overflow following stimulation.

It was shown that the major component of the DEAM fraction which effluxed from the PBZ-treated arteries was DOPEG.

DISCUSSION

As there were some quantitative differences between the effluxes of tritium-labelled material from arteries incubated in dl-^{3}HNA and l-^{3}HNA, particularly in regard to the resting effluxes, the results obtained from those arteries incubated in racemic NA will form the basis of this discussion. Where significant differences existed between the results for the two incubation media the efflux from arteries incubated in l-NA will be considered separately.

1. Overflow of tritium-labelled material

In both series of experiments reported in this study, the major proportion (approximately 90%) of the tritiated material released from the untreated artery prior to and as a result of stimulation diffused into the medium bathing the adventitial surface. Similar results have been reported by Allen et al (1973) and Bevan and Su (1974) for the rabbit ear artery, and by Su and Bevan (1971) for the rabbit pulmonary artery.

Bevan and Su (1974) used superfused ear artery segments and found that the ratio of tritium which overflowed into the extraluminal superfusate relative to the intraluminal perfusate was

increased during the first 2 minutes of stimulation from its prestimulation value of 4:1, to 22:1. In the present study, although the prestimulation ratio was greater (between 9.1 and 12.1), the ratio during the 4 minutes of stimulation (Series 1) was almost identical (20:1) with that of Bevan and Su. These workers did not report a post-stimulation ratio although they found that the ratio declined to approximately 6:1 during continuous stimulation. They explained the enhanced overflow of tritium from the adventitial surface, and the delayed overflow from the intimal surface, in terms of the higher coefficient of diffusion in the adventitia relative to the media (Bevan and Török, 1970; Bevan and Su, 1974).

Further evidence that the media represented a diffusion barrier was provided by the histochemical studies, in which it was apparent that relatively little NA reached the nerve terminals in arteries following its application to the intimal surface, unless extraneuronal uptake or the metabolizing enzymes were inhibited (Chapter 5). As it appeared likely that extraneuronal uptake of NA was one of the factors contributing to the diffusion barrier, it was of interest to determine whether this uptake exerted a similar influence on the diffusion of released NA. In this connection the results obtained with PBZ are relevant.

The effect of PBZ on the overflow of tritium from the resting artery was variable, in the sense that it decreased the intraluminal and extraluminal overflows in the first series of experiments but usually increased these overflows in the second (Table 8.5). These results are difficult to explain. However, the second series of experiments might be more reliable as the problems associated with

impurities in the incubates were better understood. The effect of PBZ in the second series of experiments was in accord with the study of Cubeddu et al (1974), in which PBZ (30 μmol l^{-1}) increased the spontaneous efflux of tritium from cat spleens which had been perfused previously with l-^{3}HNA. An important qualification, however, was that in Cubeddu's experiments PBZ was present throughout, whereas in the present study the arteries were treated with PBZ prior to, but not during, the prestimulation collection periods.

It will be noted that of all the actions of PBZ, namely inhibition of the neuronal uptake of NA, of the extraneuronal uptake and of the pre- and post-synaptic alpha receptors, only its action on extraneuronal uptake could influence the relative extraluminal/intraluminal overflows in the resting artery. Hence it was significant that this ratio was not altered by PBZ, as it implied that extraneuronal uptake of NA was not a factor limiting the resting efflux of tritium. It must be remembered, however, that only approximately 30% of the tritiated material which effluxed from the neuronal stores was NA.

In contrast, the effect of PBZ on the efflux of tritium following nerve stimulation provided evidence that extraneuronal uptake influenced the diffusion of NA through the artery wall. Under these conditions PBZ increased the extraluminal overflow between 2 and 3-fold, the intraluminal overflow approximately 5-fold, and increased the rate of intraluminal overflow. Whilst the enhanced extraluminal overflow could be attributed to the inhibitory action of PBZ on the neuronal uptake system, the

other changes supported the concept that extraneuronal uptake constituted one component of the NA diffusion barrier in the tunica media. An added complication in these experiments was the possible effect of PBZ in limiting the constrictor response of the artery during sympathetic nerve stimulation. The influence of constriction on the diffusion of NA will be considered in detail in Chapter 11.

As mentioned at the outset of this discussion, differences between the overflow of tritium from arteries incubated in dl- and l-^{3}HNA would be considered separately. One such difference was the somewhat greater effect of PBZ on the resting efflux from arteries incubated in l-NA (Table 8.5). A possible explanation for this finding was that the retention of NA in the storage granules was greater in those arteries incubated in l- than dl-NA, and that PBZ increased the leakage of stored NA into the axoplasm. Although the present study did not include an evaluation of the amount of NA retained in the granules following incubation of the arteries in l- and dl-NA, vesicular retention is thought to be stereospecific for l-NA (page 1.10). The metabolite studies, in which it was shown that the relative proportions of NA and its metabolites which overflowed from untreated arteries were not altered by PBZ, supported the concept that the enhanced overflow of tritium in the presence of PBZ was a result of an enhanced neuronal efflux rather than a consequence of inhibition of uptake. This enhanced efflux could be explained if PBZ increased the leakage of NA from the storage granules into the axoplasm, from which it diffused either as unchanged NA or its deaminated metabolites.

2. Composition of the overflow

In the absence of drug treatment the deaminated metabolites constituted the largest fraction of the extraluminal overflow, and the only fraction which could be unequivocally detected in the intraluminal overflow from arteries prior to and as a result of nerve stimulation. The nature of these deaminated metabolites was not determined, although DOPEG was the principal metabolite of both the resting and stimulated overflows in PBZ-treated arteries. That this glycol was also the major constituent of the deaminated metabolites in untreated arteries was suggested by the findings of Head (1976). In his study Head incubated ear artery segments in ^{3}HNA, and found that in the second 30 minutes' wash after incubation approximately 70% of the tritium-labelled material which overflowed into the bathing medium was DOPEG; the remainder was NA. DOPEG was also found to be the major metabolite in the resting efflux from the rat vas deferens (Langer 1970; Graefe et al, 1973), cat spleen (Cubeddu et al, 1974), guinea pig atrium (Tarlov and Langer, 1971) and rabbit aorta (Levin, 1974).

The present study indicated that DOPEG was formed intraneuronally. The evidence for this was that the amount of DOPEG which overflowed from the PBZ-treated arteries was greatly reduced in paired arteries which were incubated in the presence of the neuronal uptake inhibitor cocaine. In another study Levin (1974) separated the tunica adventitia from the tunica media in the rabbit aorta, incubated them separately in NA, and demonstrated that DOPEG formed in the adventitia (which contains nerve terminals) but

did not form in the media in which these terminals are absent. The metabolic study of Head (1976) also indicated that the origin of DOPEG in the rabbit ear artery was neuronal.

Although a fraction containing MOPEG and VMA was apparently isolated from the efflux in PBZ-treated arteries, it was felt that this might have reflected an artefact of the column fractionation procedure employed to separate the metabolites. This was confirmed by Head (1976) in experiments in which the metabolites were also separated by a thin layer chromatographic procedure. Partly as a result of these experiments Head concluded that COMT appeared to be an extraneuronal enzyme in the ear artery.

The non-appearance of NA in the intraluminal overflow from untreated arteries prior to stimulation could be attributed to its uptake, binding and metabolism in smooth muscle cells; however it could also be explained by the small influx of tritium-labelled materials into the lumen. In fact, if the ratio of the tritium activity in the lumen attributable to NA relative to the total tritium activity ($\frac{^3HNA}{^3H \text{ activity}}$) were the same as the equivalent ratio for the extraluminal overflow, then the expected activity of the ^3HNA (based on the total intraluminal overflow of tritium) would be below the limits of detectability. In contrast, although the overflow of tritiated material into the lumen following stimulation was still small, there was sufficient for the detection of NA if the ratio $\frac{^3HNA}{^3H \text{ activity}}$ were the same as that for the extraluminal overflow. That it was not detected suggested that the diffusion of NA across the media was impeded. Further support for this argument was provided by the findings that in

PBZ-treated arteries the relative proportions of NA and the deaminated metabolites in the extraluminal and intraluminal overflows following nerve stimulation were similar.

In the present experiments stimulation increased the extraluminal overflow of deaminated metabolites in untreated arteries at least two-fold, and the overflow of NA between three and four-fold. NMN was detected unequivocally, and constituted approximately 10% of the total overflow. In PBZ-treated arteries the overflow of deaminated metabolites was not increased by stimulation, NMN was not detected, and the NA overflow was six to eight-fold greater than the prestimulation level. The increased proportion of deaminated metabolites in untreated arteries following nerve stimulation can be explained in several ways. The simplest explanation is that during stimulation part of the released NA was taken up into the cytoplasm of the neurone, where it was metabolized to DOPEG which subsequently diffused through the neuronal membrane into the extracellular space. However, this reasoning does not account for the presence of deaminated metabolites in the overflow following stimulation in PBZ-treated arteries. A more likely explanation for the presence of these metabolites was that a small amount of NA leaked from the storage vesicles into the axoplasm, where it was deaminated by MAO and from which it effluxed as the metabolite. If this explanation is correct then the results are also in accord with the postulate that NA is not released into the cytoplasm of the neurone, but is released at its membrane by a process of exocytosis.

Further evidence that the extraneuronal uptake of NA contributed to the diffusion barrier in the artery wall was provided by the findings of the metabolite studies, in which it was shown that PBZ prevented the formation of NMN and permitted the detection of NA in the intraluminal overflow.

Although there was no significant difference between the relative amounts of the deaminated metabolites, NA and NMN which overflowed from arteries incubated in dl-NA compared with l-NA, the proportion of deaminated metabolites in the overflow from arteries incubated in the laevo-isomer was slightly greater. As DOPEG constituted 70% of the deaminated metabolites (in PBZ-treated arteries) it was of interest that Head (1976) found that the amount of DOPEG which accumulated in the extraluminal bathing medium during incubation of ear artery segments with l-NA was greater than with dl-NA. Similar observations were reported by Graefe et al (1973) in the rat vas deferens and by Levin (1974) in the rabbit aorta. It is thought that this might be the result of the greater vesicular binding of l-NA relative to d-NA.

SUMMARY

The results presented in this chapter indicated that prior to and as a result of stimulation of the sympathetic nerves, the major portion of the transmitter and its metabolites released from the nerves diffused through the tunica adventitia of the artery. Considerably less reached the lumen following diffusion across the tunica media and, in fact, only the deaminated metabolites reached the lumen in sufficient quantity to be detected unequivocally.

The distribution of metabolites which effluxed from the untreated and PBZ-treated arteries demonstrated qualitatively that neuronal and extraneuronal uptake of NA occurred following release of the transmitter. However, the action of the uptake systems could not be quantified because of the proposed effect of PBZ on the inhibitory presynaptic alpha receptors (page 1.5). The results emphasized the importance of MAO in the metabolism of released transmitter, and the apparent lesser role of O-methylation as assessed by the small amounts of NMN which appeared in the overflow.

Further evidence that uptake of NA by the smooth muscle cells limited its diffusion across the tunica media was presented. However, even when this uptake system was blocked, considerably more NA effluxed through the adventitia than the media. It was suggested that this phenomenon was a manifestation of the greater coefficient of diffusion in the tunica adventitia and the arrangement of the sympathetic nerves at the adventitia-media border.

CHAPTER 9

FACTORS INFLUENCING THE INTRALUMINAL OVERFLOW OF ENDOGENOUS NORADRENALINE AND ITS METABOLITES IN THE OIL-IMMERSED RABBIT EAR ARTERY

CHAPTER 9

The study undertaken in the preceding chapter indicated that inhibition of the neuronal and extraneuronal uptake of NA increased the overflow of unchanged NA from the rabbit ear artery following its release by sympathetic nerve stimulation, and diminished the overflow of metabolites. In fact when the uptake processes were inhibited, the overflow of metabolites in the resting artery was not increased by stimulation. These results might have been complicated by an action of PBZ on the postulated presynaptic inhibitory feedback mechanism, so that facilitation of transmitter release, as well as inhibition of the uptake systems, might have contributed to the enhanced overflow of NA.

The main purpose of the experiments described in the present chapter was to define more completely the relative roles of the two uptake processes in modifying the diffusion of transmitter across the artery wall. For these experiments cocaine was used to inhibit the neuronal uptake system, DOCA to inhibit the smooth muscle uptake system and PBZ to inhibit both simultaneously. As PBZ also prevented vasoconstriction, the effects of cocaine and DOCA were examined in the presence of the post-synaptic alpha receptor antagonist, phentolamine. Hence it was possible to investigate the effects of cocaine and DOCA on the release and diffusion of transmitter without the complication of increased vascular tone. As phentolamine also antagonized the presynaptic alpha receptors in the rabbit ear artery

(Story, D.F; personal communication), it was used to investigate the effect of the presynaptic inhibitory feedback mechanism on the release of NA.

The influence of collagen and elastic tissue on the diffusion of NA in the artery wall was investigated using oxytetracycline, a drug which according to Powis (1973) inhibited the binding of NA to these tissues.

In one experiment the tritium-labelled material which overflowed from the arteries was separated by Dowex 50 column chromatography into fractions containing the deaminated metabolites (DEAM), NA and NMN. Thus it was possible to gain a more complete understanding of the influences of the uptake processes and the intracellular enzymes, MAO and COMT, on the diffusion of NA in this vascular preparation.

A further complication in the experiments described in the preceding chapter was that the major portion of the released transmitter and its metabolites diffused rapidly through the tunica adventitia of the artery, and overflowed into the extraluminal bathing medium, whereas considerably less diffused through the tunica media to reach the lumen. Because the activity of the tritium-labelled material which overflowed intraluminally was so low it seemed worthwhile to restrict the direction of the diffusion of transmitter after its release, so that it became unidirectional from the nerve terminals to the lumen. As NA and its major metabolites did not appear to be soluble in liquid paraffin, a technique was devised to permit this unidirectional diffusion in which the outside surface

of the artery was bathed in liquid paraffin. Previously, Kalsner and Nickerson (1968) had described the use of liquid paraffin and reported that:

a. the responses of aortic strips to NA were unchanged by paraffin, and

b. providing adequate gas exchange was maintained, these strips could be immersed in paraffin for at least 120 minutes without change in basal tone.

METHODS

Pairs of arteries were placed in small organ baths and after equilibration were incubated in ^{3}HNA (usually dl; see text) for 60 minutes as described previously (pages 2.2 and 4.2). The intraluminal perfusate (flow rate - 4.5 ± 0.5 cm^3 min^{-1}) was stopped for the period of incubation.

Subsequently, the arteries were washed in Krebs' solution for 40 minutes before the addition of drugs. Cocaine, DOCA, phentolamine and oxytetracycline were added to the extraluminal and intraluminal Krebs' solution, and remained in the intraluminal perfusion medium throughout the experiment. PBZ was added extraluminally only. After at least 20 minutes the intraluminal flow rate was reduced to 0.23 cm^3 min^{-1}, and 5 minutes later the aqueous bathing medium was replaced with gassed, liquid paraffin at 37°C. After a further 5-10 minutes the perfusate collections commenced. Throughout the experiment the perfusate was collected at regular intervals in vials containing Bray's scintillant, and the tritium activity in each was

determined (page 4.12). The sympathetic nerves in the arteries were stimulated by square wave pulses at a frequency of 5 Hz for a period of 5 minutes. The internal-external electrode arrangement was used (page 2.5).

TERMINOLOGY

The term stimulation-induced overflow (SIO) was used to denote the activity of the tritium-labelled material which overflowed into the luminal perfusate in the 20 minutes following the onset of stimulation. It was calculated from the formula:

$$SIO = S + PS - R$$

where:

S = tritium activity of the overflow (dpm mg^{-1}) during the 4 minutes' period of stimulation

PS = tritium activity of the overflow (dpm mg^{-1}) during the 16 minutes' period after stimulation ceased,

R = tritium activity of the overflow expected during the 20 minutes' period prior to stimulation. This figure was derived from the overflow from the resting artery which occurred in 8 minutes. Such an extrapolation was justified as observations confirmed that the rate of tritium overflow from resting arteries, approximately 70 minutes after incubation, was relatively constant.

RESULTS

1. Characteristics of the paraffin-bathed artery

The effects of paraffin on the constriction produced by nerve stimulation, and on the overflow of tritium-labelled material into the perfusate, were investigated in seven untreated arteries. Each artery was stimulated three times. For the first and third stimulation periods paraffin was present extraluminally. During the second stimulation the artery was bathed in Krebs' solution.

The mean rise in perfusion pressure recorded during stimulation of the Krebs' bathed arteries was not significantly different from that recorded when the arteries were bathed in liquid paraffin (Table 9.1). Furthermore, the shapes of the responses were qualitatively similar. Typical responses are indicated in Figure 9.1.

The overflow of tritium-labelled material into the luminal perfusate is indicated for each stimulation period in Figure 9.2. For comparison, the overflow of tritium-labelled material into the luminal perfusate is indicated for another series of experiments in which paraffin was maintained in contact with the outside of the arteries for all three periods of stimulation. It will be seen that paraffin considerably enhanced the overflow of tritium-labelled materials into the lumen. The kinetics of the overflow of tritium-labelled material in paraffin-bathed arteries resembled the luminal overflow in aqueous-bathed preparations, in that the peak overflow was not observed until after the period of stimulation (Figure 8.3). However, the rate of the subsequent decline from this peak was slower in the paraffin-bathed arteries.

ARTERY	BATHING MEDIUM		
	Paraffin	Krebs' solution	Paraffin
1	30.0	33.0	30.0
2	5.1	6.1	3.3
3	15.7	16.0	16.3
4	0.5	1.3	1.5
5	0.7	0.3	0.3
6	8.8	8.4	6.0
7	2.8	1.9	2.0
Mean	9.1	9.6	8.5
(±S.E.M.)	(±4.3)	(±4.4)	(±4.1)

TABLE 9.1 Rise in perfusion pressure ($x10^3$ Nm^{-2}) during stimulation of 7 arteries alternatively bathed in liquid paraffin, Krebs' solution and liquid paraffin.

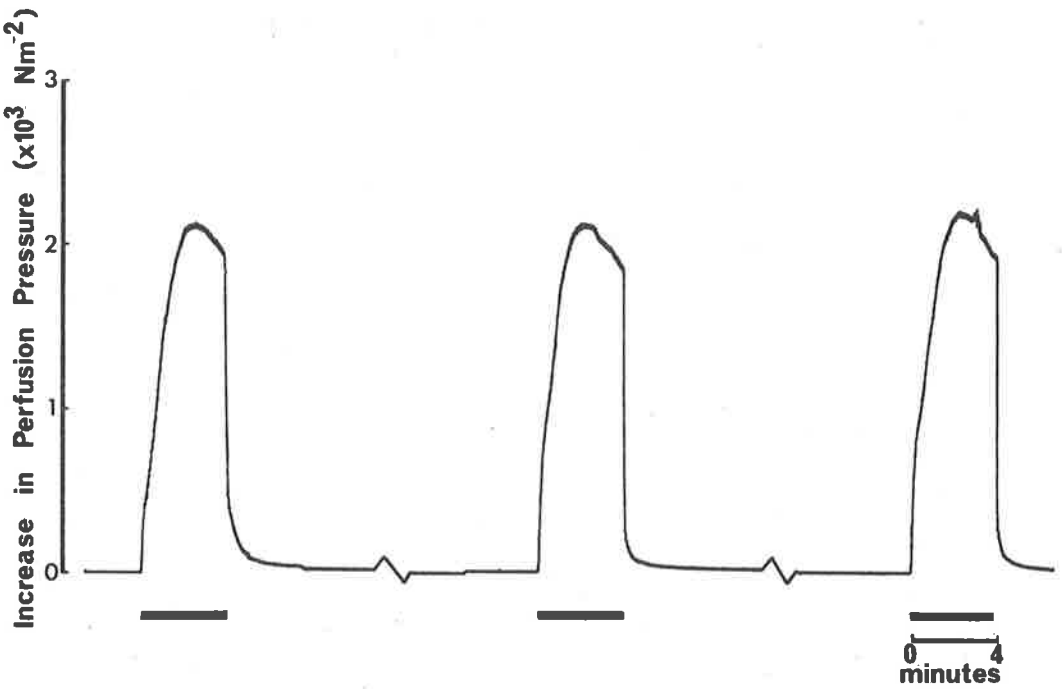
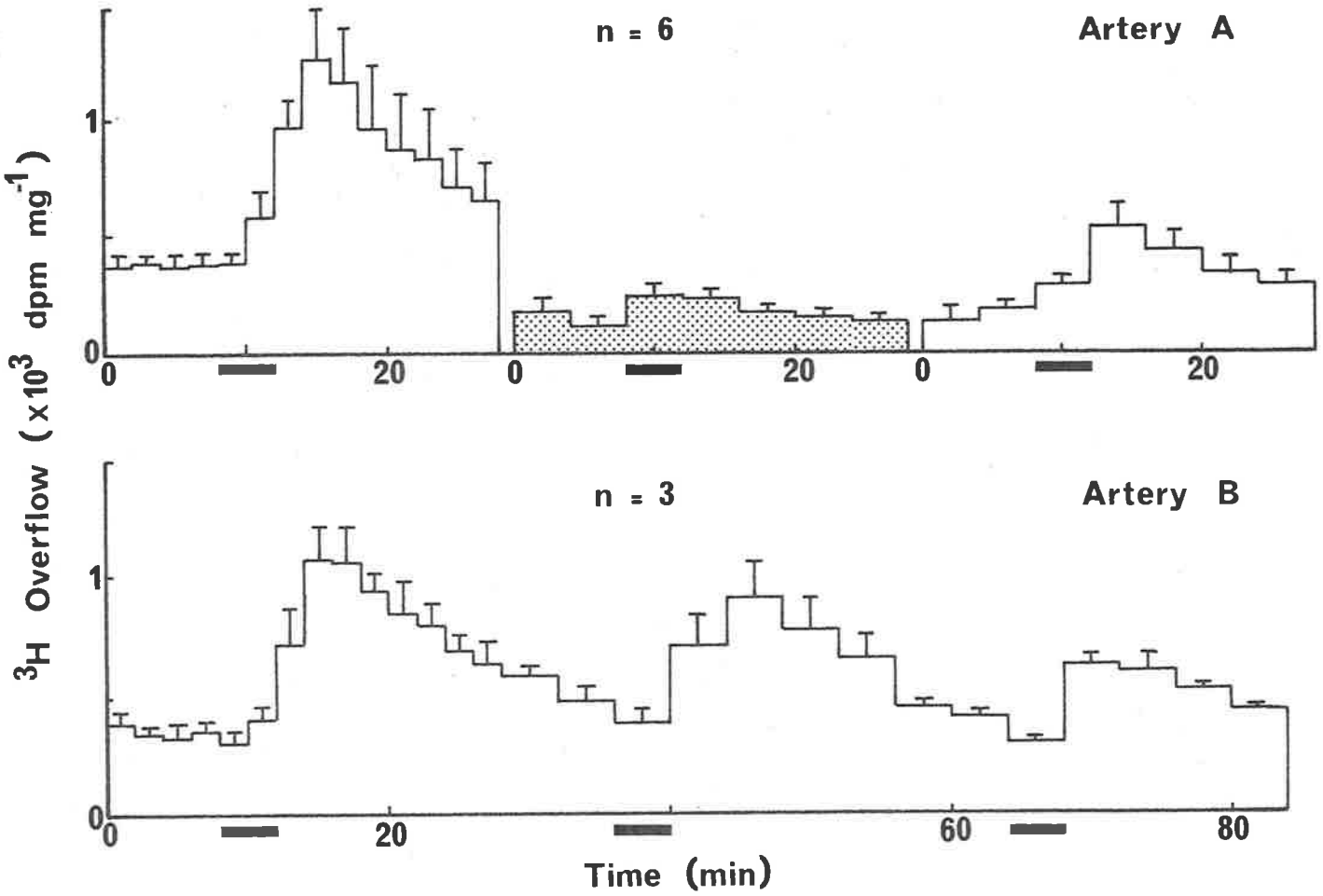


FIGURE 9.1 The effect of liquid paraffin on the constrictor response of the rabbit ear artery to nerve stimulation. For the first and third stimulations the adventitial surface of the artery was bathed in liquid paraffin, and for the second it was bathed in Krebs' solution.

Ordinate: rise in perfusion pressure ($x10^3$ Nm^{-2})



n - represents the number of arteries used
▬ - stimulation period (4 min; 5 Hz)

FIGURE 9.2 The mean overflow of tritium-labelled material (+S.E.M.) in untreated arteries prior to and as a result of nerve stimulation. The figure illustrates the effect of replacing the extraluminal paraffin with Krebs' solution (hatched profile, Artery A). Extraluminal paraffin was maintained for all stimulation periods in Artery B. Arteries A and B were unpaired, and were incubated in different batches of dl-^{3}HNA.

Ordinate: tritium activity (dpm mg^{-1}) per 2 minutes of collection

Abscissa: duration of collection (min)

Comparison of the overflow during the second period of stimulation with the overflow during the first and third periods showed that the effects of paraffin did not persist after the paraffin had been washed out, that is the effects of paraffin on the luminal overflow appeared to be completely reversible.

Except where an aqueous phase was visualized in the liquid paraffin, there was no evidence of any significant loss of tritiated material into the extraluminal oil. Thus in 12 arteries, the tritium content of the extraluminal bathing medium was less than 0.15% of the total overflow into the luminal perfusate during comparable periods.

It was concluded from these experiments that the use of paraffin as a bathing medium did not adversely affect the behaviour of the isolated rabbit ear artery. Furthermore, it increased the magnitude of the luminal overflow of tritiated material to a level which permitted quantitative analysis.

2. Effects of drugs on the overflow of tritium-labelled material

i. PBZ

Figure 9.3 summarizes experiments in which the luminal overflow of tritium in PBZ-treated arteries was compared with paired, untreated arteries. It will be noted that PBZ enhanced the resting overflow by approximately 30%. Following the onset of stimulation PBZ greatly increased the rate of tritium overflow, so that the peak overflow occurred earlier than in the untreated control artery segments (namely, during the second half of the stimulation period). The

FIGURE 9.3 The effects of PBZ (33 μmol l^{-1}) on the mean overflow of tritium-labelled material into the lumen of arteries prior to, during the following nerve stimulation. For convenience some standard errors of the means have been omitted. Arteries were incubated in dl-^{3}HNA or l-^{3}HNA as indicated.

n - number of paired arteries

R - estimated mean overflow in the 20 minutes' period prior to stimulation

SIO - stimulation-induced overflow (for details of R and SIO, see page 9.4)

▬ - stimulation period (4 min; 5 Hz)

Ordinates: (left) tritium activity (dpm mg^{-1}) per 2 minutes of collection

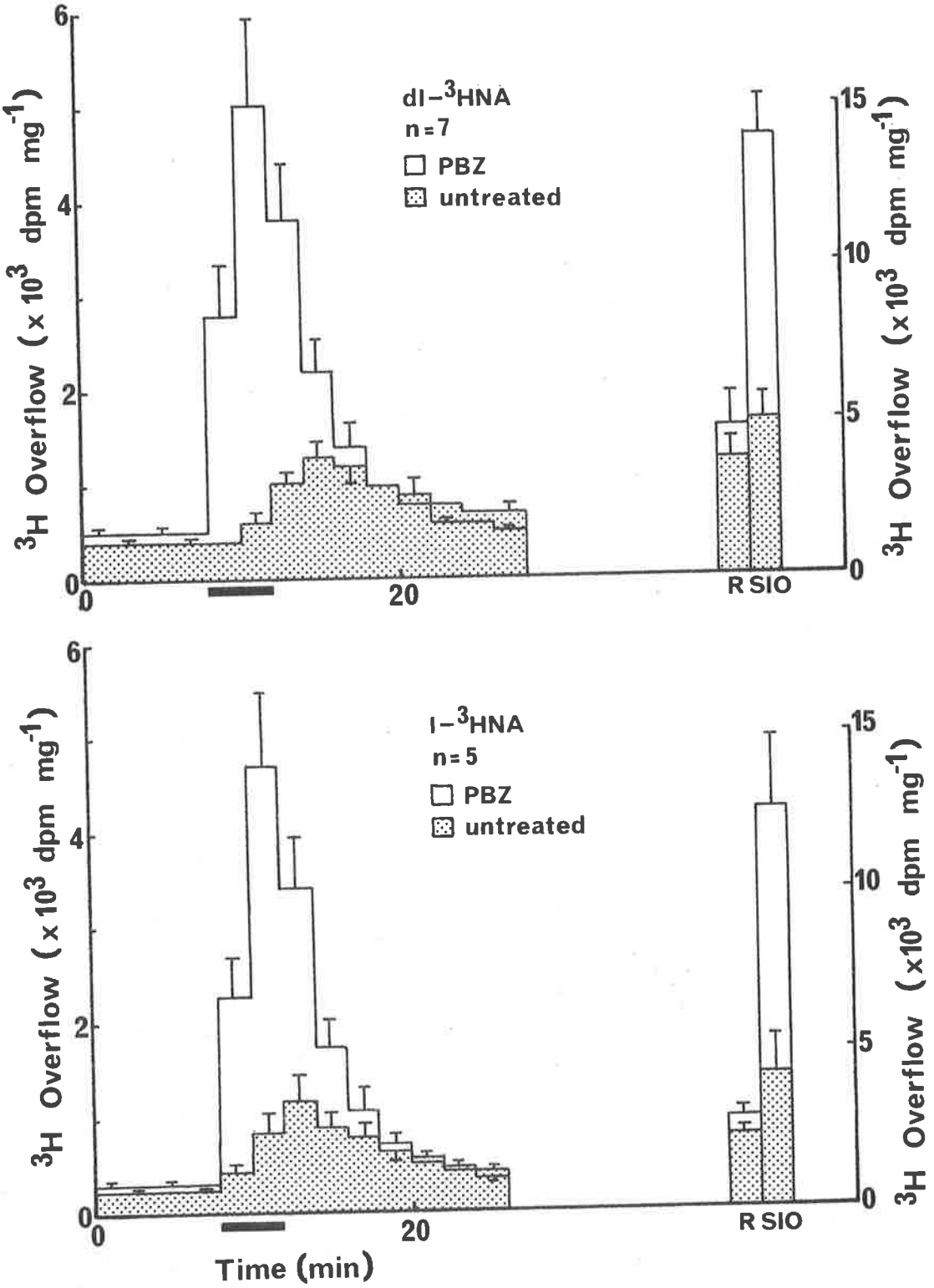
(right) tritium activity (dpm mg^{-1}) per 20 minutes of collection

Abscissa: duration of collection (min)

Tests of significance will be presented in this Chapter in the form indicated below. The significance of the effects of PBZ on the prestimulation (R) and the stimulation-induced overflows (SIO) are indicated.

Tests of significance: paired t-test (PBZ v untreated)

dl-^{3}HNA		l-^{3}HNA	
R	$p > 0.05$	R	$p < 0.05$
SIO	$p < 0.01$	SIO	$p < 0.005$


dl-3HNA
n=7
PBZ
untreated
3H Overflow (x 10^3 dpm mg^-1)
R SIO
l-3HNA
n=5
PBZ
untreated
3H Overflow (x 10^3 dpm mg^-1)
R SIO
Time (min)

decline from this peak was also much more rapid during the subsequent 6 minutes than in the untreated arteries, after which the rates of decline were more comparable. When estimated over 20 minutes, the SIO in the presence of PBZ was increased by a factor of 2.9.

The above effects of PBZ were similar, irrespective of the incubate used (dl-^{3}HNA or l-^{3}HNA) (Figure 9.3 and Table 9.2).

ii. Cocaine

The effects of cocaine (3 and 15 μmol l^{-1}) on the overflow of tritium prior to and as a result of stimulation are shown in Figure 9.4. In these experiments the same artery was exposed to the two concentrations of cocaine; for the second stimulation the higher concentration was used.

The characteristics of the overflow in the untreated arteries were similar to those described previously (page 9.5). Although the peak overflows following the onset of stimulation were reached at about the same time in both the untreated and cocaine-treated arteries, the initial rate of decline from this maximum was slower in the cocaine-treated arteries. Cocaine also decreased both the resting and stimulation-induced overflows by 26% and 33% respectively.

During the repeat stimulation the intraluminal overflow of tritium, in both untreated and cocaine-treated arteries, was less than that during the first period of stimulation. Although the kinetics of the overflow following the onset of nerve stimulation were similar for both concentrations of cocaine used, the mean SIO was only depressed by 13% in the presence of the higher concentration

Collection		Incubate	
Period	Number	dl-^{3}HNA	l-^{3}HNA
Prestimulation	4	1.3	1.2
Stimulation	5	7.6	5.4
	6	8.1	5.6
Post-stimulation	7	3.9	2.9
	8	1.7	2.0
	9	1.2	1.4
	11	0.9	0.9
	14	0.8	0.8
SIO		2.9	2.9

TABLE 9.2 The effect of PBZ on the overflow of tritiated material from arteries incubated in dl-^{3}HNA and l-^{3}HNA. The figures represent the ratios of the mean tritium activity in $\frac{\text{PBZ-treated}}{\text{untreated}}$ arteries for 8 collection periods.

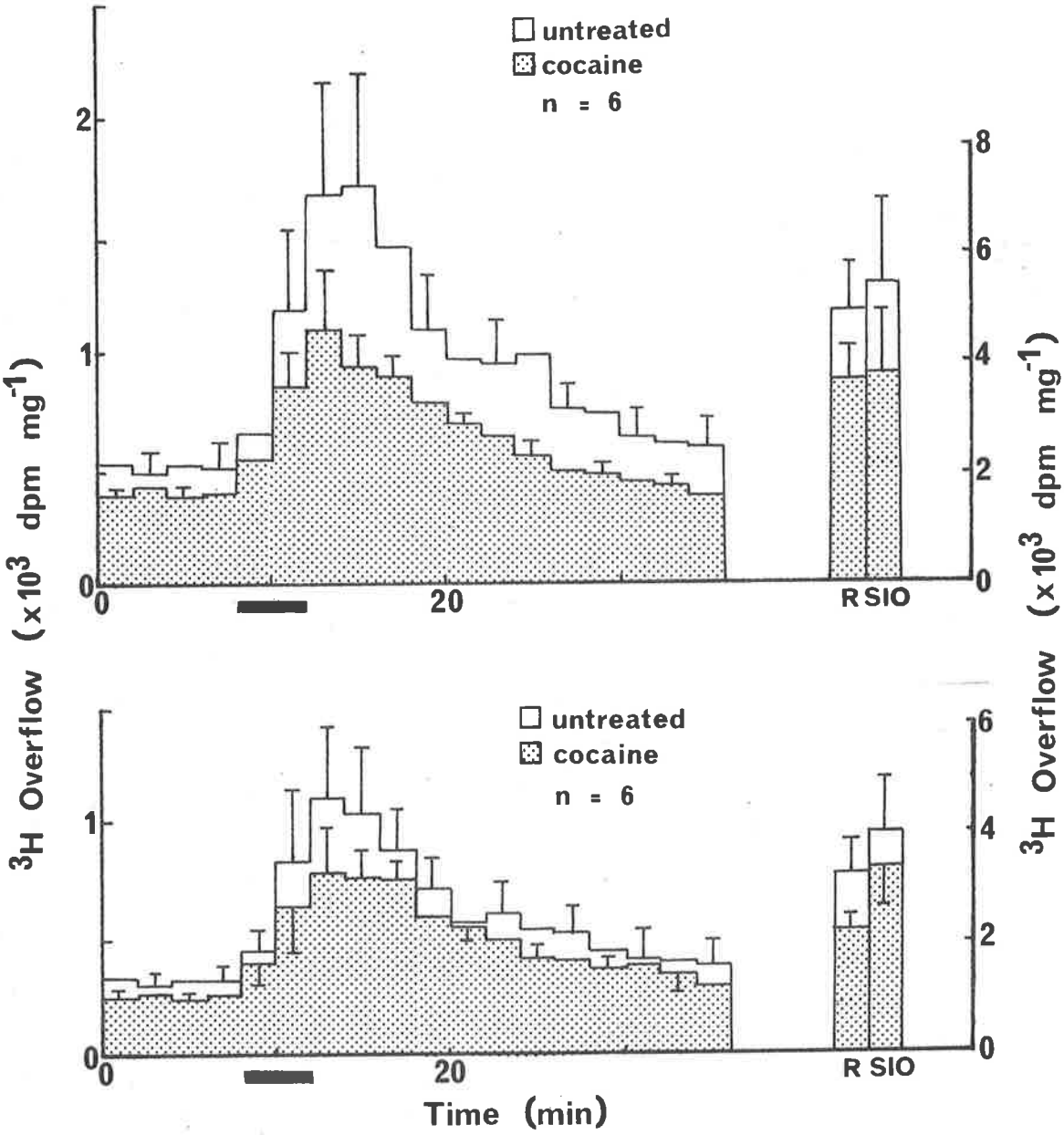


FIGURE 9.4 The effects of cocaine on the intraluminal overflow of tritium-labelled material prior to, during and following stimulation. For the first stimulation period (upper panel) one of each pair of arteries was treated with cocaine (3 μmol l^{-1}), and for the second period (lower panel) the concentration of cocaine was increased to 15 μmol l^{-1}. Arteries were incubated in dl-^{3}HNA. Details of symbols used are included in Figure 9.3.

Tests of significance: paired t-test (cocaine v untreated)

cocaine (3 μmol l^{-1})		cocaine (15 μmol l^{-1})	
R	$p < 0.1$	R	$p < 0.1$
SIO	$p < 0.2$	SIO	< 0.4

of cocaine. On the other hand, the magnitude of the reduction in the overflow from the resting arteries produced by cocaine (3 μmol l^{-1}) was unaffected at the higher concentration.

iii. Cocaine and DOCA

The effects of DOCA (27 μmol l^{-1}), and DOCA plus cocaine (3 μmol l^{-1}) on the overflow of tritiated material were examined in paired arteries, and although paired comparisons did not include untreated arteries, unpaired comparisons were possible as the incubates used in the two series of experiments were prepared from the same stock solution of dl-^{3}HNA (Figure 9.5).

Based on comparisons with untreated arteries (unpaired) combined cocaine and DOCA-treatment led to a decrease in the mean SIO of 43%, and DOCA-treatment alone resulted in a decrease of 20%. DOCA also increased the rate of tritium overflow following the onset of stimulation, so that the peak overflow occurred earlier.

In DOCA-treated arteries, cocaine decreased the mean overflow of tritium into the perfusate during stimulation, and for the first two collections following stimulation. The mean decrease in the SIO was 29%. Cocaine also altered the kinetics of luminal overflow by increasing the time taken for the peak value to be reached.

iv. Phentolamine

The mean overflow of tritiated material for each collection period from 5 phentolamine-treated and 5 paired, untreated arteries is shown in Figure 9.6. It will be noted that phentolamine (6 μmol l^{-1}) increased the rate of luminal overflow of tritium

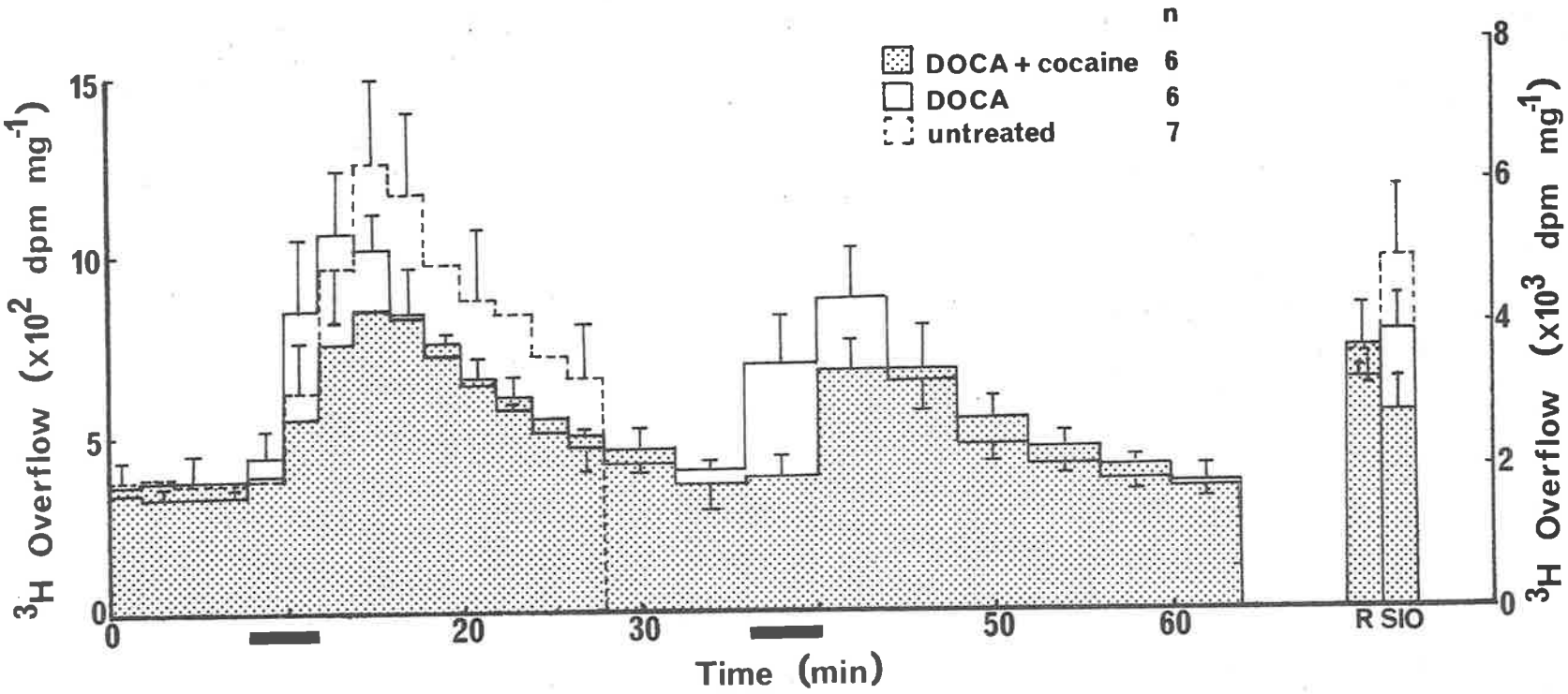


FIGURE 9.5 The effects of cocaine (3 µmol l^{-1}) on the intraluminal overflow of tritium-labelled material from arteries treated with DOCA (27 µmol l^{-1}) prior to and following the onset of nerve stimulation. Two stimulation periods are included. For comparison, the overflow of tritium-labelled material from the seven untreated arteries used in Figure 9.3 are included. All arteries were incubated in dl-^{3}HNA. Note: R and SIO are for the first stimulation period. Details of other symbols used are included in Figure 9.3.

Tests of significance:

paired t-test

DOCA v DOCA + cocaine	R	$p > 0.05$	
	SIO	$p < 0.05$	

unpaired t-test

DOCA + cocaine v untreated	R	$p > 0.05$	
	SIO	$p < 0.05$	
DOCA v untreated	R	$p > 0.05$	
	SIO	$p > 0.05$	

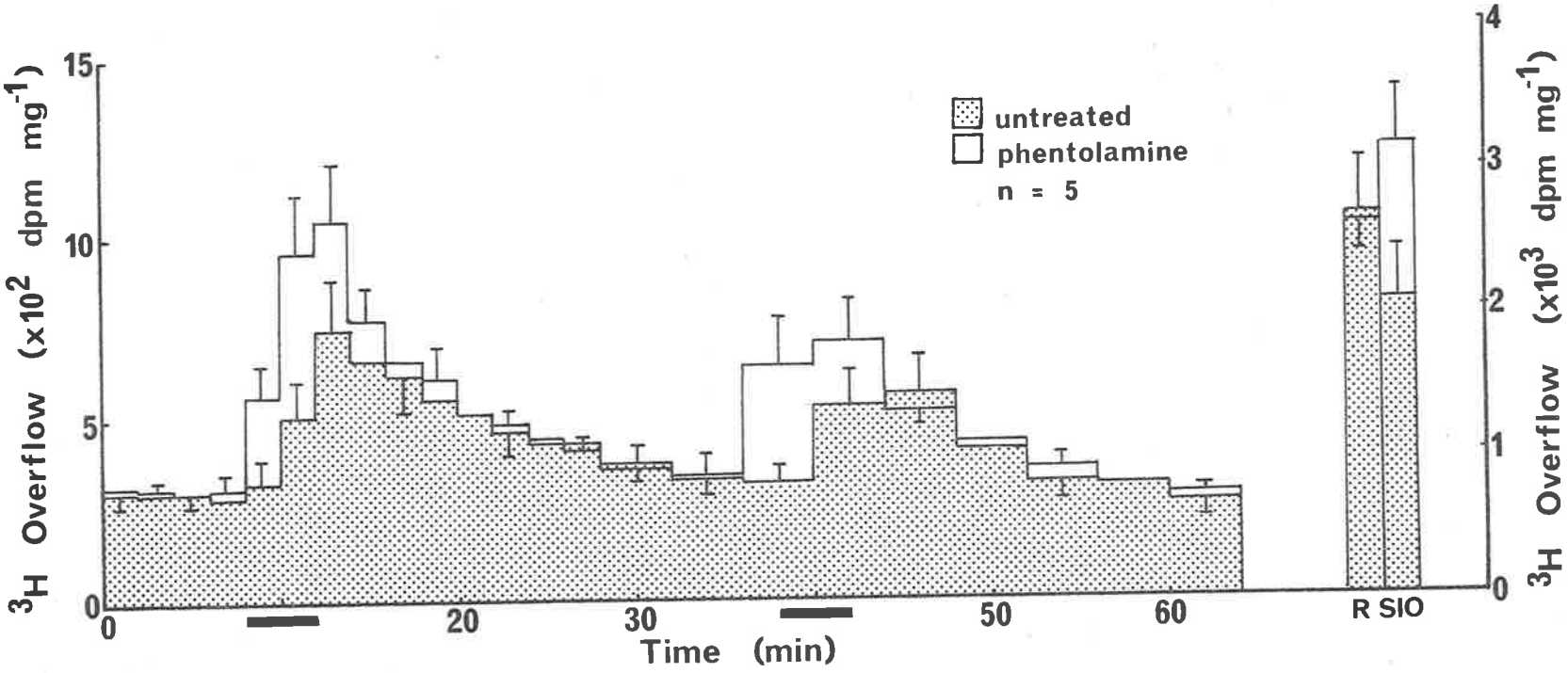


FIGURE 9.6 The effects of phentolamine (6 µmol l^{-1}) on the intraluminal overflow of tritium-labelled material prior to and following the onset of nerve stimulation. Two periods of stimulation are indicated. Arteries were incubated in dl-^{3}HNA. Note: R and SIO are for the first stimulation period. Details of symbols used are included in Figure 9.3.

Tests of significance: paired t-test (phentolamine v untreated)

R $p > 0.05$

SIO $p < 0.005$

during the period of stimulation, although the peak overflow in both arteries was not reached until after stimulation had ceased.

Phentolamine also significantly enhanced the magnitude of the mean SIO by 53%. Although the absolute overflow was decreased during the second period of stimulation phentolamine still facilitated the overflow.

v. *Cocaine in phentolamine-treated arteries*

The influence of cocaine (3 and 15 μmol l^{-1}) on the overflow of tritium in phentolamine-treated (6 μmol l^{-1}) arteries is shown in Figure 9.7. The only difference between the profiles for the phentolamine-treated and the phentolamine and cocaine-treated (3 μmol l^{-1}) arteries during the first period of stimulation was that the overflow in the cocaine-treated arteries was slightly less, and the peak overflow occurred faster, than in those arteries treated with phentolamine alone. Cocaine appeared to be without effect on the SIO.

When the concentration of cocaine was increased to (15 μmol l^{-1}) for the second stimulation period relatively more tritium reached the lumen, particularly in the stimulation and first recovery collections. However, although cocaine now increased the mean SIO by 18%, this increase was not significant.

Comparison of these results with those obtained in the previous cocaine experiments, in which constriction of the artery was not prevented, showed that phentolamine modified the overflow of tritium in cocaine-treated arteries by a) increasing the magnitude

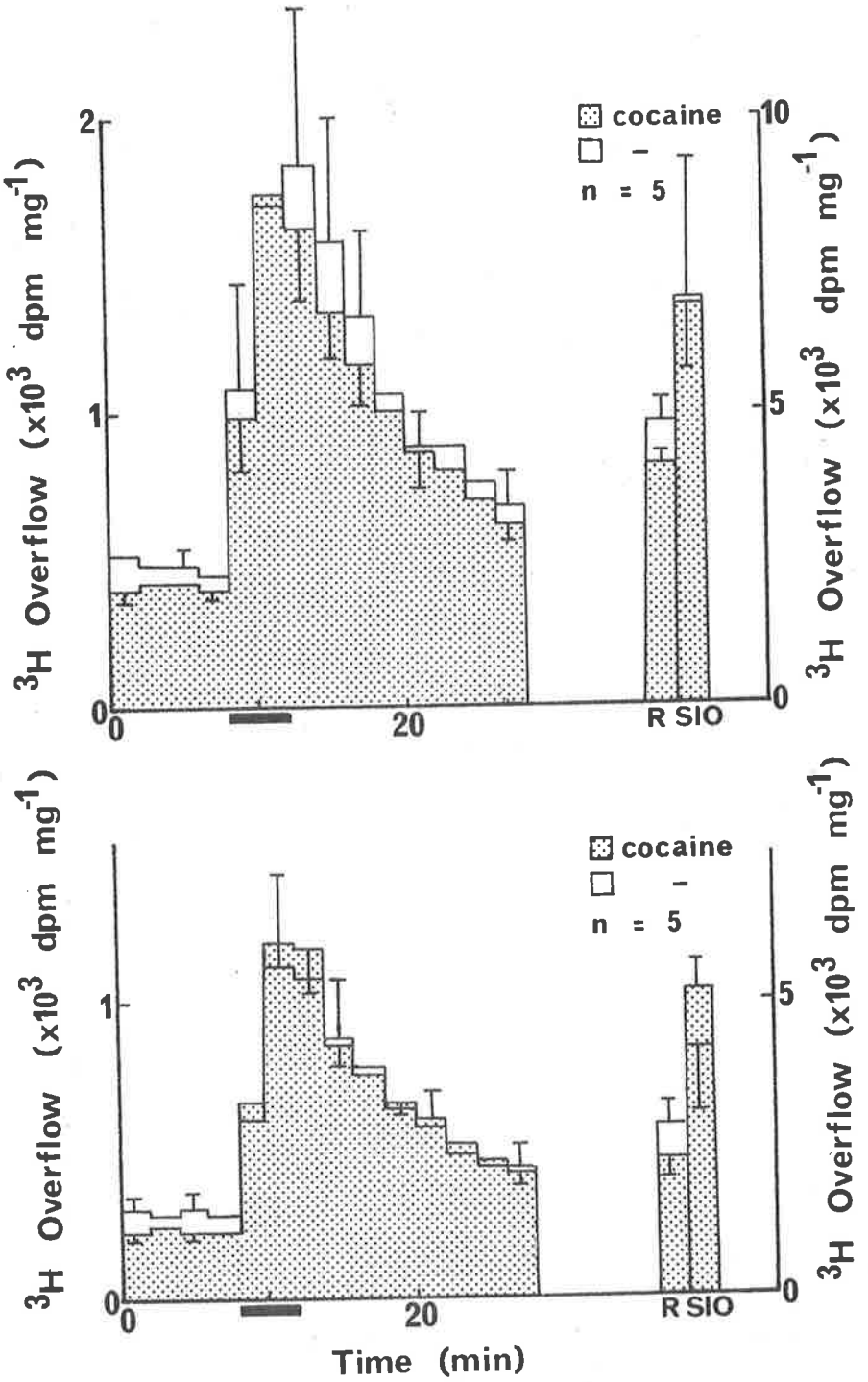


FIGURE 9.7 The effects of cocaine on the intraluminal overflow of tritium-labelled material from phentolamine-treated arteries prior to and following the onset of nerve stimulation. For the first stimulation period (upper panel) one of each pair of arteries was treated with cocaine (3 μmol l^{-1}), and for the second period (lower panel) the concentration was increased to 15 μmol l^{-1}. Arteries were incubated in dl-^{3}HNA.

Details of symbols used are included in Figure 9.3

Tests of significance: paired t-test (cocaine plus phentolamine v phentolamine)

	stimulation 1	stimulation 2
R	$p < 0.05$	$p < 0.05$
SIO	$p > 0.1$	$p > 0.1$

of the SIO, and b) reducing the time taken for the peak overflow to be reached. It will be noted that cocaine still tended to depress the resting overflow in the phentolamine-treated arteries.

vi. Cocaine and DOCA in phentolamine-treated arteries

In the presence of cocaine (3 µmol l^{-1}) and DOCA (27 µmol l^{-1}) the rate of luminal overflow following the onset of stimulation was increased in phentolamine-treated arteries (6 µmol l^{-1}), so that the peak overflow occurred earlier (that is, during the period of stimulation), and the initial rate of decline was more rapid. In contrast to their inhibitory effect in the absence of phentolamine, cocaine and DOCA now caused a significant increase in the mean SIO of 48% (Figure 9.8). However, the magnitude of the SIO was still below that produced by PBZ (Figure 9.14).

In case the concentration of cocaine might have been too low to completely inhibit neuronal uptake of NA, it was decided to repeat the preceding experiments using a higher concentration of cocaine (90 µmol l^{-1}). Of thirteen arteries treated with phentolamine, four were treated also with cocaine and six with cocaine and DOCA. Two of the phentolamine-treated arteries and two of the cocaine, DOCA and phentolamine-treated arteries were stimulated a second time, and samples of the luminal perfusate collected prior to and following the onset of stimulation were fractionated on Dowex 50 (Na^+) ion exchange resin columns (page 4.7). This fractionation was undertaken, because in theory inhibition of the uptake processes should prevent the appearance of metabolites other than those derived from the intraneuronal metabolism of NA

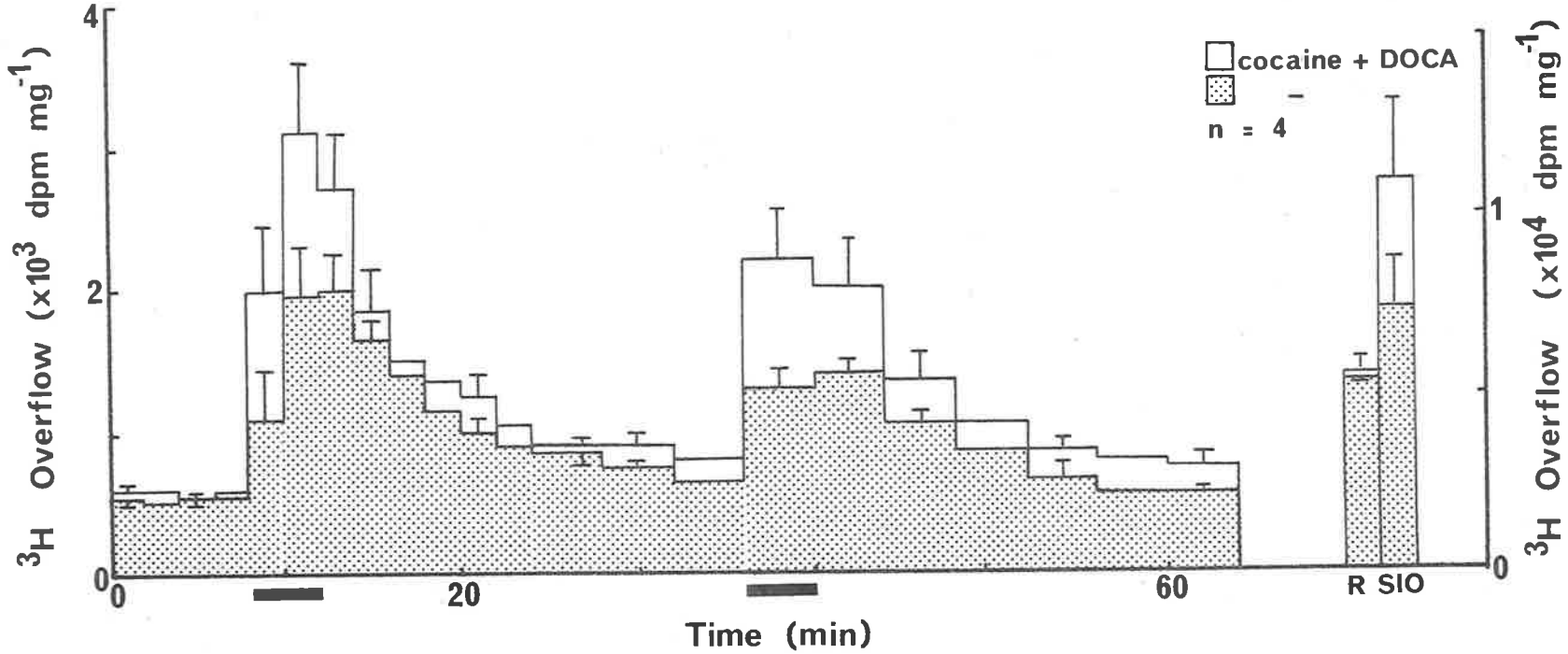


FIGURE 9.8 The effects of cocaine (3 µmol l^{-1}) plus DOCA (27 µmol l^{-1}) on the overflow of tritium-labelled material from phentolamine-treated arteries (6 µmol l^{-1}) prior to and following the onset of nerve stimulation. Two stimulation periods are shown. Arteries were incubated in dl-^{3}HNA. Note: R and SIO are for the first stimulation period. Details of symbols used are included in Figure 9.3.

Tests of significance: paired t-test (cocaine, DOCA and phentolamine v phentolamine)

R $p > 0.05$

SIO $p < 0.01$

which might have leaked out of the storage vesicles. Thus an analysis of the distribution of NA and its metabolites might indicate whether cocaine (90 μmol l^{-1}) was completely inhibiting the uptake of NA into the nerves.

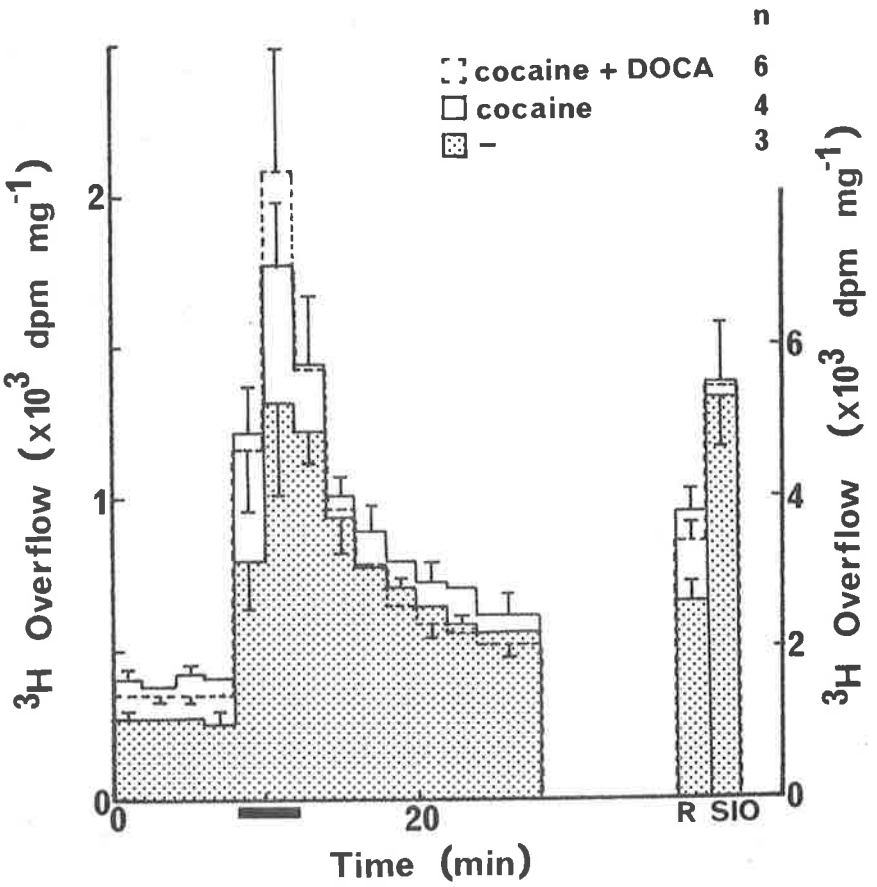
The effects of cocaine and cocaine plus DOCA on the overflow of tritium in phentolamine-treated arteries are shown in Figure 9.9. Although the kinetics of the overflow of tritium for each treatment were similar to those described for the previous experiments (page 9.9) in which the lower concentrations of cocaine were used, there were important differences in the relative magnitudes of the stimulation-induced overflow. In this series cocaine again failed to increase the mean SIO, and although cocaine and DOCA increased this overflow by 18% the increment was considerably less than the 48% increase produced by cocaine and DOCA in the earlier experiments (page 9.10).

In the phentolamine-treated arteries, NA and NMN could not be demonstrated in the resting overflow, and even in the stimulation-induced overflow the tritium activity associated with the NA fraction was very low and NMN was not identified (Figure 9.10).

Cocaine (90 μmol l^{-1}) and DOCA had no obvious effect on the composition of the resting overflow, but had a dramatic effect on the stimulated overflow; the major fraction was not unchanged NA (57%), and the remainder consisted of deaminated metabolites.

vii. PBZ in phentolamine-treated arteries

As the effect of cocaine and DOCA on the overflow of tritiated material was augmented by phentolamine and vice versa, it was of



n - represents the number of unpaired arteries used

FIGURE 9.9 The effects of cocaine (90 µmol l^{-1}), and cocaine (90 µmol l^{-1}) plus DOCA (27 µmol l^{-1}) on the overflow of tritium-labelled material in phentolamine-treated arteries (6 µmol l^{-1}) prior to and following the onset of nerve stimulation. The arteries used were incubated in dl-^{3}HNA.

Details of symbols used are included in Figure 9.3.

Tests of significance: unpaired t-test

	R	SIO
Cocaine* v phentolamine	$p < 0.05$	$p < 0.5$
Cocaine* v cocaine + DOCA*	$p < 0.2$	$p < 0.5$
Cocaine + DOCA* v phentolamine	$p < 0.1$	$p < 0.5$

** plus phentolamine*

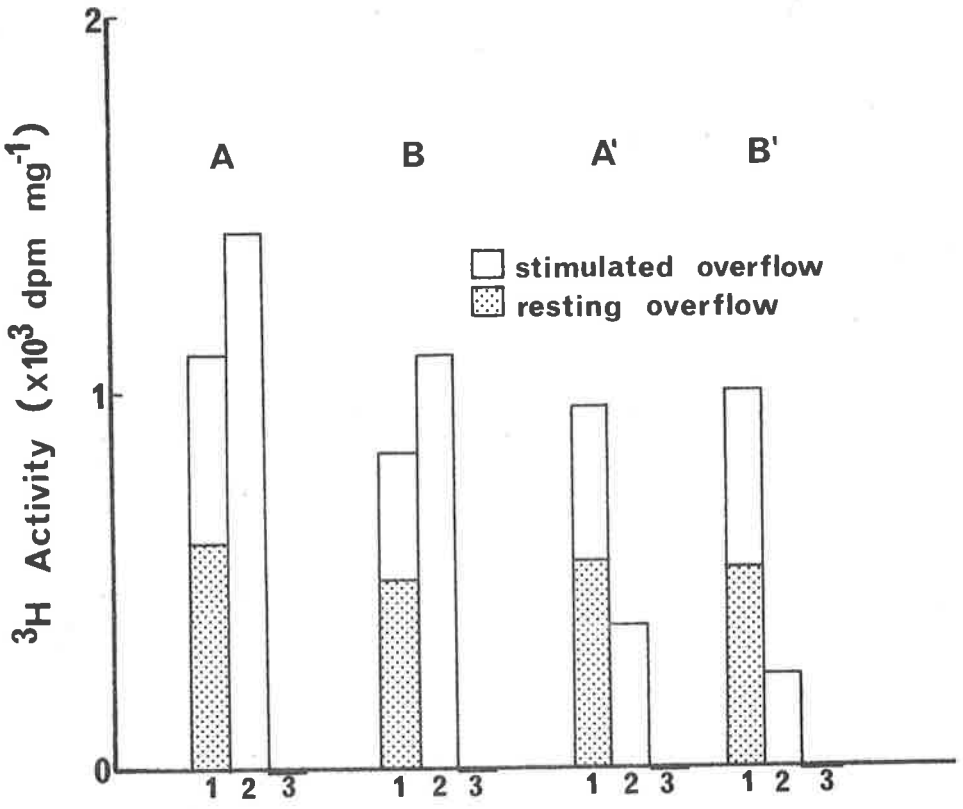


FIGURE 9.10 The distribution of NA and its metabolites in the intraluminal overflow prior to and following nerve stimulation in pairs of arteries from two rabbits (A and B). One artery in each pair (A or B) was treated with phentolamine (6 µmol l^{-1}), cocaine (90 µmol l^{-1}) and DOCA (27 µmol l^{-1}). The second artery (A' or B') was treated with phentolamine alone. Arteries were incubated in dl-^{3}HNA.

The three fractions separated by Dowex 50 chromatography consisted of deaminated metabolites (1), NA (2) and NMN (3).

Ordinate: tritium activity (dpm mg^{-1}) in each fraction

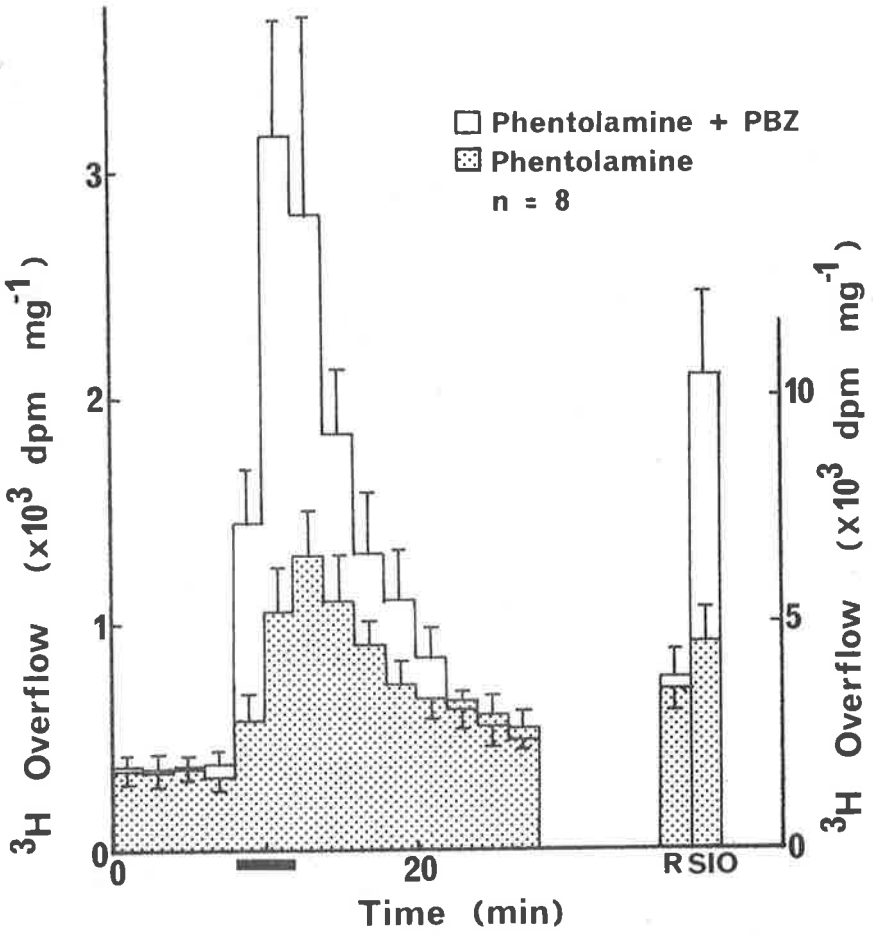
interest to establish whether a) the effect of PBZ was enhanced in the presence of phentolamine, and b) the overflow in phentolamine-treated arteries was increased by PBZ.

Three paired and two unpaired arteries were treated with phentolamine (6 μmol l^{-1}) and one artery in each pair and one of the unpaired arteries were bathed in PBZ (33 μmol l^{-1}) as well.

The effects of PBZ on the kinetics of overflow in phentolamine-treated arteries (Figure 9.11) were essentially the same as its effects in untreated arteries (that is, the peak occurred earlier and the decline from this peak was more rapid) (Figure 9.3). Furthermore, in the presence of phentolamine PBZ still caused its characteristic increase in the stimulation-induced overflow of tritium-labelled material. Although the absolute magnitude of this increase was of the same order as that which occurred in the absence of phentolamine (Figure 9.14), the percentage increase was less (130% compared with 186%). This difference resulted because the overflow in the phentolamine-treated arteries was greater than that in untreated arteries.

viii. Oxytetracycline in untreated and PBZ-treated arteries

As indicated previously, the binding of NA to elastin and collagen in the artery wall might influence the overflow of transmitter into the lumen of the rabbit ear artery. To test this possibility, and to see whether PBZ might interfere with this binding, five pairs of arteries were incubated in dl-^{3}HNA. For the first stimulation one of each pair was treated with



n - represents the number of arteries used; three paired and two unpaired

FIGURE 9.11 The effects of PBZ on the overflow of tritium-labelled material from phentolamine-treated arteries prior to, during and following nerve stimulation. Arteries were incubated in dl-^{3}HNA. Details of symbols used are included in Figure 9.3.

Tests of significance: unpaired t-test (PBZ in phentolamine-treated arteries)

R $p < 0.4$

SIO $p < 0.05$

oxytetracycline (94 μmol l^{-1}), and for the second both arteries were treated also with PBZ (33 μmol l^{-1}).

As shown in Figure 9.12, oxytetracycline caused a slight but not significant increase in the SIO in both the untreated and PBZ-treated arteries. However, it had little effect on the kinetics of the overflow of tritiated material.

3. Summary of results

The effects of the various treatments on the resting overflows are summarised in Figure 9.13. This diagram indicated that:

a. except at a concentration of 90 μmol l^{-1}, cocaine depressed the resting overflow in untreated and phentolamine-treated arteries; phentolamine itself did not alter the resting overflow,

b. combined cocaine and DOCA treatment did not affect the resting overflow, except when the concentration of cocaine was increased to 90 μmol l^{-1},

c. PBZ increased the resting overflow compared with untreated arteries; this effect was not observed in phentolamine-treated arteries.

Not shown in the summary was the tendency for the resting overflow to decline much more slowly in paraffin-bathed preparation than was previously observed in the Krebs' bathed preparations.

Figure 9.14 summarises the effects of the various drugs on the stimulation-induced overflows. The composite figure also includes the

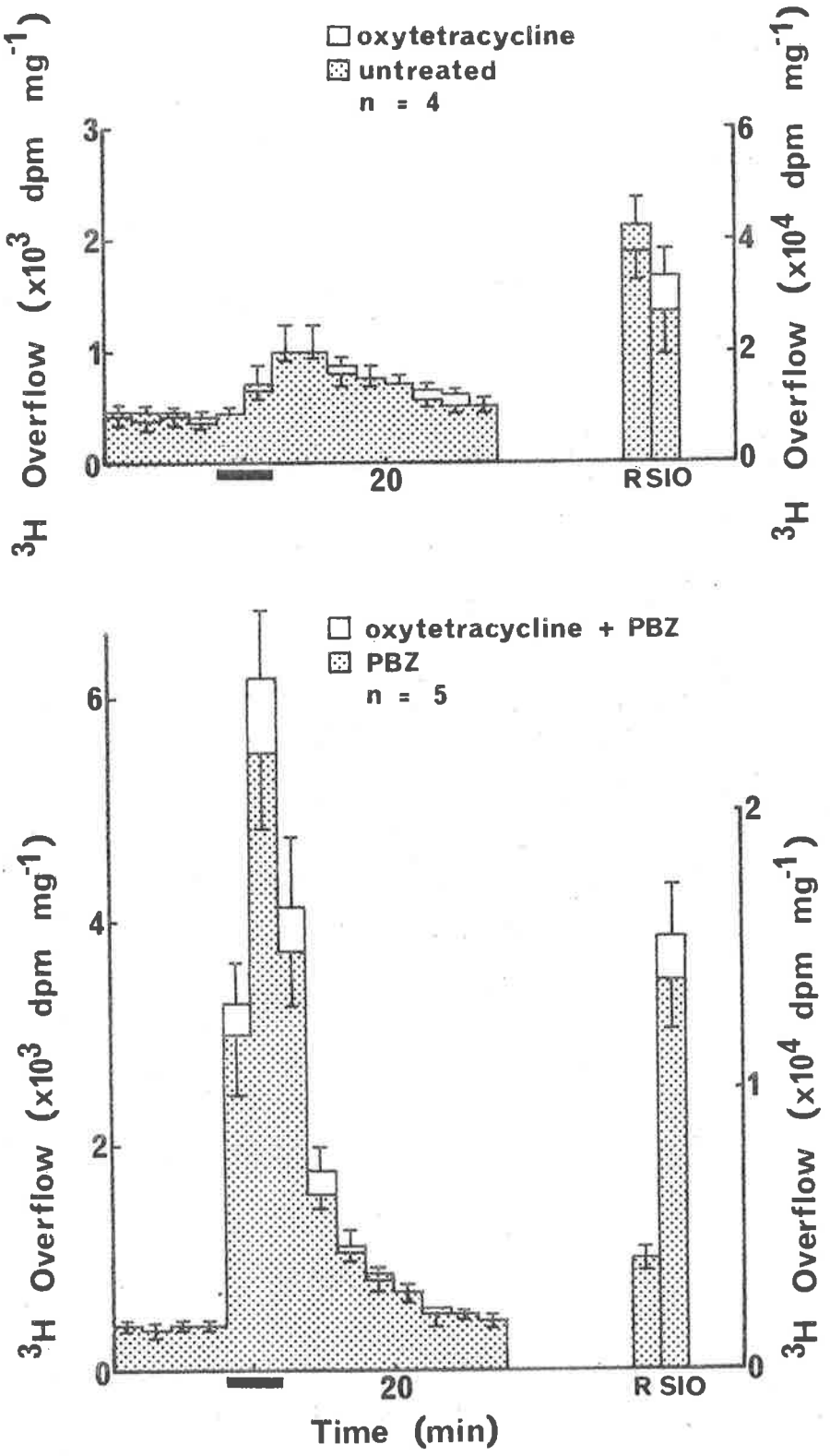


FIGURE 9.12 The effects of oxytetracycline (94 µmol l^{-1}) on the overflow of tritium-labelled material from untreated and PBZ-treated arteries (33 µmol l^{-1}) prior to and following the onset of nerve stimulation. Arteries were incubated in dl-^{3}HNA. Details of symbols used are included in Figure 9.3.

Tests of significance: paired t-test

	R	SIO
Oxytetracycline v untreated	$p < 0.2$	$p < 0.1$
Oxytetracycline in presence of PBZ	$p < 0.5$	$p < 0.2$

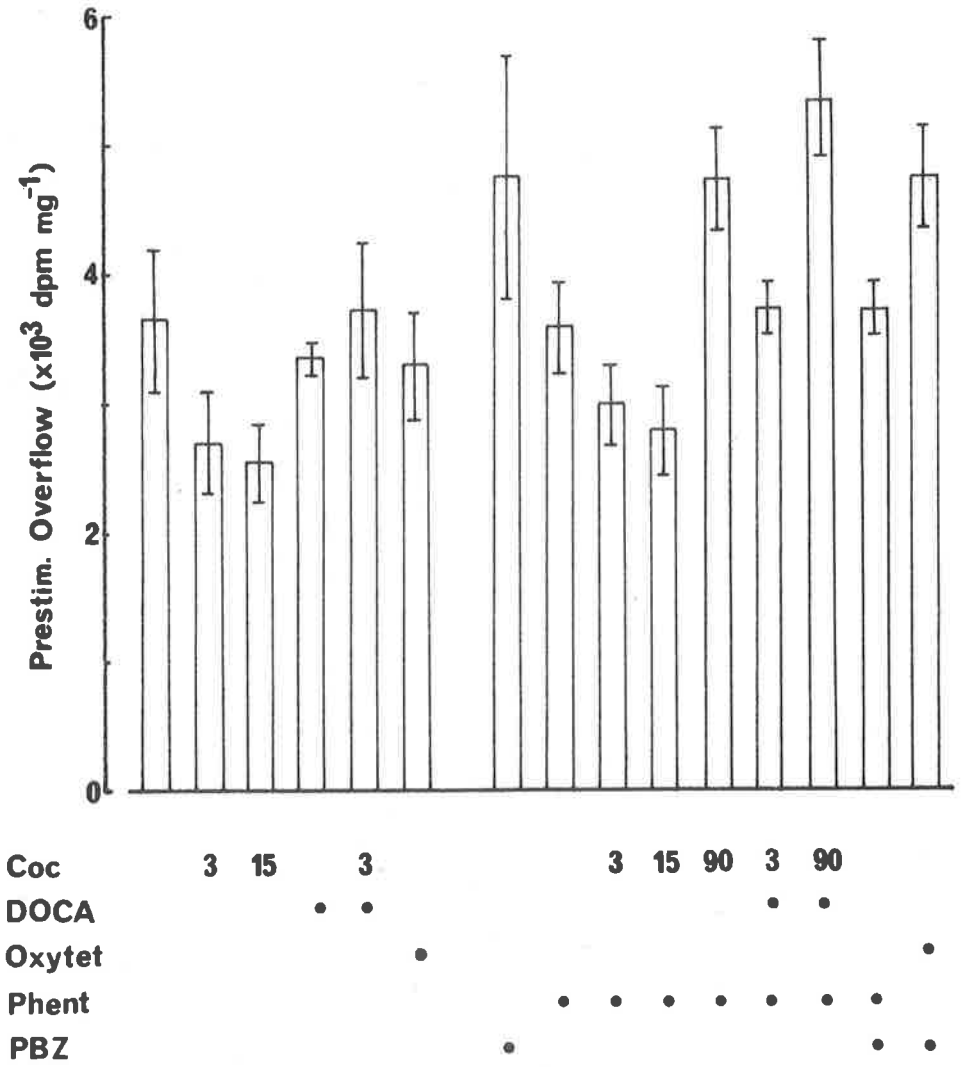


FIGURE 9.13 The effects of several drugs on the mean overflow of tritium-labelled material from arteries in the 20 minutes prior to nerve stimulation. For comparative purposes the mean overflows were standardized against the mean overflow in untreated arteries. All arteries were incubated in dl-^{3}HNA.

Symbols used represent:

Coc - cocaine (3,15 or 90 µmol l^{-1})

DOCA - deoxycorticosterone acetate (27 µmol l^{-1})

Oxytet - oxytetracycline (94 µmol l^{-1})

Phent - phentolamine (6 µmol l^{-1})

PBZ - phenoxybenzamine (33 µmol l^{-1})

• - drug present

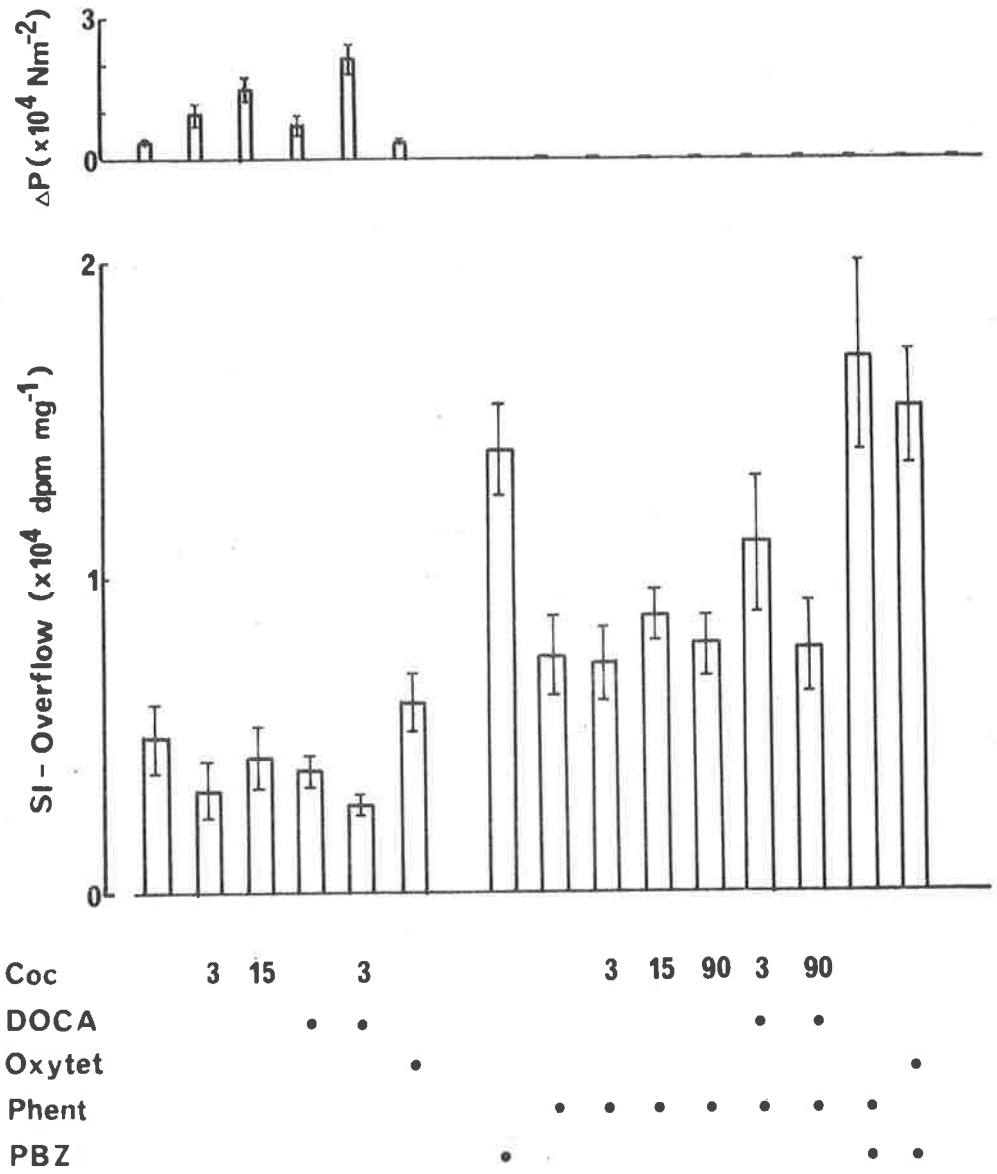


FIGURE 9.14 The effects of several drugs on the mean stimulation-induced overflow of tritium-labelled material into the lumen of rabbit ear arteries. For comparative purposes the mean overflows were standardized against the mean overflow in untreated arteries. All arteries were incubated in dl-^{3}HNA. The mean rises in perfusion pressure (ΔP) for the corresponding overflows are indicated in the upper portion of the figure.

For details of symbols used see Figure 9.13.

effects of the various agents on the constrictor responses of the arteries to nerve stimulation.

It will be noted that cocaine caused a small but significant increase in the constrictor response, and although DOCA alone had little effect the combination of DOCA and cocaine caused a marked increase (3.5-fold). Oxytetracycline did not increase the magnitude of the response relative to untreated arteries. Constrictor responses, other than occasional initial transient increases in perfusion pressure, were not detected in phentolamine or PBZ-treated arteries.

A feature of Figure 9.14 was that the stimulation-induced overflow of tritiated material in those arteries which constricted was less than in those in which constriction was prevented by an alpha receptor antagonist. Thus in cocaine and DOCA-treated arteries the SIO value was lowest while the response was greatest. When the responses were abolished by phentolamine, cocaine (3, 15 or 90 μmol l^{-1}) had little effect on the overflow of tritium-labelled material, but the combination of cocaine (3 μmol l^{-1}) plus DOCA caused a significant increase.

Two other features should be mentioned. One was that the effect of cocaine plus DOCA on the SIO from phentolamine-treated arteries was decreased when the concentration of cocaine was increased from 3 to 90 μmol l^{-1}, and the other was that the combined effect of phentolamine, cocaine (all concentrations) and DOCA did not increase the mean SIO as much as PBZ.

DISCUSSION

The oil-bathed artery had certain technical advantages over the aqueous-bathed preparation in the present experiments. Firstly, the use of liquid paraffin simplified the collection procedures and permitted continuous collection of the tritium overflow and secondly, and perhaps more importantly, the extraluminal paraffin caused the overflow of transmitter to become unidirectional, so that the tritium activity of the material which overflowed into the luminal perfusate was increased to a level which permitted reliable assay.

The pharmacological data presented in this chapter indicated that the functional activity of the artery was not impaired when its adventitial surface was bathed in paraffin. This evidence included observations that:

a. in untreated arteries the magnitude of the constrictor response produced by sympathetic nerve stimulation and the time required for the perfusion pressure to return to its prestimulation level after stimulation stopped were not affected by bathing the arteries in paraffin,

b. the actions of drugs appeared to be unchanged; thus cocaine, and DOCA in the presence of cocaine potentiated the responses in paraffin-bathed arteries as described in aqueous-bathed preparations (de la Lande and Rand, 1965; de la Lande et al, 1967b; Johnson, 1975), and PBZ and phentolamine antagonized the constrictor responses mediated by the post-synaptic alpha receptors.

In earlier experiments undertaken in the same laboratories as the author, de la Lande and Jellett (1972) found that the responses of arteries to sustained application of exogenous NA were also unaffected by paraffin.

In accord with the assumption that the overflow of tritium became unidirectional through the tunica media in the paraffin-bathed preparations, the intraluminal efflux of tritium in the resting arteries was increased by paraffin at least 3-fold. An interesting finding was that this overflow was less than the combined overflow (extraluminal plus intraluminal) in the aqueous-bathed preparations, amounting to only 40% of the latter. This difference between the two preparations was reduced in the presence of PBZ, so that the intraluminal overflow in the paraffin-bathed arteries was now 80% of the combined overflow in the aqueous-bathed preparations. Hence, a possible explanation for the smaller overflow in the untreated paraffin-bathed arteries was that the neuronal re-uptake and the extraneuronal uptake of the NA released in the resting artery were greater, when its diffusion was restricted mainly to the tunica media with its lower coefficient of diffusion (page 1.26). The finding of the present study that the overflow of tritium following sympathetic nerve stimulation declined to the prestimulation level much more slowly in paraffin than in aqueous-bathed preparations, also highlighted the slower diffusion of NA in the tunica media relative to the tunica adventitia. For example in the aqueous-bathed untreated artery, in which most of the transmitter overflowed through the adventitia, the prestimulation level was reached approximately 8 minutes after stimulation stopped, whereas, in the paraffin-bathed

artery the stimulation-induced overflow did not return to its prestimulation level for about 20 minutes.

Although differences in the affinities of the uptake systems and the inactivating enzymes for d-NA and l-NA have been described (Chapter 1), the kinetics and magnitude of the stimulation-induced overflow in arteries incubated in dl-NA and l-NA appeared to be little different. Similarly, the action of PBZ on the overflow was unaffected by the nature of the isomers.

As was evident in some of the aqueous-bathed arteries (Chapter 8), PBZ increased the resting overflow of tritium-labelled materials in paraffin-bathed arteries by approximately 30%, irrespective of the isomer used for incubation. Possible mechanisms for this action of PBZ on the resting overflow were discussed previously (page 8.15).

PBZ enhanced the stimulation-induced overflow by a factor of 2.9. This effect is explicable in terms of the well known inhibitory actions of PBZ on the neuronal and smooth muscle uptake of NA, and the more recently described action of PBZ on the presynaptic inhibitory alpha receptors.

To test the contribution of the uptake processes alone, the effects of an inhibitor of neuronal uptake (cocaine) and of an inhibitor of smooth muscle uptake (DOCA) were examined. Surprisingly, cocaine and DOCA separately as well as in combination tended to decrease the stimulation-induced overflow, while at the same time potentiating the constrictor responses. This apparent paradox will be referred to later (page 9.21). However in the presence of phentolamine, the combination of cocaine and DOCA increased the

overflow of tritiated material following nerve stimulation. Since phentolamine abolished the constrictor response to stimulation it is possible that vasoconstriction might have been responsible for the failure of cocaine and DOCA to increase the stimulation-induced overflow. However, as phentolamine also antagonized the presynaptic alpha receptors (Story, D.F., private communication), the failure of cocaine and DOCA to increase the overflow might also have been due to the effects of released NA on these inhibitory receptors.

Phentolamine itself significantly increased the magnitude of the stimulation-induced overflow of tritium-labelled material in untreated arteries. However, in this instance there was some evidence which suggested that inhibition of the vasoconstrictor response was responsible for this result, at least in part, since the rate of overflow during nerve stimulation was also enhanced. The significance of the vasoconstrictor response is analysed further in Chapter 11.

Irrespective of the mechanism of the action of phentolamine, the importance of the uptake systems to the diffusion of released transmitter was indicated in phentolamine-treated arteries by the findings that cocaine plus DOCA increased the magnitude of the SIO, and accelerated the rate at which the tritiated materials overflowed into the lumen. PBZ also increased the SIO in phentolamine-treated arteries. The magnitude of this increase (130%) was greater than that produced by cocaine and DOCA (48%). This discrepancy can be explained if PBZ has a greater effect on the release process than phentolamine, or the effects of cocaine and DOCA on the neuronal and smooth muscle uptake systems are less than those of PBZ. The latter possibility received support from the subsequent finding that the concentration

of cocaine used (3 μmol l^{-1}) did not completely inhibit the neuronal uptake of ^{3}HNA (Chapter 10). The choice of 3 μmol l^{-1} was based on the pharmacological and histochemical evidence that at this concentration cocaine inhibited the neuronal uptake of NA (de la Lande et al, 1974), and caused near maximum potentiation of the vasoconstrictor response to NA (Figure 10.2). However, in the collaborative study described in the previous chapter (pages 8.4 and 8.11), it was found that stimulation of an artery which had been previously incubated in cocaine (3 μmol l^{-1}) and ^{3}HNA caused a marked overflow of tritium-labelled material. It was necessary to increase the concentration of cocaine to 90 μmol l^{-1} to prevent this effect. In separate experiments it was shown that cocaine (3 μmol l^{-1}) inhibited the neuronal uptake of NA by only 60% (page 10.5).

Hence, some of the experiments which investigated the effects of cocaine and DOCA in phentolamine-treated arteries were repeated using a higher concentration of cocaine (90 μmol l^{-1}). Cocaine still failed to increase the SIO, and the overflow in the DOCA and cocaine-treated arteries was less than that in the preceding experiments when cocaine was used at the lower concentration (Figure 9.9). The explanation for this proved to be that cocaine itself inhibited the release of transmitter at the concentration used. This evidence is presented in Chapter 10.

Based on the effects of PBZ in phentolamine-treated arteries in which PBZ increased the SIO by 130%, it would appear that more than one-half of the NA released in phentolamine-treated arteries is normally taken up by the neuronal and extraneuronal uptake systems. Furthermore, it seems likely that the neuronal system is

quantitatively more important. This follows from the analyses of the tritium-labelled material which effluxed from phentolamine-treated arteries following stimulation, which indicated that although a small quantity of NA (approximately 25%) reached the luminal perfusate most of the efflux consisted of deaminated metabolites. Significant amounts of NMN were not detected. The absence of NMN was perhaps surprising as it was shown in a separate study that NMN was the major extraneuronal metabolite of exogenous NA in the rabbit ear artery (Head, 1976).

A possible explanation for the present findings was that the amount of NA which was recaptured by neuronal uptake was increased when its diffusion through the adventitia was prevented by paraffin. Hence, as much of the NA which is taken up by nerves is metabolized by MAO (Head, 1976), the overflow of deaminated metabolites increased. Presumably the non appearance of NMN reflected the small amount of NA which actually diffused into the smooth muscle layers.

Predictably the proportion of NA in the stimulation-induced overflow was increased considerably when the uptake processes were inhibited simultaneously by cocaine and DOCA. However, it was difficult to explain why the tritium activity associated with the fraction containing the deaminated metabolites which effluxed from the resting arteries was increased by stimulation, unless cocaine still failed to completely inhibit neuronal uptake.

The overflow from the resting phentolamine-treated arteries, whether cocaine and DOCA-treated or not, consisted of deaminated metabolites. These results can be viewed as providing further

evidence that the deaminated metabolites were derived intraneuronally, probably by leakage of NA from the storage granules followed by metabolism by MAO.

The metabolic data also showed that the effects of inhibiting the uptake processes were very much greater on the overflow of NA than on the total overflow of tritium-labelled material. This factor might help to explain the apparent paradox that in arteries not treated with phentolamine, DOCA and cocaine did not increase the overflow of tritium although markedly potentiating the constrictor response. The metabolic data pointed to the possibility that the proportion of NA in the overflow from cocaine plus DOCA-treated arteries was increased, despite the observed decrease in the total tritium overflow.

SUMMARY

The evidence presented in this chapter indicated that the neuronal and smooth muscle systems were important factors regulating the overflow of released transmitter into the lumen of the rabbit ear artery. Inhibition of both processes in phentolamine-treated, non-constricting arteries increased the amount of tritiated material which reached the lumen, and prevented metabolism of a large proportion of the released NA. The quantitative importance of the processes in constricting arteries was difficult to determine because of the possibility that the contractile process itself influenced the overflow. However, even in constricting arteries it was evident that DOCA accelerated the overflow of NA. This evidence suggested that in untreated arteries the uptake of NA by smooth muscle cells retarded the diffusion of transmitter

across the wall, presumably as a consequence of the slow release of NA or its metabolites.

Two of the problems which arose from the results of this chapter were investigated further and are described in succeeding chapters. These problems were:

a. the effect of cocaine on the release of transmitter, and

b. the influence of constriction on the diffusion of NA in the artery wall.

CHAPTER 10

THE INFLUENCE OF COCAINE ON THE NEURONAL UPTAKE OF NORADRENALINE AND ON THE RELEASE OF TRANSMITTER

CHAPTER 10

As foreshadowed in Chapter 9, the purpose of the experiments described in this chapter was to define the effects of cocaine on the release of transmitter, uncomplicated by its associated effects on neuronal uptake. In addition, some quantitative data on the effects of cocaine on neuronal uptake will be presented. The latter experimentation was not part of this study, although initiated by it, and the results are presented with the kind permission of R.J. Head.

In the present study the effects of graded concentrations of cocaine on the stimulation-induced overflow of NA in arteries were examined under conditions in which the neuronal and smooth muscle uptake of NA and the presynaptic alpha adrenoreceptors were inhibited by PBZ. By this means it was hoped that the luminal overflow of NA would closely approximate the amount of transmitter released, so that any effect of cocaine on the overflow would represent a direct action on the release mechanism.

In a second series of experiments the effects of cocaine on the contractile responses of arteries to sympathetic nerve stimulation and to extraluminal NA were studied. These experiments provided a pharmacological appraisal of the effects of cocaine on the release of endogenous NA.

METHODS

Short-eared rabbits, bred in the Institute of Medical and Veterinary Science, Adelaide, were used in these experiments because of a prolonged shortage of the semi-lop-eared variety.

1. Effects of cocaine on the stimulation-induced release of transmitter

Pairs of arteries were double-cannulated and placed in small organ baths according to the method described on page 2.2. Following incubation in l-^{3}HNA, they were washed in and perfused with Krebs' solution for 40 minutes prior to treatment with PBZ (33 µmol l^{-1}). After a further 10 minutes cocaine, at a concentration of 3, 30 and 90 µmol l^{-1}, was added to the extraluminal and intraluminal Krebs' solution and remained in the intraluminal fluid throughout the experiment. Twenty minutes after the addition of cocaine the luminal flow rate was reduced to 0.23 cm^{3} min^{-1} in both arteries, and the extraluminal solution was replaced by liquid paraffin. Five to ten minutes later three aliquots of the luminal perfusate were collected at intervals of 4 minutes. A fourth collection was made during nerve stimulation (5 Hz, 4 minutes), and was followed by five post-stimulation collections each of 4 minutes' duration. At the conclusion of the experiments the arteries were weighed and placed in HCl to extract the residual tritium-labelled material (page 4.14).

2. Effects of cocaine on the responses of arteries to electrical stimulation and exogenous NA.

Pairs of arteries from short-eared rabbits were double-cannulated and placed in 15 cm^{3} capacity organ baths according to the method

described on page 2.3. In one artery the effects of repetitive nerve stimulation were studied.* When the heights of the contractile responses became uniform, cocaine (3 μmol l^{-1}) was added to the extraluminal Krebs' solution. Subsequently, the concentration of cocaine was increased to 30 and then 90 μmol l^{-1}. In some arteries the concentration was increased to 150 and 300 μmol l^{-1}. Cocaine was washed out of the arteries, and stimulation continued until the heights of the contractile responses declined and became uniform.

Subsequently l-NA was added extraluminally, initially as a single test dose, and then cumulated to provide a dose-response curve. The effects of cocaine (3 - 300 μmol l^{-1}) on these responses to exogenous NA were examined, and the potentiation produced by each concentration of cocaine was calculated in terms of the shift to the left of the dose response curves at 8×10^3 Nm^{-2} (that is, 60 mm of Hg). A sensitivity ratio (S.R.) was calculated as shown below, and the mean S.R. was calculated as a geometric mean.

$$\text{S.R.} = \frac{\text{concentration of NA required to produce a rise in perfusion pressure of } 8 \times 10^3 \text{ Nm}^{-2} \text{ in an untreated artery}}{\text{concentration of NA required to produce the same rise in a cocaine-treated artery}}$$

In the second artery the experimental order was reversed, that is the effects of cocaine on the responses to NA were examined before its effects on nerve stimulation.

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* *Square wave pulses at a supra-maximal voltage (80 volts), with a pulse duration of 0.5 msec, a most common frequency of 2 Hz and a train length of 10 secs, were delivered through platinum field electrodes. This train of pulses was repeated every 100 seconds.*

RESULTS

1. Effects of cocaine on the stimulation-induced release of transmitter

The effects of cocaine (3, 30 and 90 μmol l^{-1}) on the overflow of ^{3}HNA in PBZ-treated arteries are shown in Figure 10.1.

At concentrations of 3 and 30 μmol l^{-1} cocaine significantly depressed the mean stimulation-induced overflow* of NA by 15% and 20% respectively. At the highest concentration of cocaine used (90 μmol l^{-1}) the overflow of NA was reduced by 40%.

Comparison of the prestimulation overflows in the three experiments indicated that cocaine did not influence the resting efflux of tritium-labelled material at any of the concentrations used.

2. Effects of cocaine on the responses of arteries to electrical stimulation and exogenous NA.

The influence of cocaine on the responses of arteries to nerve stimulation are summarized in Figure 10.2. At concentrations of 3, 30 and 90 μmol l^{-1} cocaine unequivocally potentiated this response to nerve stimulation, although this potentiation was decreased at 90 μmol l^{-1}. At 150 μmol l^{-1} cocaine depressed the response to one half of that in untreated arteries, and at the highest concentration used (300 μmol l^{-1}) cocaine abolished the responses to stimulation.

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* *Stimulation-induced overflow (SIO) was calculated according to the method outlined on page 9.4.*

FIGURE 10.1 The effects of cocaine (3, 30 and 90 μmol l^{-1}) on the intraluminal overflow of tritium-labelled material in PBZ-treated arteries prior to, during and following nerve stimulation.

Ordinate: tritium activity (dpm mg^{-1}) per 4 minutes of collection

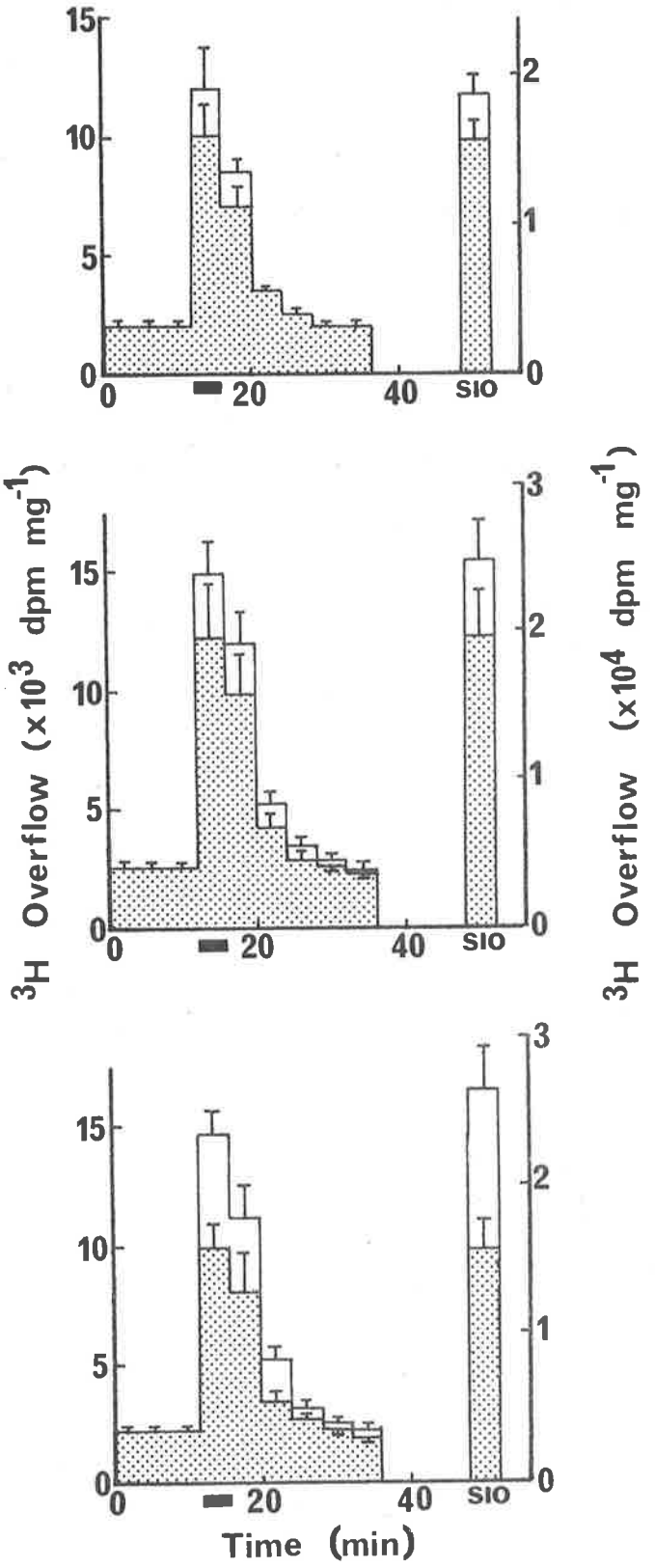
Abscissa: duration of collection (min)

Tests of significance: paired t-test

Cocaine significantly enhanced the SIO at all concentrations.

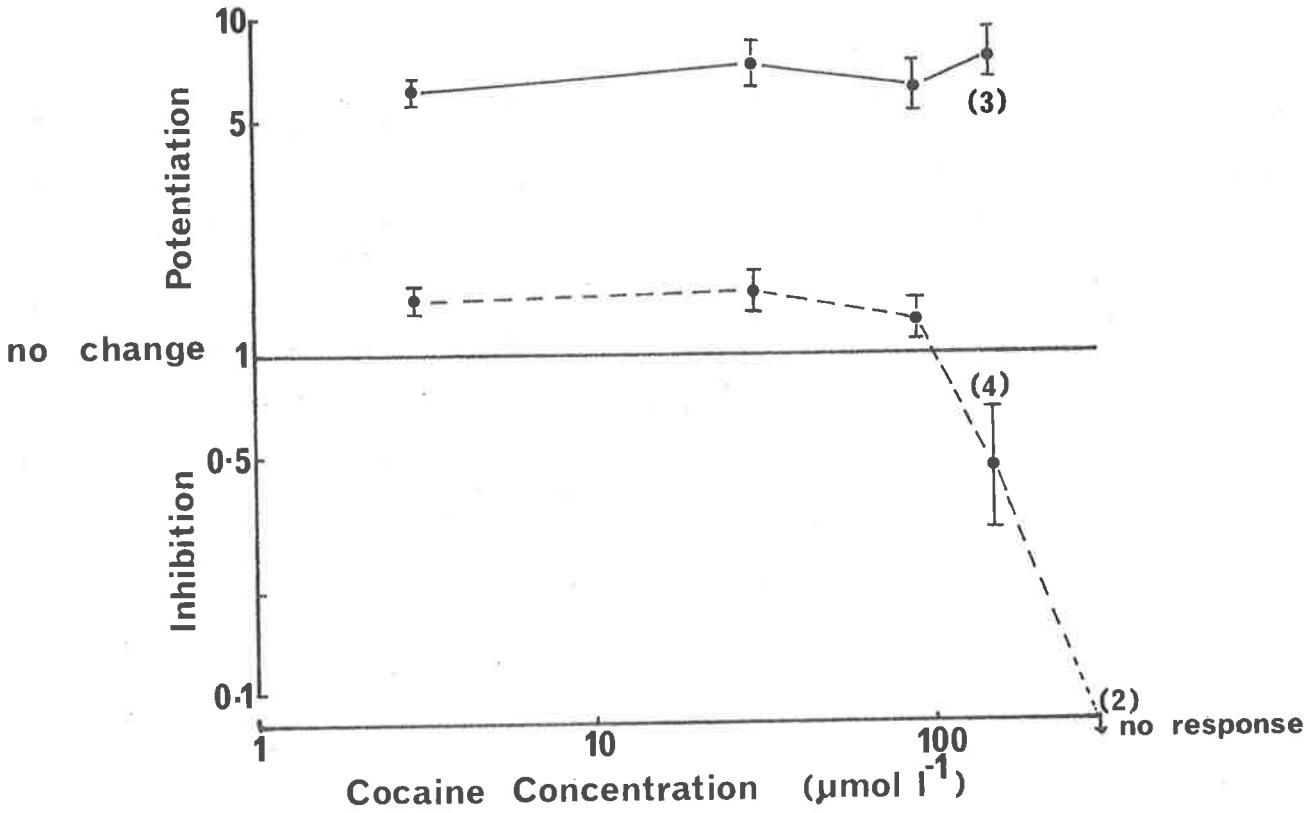
$p < 0.01$ at 3 and 90 μmol l^{-1}

$p < 0.05$ at 30 μmol l^{-1}



n - *represents the number of pairs of arteries used*

▬ - *stimulation period (4 min; 5 Hz)*



n = 6, except where indicated by the figures in brackets

FIGURE 10.2 The influence of cocaine on the responses of the rabbit ear artery to nerve stimulation (●- -●), and to extraluminally applied NA (●——●). The value at each point represents the geometric mean (±S.E.M.) of the sensitivity ratios for extraluminal NA (page 10.3), or the geometric mean (±S.E.M.) of the ratios of the responses of the arteries to nerve stimulation in the presence of cocaine relative to the responses in the absence of cocaine. Potentiation indicates that cocaine enhanced the response, and inhibition that it depressed the response.

Tests of significance: paired t-test

Cocaine (3 µmol l^{-1}) potentiated the constrictor responses of the arteries to extraluminal NA ($p < 0.001$). This potentiation was not enhanced at higher concentrations of cocaine (30 and 90 µmol l^{-1}) ($p > 0.05$).

The constrictor responses of the arteries to nerve stimulation were potentiated by cocaine (3 µmol l^{-1}) ($p < 0.01$). This potentiation was significantly depressed when the concentration was increased to 90 µmol l^{-1} ($p < 0.05$).

Cocaine at all concentrations used in these experiments potentiated the constrictor responses to extraluminally applied NA (Figure 10.2). These results indicated that, although cocaine (3 μmol l^{-1}) significantly potentiated the response of the artery to NA, increasing the concentration of cocaine did not further increase this potentiation.

The effects of cocaine on the constrictor responses of the arteries to extraluminal NA and to nerve stimulation were reversible when the drug was washed out.

3. Effects of cocaine on neuronal uptake

The data in Table 10.1 is reproduced by kind permission of R.J. Head (see Head, 1976). In these experiments segments of rabbit ear artery were incubated in l-^{3}HNA for 30 minutes at a concentration of either 1.2 or 0.12 μmol l^{-1}; eleven segments were untreated and twenty were pre-treated with, and incubated in the presence of, cocaine (3 or 30 μmol l^{-1}).

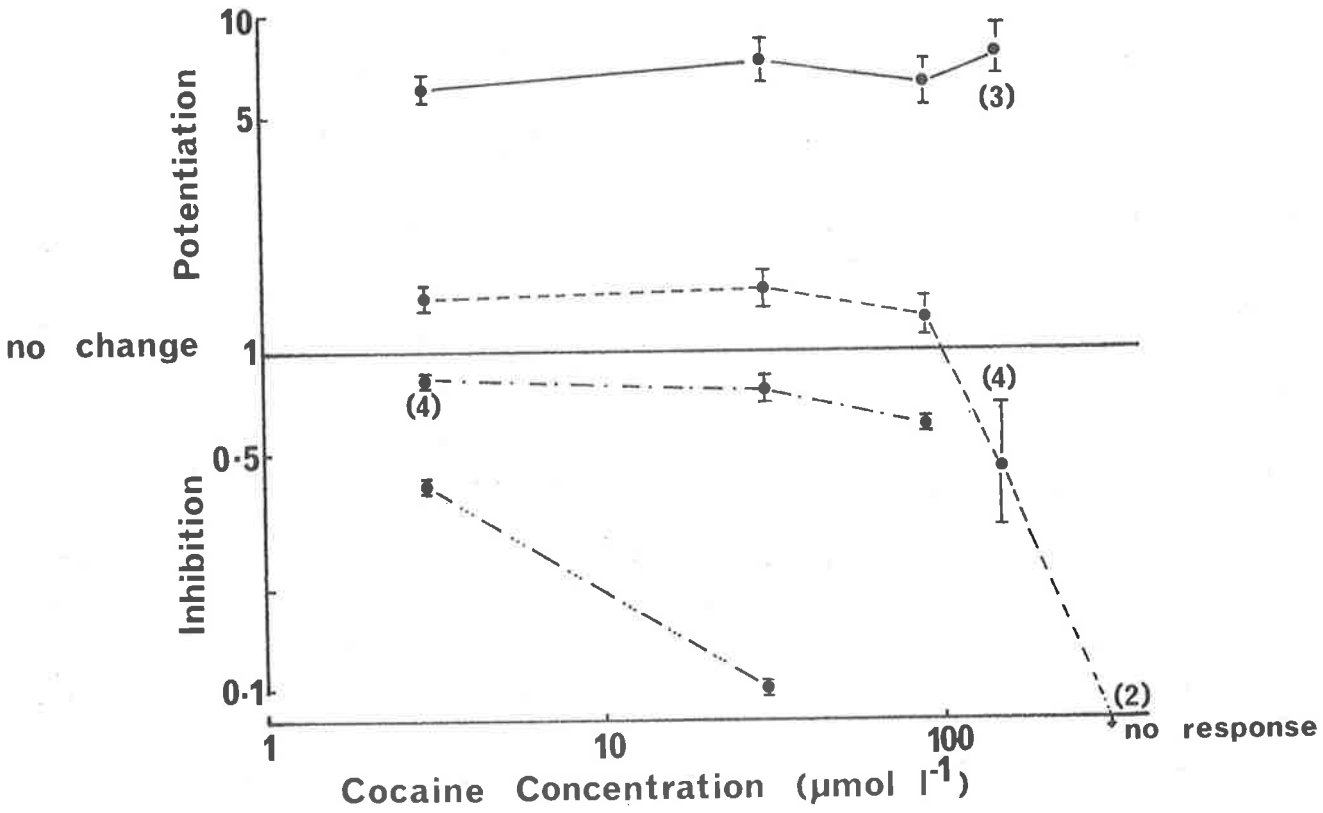
The results showed that cocaine (3 μmol l^{-1}) inhibited the neuronal uptake of ^{3}HNA by 60% when the concentration of NA in the incubate was 1.2 μmol l^{-1}, and by 66% when the incubate concentration was 0.12 μmol l^{-1}. At 30 μmol l^{-1} cocaine inhibited 90% and 91% of neuronal uptake at the two concentrations of ^{3}HNA used.

The effects of cocaine on the responses of arteries to nerve stimulation and extraluminal NA, on the neuronal uptake of ^{3}HNA, and on the overflow (release) of transmitter in ear arteries are summarized in Figure 10.3. The results presented in this composite

Cocaine (μmol l^{-1})	Uptake of ^{3}HNA (nmol g^{-1}) A	B
0	8.7 ± 2.0 (n = 5)	2.0 ± 0.3 (n = 6)
3	3.5 ± 0.4 (n = 5)	0.7 ± 0.2 (n = 5)
30	0.9 ± 0.2 (n = 5)	0.2 ± 0.03 (n = 5)

n - represents the number of artery segments incubated in NA

TABLE 10.1 The effects of cocaine on the neuronal uptake of ^{3}HNA. Arteries were incubated in l-^{3}HNA for 30 minutes at a concentration of either 1.2 μmol l^{-1} (A) or 0.12 μmol l^{-1} (B). Arteries were treated with cocaine (3 and 30 μmol l^{-1}) as indicated.



n = at least 5 artery segments, except where indicated by the figures in brackets

FIGURE 10.3 The influence of cocaine on:

a. The response of rabbit ear arteries to extraluminally applied NA (●——●), and to nerve stimulation (●- -●) (see Figure 10.2).

b. The release of ^{3}HNA in PBZ-treated arteries (●-·-●) (see Figure 10.1). Cocaine inhibited the release of NA at the three concentrations used.

c. The neuronal uptake of ^{3}HNA in ear arteries (●-···-●) (see Table 10.1). Cocaine inhibited the neuronal uptake of NA by 60% and 90% at the two concentrations used.

diagram indicated that although it was likely that cocaine blocked neuronal uptake more effectively at a concentration of 90 μmol l^{-1} than at 3 μmol l^{-1}, the higher concentration depressed the release of transmitter. This inhibitory action of cocaine on the release of transmitter could explain why the responses of cocaine-treated (3 and 30 μmol l^{-1}) arteries to nerve stimulation were greater than the responses in arteries treated with cocaine at the higher concentration (90 μmol l^{-1}), despite the greater inhibition of neuronal uptake provided by the higher concentration.

DISCUSSION

These results confirmed the possibility raised by the data in the preceding chapter that cocaine, at a concentration of 90 μmol l^{-1}, depressed the release of NA. As this influence of cocaine on release was demonstrated in the presence of PBZ it cannot be attributed to an effect of cocaine on the neuronal and smooth muscle uptake of NA nor to an effect which involved the pre or post-synaptic alpha adrenoreceptors.

An unexpected finding in these experiments was that this inhibition of transmitter release was not confined to the highest concentration of cocaine used, but extended to the lowest (3 μmol l^{-1}). As the concentration of cocaine employed to inhibit neuronal uptake in most pharmacological experiments is usually at least 3 μmol l^{-1}, it is clear from the present experiments that the action of cocaine on neuronal uptake, at least in the rabbit ear artery, is less specific than previously supposed.

It might be argued that this effect of cocaine on the release process was shown only under conditions of PBZ-treatment, or that it was a manifestation of the isotopic technique used. Hence, it was significant that in the pharmacological experiments cocaine unequivocally depressed the responses to nerve stimulation at a concentration less than twice the highest concentration used in the isotopic experiments.

The potentiating effect of cocaine on the responses to nerve stimulation in these arteries was maximal at 30 μmol l^{-1}, was less marked at 90 μmol l^{-1}, and was reversed at higher concentrations. This finding is readily interpreted in terms of the opposing effects of cocaine on adrenergic nerves; namely,

a. potentiation of responses produced by inhibition of reuptake, and

b. depression of responses as a result of inhibition of the release of NA.

Presumably, at concentrations below 30 μmol l^{-1} the former effect predominated, while at concentrations above this the latter effect became progressively more important.

In support of the above argument it was noted that there was a tendency for the responses of arteries to exogenous NA to be potentiated more in the presence of cocaine at a concentration of 30 μmol l^{-1} than at 3 μmol l^{-1}. This can be explained by the finding that inhibition of neuronal uptake was greater at the higher concentration of cocaine. Although this potentiation was less at 90 μmol l^{-1}, the decline in response height was not found at the

highest concentration of cocaine used (150 μmol l^{-1}). These results support the concept that the inhibitory effects of cocaine were a manifestation of its action on neuronal tissues rather than on smooth muscle cells.

It could be argued that at higher concentrations cocaine exerted some inhibitory effect on the smooth muscle cells, which was compensated by its greater inhibitory effect on neuronal uptake. Such an argument implies that the inhibitory effect of cocaine on NA release was a manifestation of a non-specific inhibitory effect on excitable tissues. This is unlikely since the release of tritiated material in the resting artery was unaffected at all concentrations of cocaine tested.

The present findings help to explain the apparent paradox that the stimulation-induced overflow in PBZ-treated arteries was greater than the equivalent overflow from arteries in which neuronal uptake, smooth muscle uptake and the presynaptic alpha adrenoreceptors were inhibited by cocaine, DOCA and phentolamine respectively (page 9.10). Presumably at least part of the reason for the observed discrepancy in the overflow of tritiated-material was that cocaine depressed the release of transmitter even at concentrations which only partially inhibited neuronal uptake. At higher concentrations, although inhibition of neuronal uptake was enhanced, the release was substantially depressed. In the following chapter the influence of the neuronal and smooth muscle uptake systems on the movement of NA through the artery wall will be investigated under conditions in which the release process was not a complicating factor.

CHAPTER 11

FACTORS INFLUENCING THE DIFFUSION OF EXOGENOUS NORADRENALINE ACROSS THE ARTERY WALL

CHAPTER 11

In preceding chapters evidence was presented which indicated that the amount of NA which overflowed from the rabbit ear artery following its release by nerve stimulation was reduced by neuronal and extraneuronal uptake. Metabolism by MAO and COMT following uptake also reduced the overflow of NA. However it appeared that these studies might be complicated by the presynaptic alpha adrenoreceptor modulation of transmitter release and the constrictor response of the artery during nerve stimulation.

The purpose of the work undertaken in this chapter was:

a. To examine the roles of neuronal and smooth muscle uptake of NA uncomplicated by factors which might influence the release process. In these experiments ^{3}HNA was applied to the adventitial surface of artery segments under conditions which closely resembled those of stimulation; that is, the NA was applied for 4 minutes and subsequently the artery was bathed in liquid paraffin for the duration of the intraluminal overflow (influx). The amount of tritium-labelled material which influxed was monitored, and the influence of various drug treatments on the magnitude and kinetics of this overflow was examined.

b. To examine the influence of constriction on the influx of tritiated material. This was done by investigating the effects of phentolamine on the characteristics of the influx, and by

examining the differences in the diffusion of NA when constricting and non-constricting concentrations of NA were used.

c. To determine the composition of the tritiated material following its diffusion across the artery wall. These experiments provided a further guide to the influence of the uptake processes on the inactivation of transmitter.

METHODS

Paired arteries were placed in small organ baths and perfused with Krebs' solution for at least 60 minutes as described in Chapter 2.

With the exception of PBZ, drugs were added to the Krebs' solution bathing the adventitia and perfusing through the lumen for a minimum period of 30 minutes prior to the application of NA, and were maintained during this application. Usually PBZ was added to the external bathing medium only.

In the majority of experiments, l-^{3}HNA was used after purification on Dowex 50 ion exchange resin (for details see page 4.11). Details of the method of preparation of NA were given previously (page 4.3). Variations in the concentration of NA will be referred to in the text.

Two types of study were undertaken. In one, the NA was applied to the outside of the arteries for periods varying from 8 to 60 minutes and the influx of tritium into the luminal perfusate was determined by scintillation spectrometry following collection of the perfusate in Bray's scintillant. In other experiments the period of application of NA was only 4 minutes, after which the Krebs' solution was replaced with liquid paraffin. This procedure was used to mimic

the conditions under which the stimulation-induced overflow had been studied in Chapter 9. When more than one application of NA was made to an artery, the liquid paraffin was removed and the artery and organ bath were flushed with Krebs' solution containing the appropriate drugs for at least 30 minutes.

In some experiments the composition of the intraluminal influx was analysed by chromatographic separation on Dowex 50 ion exchange resin columns (page 4.7).

Usually the tritium activity in each aliquot of perfusate collected was standardized against a constant tritium activity in the diffusion medium; that is, the activity in the Krebs' solution containing ^{3}HNA which was applied to the adventitial surface of the artery. Although the rate of tritium influx in these experiments was based on the mass of the artery, the distance between the ends of the cannulae was also measured.

In some experiments the artery diameter was determined as well, to permit an estimation of the diffusion coefficient. For this measurement a travelling microscope with a vernier scale was used. Comparative measurements were made between the artery diameter and the diameter of a stainless steel wire of known dimension, placed in the organ bath in the same plane as the artery. The thickness of the artery wall was calculated indirectly from the values obtained for length, radius and mass, according to the following formulae:

$$m = \pi l (r_o^2 - r_i^2)$$

where m = mass

l = length

r_o = outside radius

r_i = inside radius (i.e., radius of the lumen).

This formula, which assumed that the specific gravity of the artery was 1.0, permitted an estimation of r_i for substitution in the equation:

$$d = r_o - r_i$$

where d = wall thickness.

The diffusion coefficient for NA in the rabbit ear artery was estimated from the diffusion equation for movement of a substance through a flat layer of tissue (Bevan and Su, 1974):

$$M = \frac{kA (Cp - Cb)t}{d}$$

where M = mass of NA which diffused into the lumen

A = surface area of the artery

d = wall thickness

Cp = concentration of NA on the outside of the artery

Cb = concentration of NA in the perfusate

t = time of diffusion

k - coefficient of diffusion of NA in the artery wall.

Cb was assumed to be zero as the concentration of NA in the lumen was very much less than its concentration in the bathing medium.

The above equation was used by Bevan and Su (1974) to estimate the concentration of NA at its site of release, that is, in the synaptic cleft. These workers used the diffusion coeffient previously determined by Bevan and Waterson (1971) for the rabbit ear artery.

Drugs used in these experiments were:

PBZ (33 μmol l^{-1})

Cocaine (90 μmol l^{-1})

DOCA (27 μmol l^{-1})

phentolamine (0.3 - 0.6 μmol l^{-1})

RESULTS

1. Sustained application of NA

Since it was possible that an intraluminal flow rate as low as 0.25 cm^3 min^{-1} might limit the rate of diffusion of tritiated material across the artery wall, the influxes of tritium into the lumen at this rate and at 1.0 cm^3 min^{-1} were compared. Comparisons were made in paired, untreated and PBZ-treated arteries using dl-^{3}HNA (0.27 μmol l^{-1}).*

The results which are summarized in Figure 11.1 failed to reveal any obvious difference between the influxes at the different flow rates. It will be noted that in the untreated arteries the initial rapid influx of tritium was followed by a much slower influx, so that the rate appeared to be approaching a steady-state at 30 to 60 minutes.

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* *The sample of dl-^{3}HNA used was not purified prior to use. The concentration of NA in the stock solution was assayed by the fluorometric method (page 2.9) at 75% of that stated by the manufacturer. The preparation of the NA solution for diffusion was outlined previously (page 4.3).*

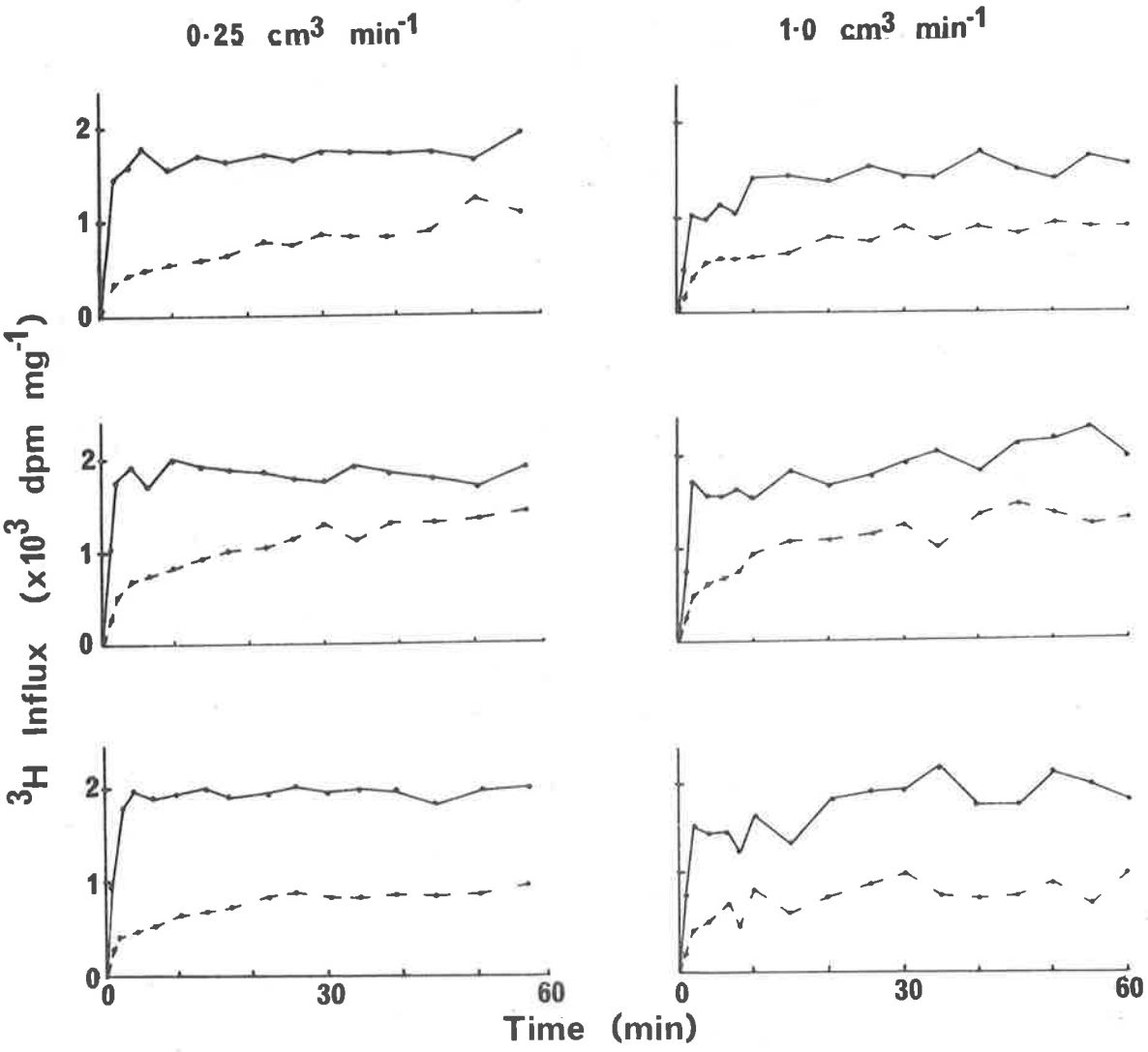

FIGURE 11.1 The influx of tritium-labelled material in untreated (⊸) and PBZ-treated (⟜⊸) arteries during the sustained application of extraluminal dl-^{3}HNA (0.27 µmol l^{-1}). The arteries were perfused intraluminally at a flow rate of 0.25 cm^{3} min^{-1} or 1.0 cm^{3} min^{-1}.

Ordinate: influx of tritiated material (dpm mg^{-1}) per two minutes

Abscissa: duration (min) of NA application and collection

Following PBZ-treatment, the overflow of tritium was enhanced and the rate of entry was faster, so that the steady-state was approached within approximately 6 minutes. The effect of PBZ on the rate of influx was seen in another way. When the data from the six experiments was pooled, it was found that the time taken for the overflow to reach half its peak value (where peak value = mean overflow of the last two collections) was 1.0 ± 0.2 min and 3.7 ± 0.5 min in the PBZ-treated and untreated arteries respectively.

Although the diameters of the arteries were not measured in these experiments, they were in some later experiments which permitted an estimate of the diffusion coefficient in PBZ-treated arteries. For these experiments PBZ was added to both the intraluminal and extraluminal solution and remained in the Krebs' solution during the application (16 minutes) of l-^{3}HNA (0.18 μmol l^{-1}). The overflow of tritium into the luminal perfusate during the first 8 minutes and during the two succeeding periods of 4 minutes was measured. As the tritium activities in the latter collections were virtually identical, the diffusion coefficient for each artery was calculated from the mean activity in these two collections (Table 11.1). The mean diffusion coefficient for l-^{3}HNA in four PBZ-treated arteries was $0.54 \pm 0.02 \times 10^{-6}$ cm^{2} sec^{-1}.

2. Application of NA followed by paraffin

i. Untreated and PBZ-treated arteries

The characteristics of the influx of tritiated material in untreated arteries exposed to dl-^{3}HNA (0.27 μmol l^{-1}) and

ARTERY	WALL THICKNESS (mm)	COEFFICIENT OF DIFFUSION ($\times 10^{-6}$ cm^2 sec^{-1})
1	0.16	0.48
2	0.16	0.52
3	0.21	0.57
4	0.18	0.58
Mean (±S.E.M.)	0.18 (±0.01)	0.54 (±0.02)
Bevan & Waterson	0.16	1.4

TABLE 11.1 Wall thicknesses and diffusion coefficients in four PBZ-treated rabbit ear arteries. The coefficients were determined during the steady-state influx of tritium which occurred between 8 and 16 minutes after the diffusion of NA across the artery wall commenced. Comparative figures derived by Bevan and Waterson (1971) in untreated rabbit ear arteries are included.

l-^{3}HNA (0.18 μmol l^{-1}) are indicated in Figure 11.2.* As outlined in the methods section, NA was applied to the adventitial surface of the artery for 4 minutes, after which it was replaced with liquid paraffin.

The flux of tritium-labelled material into the lumen of untreated arteries differed in a number of respects in the two series of experiments. It will be noted that the diffusion of tritium into the lumen reached a peak during the second two minutes of the application of dl-NA and declined steadily after its removal. However, even 28 minutes after the NA was removed the influx of tritium was still slightly more than 20% of the peak value. When l-NA was applied to the adventitial surface of untreated arteries the peak influx was not observed until after the NA was removed. The influx was maintained at this level for about 8 minutes and then declined more slowly than in the dl-series, so that 28 minutes after the NA was withdrawn the influx of tritium was approximately 40% of the peak value. The magnitude of the influx in the first ten minutes was 4-fold greater in the dl- than in the l-series.

In both series of experiments, PBZ markedly increased the magnitude of the luminal influx of tritiated material; however, the effect of PBZ was more marked in the l-series (Figure 11.2 and Table 11.2). Comparison of the two histograms in Figure 11.2 indicated that the greater effect of PBZ really reflected the smaller

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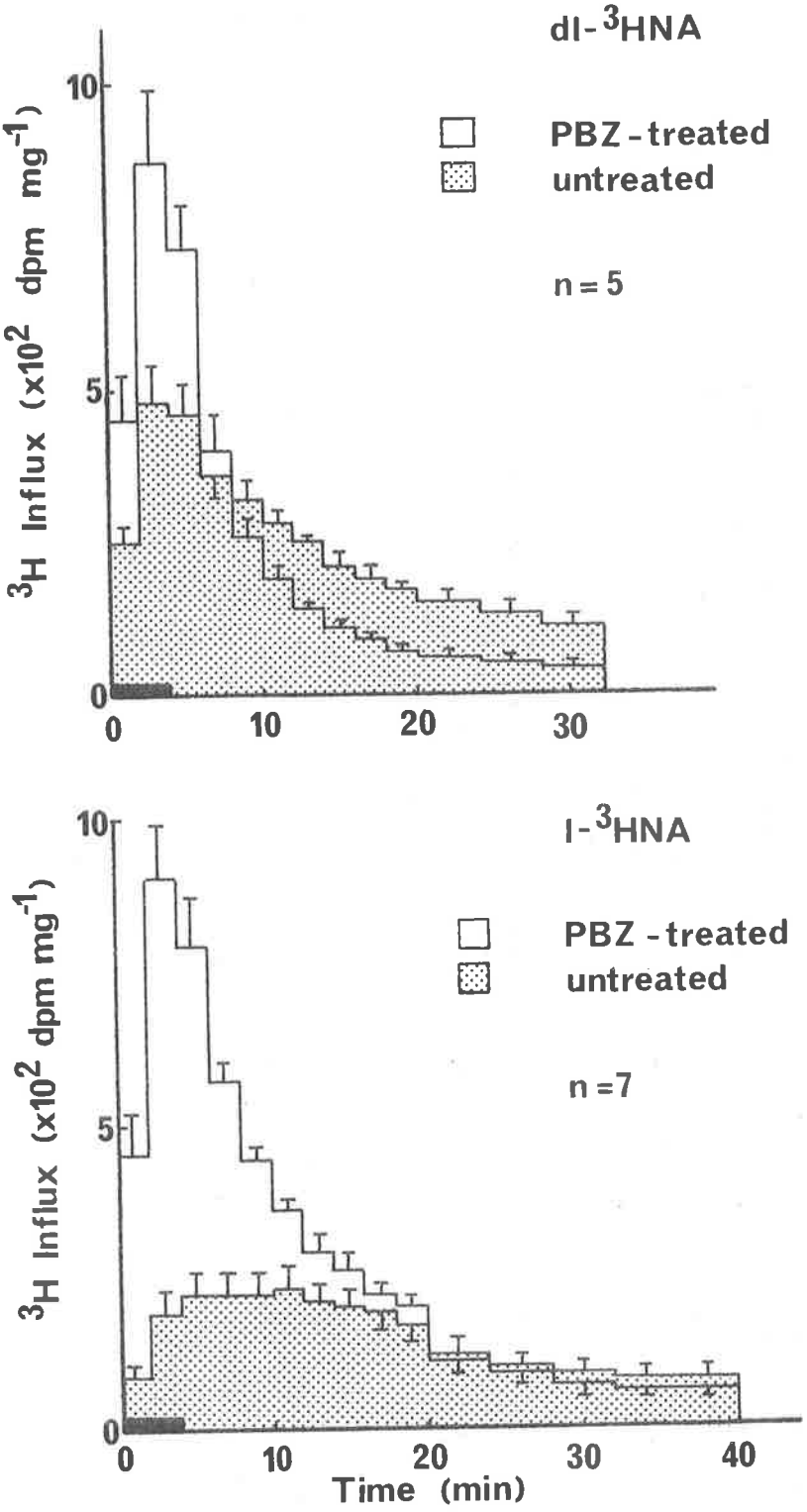
* *see footnote page 11.5.*



n - represents the number of paired arteries used

▬ - *represents the period of application of ^{3}HNA to the adventitial surface of the artery*

FIGURE 11.2 The effects of PBZ on the diffusion of dl-^{3}HNA (0.27 µmol l^{-1}) and l-^{3}HNA (0.18 µmol l^{-1}) across the wall of isolated arteries.

Ordinate: tritium activity (dpm mg^{-1}) per 2 minutes of collection

Abscissa: duration of collection (min)

The influx in each artery was standardized against a constant tritium activity in the diffusion medium of 2.9×10^{6} dpm cm^{-1}.

Time of Collection (min)	SERIES 1 (dl-^{3}HNA)	SERIES 2 (l-^{3}HNA)
4	1.8 (2.1 - 1.6)	5.3 (5.8 - 5.0)
6	1.8 (2.0 - 1.6)	4.7 (5.2 - 4.3)
10	1.5 (1.7 - 1.3)	3.6 (4.0 - 3.3)
20	1.1 (1.3 - 1.0)	2.4 (2.7 - 2.2)

TABLE 11.2 The effect of PBZ on the influx of tritium-labelled material 4, 6, 10 and 20 minutes after the application of dl-^{3}HNA and l-^{3}HNA commenced. The NA was applied to the adventitial surface of each artery for 4 minutes.

The figures in the table represent the geometric means (+S.E.M. -S.E.M.) of the tritium activity in the luminal perfusate in PBZ-treated arteries relative to untreated arteries.

Tests of significance: unpaired t-test

The mean ratios in Series 2 were significantly greater than those in Series 1 at all collection times ($p < 0.05$).

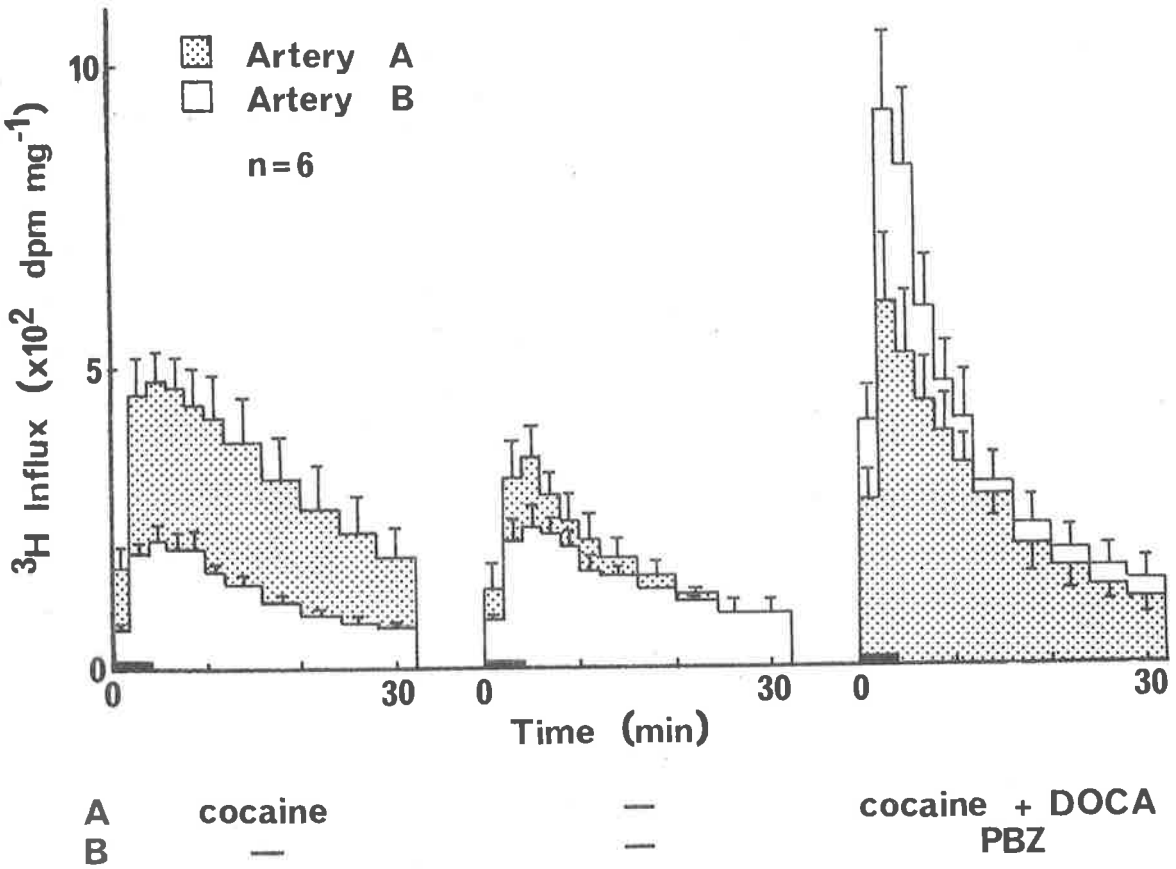
influx of tritium following exposure of untreated arteries to l-NA compared with dl-NA. In the PBZ-treated arteries, the maximum influx of tritium occurred during the application of the NA and declined rapidly, so that 28 minutes after the NA was removed from the outside of the arteries the influx was less than 10% of the peak value.

ii. Cocaine and cocaine plus DOCA-treated arteries

The experimental design in this study was varied to allow repeated applications of l-^{3}HNA (0.18 µmol l^{-1}). As can be seen in Figure 11.3, there was no significant difference between the influx profiles obtained for the successive applications of NA in untreated arteries. As similar results were obtained in a subsequent series of experiments in which the arteries were perfused with phentolamine (Figure 11.6), it was decided that this method of repeating the application of NA was justified.

As indicated in Figure 11.3, the kinetics and magnitude of the tritium influx in untreated arteries were similar to those described previously (Figure 11.2; l-series).

In contrast to the failure of the untreated arteries to constrict, the cocaine and the cocaine plus DOCA-treated arteries consistently responded to the NA. The mean increase in perfusion pressure (steady-state) during the application of NA to the drug-treated arteries was 21 ± 12 x 10^{2} Nm^{-2} and 52 ± 19 x 10^{2} Nm^{-2} respectively.



n - represents the number of pairs of arteries used

▬ - *represents the period of application of 1-^{3}HNA (0.18 µmol l^{-1}) to the adventitial surface of the artery*

FIGURE 11.3 The effects of cocaine (90 µmol l^{-1}), cocaine plus DOCA (27 µmol l^{-1}) and PBZ (33 µmol l^{-1}) on the influx of tritium-labelled material in untreated arteries.

Ordinate: tritium activity (dpm mg^{-1}) per 2 minutes of collection

Abscissa: duration of collection (min)

The influx in each artery was standardized against a constant tritium activity in the diffusion medium of 2.9 x 10^6 dpm cm^{-3}.

The influence of these drugs on the influx of tritium-labelled material is shown in Figure 11.3. Cocaine increased the magnitude of the influx, but the rate of decline from the peak value remained slow. An unexpected finding was that the action of cocaine had not completely disappeared 30 minutes after its washout from the intraluminal and extraluminal media. This was seen by comparing the influx of tritium during the second application of NA in this artery (A) with the influx in the paired, untreated artery (B).

The addition of DOCA to cocaine-treated arteries tended to increase the magnitude of the peak influx of tritium, but had a more dramatic effect on the kinetics of the influx. As indicated in Figure 11.3, the attainment of the peak value, and more particularly the rate of decline of the influx from this peak, was more rapid in the presence of DOCA. One of the six arteries treated with cocaine and DOCA did not display these effects. In this artery the influx peaked during the application of NA and remained reasonably constant for the next 12 minutes (Figure 11.4). The magnitude of the peak overflow was considerably less than the mean overflow for the six arteries. It was noted that the perfusion pressure during the application of NA in this artery was more than three times greater than the mean rise in perfusion pressure for the other five arteries.

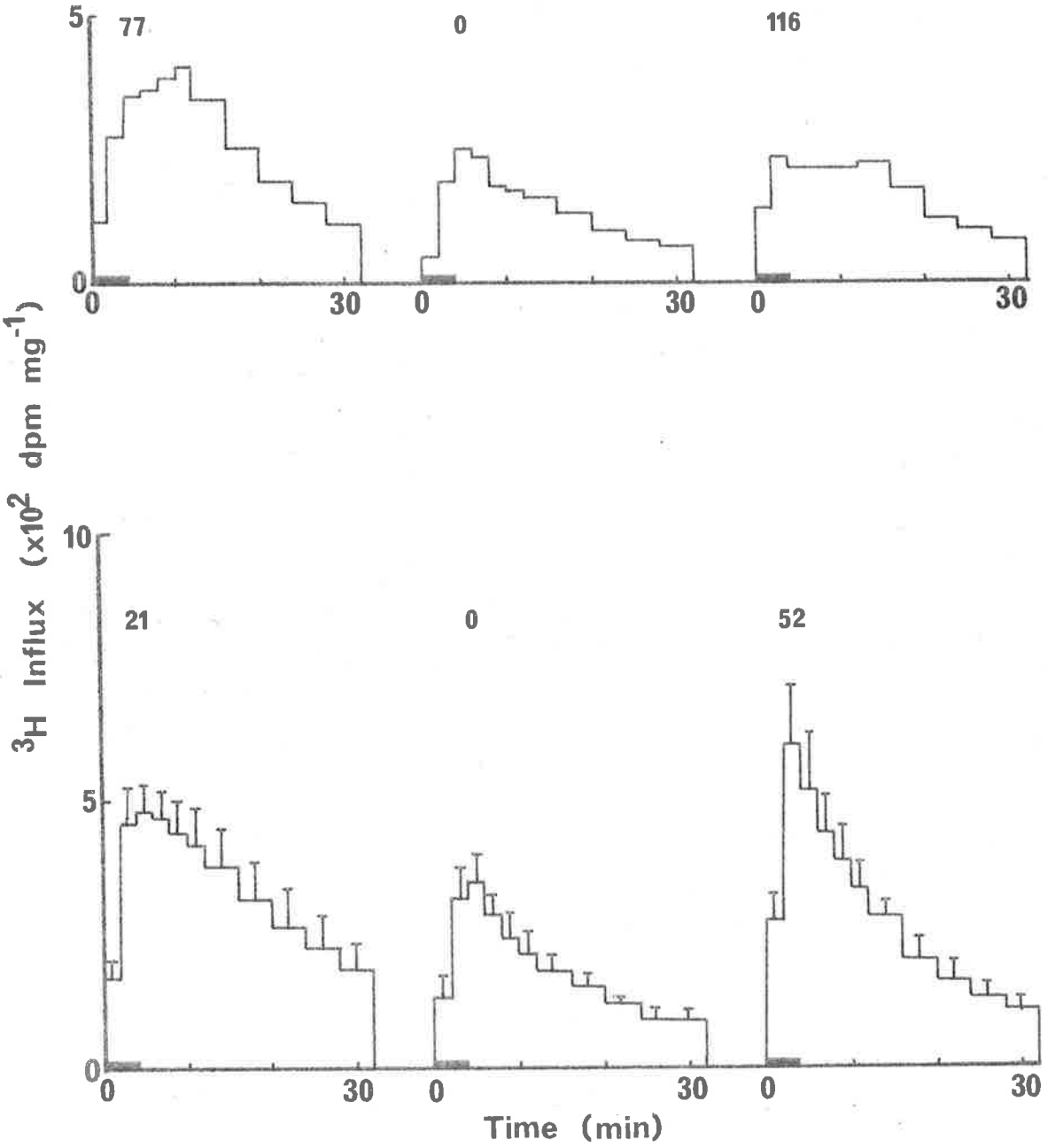
Although the magnitude of the tritium influx in paired PBZ-treated arteries was greater than that in the cocaine and DOCA-treated arteries, the kinetics of the overflow were similar; that is, the activity of the tritiated material which overflowed into the lumen reached a peak during the application of ^{3}HNA and declined rapidly following its removal.

FIGURE 11.4 The effects of cocaine (90 μmol l^{-1}) and cocaine plus DOCA (27 μmol l^{-1}) on the influx of tritium-labelled material in untreated arteries. The lower three histograms represent the mean influxes (+S.E.M.) in six arteries, and the upper histograms the influxes in one of these arteries in which the increase in the perfusion pressure during the application of 1-^{3}HNA (plus drugs) was substantially higher than the mean increase for the six arteries used.

Ordinate: tritium activity (dpm mg^{-1}) per 2 minutes of collection

Abscissa: duration of collection (min)

The influx in each artery was standardized against a constant tritium activity in the diffusion medium of 2.9×10^{6} dpm cm^{-3}.



▬ - *represents the period of application of 1-^{3}HNA (0.18 µmol l^{-1}) to the adventitial surface of the artery*

The figure above each histogram refers to the rise in perfusion pressure (x 10^2 Nm^{-2}) during the extraluminal application of 1-^{3}HNA

The influences of the various drugs on the magnitudes of the tritium influx, 2, 4, 6 and 20 minutes after diffusion commenced, are shown in Figure 11.5. The more rapid kinetics of the influx which prevailed in the cocaine plus DOCA, and PBZ-treated arteries are illustrated by the greater influx, relative to untreated and cocaine-treated arteries, after 2 and 4 minutes than after 6 and 20 minutes. It is noteworthy that the total influx of tritium into the lumen after 20 minutes was of the same order in cocaine and cocaine plus DOCA-treated arteries. The greatest differences in the influxes after 20 minutes were between the untreated arteries and the various drug-treated arteries.

iii. Cocaine and cocaine plus DOCA in phentolamine-treated arteries

To avoid the possible influence of constriction on the luminal influx of tritiated material, the foregoing experiments were repeated in the presence of phentolamine. The results are shown in Figure 11.6. As expected, the perfusion pressure did not increase during the application of NA in any of the arteries.

Phentolamine itself increased the magnitude of the peak influx of tritium (approximately 50%), and increased the kinetics of influx relative to unpaired, untreated arteries (Figure 11.3), so that the peak occurred earlier and the decline from this peak was more rapid.

In the presence of phentolamine, the action of cocaine on the diffusion of tritium was markedly altered; the magnitude of the peak influx was greater and the decline from this peak value was faster. The influx of tritium in cocaine and phentolamine-treated arteries more closely resembled that in PBZ-treated arteries.

TESTS OF SIGNIFICANCE:

The levels of significance of the effects of the various drug treatments on the influx of tritium-labelled material are indicated for the four collection times illustrated in Figure 11.5 ($p <$).

paired t-test

		Cocaine	Cocaine + DOCA	PBZ
Untreated	2	0.02	0.01	0.002
	4	0.01	0.02	0.001
	6	0.01	0.02	0.002
	20	0.05	0.02	0.005
Cocaine	2		0.05	0.02
	4		0.1	0.02
	6		0.1	0.02
	20		0.1	0.05
Cocaine + DOCA	2			0.1
	4			0.05
	6			0.05
	20			0.05

paired t-test

		Phentolamine		
		Cocaine	Cocaine + DOCA	PBZ
Phentolamine	2	0.05	0.005	0.005
	4	0.02	0.005	0.005
	6	0.02	0.005	0.005
	20	0.005	0.005	0.005
Phentolamine + Cocaine	2		0.05	0.1
	4		0.1	0.1
	6		0.2	0.2
	20		0.4	0.4
Phentolamine + Cocaine + DOCA	2			0.4
	4			0.4
	6			0.5
	20			0.5

unpaired t-test

			Phentolamine	
		Phentolamine	Cocaine	Cocaine + DOCA
Untreated	2	0.05		
	4	0.02		
	6	0.05		
	20	0.05		
Cocaine	2		0.05	
	4		0.02	
	6		0.02	
	20		0.3	
Cocaine + DOCA	2			0.05
	4			0.05
	6			0.05
	20			0.2

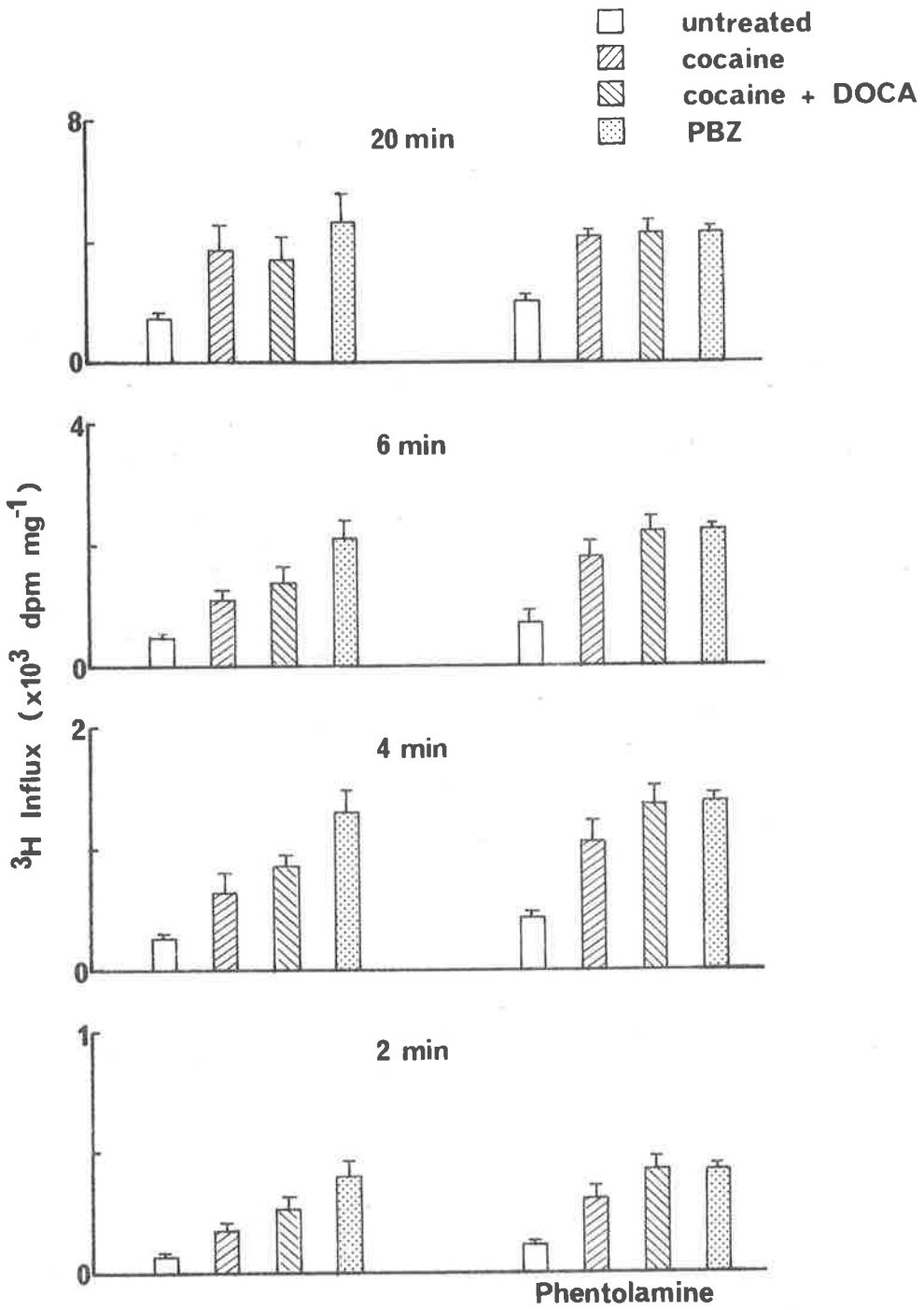
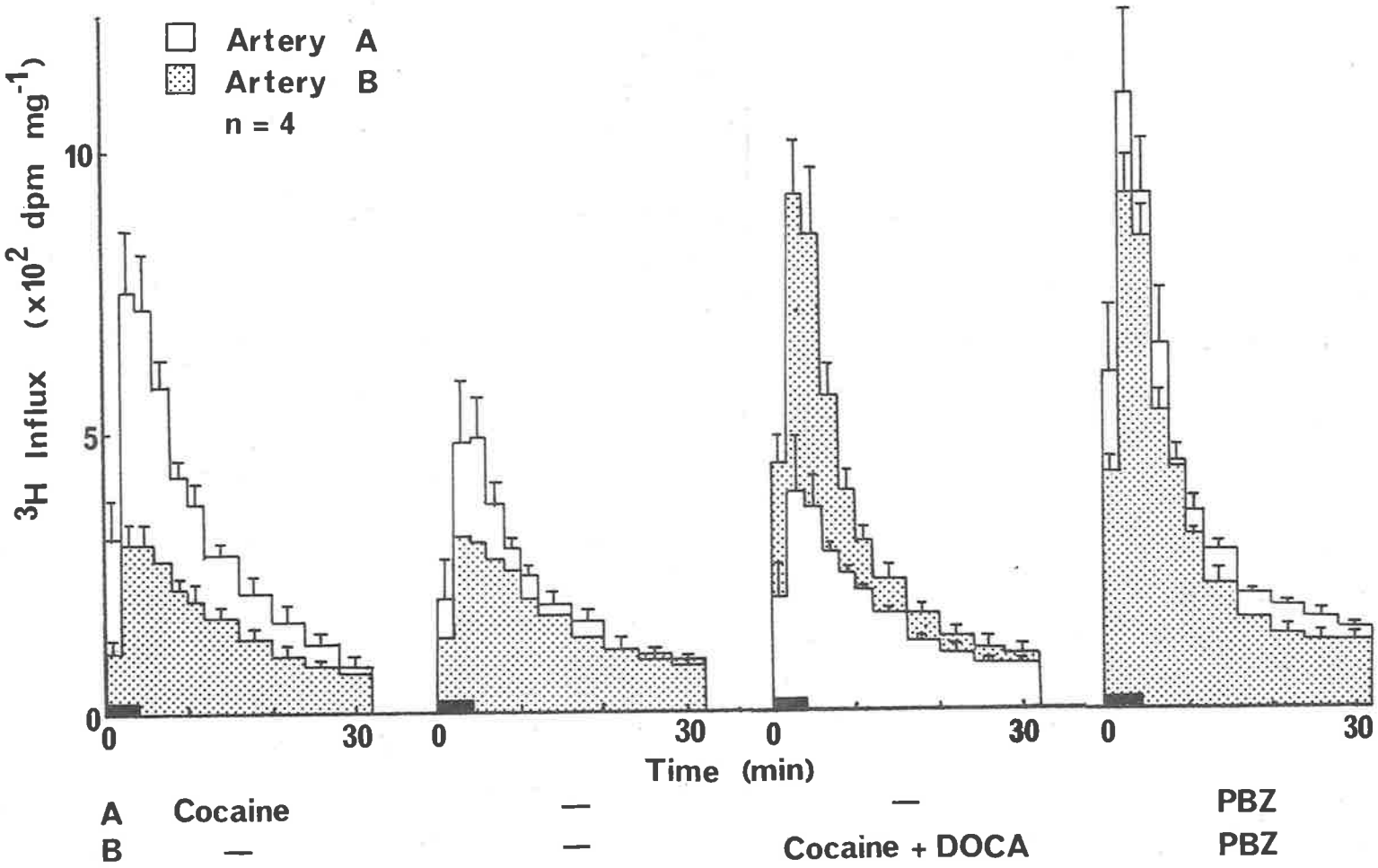


FIGURE 11.5 The effects of cocaine (90 µmol l^{-1}), cocaine plus DOCA (27 µmol l^{-1}) and PBZ (33 µmol l^{-1}) on the influx of tritiated material into the lumen of untreated and phentolamine-treated (0.3 µmol l^{-1}) arteries. The mean influx of tritium (+S.E.M.) 2, 4, 6 and 20 minutes after diffusion commenced is indicated for each treatment regime.

Ordinate: tritium activity (dpm mg^{-1}) in the luminal perfusate

The influx in each artery was standardized against a constant tritium activity in the diffusion medium of 2.9 x 10^6 dpm cm^{-3}.



n - represents the number of pairs of arteries used
▬ *- represents the period of application of 1-^{3}HNA (0.18 µmol l^{-1}) to the adventitial surface of the artery*

FIGURE 11.6 The effects of cocaine (90 µmol l^{-1}), cocaine plus DOCA (27 µmol l^{-1}) and PBZ (33 µmol l^{-1}) on the influx of tritium-labelled material in phentolamine-treated (0.3 µmol l^{-1}) arteries.
Ordinate: tritium activity (dpm mg^{-1}) per 2 minutes of collection
Abscissa: duration of collection (min)
The influx in each artery was standardized against a constant tritium activity in the diffusion medium of 2.9 x 10^6 dpm cm^{-3}.

The further effect of DOCA on the influx of tritium in cocaine and phentolamine-treated arteries was to increase the magnitude of the influx in the first 6 minutes, so that the kinetic profile was virtually indistinguishable from that of PBZ.

The effects of cocaine, cocaine plus DOCA and PBZ on the magnitude of the influx of tritium-labelled material in untreated and phentolamine-treated arteries are summarized in Figure 11.5.

It will be noted that:

a. treatment with PBZ, DOCA and/or cocaine significantly increased the influx of tritiated material in untreated and phentolamine-treated arteries for the four periods shown,

b. phentolamine increased the influx of tritium in untreated, cocaine-treated and cocaine plus DOCA-treated arteries during the four periods indicated. This difference was significant except for the 20 minutes' collections in cocaine and cocaine plus DOCA-treated arteries,

c. the amount of tritium which reached the lumen was identical for PBZ-treated arteries and cocaine, DOCA and phentolamine-treated arteries for the four collection periods represented.

As a further index of the influence of constriction on the influx of tritium-labelled materials pairs of arteries, each treated with cocaine plus DOCA, were exposed to two different concentrations of l-^{3}HNA (0.06 and 0.3 μmol l^{-1}). The lower concentration was selected

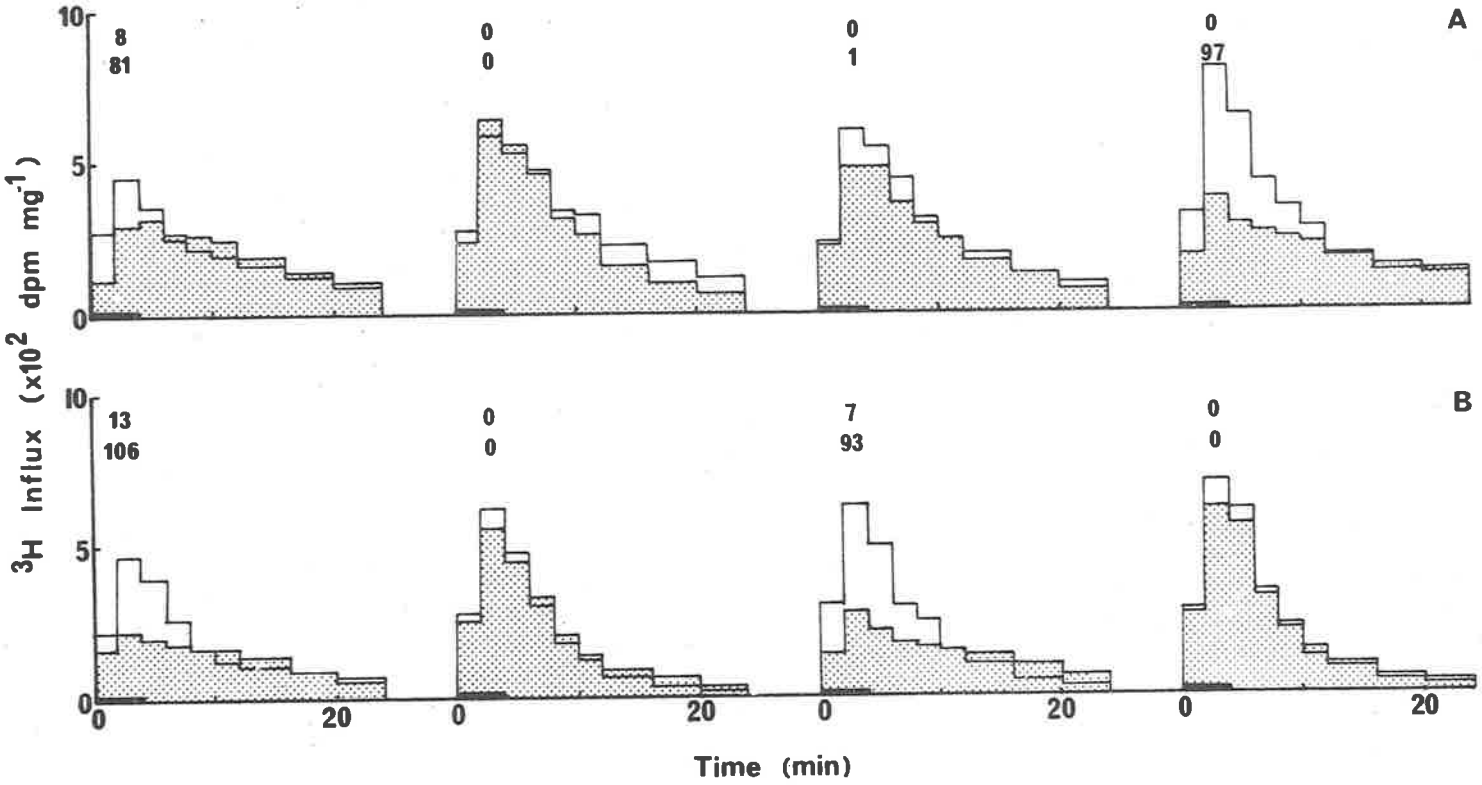
on the basis that if it produced a constrictor response at all the response would be small. The higher concentration was selected as it was expected to produce a substantial constrictor response in the arteries. Unfortunately, in one artery the higher concentration only produced a very small response. For this reason, the influx profiles from each artery and the steady-state constrictor responses produced by the NA are shown in Figure 11.7.

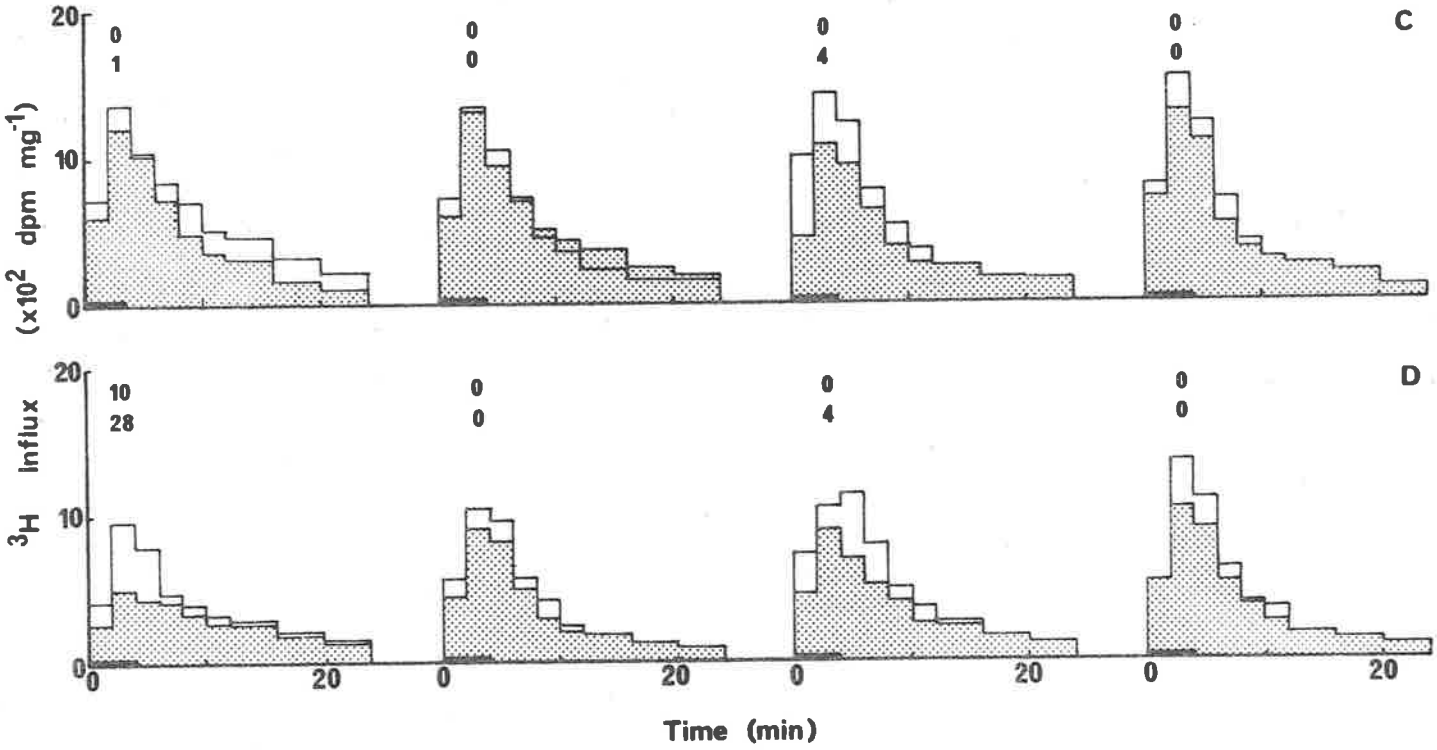
It will be seen that, when the concentration of NA present in the bathing medium was low, the kinetic profiles of the influx of tritium-labelled material were less affected by either phentolamine or PBZ-treatment than were the profiles obtained with the higher concentration of NA. That is, when the constriction caused by the higher concentration of NA was prevented by phentolamine or PBZ, the magnitude of the peak influx was increased and the decline from this peak was more rapid. The one artery which failed to show these effects of phentolamine and PBZ also failed to respond by constriction to the higher concentration of NA.

3. Influx of NA and metabolites

The proportions of NA, NMN and deaminated metabolites (DEAM) in the luminal perfusate following the diffusion of NA across the wall of phentolamine-treated arteries are shown in Figure 11.8. The influences of cocaine and cocaine plus DOCA on the relative proportions of the three fractions are also indicated.

In phentolamine-treated arteries which were exposed to l-^{3}HNA (0.18 µmol l^{-1}), only that fraction which contained deaminated


A
B
8
81
0
0
0
1
0
97
13
106
0
0
7
93
0
0
10
5
0
20
Time (min)
^3H Influx ($x10^2$ dpm mg^{-1})



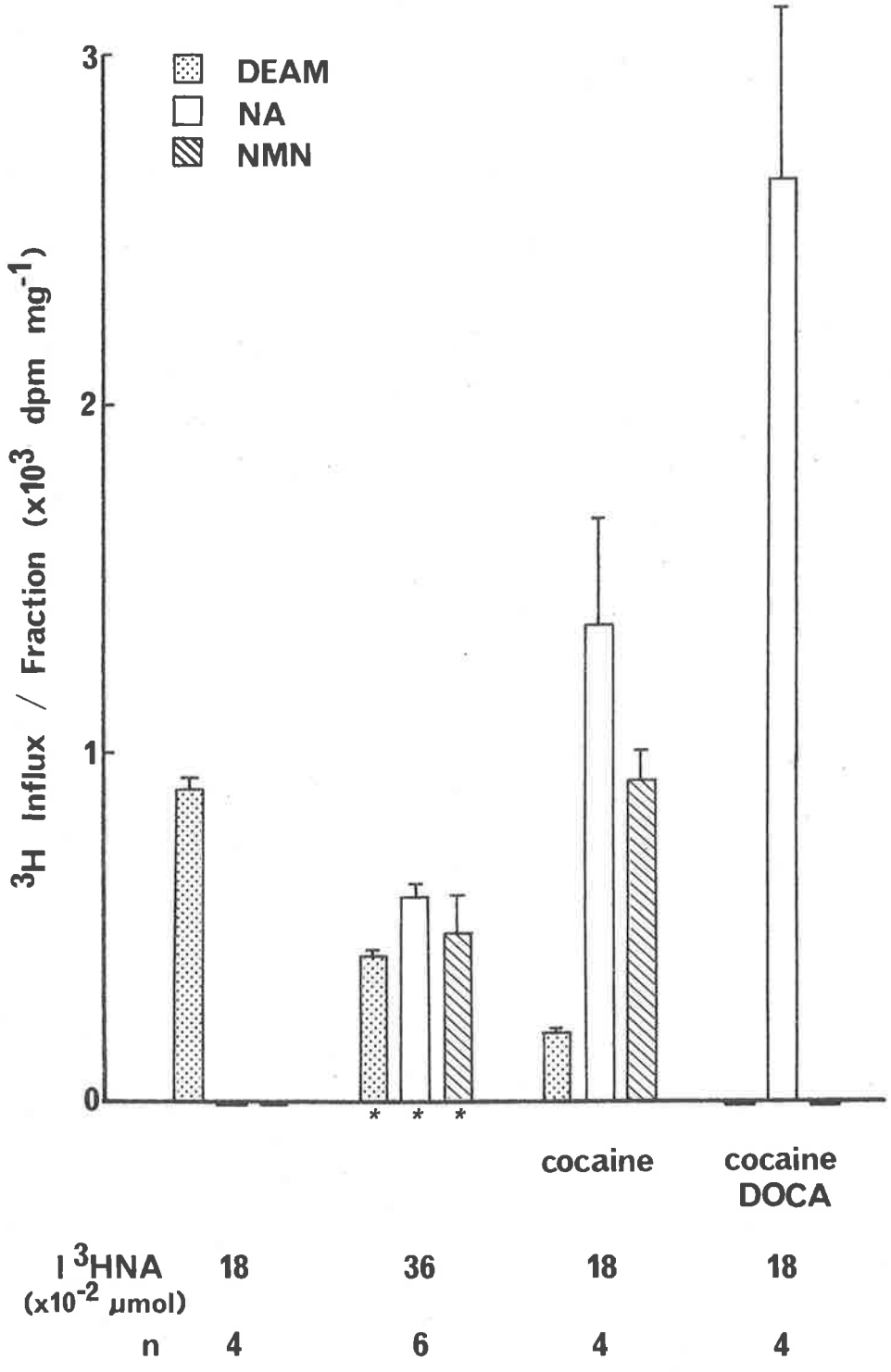
▬▬ - *represents the period of application of 1^{-3}HNA (0.3 µmol 1^{-1}; hatched histogram) and 1^{-3}HNA (0.06 µmol 1^{-1}; open histogram) to four pairs of ear arteries.*

FIGURE 11.7 The effects of vasoconstriction on the diffusion of ^{3}HNA across the wall of the rabbit ear artery. For the first three applications of NA all arteries were treated with cocaine (90 µmol 1^{-1}) and DOCA (27 µmol 1^{-1}). Phentolamine (0.6 µmol 1^{-1}) was added as well for the second application. For the fourth application the arteries were treated with PBZ (33 µmol 1^{-1}) except artery A (hatched profile only) which was treated with cocaine and DOCA.
The figures above each histogram refer to the rise in perfusion pressure (x 10^{2} Nm^{-2}) during the application of NA. The upper figure refers to the response during the application of 1^{-3}HNA (0.06 µmol 1^{-1}) and the lower figure during the application of 1^{-3}HNA (0.3 µmol 1^{-1}).

FIGURE 11.8 The effects of cocaine (90 μmol l^{-1}) and DOCA (27 μmol l^{-1}) on the nature of the tritium-labelled material which overflowed into the luminal perfusate following the diffusion of l-^{3}HNA across the wall of phentolamine-treated (0.3 μmol l^{-1}) arteries. NA was applied to the adventitial surface of each artery for 8 minutes, and the influx was separated by Dowex 50 chromatography into fractions containing deaminated metabolites (DEAM), NA and normetanephrine (NMN).

Ordinate: activity (dpm mg^{-1}) of the tritium-labelled material which influxed into the luminal perfusate during the application of NA. These activities were standardized against a tritium activity in the diffusion medium of 2.9×10^{6} dpm cm^{-3}, except as noted opposite.

In two experiments, the influx of tritium-labelled material was analysed following the diffusion of l-^{3}HNA (0.18 μmol l^{-1}) across the wall of untreated arteries (i.e., not treated with phentolamine). NA and NMN could not be detected, and the tritium activity of the DEAM was less than twice the expected background activity.



n - represents the number of arteries used

** - the tritium activity in each of these fractions was standardized against an extraluminal activity of 5.8 x 10^6 dpm cm^{-3}.*

metabolites (DEAM) could be unequivocally demonstrated. When the NA concentration applied to the adventitial surface of these arteries was increased to 0.36 µmol l^{-1}, the total tritium influx increased by 75% and in addition to DEAM (28%), NA (39%) and NMN (33%) were now detected in the influx in four of the six arteries used; in the other two, the presence of NA and NMN was equivocal because of the low tritium activity.

In the presence of cocaine the influx of deaminated metabolites was equivocal, as the tritium activity associated with the fraction containing DEAM was only 50% to 100% above the expected background level of all four arteries. Considerable amounts of NA and NMN now reached the lumen. Following the addition of DOCA to these cocaine-treated arteries only the NA fraction could be positively identified; the tritium activity in the other two fractions was less than 50% above the expected background activity. Furthermore, the amount of NA which reached the lumen was approximately twice that obtained in the absence of DOCA.

DISCUSSION

One of the reasons why the flow rate of 0.23 cm^3 min^{-1} was selected for these studies was that the concentration of tritium-labelled materials which accumulated in the perfusate was usually sufficient for reliable assay. Thus it was an important finding in the present experiments that this flow rate, when compared to a rate of 1.0 cm^3 min^{-1}, was not a limiting factor to the diffusion of NA across the artery wall (at least in untreated and PBZ-treated arteries).

Another important consideration in the design of the experiments described in the chapter was whether the diffusion characteristics of d-NA and l-NA were different. As the results indicated (Figure 11.2), the diffusion characteristics of dl-NA and l-NA were virtually identical in PBZ-treated arteries, but in untreated arteries considerably more tritium reached the lumen following the application of dl-NA than l-NA.

These results can be explained by the findings of Stjärne and Euler (1965) that the vesicular retention of NA was stereospecific for l-NA, and those of Trendelenburg and Draskóczy (1970) that the amount of NA retained by the neuronal tissues was very small when vesicular retention was inhibited. Hence, in the untreated artery it is possible that the smaller influx of tritium-labelled material following the diffusion of l-^{3}HNA, when compared with dl-^{3}HNA, reflected the greater neuronal uptake and retention of l-NA. Although there is evidence in some tissues that COMT and smooth muscle uptake of NA are not stereospecific, and that MAO only exhibits a small degree of stereospecificity (Chapter 1), metabolism and extraneuronal uptake might have further influenced the relative diffusion of d-NA and l-NA in the rabbit ear artery. The PBZ studies lend support to the concept that one or more mechanisms in the artery wall are stereospecific for l-NA, as the tritium influx profiles in PBZ-treated arteries following diffusion of l-^{3}HNA and dl-^{3}HNA were virtually indistinguishable. A possible complication in these experiments was that the dl-NA was not purified prior to use, and hence the greater influx of tritium in the dl-NA studies might be partly the result of impurities. To avoid these problems, purified samples of l-NA were

used in all other experiments except those in which the application of NA was sustained for 60 minutes.

The influence of the neuronal and smooth muscle uptake systems on the diffusion of NA across the artery wall was illustrated by those experiments in which NA diffusion was sustained for 60 minutes in untreated and PBZ-treated arteries. Whereas in the untreated preparations the steady-state influx of tritium, if attained at all, was reached very slowly, PBZ decreased the time required for this to develop and greatly enhanced the magnitude of the influx. Hence, as none of the untreated arteries constricted during the application of NA, these effects of PBZ were mostly, if not entirely, a consequence of the inhibition of the neuronal and smooth muscle uptake systems.

Since it has been suggested that NA is not firmly bound in arterial smooth muscle cells following its uptake (Gillespie, 1968), the smaller amount of tritium which reached the lumen during steady-state influx in untreated arteries was probably mainly a consequence of the neuronal uptake of NA. On the other hand, the delayed attainment of the steady-state influx in the untreated arteries might be a manifestation of the increasing saturation of the uptake systems, and the retarded efflux of metabolites from the nerves and smooth muscle cells. However as the diffusion medium used in these experiments was not purified prior to use, and as approximately one third of the NA was the dextro isomer, it is probable that the magnitude of the influx in the untreated arteries was not a true reflection of the factors influencing the diffusion of NA in the artery wall.

It was also of interest that the diffusion coefficient (0.54×10^{-6} cm^2 sec^{-1}) calculated during the steady-state influx of tritium in PBZ-treated arteries was less than that determined by Bevan and Waterson (1971) in untreated arteries (1.4×10^{-6} cm^2 sec^{-1}). Bevan and Waterson estimated this coefficient from the formula used by Bevan and Török (1970) to calculate the diffusion coefficient for ^{3}HNA in the rabbit aorta. In their experiments Bevan and Waterson measured the amount of NA in the extracellular space following the application of ^{3}HNA to the intimal surface of the arteries. It was not possible to determine whether the lower diffusion coefficient in the present experiments was a consequence of an action of PBZ, or whether it was the result of the different experimental procedures.

Additional evidence that the uptake processes influenced the diffusion of NA across the artery wall was provided by the finding that PBZ increased the rate and magnitude of the influx of tritium-labelled materials, following the application of l-^{3}HNA to the adventitial surface of arteries for 4 minutes. Since it was not possible to make an assessment of the relative contribution of neuronal uptake and extraneuronal uptake to the diffusion characteristics in these experiments, further studies were carried out with the relatively specific inhibitors of neuronal and extraneuronal uptake, namely cocaine and DOCA.

Comparison of the effects of cocaine, and of cocaine plus DOCA indicated that the main influence of cocaine was to increase the magnitude of the influx, whereas the addition of DOCA accelerated the attainment of the peak influx, and the subsequent decline from this peak. Unfortunately, these results were complicated by the

sensitizing actions of cocaine and DOCA, so that although the concentration of NA was subconstrictor in the untreated arteries it caused varying degrees of constriction in a number of these drug-treated arteries. When the constriction was small, the influx of tritium in cocaine plus DOCA-treated preparations usually resembled that in PBZ-treated arteries. However when the constriction was greater, the magnitude of the influx was considerably less and the kinetics were slower. These observations indicated that constriction itself might have altered the characteristics of the diffusion of NA across the artery wall. This possibility was supported by the finding that phentolamine increased the magnitude and rate of influx of tritium in cocaine-treated arteries. The addition of DOCA appeared to increase the rate of influx of tritium-labelled material, so that the influx profile was identical to the profile in PBZ-treated arteries. Thus, as noted previously in the non-phentolamine-treated preparations, the major effect of DOCA in cocaine-treated arteries was to increase the rate of diffusion of NA from the adventitia to the lumen.

One of the questions raised by these results was whether the effects of phentolamine were due to the elimination of the constrictor response to NA, or whether phentolamine itself augmented the effects of cocaine or DOCA by interfering with the uptake or binding of the amine. Inhibition of neuronal uptake and extraneuronal uptake of NA by phentolamine has been reported by Cubeddu et al (1974) in the isolated cat spleen, but the concentration of the drug required to inhibit the processes was much higher than that used in these experiments.

The simple direct experiment of comparing the effect of constriction on the influx of a neutral material like sorbitol, which is restricted to the extracellular space, has not yet been carried out. However, less direct evidence presented in this chapter suggested that phentolamine exerted its effects by abolishing constriction. This evidence was obtained from experiments in which two concentrations of NA were applied to the adventitial surface of cocaine plus DOCA-treated arteries. Thus:

a. when the concentration of NA either failed to constrict the artery or induced only a small constriction, the influx of tritium was virtually unaffected by phentolamine,

b. when the concentration of NA was sufficient to produce a substantial response, the influx of tritium was reduced unless phentolamine inhibited constriction,

c. irrespective of the concentration of NA applied to the artery, the tritium influx profiles in cocaine plus DOCA-treated arteries (providing there was no constriction), in cocaine plus DOCA and phentolamine-treated arteries and in PBZ-treated arteries were similar. Note that the influxes were standardized to a constant extraluminal tritium activity, irrespective of the actual concentration of NA used.

It was concluded from the studies discussed so far, that neuronal uptake of NA was the single most important factor determining the magnitude of the influx of tritium-labelled material following the diffusion of ^{3}HNA across the artery wall. These results are consistent with evidence that NA is taken up and bound firmly within vesicles in the cytoplasm of the nerve.

The results also indicated that the influence of extraneuronal uptake was primarily on the rate of diffusion of NA across the artery wall. Such a finding was consistent with an extraneuronal compartment which possessed only a limited capacity to store NA compared with neuronal tissue. Studies in other tissues, particularly the cat nictitating membrane and rabbit aorta (Langer, 1970; Levin, 1974), demonstrated that the extent to which unchanged amine could be stored in the extraneuronal compartment was limited by its rapid O-methylation to normetanephrine. Assuming that NMN effluxes from these stores relatively rapidly, the same mechanism in the rabbit ear artery, namely O-methylation, might account for the relatively minor effect of DOCA on the total tritium influx, once the neuronal uptake of ^{3}HNA had been eliminated by cocaine. This was verified by analysis of the tritiated-material which overflowed into the lumen of cocaine-treated and cocaine plus DOCA-treated arteries following the diffusion of ^{3}HNA.

Chromatographic analysis of the intraluminal influx indicated that in untreated arteries,* although the presence of deaminated metabolites was unequivocal, the existence of NA and NMN was doubtful, even when the higher concentration of NA (0.36 µmol l^{-1}) was used. In cocaine-treated arteries, the presence of DEAM in the influx was equivocal, as in all four arteries the tritium activity associated with this fraction was less than twice the expected background activity. Substantial amounts of NA and NMN were assayed.

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* *All arteries in this particular study were treated with phentolamine to prevent constriction and to maximize the influx of tritium, but will be referred to as untreated for convenience.*

These findings implied that in untreated arteries most of the NA which diffused into the tunica adventitia was taken up by the sympathetic nerves and was bound firmly. Presumably the small amount of DEAM which reached the lumen of untreated arteries was derived from intraneuronal metabolism of NA as it entered the cytoplasm following uptake or following leakage from the storage granules. As indicated previously, in many tissues intraneuronal metabolism occurs primarily by MAO to the inactive glycol, DOPEG (page 8.18).

In the presence of cocaine and DOCA, NMN and the deaminated metabolites were virtually eliminated, so that the influx was almost all NA. This finding confirmed that when neuronal uptake was blocked, a substantial proportion of the increased amount of NA which diffused into the tunica media was metabolized. Clearly, this metabolism involved extraneuronal COMT. An involvement of extraneuronal MAO was doubtful, although it could not be entirely eliminated, as the very small and equivocal amount of DEAM detected in the cocaine-treated arteries was reduced in the cocaine and DOCA-treated arteries.

The relevance of the preceding findings to the fate of released transmitter, and the physiological response of the ear artery to NA should be considered.

As shown in Chapter 9, PBZ enhanced the stimulation-induced overflow of transmitter in paraffin-bathed arteries by 186% (page 9.6). Since the conditions under which this overflow was studied were analogous to the diffusion studies (this chapter), it can be concluded that the contribution of the two uptake processes to the enhanced overflow of released NA was represented by the increased

influx (140%) in PBZ-treated arteries in the diffusion studies (1-series; Figure 11.2). Assuming that the difference (46%) represented the actions of PBZ on the release process and on vasoconstriction, namely blockade of the pre- and post-synaptic alpha receptors, it is clear that the uptake processes were of greater importance than the combined effects of the inhibitory feedback mechanism and the constrictor response in determining the amount of transmitter which overflowed into the lumen in untreated arteries. It was of interest that this estimate of the actions of PBZ on the alpha adrenoreceptors was little different from that derived by comparing the effects of PBZ on the stimulation-induced overflow in untreated and phentolamine-treated arteries (42%). An alternative measure of the contribution of the uptake processes to the removal of released transmitter was provided by the enhanced overflow in phentolamine-treated arteries in the presence of PBZ (130%). For this estimation it was assumed that phentolamine did not appreciably influence uptake and that it abolished the presynaptic mechanism.

A second application of the diffusion experiments was their relationship to other pharmacological studies, in which cocaine caused a ten-fold increase in the sensitivity of the rabbit ear artery to extraluminal NA (de la Lande and Waterson, 1967), and the addition of DOCA caused a further two-fold increase (Johnson, 1975). The present results indicated that the amount of NA which reached the tunica media in phentolamine-treated arteries was approximately eight to ten-fold greater in cocaine-treated than non-cocaine-treated arteries. This difference was derived by comparing the total amount of NA and NMN which reached the lumen in the two series of experiments (Figure 11.8).

For this comparison, the NA and NMN figures for the non-cocaine-treated arteries were derived from the influx following the application of NA at a concentration of 0.36 μmol l^{-1}. Use of these figures implied that the diffusion of NA was unaffected by the different concentrations used (0.18 and 0.36 μmol l^{-1}). As a simple approximation, it might be assumed from the above data that an eight to ten-fold increase in the amount of exogenous NA which reached the smooth muscle cells represented a ten-fold increase in sensitivity. A major difficulty with this type of interpretation was that it was not possible to determine how quickly the NA was O-methylated once it reached the tunica media. Therefore, that portion of the influx which entered the media as NA, but reached the lumen as NMN, might not have contributed to the sensitization process.

Similarly, this difficulty applied to the interpretation of the sensitization produced by DOCA in cocaine-treated arteries. However, it was of interest that the amount of unchanged NA which reached the lumen in the DOCA-treated arteries was twice that in the non-DOCA-treated preparations.

In summary, the findings of this chapter permitted quantitation of the two uptake processes, and indicated that the neuronal uptake of NA was the single most important factor influencing the diffusion of NA from the tunica adventitia to the lumen in the rabbit ear artery. The influence of the smooth muscle uptake of NA was primarily on the rate of diffusion of NA across the artery wall. Intraneuronal metabolism by MAO, and extraneuronal metabolism by COMT following smooth muscle uptake reduced the amount of NA which diffused across the artery wall.

The results also indicated that the vasoconstrictor response of the artery limited the diffusion of exogenous NA. Further experimentation will be required to quantitate the effects of vasoconstriction and inhibition of transmitter release on the overflow of NA released by nerve stimulation.

CHAPTER 12

GENERAL DISCUSSION

CHAPTER 12

The present study was prompted by earlier observations in this laboratory that a factor or factors in the wall of the rabbit ear artery constituted a barrier to the unrestricted diffusion of exogenous NA. When the work for this thesis commenced it was apparent that the neuronal plexus restricted the diffusion of exogenous NA and there was some evidence which suggested that the tunica media also contributed to this barrier (Chapter 1). Basically, two experimental approaches were adopted to examine the mechanisms which influenced the diffusion of NA in the artery wall; one involved an investigation of the release and overflow of transmitter, and the other the diffusion of exogenous NA. Some information was also provided by the vasoconstrictor response studies.

In the earlier chapters in this thesis some of the problems encountered with the various experimental techniques were outlined. One of these involved the failure of fluorescence histochemistry to demonstrate diffusion of NA following its release by nerve stimulation. A number of reasons for this failure are now apparent, and include the rapid and preferential diffusion of released NA through the tunica adventitia of untreated arteries. Hence, if these experiments were repeated it would be of advantage to restrict the extraluminal diffusion of NA by the use of liquid paraffin. Under these conditions it might be possible to visualize NA in the tunica media following its release by nerve stimulation, and to examine qualitatively the effects

of the uptake processes and metabolism on the diffusion of transmitter through the tunica media.

A further problem encountered in the early stages of this project was the very small amount of endogenous NA which overflowed from the adventitial surface of artery segments, even following PBZ-treatment. An added complication in these experiments was the finding that NMN and MN, the O-methylated metabolites of NA and A, were demethylated during nerve stimulation, and that under certain conditions NA itself was oxidized to an aminochrome. Whether these processes also occurred in the artery wall following the release of NA and its metabolism by COMT is not known, although it is perhaps unlikely as these electrolytic changes occurred at the surface of the anode. In the response studies (Chapter 6), in which MN was used to inhibit the smooth muscle uptake of NA, biological assay of the superfusate containing MN did not indicate an altered level of vasoactivity following the passage of current between the electrodes, which suggested that the potentiation produced by nerve stimulation in the presence of MN was a direct effect of the drug, and not an effect of its demethylated product NA.

An important consideration in the isotopic studies was the purity of the NA samples, and the possible influence of impurities on the diffusion and overflow of NA in the rabbit ear artery. Although impurities were present in the stock solutions of NA they did not appear to be retained in the artery following incubation (Chapter 4). Other studies reported in this thesis justified the use of ^{3}HNA despite the apparent impurities. For example, cocaine exerted its well known inhibitory action on the neuronal uptake of NA (page 10.5),

nerve stimulation did not increase the overflow of tritium-labelled material from arteries which were incubated in cocaine (90 μmol l^{-1}) and ^{3}HNA (page 8.11), and the stimulation-induced overflow in PBZ-treated arteries comprised ^{3}HNA (page 8.11). In the latter studies, although it was apparent that the overflow also contained deaminated metabolites, evidence was presented that this was unrelated to nerve stimulation, and probably reflected the efflux of intraneuronal metabolites of NA. In the isotopic diffusion studies (Chapter 11), all but one of the samples of ^{3}HNA used were purified prior to use, and column chromatography (Dowex 50 Na^{+}) of the prepared diffusion medium (that is, Krebs' solution containing the purified ^{3}HNA) indicated that in excess of 95% of the tritium was associated with the NA fraction.

That the artery wall influenced the diffusion of released transmitter was indicated in those experiments in which the relative extraluminal and intraluminal overflows of tritium-labelled materials were determined (Chapter 8). In untreated arteries the extraluminal overflow of tritium was considerably greater than the intraluminal, and was enhanced more than the intraluminal overflow during the period of stimulation. In PBZ-treated arteries relatively more tritiated material reached the lumen, although the extraluminal overflow was still greater than the intraluminal. In addition, the kinetics of the luminal overflow were altered, so that the peak overflow occurred earlier. These results pointed to the influence of extraneuronal uptake on the diffusion of transmitter from the neuronal plexus to the lumen, and to the lower coefficient of diffusion in the tunica media relative to the tunica adventitia.

The metabolic studies undertaken in Chapter 8 also helped to define the influence of neuronal and extraneuronal uptake of NA on the overflow of released transmitter, and will be referred to later. However, the relative significance of these uptake processes was more clearly defined in subsequent experiments in which the stimulation-induced overflow of transmitter in paraffin-bathed arteries, and the influx of exogenous NA following its application to the adventitial surface of artery segments were investigated. It was apparent in these studies that a number of factors influenced the release and diffusion of NA in the rabbit ear artery, and in some instances it was possible to quantify the contribution of the particular factor.

Neuronal uptake

The significance of the neuronal uptake process to the diffusion of exogenous NA across the artery wall, and to the movement of endogenous NA away from its site of release, was demonstrated clearly in the present experiments. Previously, the existance of the uptake process in the rabbit ear artery had been demonstrated in studies in which it was shown that inhibition of neuronal uptake potentiated the responses of artery segments to extraluminal NA about ten-fold. As these results implied that the nerves had extracted approximately 90% of the NA before it reached the underlying smooth muscle it was of interest to determine whether this could be verified in the present experiments.

The overflow studies in paraffin-bathed arteries provided a means of quantifying the effects of neuronal uptake on the efflux of

released transmitter. On the assumption that the actions of PBZ and phentolamine on the pre- and post-synaptic alpha receptors were similar, the data presented in Chapter 9 indicated that inhibiting the neuronal and extraneuronal uptake of released NA increased the overflow of tritium-labelled material by 130% (Figure 9.11). These results implied that the uptake and retention of NA in the rabbit ear artery inhibited 56% of the released transmitter from overflowing into the luminal perfusate within 20 minutes of the onset of nerve stimulation. However, as the results of the isotopic diffusion studies indicated that the uptake and retention of NA or its metabolites in extraneuronal sites did not contribute to the loss of NA as it diffused across the walls of phentolamine-treated arteries (Figure 11.5), it is suggested that the retention of NA by the nerves alone accounted for the decreased stimulation-induced overflow (56%) noted above.

A second component of neuronal uptake must be considered as it was apparent that some of the NA which was taken up by the nerves was metabolized and sequestered as the tritiated metabolite. If it is assumed that the results of the metabolite studies on only two phentolamine-treated arteries were typical (Figure 9.10), and if the simplifying assumption is made that the deaminated metabolites were formed intraneuronally and NMN extraneuronally (page 12.9) then it can be calculated that approximately 80% of the NA which was released in phentolamine-treated arteries was taken up by the nerve terminals; 70% was retained, presumably in storage vesicles, and 30% was deaminated.

The data derived from the diffusion experiments demonstrated the role of neuronal uptake under circumstances in which the complications of the release process were eliminated. When the constrictor responses of the arteries to NA were prevented by phentolamine, the mean influx of tritium-labelled material in the 20 minutes following the application of ^{3}HNA to the adventitial surface of these arteries was increased by cocaine (52%), but not further increased by cocaine and DOCA (54%). This implied that the retention of tritiated material in the artery wall was almost if not entirely neuronal. On the assumption that the deaminated metabolites were formed intraneuronally it was apparent that neuronal uptake reduced the amount of NA which diffused across the wall of phentolamine-treated arteries by nearly 70%. 78% of this reduction was the result of intraneuronal binding, and 22% the result of intraneuronal metabolism. These figures were similar to those derived from the stimulation-induced overflow studies described above.

The use of cocaine as an inhibitor of the neuronal uptake of NA presented a number of difficulties. The pharmacological data presented in Figure 10.2 indicated that cocaine had little or no effect on the release of transmitter at concentrations of 3 or 30 μmol l^{-1}, and hence it was surprising that the stimulation-induced overflow in arteries, in which the neuronal and extraneuronal uptake of NA were inhibited by cocaine and DOCA and the alpha receptors by phentolamine, was considerably less than that in arteries in which these processes were inhibited by PBZ. This result was explained subsequently by the findings that cocaine (3 μmol l^{-1}) depressed the stimulation-induced release of transmitter (at least in PBZ-treated

arteries), and only decreased the neuronal uptake of NA by 60%. Indirect evidence that cocaine was interfering with the release process was provided by the diffusion studies in which the influx of NA was identical in cocaine (90 μmol l^{-1}), DOCA and phentolamine-treated arteries and in PBZ-treated arteries.

Clearly, the use of cocaine to inhibit the neuronal uptake of NA in the overflow studies presented a dilemma as it inhibited the release of transmitter at concentrations which did not completely inhibit neuronal uptake.

Extraneuronal uptake

The significance of the extraneuronal uptake process and O-methylation to the diffusion of NA across the artery wall was shown by the isotopic diffusion studies in which the nature, but not the magnitude of the influx of tritium-labelled material in phentolamine and cocaine-treated arteries was altered when the uptake of NA by smooth muscle cells was inhibited. That the magnitude of the influx was unaltered was in accord with the concept that NA was not firmly bound in smooth muscle cells, but was sequestered either as unchanged NA or as a metabolite of NA. As NMN comprised 40% of the influx in cocaine and phentolamine-treated arteries it was apparent that at least 40% of the NA which entered the tunica media was taken up by the smooth muscle cells. Whether part of the NA which reached the lumen was taken up also by the smooth muscles cells, but effluxed from them without being metabolized, was not determined in the present experiments.

It was of interest that in both the release and diffusion experiments the initial rate of overflow or influx of tritium-labelled material in cocaine-treated arteries was increased by DOCA. Thus it would appear that the uptake of NA by smooth muscle cells retarded its diffusion across the tunica media.

The influence of the smooth muscle uptake of NA was demonstrated in another way in experiments in which the smooth muscle uptake inhibitor, MN, potentiated the constrictor responses of the ear artery to nerve stimulation, and decreased the rate of recovery from these responses. Thus it seemed likely that the uptake of NA by smooth muscle cells contributed to the inactivation of released transmitter and the termination of the constrictor response. Other results, which indicated that inhibition of COMT in MN-treated arteries did not potentiate further the constrictor responses or delay the recoveries from these responses, were in accord with the concept that inhibition of smooth muscle uptake prevented the access of NA to the O-methylating enzyme. Further, as the potentiation was less and the recovery faster in COMT-inhibited arteries than in MN-treated arteries, it was likely that only part of the effect of smooth muscle uptake on the inactivation of released transmitter was related to COMT. This evidence was in accord with the suggestions of Johnson (1975) and Graefe and Trendelenburg (1974) that two compartments might exist in smooth muscle; one sensitive to inhibition of COMT and one not.

Contrary to the findings of Powis (1973), the present experiments did not indicate a significant binding of NA to elastin and collagen. The evidence for this was that oxytetracycline, a drug which inhibited this binding, did not significantly alter the magnitude or the

kinetics of the intraluminal overflow of tritium-labelled material following nerve stimulation, and did not alter the constrictor response during stimulation. A possible explanation for the findings of the overflow studies was that the amount of NA which escaped from the neuronal plexus and diffused across the tunica media was very small. Hence it was significant that the overflow characteristics in PBZ-treated arteries, when the amount of NA which diffused across the wall was greatly increased, were little altered by oxytetracycline. Powis concluded that PBZ did not affect the binding of NA to collagen and elastin. Other experiments in this laboratory have failed to show any appreciable effect of oxytetracycline on the steady-state response of artery segments to exogenous NA (de la Lande, private communication).

Metabolism

When the present study commenced biochemical evidence for the existence and distribution of the metabolizing enzymes, MAO and COMT, in the rabbit ear artery was not available. MAO had been demonstrated extraneuronally by a histochemical technique (de la Lande et al, 1970), and intraneuronally in pharmacological studies (de la Lande and Jellett, 1972). Subsequently, it was found that extraneuronal MAO had little effect on the metabolism of NA unless the concentration of the amine was high (de la Lande and Jellett, 1972; de la Lande and Johnson, 1972). COMT was known to be present in amounts which were comparable with those in the rabbit aorta, although its distribution was uncertain (Burnstock et al, 1972). More recently it was concluded that COMT was largely extraneuronal (Head et al, 1974). In

the present studies evidence was presented which indicated that intraneuronal MAO and extraneuronal COMT influenced the diffusion of NA in the artery wall. Extraneuronal MAO did not affect the diffusion of NA at the concentration of NA used.

The influence of intraneuronal metabolism on the diffusion of released NA was discussed previously (page 12.5). In phentolamine-treated arteries it was apparent that almost 25% of the released transmitter consisted of deaminated metabolites. That these metabolites were formed intraneuronally was suggested by the results of the diffusion studies in which the formation of deaminated metabolites was prevented in arteries in which neuronal but not extraneuronal uptake was inhibited. These observations were in accord with the findings of Head (1976) and Levin (1974) that deaminated metabolites were formed intraneuronally in the rabbit ear artery and aorta respectively. DOPEG was the major intraneuronal metabolite in both vessels. DOPEG was also the main intraneuronal metabolite in the overflow from resting and stimulated PBZ-treated arteries (Chapter 8). As MOPEG and VMA appeared to be overestimated and as NMN was not detected in these PBZ-treated arteries, this study was in accord with the conclusion of Head (1976) that COMT was not present intraneuronally.

The response studies described in Chapter 6 also pointed to the absence of intraneuronal COMT, as neither the constrictor response to nerve stimulation nor the recovery from this response was altered by inhibition of COMT in arteries in which the smooth muscle uptake of NA was inhibited by MN. As COMT inhibition failed to alter the

constrictor response or rate of recovery of MAO-inhibited, MN-treated arteries it appeared that intraneuronal COMT was not masked by the effects of MAO.

The role of extraneuronal metabolism in the diffusion of NA through the tunica media was demonstrated clearly in the overflow and diffusion studies (Chapter 11). In the latter experiments approximately 45% of the NA which entered the tunica media was metabolized to NMN. This figure was derived from the influx of NA and NMN in phentolamine-treated arteries. A more direct estimate of the amount of NA which was O-methylated in the media was provided by the influx of NMN (40%) in phentolamine-treated arteries in which the neuronal uptake of NA was inhibited by cocaine. That is, 40% of the NA which entered the tunica media was metabolized by COMT. That this metabolism occurred within the smooth muscle cells was demonstrated by the absence of metabolites in the influx in cocaine and DOCA-treated arteries.

The absence of deaminated metabolites in cocaine and phentolamine-treated arteries indicated that extraneuronal metabolism by MAO did not occur, at least at the NA concentration used in these experiments (0.18 μmol l^{-1}).

The influence of extraneuronal COMT on the metabolism of NA in the tunica media helped to explain the failure of intraluminally applied NA to restore fluorescence in the nerve terminals of reserpine-depleted and MAO-inhibited arteries (Chapter 5). On the assumption that the metabolism of NA as it diffused from the lumen to the neuronal plexus was similar to that in the opposite direction,

it can be predicted that between 55% and 60% of the NA would cross the media unchanged. However, as de la Lande et al (1974) found that fluorescence was restored in reserpine pretreated arteries when the concentration of extraluminal NA was only 40% of that used in the experiments described in this thesis, it would appear that another factor, such as the marked vasoconstrictor response to intraluminal NA, might be involved. It would be interesting therefore to repeat these studies in the presence of phentolamine.

It was also of interest that approximately equal amounts of NMN and deaminated metabolites reached the lumen of phentolamine-treated arteries following the diffusion of NA across the artery wall (Figure 11.8). Although Levin (1974) described a similar distribution of metabolites following the incubation of strips of rabbit aorta in ^{3}HNA, Head (1976) reported that 90% of the metabolites formed following the incubation of rabbit ear artery segments in NA were deaminated. Head suggested that the difference between his findings and those of Levin might reflect the different routes of application of the NA. That is, as Head applied the NA to the outside of the segments it would need to cross the neuronal plexus before O-methylation occurred, whereas in Levin's experiments the NA was applied to both sides of the strips. However, a possible complication in Head's studies was that the vasoconstrictor responses of the arteries might have prevented much of the NA from diffusing into the tunica media. Hence, at least part of the explanation for the discrepancy between the findings of the present study and those of Head might have been the enhanced access of NA to the tunica media in the present experiments, as vasoconstriction was prevented by phentolamine.

Constrictor response

The influence of vasoconstriction on the movement of NA through the artery wall was indicated in the isotopic diffusion studies, in which phentolamine was used to inhibit the post-synaptic alpha receptors and cocaine and DOCA to inhibit the neuronal and smooth muscle uptake of NA. The results of these experiments indicated that phentolamine increased the influx of tritium-labelled material in untreated, cocaine-treated, and cocaine and DOCA-treated arteries by approximately 60% in each of the collections during the first 6 minutes of diffusion (Chapter 11). During the fifth collection period, that is the collection which commenced 4 minutes after the NA was removed from the adventitial surface of the artery, the amount of material which influxed in the cocaine and cocaine plus DOCA-treated arteries was not increased by phentolamine, and in the untreated arteries phentolamine only increased the influx by 10%. These results implied that the movement of NA through the artery wall during and immediately following the application of NA to the adventitial surface was facilitated by phentolamine. It is tempting to postulate that this effect was the result of phentolamine's action in inhibiting the constrictor response during the application of the NA. However, even though the constriction in the cocaine and cocaine plus DOCA-treated arteries was substantial, there was no rise in the perfusion pressure during the application of NA to the untreated arteries. Whether the diffusion coefficient for a neutral substance like sorbital would also have been altered in these untreated arteries was not investigated, but the simultaneous application of ^{14}C-sorbitol and ^{3}HNA in phentolamine-treated and untreated arteries might provide useful information about the action of phentolamine.

As it was possible that the above effects of phentolamine were the result of its actions on mechanisms other than the post-synaptic alpha receptors, a further series of experiments was designed in which the concentration of NA applied to one of each pair of arteries was expected to elicit either a very small constrictor response or none at all, and the concentration applied to the other was expected to elicit a marked constriction (Figure 11.7). Both arteries were treated with cocaine and DOCA to inhibit the uptake of NA by neuronal and smooth muscle tissues. In the three experiments in which the higher concentration of NA produced the desired vasoconstriction, the amount of NA which reached the lumen during the first six minutes of the diffusion was approximately 40% less than the equivalent influx from the arteries in which the lower concentration of NA was used. During the second application of NA, when both arteries of each pair were treated with phentolamine as well, this reduction was only 8%. That is, when phentolamine inhibited the constrictor response the influx of NA in both arteries was similar.

Clearly, the influx of NA in cocaine and DOCA-treated arteries was enhanced when the constrictor response was either minimized or prevented, irrespective of whether this was achieved by reducing the concentration of NA applied to the adventitial surface of the arteries or by treating the arteries with phentolamine. Thus it is suggested that vasoconstriction is a factor limiting the diffusion of NA across the wall of the rabbit ear artery.

Release of NA

The release of transmitter in the resting rabbit ear artery was demonstrated in Chapter 8, in which it was shown that deaminated metabolites formed the major proportion of the overflow prior to nerve stimulation. As this proportion was unaltered when the artery was treated with PBZ, and as the absolute amount of the deaminated metabolites was greatly reduced when the arteries were incubated in the presence of cocaine, it was concluded that the metabolites were formed intraneuronally prior to their efflux from the nerve terminals. A possible explanation for these findings was that some of the NA stored in the intraneuronal vesicles leaked into the cytoplasm where it was deaminated by MAO prior to efflux. The same experiments also provided evidence which was in accord with the postulate that NA is released from the neurones during nerve stimulation by a process of exocytosis rather than by intraneuronal release and diffusion across the axoplasm.

The possible influence of several inhibitory feedback mechanisms on the release of transmitter was reviewed earlier (page 1.5), and although several reports have appeared in the literature which indicated that a presynaptic alpha receptor mechanism influenced the release of transmitter in some tissues, its precise role in the rabbit ear artery is not clear. In the present study, the influence of the alpha receptor antagonist, phentolamine, on the stimulation-induced overflow of tritium-labelled material was examined in paraffin-bathed artery segments (Chapter 9). In these experiments the overflow in untreated arteries was 35% less than that in phentolamine-treated arteries in the first 6 minutes following the

onset of nerve stimulation. Providing that phentolamine did not inhibit the neuronal and smooth muscle uptake of NA at the concentration used, this result can be explained by the action of phentolamine on pre- and/or post-synaptic alpha receptors. It was of interest that the influx of tritium-labelled material in untreated arteries in the first six minutes of the diffusion of exogenous ^{3}HNA was also approximately 35% less than in phentolamine-treated arteries. Further studies will be required to elucidate the relative contributions of the vasoconstrictor response and the putative presynaptic alpha receptor inhibition of transmitter release. However, the present study highlights the relatively minor role of the presynaptic receptors in minimizing the overflow of transmitter in the isolated rabbit ear artery following sympathetic nerve stimulation.

Rand et al (1973) concluded that modulation of transmitter release by presynaptic alpha receptors was not apparent in the rabbit ear artery when the frequency of nerve stimulation was 5 Hz (frequency used in the present experiment). The evidence for this was derived from comparable studies in the guinea pig atria and the rabbit ear artery. When the former tissue was stimulated at a frequency of 1 Hz, the efflux of transmitter per pulse declined with increasing train length, so that the efflux following 16 pulses was 14% of the efflux with 1 pulse. This decline was largely prevented by PBZ. However, Rand and co-workers were unable to demonstrate a similar decline in the rabbit ear artery which suggested that NA did not accumulate in sufficient concentration to modulate release. In further experiments, when the arteries were stimulated for 200 seconds at frequencies from 0.05 to 50 Hz, they found that the efflux per pulse reached a peak at

5 Hz and declined at higher frequencies. Although it was suggested that sufficient NA might have accumulated to inhibit further release at these higher frequencies, it was pointed out that other mechanisms (such as failure of impulse conduction) might account for the decreased release of transmitter.

Much of the evidence in support of the action of NA in modulating transmitter release has been derived from the effects of exogenous NA on the release and overflow of transmitter. For example, it was shown that NA at a concentration of 0.05 μmol l^{-1} did not reduce the stimulation-induced overflow of tritium-labelled material from cocaine-treated rabbit ear arteries, whereas the overflow was reduced by almost 40% when the concentration of NA was increased to 0.5 μmol l^{-1} (Rand et al, 1973; Hope et al, 1975). As Bevan and Su (1973) calculated that the concentration of NA within the synapse of PBZ-treated arteries was less than 0.04 μmol l^{-1} during nerve stimulation at a frequency of 6 Hz, it is apparent why Rand et al (1973) were unable to demonstrate a decline in the efflux of NA per pulse in the rabbit ear artery when stimulated at a frequency of 5 Hz. In the same study, Bevan and Su found that the intrasynaptic concentration of NA increased to 0.6 μmol l^{-1} when the frequency of stimulation was 10 Hz. Thus it is possible that the reduced efflux per pulse which was reported at this frequency by Rand and co-workers was a consequence of the increased intrasynaptic concentration of NA, and the resultant presynaptic modulation of NA release.

These findings of Rand et al (1973) and Bevan and Su (1973) indicated that the minor effect of phentolamine on the presynaptic

inhibitory mechanism in the present study might be a consequence of the frequency of nerve stimulation and the mechanisms responsible for for the inactivation of NA in the synaptic cleft.

In summary, the findings of this thesis indicated that a number of factors affected the diffusion of exogenous NA in the rabbit ear artery (Figure 12.1). The neuronal uptake of NA was found to be the single most important factor limiting the diffusion of transmitter away from its site of release, and limiting the diffusion of exogenous NA from the adventitial surface of the artery to the lumen. It was estimated that approximately two-thirds of the NA which was taken up by the nerves was retained, while one-third was metabolized by MAO and sequestered. COMT did not appear to be an intraneuronal enzyme. At least 90% of the transmitter (NA and metabolites) which escaped from the confines of the neuronal plexus in the isolated, aqueous-bathed ear artery diffused through the tunica adventitia to the external bathing medium, and only 10% diffused through the tunica media to the lumen. It was suggested that the preferential diffusion of NA through the adventitia was a consequence of the position of the neuronal plexus, and the greater coefficient of diffusion in the adventitia. As the proportion of transmitter which overflowed extraluminally was even greater during nerve stimulation, it was probable that the vasoconstrictor responses of the arteries also limited the diffusion of NA and its metabolites through the tunica media.

Approximately 40% of the NA which escaped neuronal uptake and diffused through the tunica media of these arteries was taken up by the smooth muscle cells and metabolized by COMT to NMN. Whether

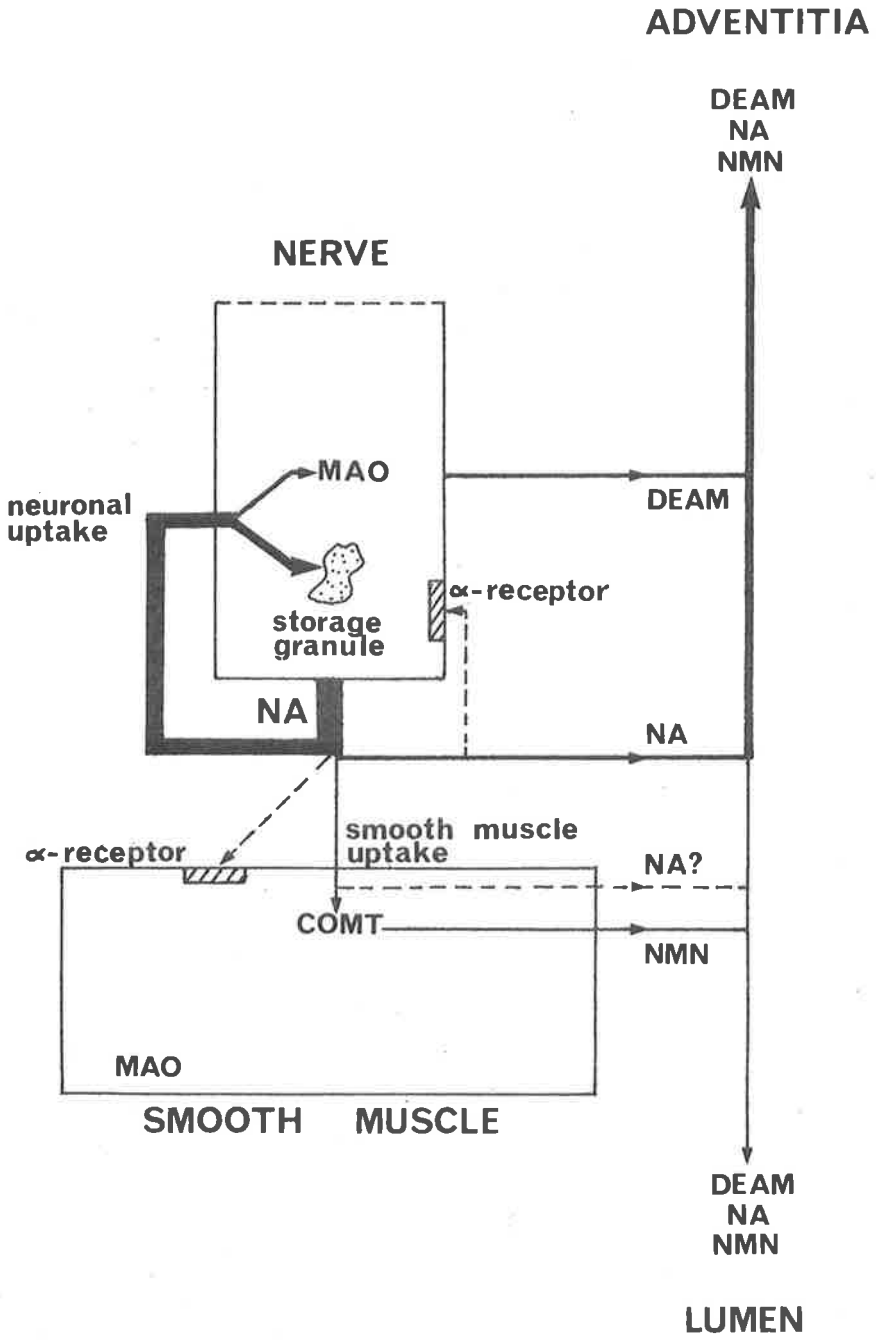


FIGURE 12.1 Diagram of the possible fate of NA released by nerve stimulation in the isolated aqueous-bathed rabbit ear artery. The thickness of the continuous lines provides an approximation of the relative significance of the uptake processes and metabolism to the fate of the released NA. The presence of NMN and NA in the intraluminal overflow was equivocal. Whether NA entered the smooth muscle cells and effluxed unchanged from them was not determined in the present experiments.

some of the NA which eventually reached the lumen after crossing the media was also taken up by the smooth muscle cells, but effluxed unchanged, could not be determined in the present study. As inhibition of the smooth muscle uptake of NA did not significantly alter the magnitude of the luminal overflow, except in its initial stages, it was concluded that the retention of NA in the smooth smooth muscle cells did not contribute to the diffusion barrier in the artery wall. Although extraneuronal MAO has been demonstrated in the rabbit ear artery, extraneuronal deaminated metabolites did not appear to be formed in this study.

At the frequency (5 Hz) most commonly used to stimulate the sympathetic nerves in the experiments reported, it appeared likely that the modulation of NA release by the presynaptic alpha receptors altered the magnitude of the overflow of NA and its metabolites less than the post-synaptic alpha receptors. Whether the presynaptic receptors had a greater influence on the release of transmitter at other frequencies of stimulation was not tested in this study.

The present study has generated a number of questions which have not been answered, and which require further investigation before the precise roles of metabolism and constriction on the diffusion of released transmitter in the rabbit ear artery wall can be defined. For example, the overflow studies in paraffin-bathed arteries should be extended to include an analysis of the metabolites which overflow into the lumen following the release of NA by nerve stimulation. In particular, it is necessary to verify that the results obtained in the present exogenous diffusion experiments represent the

relationship between the neuronal uptake of NA, the extraneuronal uptake of NA and metabolism by MAO and COMT following the release of endogenous NA.

Perhaps the most significant area for further investigation relates to the influence of vasoconstriction on the diffusion of NA away from its site of release, and on the diffusion of exogenous NA across the artery wall. The influence of constriction on the diffusion coefficient in the tunica media and on the diffusion of a neutral substance such as sorbitol should be examined, and the study should be expanded to include an analysis of metabolism in constricting and non-constricting arteries. The latter experiments might help to clarify the effects of vasoconstriction on the neuronal and extraneuronal uptake of NA.

Finally, this study could be extended to include an *in vivo* investigation of the influence of the uptake processes, the metabolising enzymes, vasoconstriction and the alpha receptor inhibitory feedback mechanism on the release, diffusion and overflow of NA and its metabolites.

APPENDICES

APPENDIX 1

PBZ AS AN INHIBITOR OF THE SMOOTH MUSCLE UPTAKE OF NORADRENALINE

The inhibitory effects of PBZ on the neuronal uptake and the smooth muscle uptake of NA, and on the post-synaptic alpha receptors were reviewed earlier (page 1.12). Although it had been demonstrated that PBZ irreversibly blocked these adrenoreceptors, and that providing it remained in contact with the tissue for sufficient time it inhibited neuronal uptake by a non-competitive mechanism (Iversen and Langer, 1969), it seemed important to test the extent to which the action of this drug on the smooth muscle uptake of NA was irreversible in the rabbit ear artery. By incubating arteries in high concentrations of NA following pre-treatment with PBZ, it was possible to use the fluorescent histochemical technique to examine this irreversibility.

METHODS

Rabbits were anaesthetized with urethane (17.7 mol kg^{-1}; IP), and single cannulated artery segments or non-cannulated segments tied at one end with a piece of cotton were removed. Cannulated segments were placed in 15 cm^3 capacity organ baths and equilibrated in Krebs' solution (page 2.3). The tied segments were suspended in organ baths.

In each experiment one tied segment remained untreated, while a second was treated with PBZ (33 µmol l^{-1}) for 20 minutes, and then rinsed in PBZ-free Krebs' solution for 60 - 90 seconds. Both pieces of

artery were frozen in an acetone/dry ice mixture. The remaining segments were treated in one of several ways as indicated in Table A1.1.

When required PBZ was applied for 20 minutes; extraluminally to all arteries and intraluminally to the cannulated segments as well. At the end of this period, NA (600 μmol l^{-1}) and ascorbic acid (300 μmol l^{-1}) were added to one artery for 10 minutes, and PBZ was washed out of the remaining segments for periods ranging from 10 to 180 minutes. After this washout of PBZ, NA and ascorbic acid (above concentrations) were added to the Krebs' solution perfusing through the lumen of the cannulated arteries or bathing the tied artery segments for 10 minutes. It should be noted that for all applications of NA the flow rate through the cannulated segments was reduced to 1.6 cm^3 min^{-1}.

In each series of experiments, one artery segment was either perfused with or immersed in Krebs' solution containing NA and ascorbic acid for 10 minutes without previous or concurrent exposure to PBZ.

Following the application of NA all arteries were washed in Krebs' solution for 60 - 90 seconds prior to freezing in an acetone/dry ice mixture, and processing by the fluorescent histochemical technique. Sections were prepared and examined in ultra-violet light as described previously (page 2.6).

RESULTS

Untreated and PBZ-treated arteries displayed monoamine fluorescence only in the nerve terminals at the media-adventitia border, whereas those arteries exposed to NA alone (that is, not pretreated with PBZ) usually displayed an intense fluorescence throughout the entire section, including the media (Table A1.1; Figures A1.1 and A1.2). Occasionally this intense fluorescence extended across only part of the media.

In comparison, those segments which had been exposed to NA following pre-treatment with PBZ showed only a weak fluorescence except in the nerve terminals. As indicated in Figure A1.2, the fluorescence in the media was confined to a network surrounding non-fluorescing fusiform-shaped spaces.

NA produced a marked conctrictor response in the untreated cannulated segments, but failed to produce this response in the PBZ-treated arteries. Although the tied segments could not be perfused, evidence of constriction was obtained from the photomicrographs by examining the diameter and shape of the lumen following NA application.

The above effects of PBZ were undiminished even after 180 minutes of PBZ washout prior to the application of NA.

DISCUSSION

These results are in accord with the findings of Gillespie and Hamilton (1966) that NA was taken up by the smooth muscle cells in the

	PBZ (min)	PBZ WASHOUT (min)						NA (min)	MEDIAL FLUORESCENCE	
n	20	0	10	30	90	120	180	10	Smooth muscle cells	Connective tissue
6									-	-
5	*	x							-	-
6								*	+	+
6	*	x						*	-	+
4	*		x					*	-	+
6	*			x				*	-	+
4	*				x			*	-	+
2	*					x		*	-	+
2	*						x	*	-	+

n - represents the number of artery segments used
** - drug present*
x - duration of PBZ washout

TABLE A1.1 The effects of PBZ (33 µmol l^{-1}) on the smooth muscle uptake of NA, and on the binding of NA to connective tissue in the tunica media of the rabbit ear artery following the application of exogenous NA (600 µmol l^{-1}). Fluorescence in the smooth muscle cells and the connective tissue of the tunica media was assessed as present (+) or absent (-).

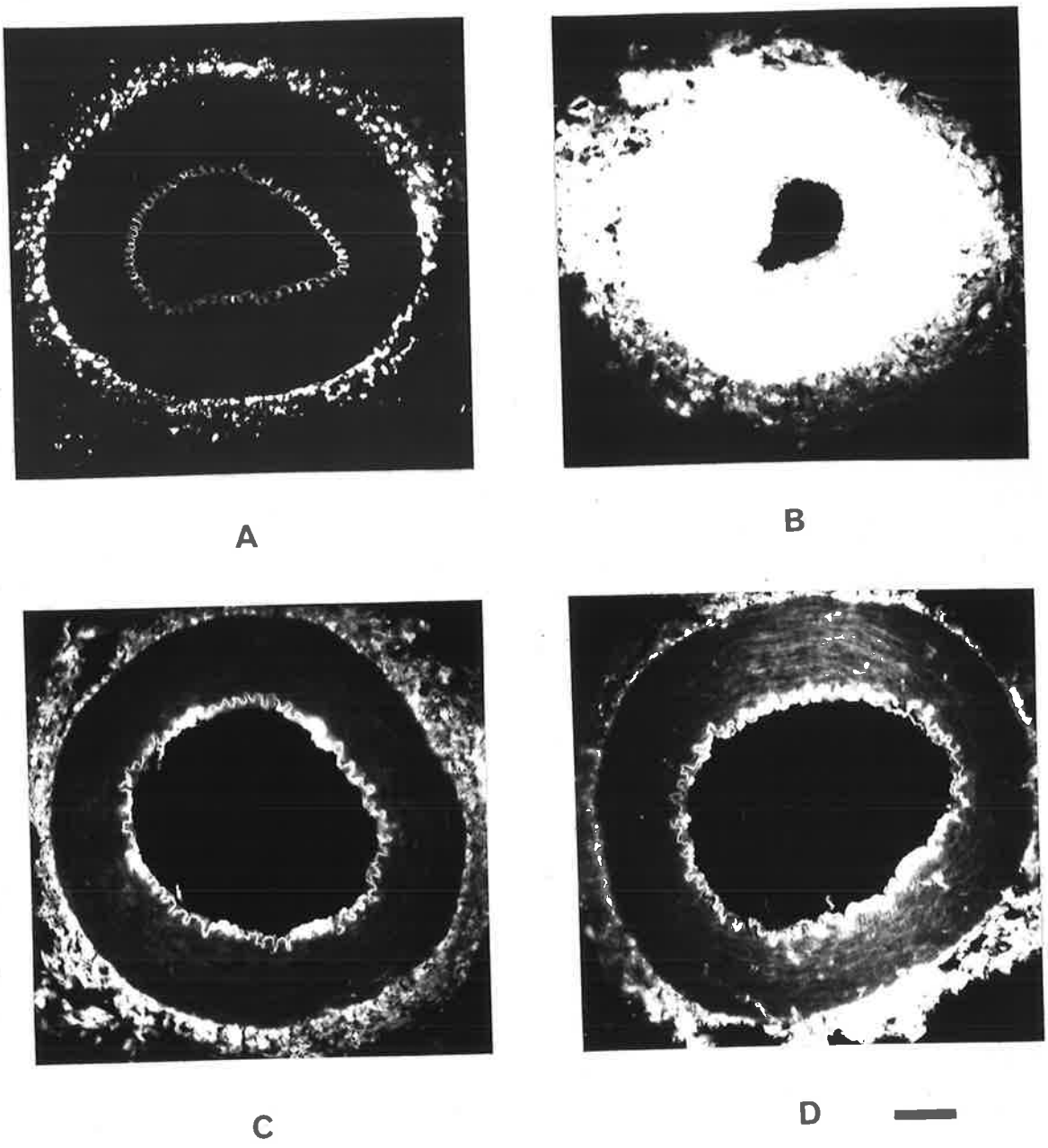


FIGURE A1.1 Transverse sections of formaldehyde-treated rabbit ear arteries, showing the binding of exogenous NA in the artery wall, and the effect of PBZ on this binding.

A. Artery treated with PBZ (33 µmol l^{-1}) only. Monoamine fluorescence was detected only in the neuronal plexus.

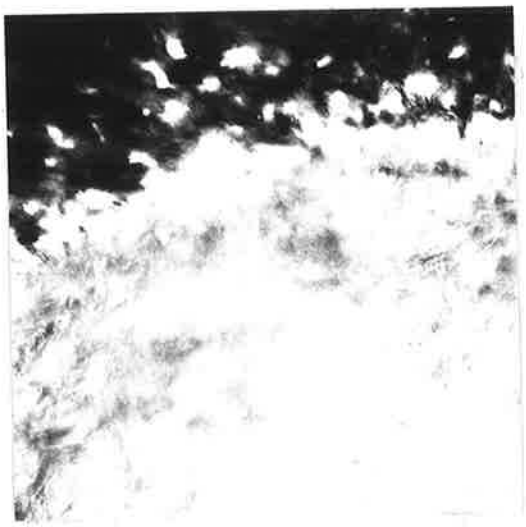
B. Non-PBZ-treated artery exposed to extraluminal NA (100 µmol l^{-1}). Intense fluorescence throughout the tunica media (see Figure A1.2).

C. Artery exposed to extraluminal NA in the presence of PBZ. Fluorescence was observed in the connective tissue elements, but not in the smooth muscle cells (see Figure A1.2).

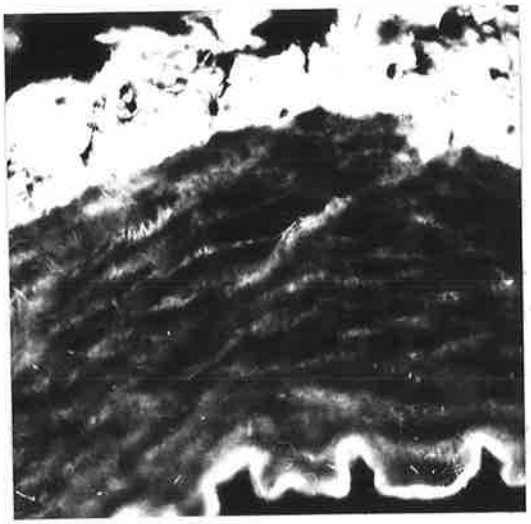
D. PBZ-treated artery exposed to extraluminal NA following the washout of PBZ for 90 minutes. Fluorescence characteristics were similar to those in C.

Scale represents 100 µm

A



B



C

FIGURE A1.2 Transverse sections of formaldehyde-treated rabbit ear arteries showing a portion of the tunica intima, tunica media, the neuronal plexus, and the tunica adventitia.

A. Untreated artery, showing monoamine fluorescence of the nerve terminals.

B. Untreated artery exposed to NA, showing intense fluorescence in the smooth muscle cells of the tunica media, and on the connective tissue throughout the artery wall.

C. PBZ-treated artery exposed to NA following the washout of the PBZ for 90 minutes. Monoamine fluorescence in the tunica media formed a network surrounding fusiform-shaped non-fluorescing spaces. Connective tissue fluorescence in the tunica adventitia was noted.

Scale represents 20 µm

rabbit ear artery, and that it could be visualized by the fluorescent histochemical technique. They are also in agreement with the findings of Avakian and Gillespie (1968) that PBZ blocked this component of the extraneuronal uptake of NA.

It is suggested that the consistent histological observation of the present study (namely, a network of fluorescence surrounding non-fluorescing fusiform spaces in the tunica media of PBZ-treated arteries) represented fluorescence of the connective tissue and the basement membrane of the smooth muscle cells. Such a suggestion is supported by the findings of Gillespie (1968, 1973) and Powis (1973), that NA binding to connective tissue elements and the basement membrane is not PBZ sensitive, and by the observation that the non-fluorescing spaces corresponded approximately in number and position with the smooth muscle cells in the ear artery. The cotton-wool appearance of the fluorescence in the media of the non-PBZ-treated arteries could be the result of NA accumulation and concentration in the smooth muscle cells following uptake (Gillespie, 1968).

The absence of fluorescence in parts of the tunica media in some arteries exposed to NA without prior treatment with PBZ can be explained by the findings of the present study that the vasoconstrictor responses of the arteries restricted the diffusion of NA across the artery wall (Chapter 11). Avakian and Gillespie (1968) also found that the rate of diffusion of NA (600 μmol l^{-1}) across the artery wall was slow, and that even after 16 minutes the intensity of fluorescence in the tunica media was not maximal.

The present study confirmed that PBZ inhibited the smooth muscle uptake of NA and the post-synaptic alpha receptors, and indicated that providing NA was administered for 20 minutes these effects were irreversible following washout of the drug for periods of up to 180 minutes.

APPENDIX 2

DRUGS AND CHEMICALS

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1. Sources of drugs and chemicals

The following drugs were used in the experiments described:

dl-adrenaline hydrochloride	Koch-Light Laboratories
l-ascorbic acid	Koch-Light Laboratories
cocaine hydrochloride	MacFarlane-Smith
3,4-dihydroxymandelic acid	Sigma
3,4-dihydroxyphenylglycol	Sigma
DOCA (4-pregnen-21-ol-3,20 dione acetate)	Steraloids
ethyl carbamate (Urethane)	Koch-Light Laboratories
4-hydroxy,3-methoxymandelic acid	Sigma
4-hydroxy,3-methoxyphenylglycol	Sigma
dl-metanephrine hydrochloride	Sigma
methoxamine hydrochloride	Burroughs-Welcome
nialamide	Pfizer
l-noradrenaline bitartrate	Koch-Light Laboratories
dl-noradrenaline bitartrate	Koch-Light Laboratories
dl-normetanephrine hydrochloride	Sigma
oxytetracycline hydrochloride	Pfizer
phenoxybenzamine hydrochloride	Smith, Kline and French
phentolamine methane sulphonate (Regitine)	Ciba
reserpine (Serpasil)	Ciba
serotonin creatine sulphate	Sigma

tetrodotoxin	Sigma
UO521 (3,4-dihydroxy-2-methyl propiophenone	Upjohn

Unless otherwise stated, all other chemicals used were AR grade.

2. Preparation of drugs

i. The catechols and UO521 were prepared in saline (155 mmol l^{-1}) containing ascorbic acid (600 µmol l^{-1}) (pH 5.4 - 5.6).

ii. DOCA and phenoxybenzamine were prepared as stock solutions in ethanol at concentrations of 54 mmol l^{-1} and 33 mmol l^{-1} respectively, and were added to warmed, gassed Krebs' solution when required for use.

iii. Nialamide was prepared by dissolving the required amount in 20 cm^3 of saline (155 mmol l^{-1}) with the aid of gentle heat. This solution was added to the appropriate volume of Krebs' solution immediately prior to use.

iv. Oxytetracycline was dissolved in the minimum quantity of HCl (100 mmol l^{-1}) and added to Krebs' solution prior to use.

v. All other drugs were prepared in saline (155 mmol l^{-1}).

vi. The concentrations of noradrenaline, 5-hydroxytryptamine and phentolamine refer to the bases; all other concentrations refer to the salts.

3. Krebs' bicarbonate solution

The Krebs' solution used throughout this study was of the following composition:

	mmol l^{-1}
NaCl	120.0
$NaHCO_3$	25.0
Glucose	5.5
KCl	4.7
$CaCl_2$	2.5
$MgCl_2$	1.1
KH_2PO_4	1.0
EDTA*	0.01

** Ethylenediaminetetra-acetic acid (disodium salt)*

$CaCl_2$ and $MgCl_2$ were added from standardized 10% stock solutions. The Krebs' solution was filtered before use.

4. Liquid-scintillation spectrometry

Three scintillants were used in this study:

i. Brays' scintillant

Brays' scintillant consisted of:

Naphthalene	600 g
PPO (2,5-diphenyloxazole)	40 g
POPOP (1,4-di{2-(5-phenyloxazolyl)} benzene)	2 g
Methanol (absolute)	100 cm^3
Ethylene glycol	20 cm^3
p-Dioxane	to make 10 l

Prior to use the naphthalene flakes (Commercial Grade) were dissolved in ethanol (absolute: Laboratory Grade), precipitated in de-ionized, distilled water and dried at room temperature following filtration.

ii. Toluene-triton scintillant

The scintillant contained Triton X - 100 (Packard) and toluene scintillant (1:2). The latter consisted of:

PPO	8.25 g
POPOP	0.25 g
Toluene	to make 1 l

iii. Toluene scintillant

The toluene scintillant consisted of:

PPO	8.25 g
POPOP	0.25 g
Toluene	to make 1 l

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