EXTRANEURONAL FACTORS IN THE CONTROL OF VASCULAR SENSITIVITY

A THESIS SUBMITTED FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

by

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CONTENTS

SUMMARY

1. Previous studies in this laboratory on the rabbit ear artery have been largely confined to the role of neuronal inactivation on the vasoconstrictor response to noradrenaline (NA). These studies indicated that neuronal uptake and subsequent deamination by neuronal monoamine oxidase (MAO) exerted a major influence on the sensitivity to NA when the latter was applied to the adventitial surface (extraluminally), but not to the intimal surface (intraluminally). One of the explanations for the relatively minor role of neuronal inactivation in the response to intraluminal NA was that extraneuronal uptake and O-methylation in the media restricted the access of this NA to the sympathetic nerve terminals at the junction of the media and adventitia.

2. The present study deals with the roles of extraneuronal uptake and enzymatic inactivation on the sensitivity of the rabbit ear artery to catecholamines.

3. A functionally important role of extraneuronal catechol-O-methyl transferase (COMT) in the constrictor response to adrenaline, and to a lesser extent, to NA, was deduced on the basis of evidence that an inhibitor of COMT (3',4'-dihydroxy-2-methyl-propiophenone (UO521)) potentiated the sensitivities to these

amines in both untreated arteries, and in arteries in which neuronal uptake was prevented by treatment with cocaine.

4. The importance of extraneuronal uptake was suggested by the ability of an inhibitor (deoxycorticosterone acetate (DOCA)) to enhance the sensitivity of the ear artery to adrenaline, and to a lesser extent, to NA. The potentiating effect of DOCA declined when the concentration of catecholamine was increased to approximately 3-5μmol l^{-1}, and was abolished in the presence of a COMT inhibitor (UO521). These findings were interpreted as evidence for the presence of a readily saturable extreneuronal mechanism which removed catecholamine from the region of the receptors only when COMT was functionally intact.

5. The inhibitory effect of DOCA on extraneuronal uptake was confirmed in biochemical studies of the extraneuronal uptake and O-methylation of ^{3}H-isoprenaline. DOCA decreased O-methylation in a manner consistent with inhibition of access of ^{3}H-isoprenaline to COMT. In COMT-inhibited arteries DOCA also decreased the accumulation of unchanged ^{3}H-isoprenaline.

6. The results of a preliminary investigation to determine whether the pharmacological actions of cocaine and DOCA were modified in arteries from rabbits with experimental hypertension are also presented.

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.

Stephen Michael Johnson
October 1975

PUBLICATIONS

A part of the material in this thesis has been published, or submitted for publication, in the following journals:

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A manuscript, incorporating most of the work described in Chapter 4, has been submitted for publication in "Blood Vessels".

Some of the work has also been presented to the Australian Society of Clinical and Experimental Pharmacologists (1971, 1973).

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PART I

STUDIES IN NORMAL ARTERIES

CHAPTER 1

INTRODUCTION

The experiments to be described in this thesis were undertaken to determine (a) whether extraneuronal mechanisms were important in the vasoconstrictor response of a muscular artery to catecholamines and, if so, (b) to assess the relationship between the neuronal and extraneuronal control of vascular sensitivity. As an extension, it was hoped to investigate whether neuronal and extraneuronal control mechanisms were modified in experimental hypertension.

At least four mechanisms may influence the concentration of noradrenaline (NA) at the receptors of effector tissues. These are (i) uptake into sympathetic nerve terminals (neuronal uptake), (ii) uptake into effector cells (extraneuronal uptake), (iii) metabolism by monoamine oxidase (MAO), and (iv) metabolism by catechol-O-methyl transferase (COMT). Background information and current concepts relating to each of these mechanisms will be discussed first, after which attention will be directed specifically to mechanisms in the artery of the rabbit ear. This is a muscular artery which is of a convenient size and morphology for pharmacological studies and has been used exclusively in the experiments described in subsequent chapters.

(i) Neuronal Uptake

Trendelenburg (1966, 1972) has reviewed the evidence that neuronal uptake is an important factor (under certain

conditions) in reducing the concentration of NA at adrenergic receptors. Much of this evidence is based on the supersensitivity in isolated tissues arising from elimination of neuronal uptake by sympathetic denervation or by the use of drugs such as cocaine. The term "presynaptic" was adopted by Trendelenburg to describe this supersensitivity, and to distinguish it from the moderate, slowly developing and less specific supersensitivity resulting from chronic interruption of the neuroeffector link (termed "postsynaptic" to indicate a true change in sensitivity of the effector cell). The more general terms "prejunctional" and "postjunctional" (Fleming et al, 1973) will be used here to distinguish between the two types of supersensitivity. The main features of the evidence were illustrated by findings that (i) the development of denervation supersensitivity to NA in the cat nictitating membrane (Langer et al, 1967) was temporarily related to the morphological degeneration of sympathetic nerve terminals (van Orden et al, 1967) and to the decline in the ability of the degenerating nerve terminals to retain exogenous NA (Smith et al, 1966). In addition, (ii) the rapidly developing supersensitivity after denervation was highly selective, and proportional in magnitude to the relative rates of neuronal uptake of different amines (Trendelenburg, 1972). For example, there was little or no prejunctional supersensitivity to amines, such as isoprenaline and methoxamine, which had no appreciable

affinity for neuronal uptake (Pluchino and Trendelenburg, 1968; Trendelenburg et al, 1970).

Prejunctional supersensitivity is also caused by drugs which inhibit neuronal uptake. Of these cocaine has been the most widely used in pharmacological studies in preference to other more potent inhibitors such as desipramine, since the latter also possesses α-receptor blocking properties (Trendelenburg, 1966). Since cocaine has been used extensively in the present study as an inhibitor of neuronal uptake, it should be emphasised that there is considerable evidence that its potentiating effects are mediated predominantly by inhibition of neuronal uptake. For example, in the cat nictitating membrane, cocaine caused greater supersensitivity to NA compared with adrenaline while having no effect on the sensitivity to methoxamine (Trendelenburg et al, 1962; 1970). This was in accord with the relative rates of neuronal uptake of these amines (Draskoczy and Trendelenburg, 1970). Agreement between the theoretical and experimental relationship between inhibition of neuronal uptake and increase in sensitivity to NA by cocaine in the cat nictitating membrane was observed by Trendelenburg et al (1972), supporting a prejunctional action of cocaine in this tissue. The potentiation of NA by cocaine was markedly reduced or abolished in tissues in which the adrenergic nerve terminals had degenerated after denervation

(Langer et al, 1967 (cat nictitating membrane); de la Lande et al, 1967a (rabbit ear artery); Green and Fleming, 1968 (cat spleen)).

Perhaps the most convincing evidence in support of a prejunctional action of cocaine stems from analysis of various factors which influence the magnitude of cocaine-induced supersensitivity. For instance, responses of the isolated perfused rabbit heart to the l-isomer of NA were potentiated by cocaine to a much greater extent than those to the d-isomer, even though the rates of uptake of the two isomers were equal. Both isomers were found to saturate neuronal uptake at concentrations of approximately 6μmol l^{-1}. However, whereas the l-isomer elicited responses at very low concentrations, the much less potent d-isomer was effective only at very high concentrations which saturated neuronal uptake. Uptake was hence able to remove a much smaller fraction of the d-isomer from the region of the receptors. Thus the difference in the potentiations of the 2 isomers was consistent with that expected on the basis of inhibition of neuronal uptake by cocaine (Draskoczy and Trendelenburg, 1968).

Another factor of significance is the relative proximities of uptake and receptor sites. It is known that the magnitude

of cocaine-induced supersensitivity varies in different tissues. Verity (1971) showed that this correlated inversely with the neuromuscular interval. Thus the effects of cocaine were in accord with the expectation that the influence of neuronal uptake in reducing the NA concentration at the receptors decreased as the distance between uptake and receptor sites increased.

In addition to a prejunctional action, a postjunctional effect of cocaine has been described. Thus cocaine potentiated the sensitivity of the rabbit aortic strip to methoxamine (Kalsner and Nickerson, 1969b) which was not taken up by sympathetic nerves (Trendelenburg et al, 1970). Furthermore, Maxwell et al (1966) described the relationship between rate of uptake and concentration at the receptors in a theoretical model to which they applied data on the potentiation of NA sensitivity and the inhibition of uptake of NA by various concentrations of cocaine in the rabbit aortic strip. The experimental data did not comply with the model. Cocaine also potentiated responses to NA in strips from which the adventitia, and hence the sympathetic nerves, had been removed (Maxwell, 1972). Hence it seems reasonable to accept a postjunctional action of cocaine in this tissue. However, the effect is small and if it is present in other more intimately innervated tissues, it is likely to be

dominated by the prejunctional action.

(ii) Neuronal MAO

There is accumulating evidence that the extent to which neuronal uptake influences the concentration of NA at its receptors depends ultimately on intraneuronal deamination and vesicular storage. Despite biochemical and histochemical evidence for the presence of neuronal MAO (Hamberger et al, 1964; Snyder et al, 1965; Jarrott and Iversen, 1971; Jarrott, 1971a) and its ability to deaminate NA taken up by sympathetic nerves (Kopin and Gordon, 1963), earlier pharmacological investigations failed to implicate a significant role of this enzyme or extraneuronal MAO in the control of sensitivity to catecholamines (Furchgott et al, 1963). However, in a subsequent investigation of inotropic responses to NA in the isolated guinea pig atria, Furchgott and Sanchez Garcia (1968) observed that although initial steady state responses in normal and MAO-inhibited atria were similar in magnitude, prolonged exposure to NA in the latter resulted in a slowly developing increase in sensitivity and in delayed recovery following washout of the NA. They termed the phenomenon "secondary sensitisation". A similar phenomenon has been described in the rabbit ear artery by de la Lande and Jellett (1972), in the isolated rabbit atria by Graefe et al (1971), and in the cat nictitating membrane by Trendelenburg (1971).

Biochemical studies in rabbit hearts indicated a decrease in the net neuronal uptake of NA after MAO inhibition (Trendelenburg and Draskoczy, 1970) which was associated with a time (and concentration)-dependent increase in efflux of NA from the neuronal cytoplasm (Graefe et al, 1971). Accordingly, Trendelenburg (1971) proposed that progressive exhaustion of intraneuronal storage capacity by an accumulation of unmetabolised NA in the axoplasm led to a gradual efflux of NA to the receptors and hence the slowly developing secondary response after MAO inhibition. This is consistent with an important role of intraneuronal MAO in inactivating NA and thus permitting net inward flux to be sustained for long periods. It is of interest that combined inhibition of MAO and vesicular retention (by treatment with reserpine) in the cat nictitating membrane caused an increase in sensitivity to a level approaching that produced by denervation (Trendelenburg, 1971). It therefore seems that the ability of neuronal uptake to limit the concentration of NA at the receptors is ultimately largely dependent on the intraneuronal fate of the amine.

(iii) Extraneuronal MAO

It has been known for a considerable period that when tyramine or tryptamine are used as substrates, the major proportion of MAO activity in tissues is extraneuronal

(Snyder et al, 1965; Iversen et al, 1966). However, the pharmacological role of this extraneuronal MAO has not been as clearly defined as that of the intraneuronal enzyme. The most detailed study appears to be that of Kalsner and Nickerson (1969a) who proposed that under certain conditions, extraneuronal MAO was of major importance in the termination of action of NA and adrenaline. These workers obtained a steady state response of the rabbit aortic strip to NA or adrenaline, after which the bathing medium (Krebs' solution) was replaced by mineral oil. The rate at which the response then declined was taken as a measure of the rate at which the amine was removed from the region of the receptors by tissue inactivating mechanisms. These rates were slowed by both a COMT and an MAO inhibitor, suggesting that the amine was inactivated by both of these enzymes.

Although O-methylation was the principal pathway for inactivation of low concentrations of catecholamines (0.055 and 0.059μmol l^{-1} adrenaline and NA, respectively), MAO provided an effective alternate route when COMT was inhibited, and assumed major importance in the inactivation when adrenaline and NA were present in high concentrations (5.5 and 5.9μmol l^{-1}, respectively). Since the effects were observed in the presence of cocaine (added during the steady state of the response and 10 to 20 minutes prior to immersion

of the strip in mineral oil), they concluded that the sites of inactivation were extraneuronal. However, these conclusions have been questioned recently by Trendelenburg (1974). He showed that in MAO-inhibited aortic strips bathed in aqueous solution, there was a late phase of slow recovery from the constrictor response to NA which was appreciably reduced if strips were treated with cocaine prior to application of NA. This implied that neuronal uptake was important in the pharmacological action of the MAO inhibitor. To reconcile these results with those of Kalsner and Nickerson, Trendelenburg suggested that the relaxation of aortic strips in Krebs' solution was influenced by efflux of NA to the receptors from tissue compartments after washout of NA. In earlier studies (see Section ii) it was shown that after MAO inhibition, slow efflux of unmetabolised NA from nerve terminals could proceed for long periods. It is conceivable that at least part of the slow relaxation observed after MAO inhibition by Kalsner and Nickerson (1969a) was due to efflux of NA which had accumulated in the nerve terminals prior to application of cocaine. In another recent study, Levin (1974) separated the media and adventitia of the rabbit aorta and incubated these, and the intact aorta, in concentrations of NA (0.03 to 3.0μmol l^{-1}) similar to those used by Kalsner and Nickerson. Deaminated products accounted for approximately 60% of the total amount of metabolites in the adventitia,

but only about 10 - 20% in the isolated media. Since most of the endogenous NA was present in the adventitia, these observations implied a major contribution of neuronal MAO to the deamination of NA, whereas the contribution of extraneuronal MAO appeared relatively minor. In view of the findings of Trendelenburg (1974) and of Levin (1974), it seems likely that the importance of extraneuronal MAO in the inactivation of adrenaline and NA was overestimated in the studies of Kalsner and Nickerson (1969a).

(iv) Neuronal COMT

Early estimates of COMT activity in homogenates of tissues from normal and immunosympathectomised mice indicated that, like MAO, COMT activity was extraneuronal in distribution (Iversen et al, 1966). Subsequently, however, Jarrott (1971b) showed that an excess of methyl donor (S-adenosyl-methionine) was necessary for maximum COMT activity. Under these conditions, COMT activity in a number of tissues, including the vas deferens of the rat and rabbit and the nictitating membrane of the cat, declined after denervation. Hence Jarrott concluded that COMT was present in the sympathetic nerves of these tissues. However this did not apply to other tissues (e.g. the heart and spleen of the mouse and the vas deferens of the guinea pig) where there was no decline in COMT activity after denervation.

At present the functional role of neuronal COMT does not appear to have been defined. An interesting suggestion was that of Rubio and Langer (1973). These workers found that 3', 4'-dihydroxyphenylglycol (DOPEG), a major metabolite of MAO formed in the nerve terminals of the guinea pig atria, inhibited tyrosine hydroxylose activity in homogenates of atria. In contrast, 3'-methoxy-4'-hydroxy-phenylglycol (MOPEG), the O-methylated product of DOPEG, was devoid of inhibitory potency. They speculated that of the products of neuronal MAO activity, DOPEG might act as a physiological regulator of NA synthesis and that neuronal COMT might play a modulating role by converting DOPEG to the inactive MOPEG.

(v) Extraneuronal COMT

There is now considerable evidence that extraneuronal COMT is of functional importance in the control of sensitivity to catecholamines. An inhibitor of COMT (4-tropolone acetamide) enhanced the sensitivity of the rabbit aortic strip to NA (Levin and Furchgott, 1970). These workers concluded that the potentiation was mediated by inhibition of extraneuronal COMT since it was unaltered in strips treated with cocaine but was prevented by an inhibitor of extraneuronal uptake (hydrocortisone, see Section vi). The conditions under which inhibition of COMT caused sensitisation

were examined in the isolated cat papillary muscle. Kaumann (1970) showed that an inhibitor of COMT (UO521) potentiated β-receptor mediated responses to NA only when the muscle was treated with cocaine to eliminate neuronal uptake. However, the pharmacological effect was not attributed to inhibition of neuronal uptake per se, but rather to the increase in sensitivity to NA. Thus when the sensitivity was decreased by treatment with a β-receptor antagonist (propranolol), the effect of UO521 was abolished. This proposal was supported by studies in the cat nictitating membrane by Trendelenburg et al (1971). UO521 potentiated responses to NA, adrenaline and isoprenaline whenever the sensitivity of the nictitating membrane was high (ED_{50} <1μmol l^{-1}), for example in denervated muscles. However, when the sensitivity of the denervated muscle was decreased by treatment with an α-receptor antagonist (phentolamine), the potentiation by UO521 was markedly reduced. These workers also provided evidence that the lack of potentiation at high amine concentration was difficult to explain in terms of limitation of access of the amine substrate to COMT, or to saturation of the enzyme. Thus when denervated muscles were incubated with ^{3}H-NA, the accumulation of ^{3}H-NA and that of ^{3}H-O-methylated metabolites were linearly related to the concentration of ^{3}H-NA in the incubation media. Trendelenburg and his associates

were hence unable to provide a firm explanation for their observations. More recent studies however, suggest that only a small, readily saturable compartment of COMT activity may influence the concentration of amines at receptor sites. Since this concept is closely related to the influence of extraneuronal uptake, it is discussed in more detail in the following section.

(vi) Extraneuronal Uptake

In studies of the fate of intravenously administered ^{3}H-NA in rats, Fischer et al (1965) noted that a fraction of the uptake into the salivary gland persisted after denervation and treatment with reserpine, supporting an earlier suggestion that some extraneuronal binding of NA occurred in this tissue (Anden et al, 1963). While perfusing the isolated rat heart with concentrations of adrenaline or NA (5.5 and 5.9μmol l^{-1}, respectively) which had previously been shown to saturate neuronal uptake, Iversen (1965) observed an abrupt and massive increase in uptake. To distinguish between the two processes, he suggested the nomenclature "uptake$_1$" for the readily saturable neuronal uptake and "uptake$_2$" for the new process which appeared to operate at concentrations of amine of approximately 5μmol l^{-1} and above. The properties of the two processes differed strikingly. For instance, in contrast to uptake$_1$,

uptake$_2$ exhibited low affinity but high capacity for binding of amine, accumulated adrenaline in preference to NA, was not stereoselective, was insensitive to potent inhibitors of uptake$_1$ (e.g. cocaine and desipramine) and was inhibited by normetanephrine and metanephrine. Furthermore, while uptake$_1$ firmly retained accumulated amine, most of the adrenaline or NA accumulated by uptake$_2$ was rapidly removed by perfusing the heart with an amine-free medium ($t_{\frac{1}{2}}$, approximately 5 minutes). Although Iversen suggested that uptake$_2$ was probably also mediated by sympathetic nerves, subsequent histochemical studies provided evidence for an extraneuronal site, predominantly associated with cardiac muscle cells of the rat heart (Clarke et al, 1969; Farnebo and Malmfors, 1969).

Extraneuronal binding of NA by collagen and elastin and accumulation of NA in the cytoplasm of smooth muscle cells following incubation of the rabbit ear artery with NA in high concentrations was described by Avakian and Gillespie (1968). Gillespie (1968) summarised the properties of smooth muscle uptake and compared them with those of uptake$_1$ and uptake$_2$ of Iversen. Close similarities between smooth muscle uptake and uptake$_2$ were apparent (Table 1.1, from Gillespie, 1968). Subsequently, Gillespie and Muir (1970) found considerable species

Table 1.1* Comparison of some properties of uptake$_1$, uptake$_2$ and smooth muscle uptake of noradrenaline.

	Uptake$_1$	Uptake$_2$	Smooth Muscle Uptake
Threshold concentration (μmol l^{-1})	<0.1	>5.9	>59
Reversibility	Not easily removed on washing	Easily removed	Easily removed
PBZ	Marked inhibition	Marked inhibition	Marked inhibition
NMN	Slight inhibition	Marked inhibition	Marked inhibition
Metaraminol	Marked inhibition	Slight inhibition	-
Cocaine	Marked inhibition	Slight inhibition	No inhibition
Cold	-	-	Marked inhibition

* Based on that of Gillespie (1968)

PBZ = phenoxybenzamine

NMN = normetanephrine

variation in the ability of smooth muscle to accumulate NA, being most prominent in the mouse and rabbit and poorly developed in the guinea pig. In addition uptake into arterial smooth muscle was generally more pronounced than in smooth muscle of other tissues. Despite the specific drug sensitivity and suppression by cooling (Table 1.1), smooth muscle uptake was relatively insensitive to oubain, glucose deprivation and anoxia (Gillespie, 1973). Hence it has been suggested that this process represents a type of facilitated diffusion rather than active transport (Burnstock et al, 1971).

Early attempts to define the physiological significance of extraneuronal uptake faced the problem that the threshold concentration required for its demonstration histochemically was high (59μmol l^{-1} for NA in the rabbit ear artery; Avakian and Gillespie, 1968). These authors suggested that such concentrations of NA might occur at the nerve endings and that extraneuronal uptake might inactivate transmitter or prolong the response to nerve stimulation. The latter suggestion was supported by observations that the rate of recovery of the rabbit ear artery following perfusion of the latter with NA became increasingly slow as the concentration of NA was increased in the range from 5.9μmol l^{-1} to 5.9mmol l^{-1}. The relationship between the delay in

recovery and the perfusion concentration was similar to that between accumulation and the perfusion concentration. Therefore it was suggested that after its accumulation, NA effluxed from the smooth muscle cells into the region of the receptors during recovery and hence prolonged the response. This concept was also supported by the relatively fast rate of relaxation of the guinea pig inferior mesenteric artery in which no extraneuronal uptake of NA was detected histochemically (Gillespie, 1968).

The concept of extraneuronal uptake of catecholamines as a threshold phenomenon was revised by Lightman and Iversen (1969) who provided evidence suggesting that uptake occurred at all amine concentrations in the rat heart. Rapid metabolism prevented the appearance of appreciable amounts of unchanged amine in the lower ranges of concentrations ($<14.8 \mu mol\ l^{-1}$ for NA and $<4.5 \mu mol\ l^{-1}$ for adrenaline). When both MAO and COMT were inhibited, the level of metabolites was stoichiometrically replaced by an accumulation of unchanged amine which was sensitive to the $uptake_2$ inhibitor, normetanephrine. At about the same time, Kalsner (1969a,b) showed that certain steroids, including hydrocortisone and deoxycorticosterone, potentiated the sensitivities of the rabbit aortic strip to catecholamines.

The effects were similar in magnitude to those produced by inhibition of COMT. Furthermore the potentiations by the steroids were not additive with those of the COMT inhibitor; each agent failed to potentiate in the presence of the other. Kalsner therefore suggested that the potentiating effects of the steroids were mediated by inhibition of COMT. Subsequently, Iversen and Salt (1970) tested the effects of a range of steroids on uptake$_2$ in the rat heart. Although the formation of metabolites was reduced by the steroids, the accumulation of unchanged amine was decreased to about the same extent. Moreover, the steroids inhibited the accumulation of NA even when metabolism was prevented by inhibition of MAO and COMT. Hence these authors concluded that the steroids acted primarily to inhibit uptake$_2$ and thus prevent access of the substrate to the enzyme, rather than to inhibit COMT directly. This new class of inhibitor has proved more suitable than others in assessing the physiological importance of extraneuronal uptake. This is because other previously described inhibitors of uptake$_2$ in the rat heart (e.g.,O-methyl derivitives of the catecholamines, normetanephrine and metanephrine; (Iversen, 1967)) possessed sympathomimetic actions, while phenoxybenzamine (Lightman and Iversen, 1969) possessed a diversity of actions including potent α-receptor blocking activity.

The investigation of the importance of extraneuronal uptake in the control of sensitivity to catecholamines to be described in subsequent chapters of this thesis was commenced in 1971, shortly after the report of Iversen and Salt (1970). It has become evident during the subsequent period that several other groups of investigators have also exploited the advantages of the steroids in evaluating the significance of extraneuronal uptake. One such study was that of Kaumann (1972), who showed that the sensitivity of cat heart muscle to isoprenaline, an amine with little affinity for neuronal uptake but relatively high affinity for extraneuronal uptake (Iversen, 1967), was markedly potentiated by hydrocortisone. Hydrocortisone also greatly reduced the potentiating effect of COMT inhibition as had been noted earlier in the rabbit aortic strip by Kalsner (1969a,b) and by Levin and Furchgott (1970). On the basis of these observations and the evidence of Lightman and Iversen (1969) and Iversen and Salt (1970) (outlined above), Kaumann proposed that hydrocortisone potentiation was mediated by preventing access of isoprenaline to extraneuronal sites of inactivation by COMT. Responses to NA were also potentiated by hydrocortisone but to a lesser extent compared with isoprenaline, and only when the influence of neuronal uptake was removed, suggesting that the latter had masked the contribution of

extraneuronal inactivation of this amine. However, elimination of neuronal uptake (e.g. by cocaine) reduced the ED_{50} concentration of NA to a level approaching that of isoprenaline in the absence of cocaine. When the ED_{50} concentration of NA in the presence of cocaine was increased approximately 50-fold (to approx. 1μmol l^{-1}) by a β-receptor antagonist (KL_{255}), the potentiating effect of hydrocortisone was abolished. Thus the sensitivity of the cat heart muscle to NA was an important factor influencing the magnitude of potentiation by hydrocortisone. Since this ED_{50} concentration of NA (1.0μmol l^{-1}) was about 250 times lower than the previously reported Km for $uptake_2$ of NA (Iversen, 1965), Kaumann postulated the existence of a hydrocortisone-sensitive extraneuronal inactivation mechanism which was of high affinity for NA but which was readily saturable. The mechanism was less easily saturated by isoprenaline. When the ED_{50} concentration of this amine was increased (by KL_{255}) to levels at which potentiation of NA was abolished, the potentiation of isoprenaline by hydrocortisone persisted, but was appreciably reduced in magnitude.

More recent studies have provided biochemical evidence in support of the concept of readily saturable extraneuronal uptake postulated by Kaumann (Bonisch and Trendelenburg, 1974).

The latter workers showed that isoprenaline accumulated in different compartments in the rat heart. One compartment was associated with high affinity but low capacity for O-methylation of isoprenaline while the second exhibited low affinity but high capacity for binding of unchanged amine. Both compartments were sensitive to inhibition by the $uptake_2$ inhibitor, corticosterone (Bonisch et al, 1974; Uhlig et al, 1974). In the cat nictitating membrane, on the other hand, Graefe and Trendelenburg (1974) have described two extraneuronal compartments, both associated with COMT but of differing activities with respect to O-methylation of NA and isoprenaline in the intact tissue. However in this tissue only one compartment, associated with COMT of high affinity but low capacity for O-methylation of catecholamines, appeared sensitive to inhibition by hydrocortisone. A major feature of their findings was the evidence that COMT was an essential requirement for the extraneuronal uptake system to influence the concentration of catecholamines at the receptors. A similar concept has been developed independently in the course of the present study and hence its implications are considered in more detail in the General Discussion to this thesis (Chapter 7).

(vii) Factors influencing the sensitivity of the rabbit ear artery to catecholamines

In the final section of this Introduction evidence that the mechanisms discussed above may modulate sensitivity to catecholamines in the rabbit ear artery will be summarised. In the ear artery, as in most small arteries, the terminal sympathetic fibres are confined to the junction of the media and adventitia (de la Lande et al, 1967a; Burnstock et al, 1970) (Fig. 1.1). Thus the smooth muscle cells in the outer media are closest to the sympathetic innervation. Such a morphology implies that the influence of neuronal uptake on the concentration of NA at its receptors will be greatest in the case of outer smooth muscle cells (close to the adventitia) and least in the case of inner smooth muscle cells (close to the intima). De la Lande et al (1967a) showed that the sensitivity of the artery to the constrictor effects of intraluminal NA was greater than that to extraluminal NA by a factor of about ten. However, in chronic-denervated or cocaine-treated arteries, this difference was greatly diminished as a consequence of a striking and selective increase in the sensitivity to extraluminal NA. These observations were interpreted as evidence that when NA diffused from the adventitia, its concentration was greatly decreased by neuronal uptake before it reached the α-receptor sites in the underlying smooth muscle. Removal of the influence of neuronal

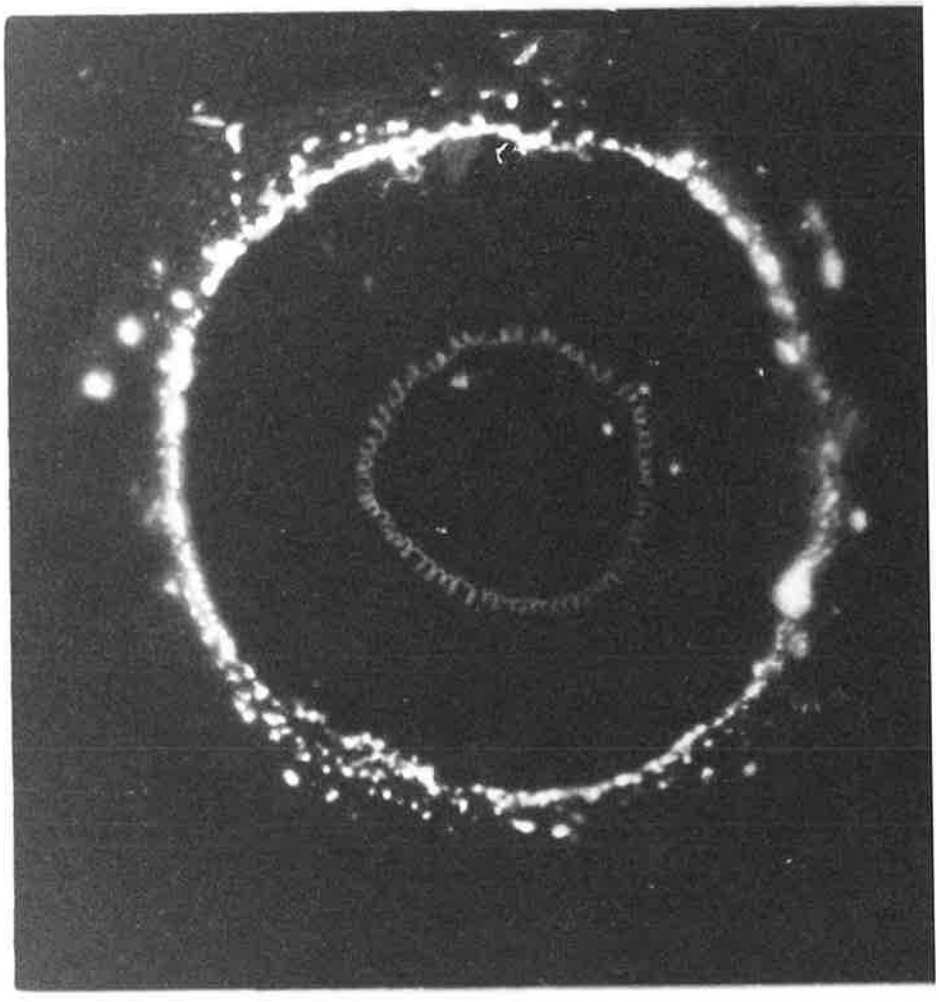


Fig. 1.1 Transverse section of the rabbit ear artery treated by the histochemical fluorescence method for demonstration of noradrenergic structures (see Methods, Chapter 2). The location of the sympathetic nerve terminals at the medial-adventitial border is indicated by the dense noradrenergic fluorescence in this region. Non-specific autofluorescence is also seen at the intima.

uptake by denervation or treatment with cocaine decreased or eliminated this loss. The onset of potentiation of extraluminal NA by cocaine when the latter was applied via the intraluminal perfusion medium occurred within 30 seconds, suggesting that cocaine diffused across the artery wall to the nerve terminals. Therefore de la Lande et al (1967a) assumed that intraluminal NA also diffused across the artery wall to the nerve terminals, and that the relatively minor potentiation of intraluminal NA by cocaine reflected the relative failure of uptake to influence the concentration which this NA achieved in the smooth muscle, rather than the relative failure of intraluminal NA (compared with extraluminal NA) to reach the nerve terminals. These features were incorporated in an hypothesis represented diagrammatically in Fig. 1.2.

This hypothesis was modified following analysis of the role of MAO in the response of the artery to NA. Using the nitrotetrazolium histochemical method, de la Lande et al (1970a) showed that the media of the artery possessed MAO activity but they were unable to detect activity specifically in the region of the nerve terminals. However, a more recent analysis, in which MAO activity was determined biochemically by measuring the oxidation of tyramine in artery homogenates, has shown a small but significant

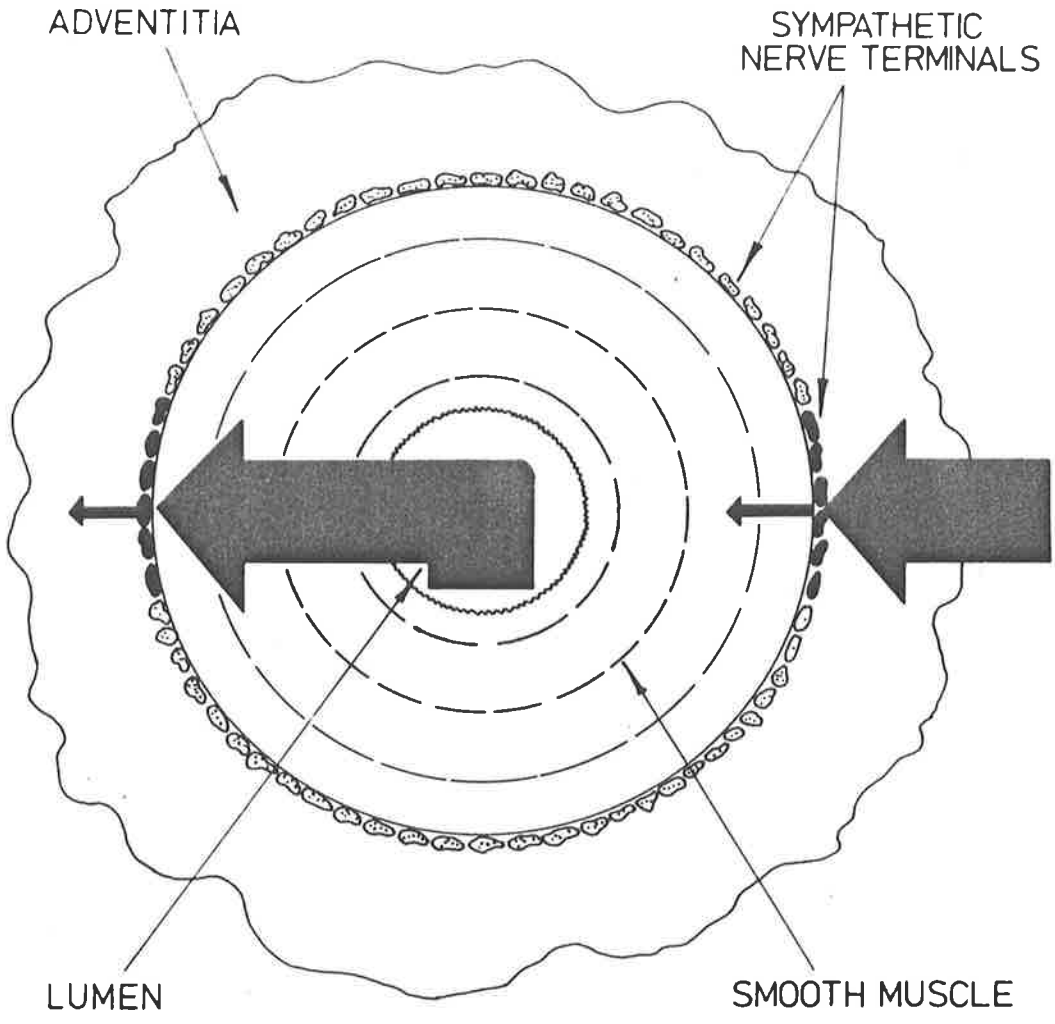


Fig. 1.2 Diagrammatic representation of the influence of uptake by the sympathetic nerve terminals on the concentration of NA in the smooth muscle of the artery. The direction of the arrows indicates the direction of diffusion of NA. Thicknesses of arrows represent concentrations of NA. The model implies free penetration of both extraluminal NA (through the adventitia) and intraluminal NA (through the media) to the sympathetic nerve terminals.

(10%) decline in MAO activity after denervation (Head et al, 1974). Hence it appears that approximately 10% of the MAO which oxidizes tyramine is located in the sympathetic nerve terminals. Previously, a pharmacological study had provided strong evidence for an important role of neuronal MAO in the constrictor response to NA (de la Lande and Jellett, 1972). The latter workers showed that after nialamide treatment the constrictor response of the artery to sustained application of extraluminal NA was characterised by a slow increase in magnitude after it had attained its initial steady state level. In addition there was a delay in the recovery from constriction after washout of the NA from the bathing medium, i.e., the artery displayed the phenomenon of secondary sensitisation described earlier by Furchgott and Sanchez Garcia (1968) in the guinea pig atria. The failure of MAO inhibition to alter appreciably the responses to extraluminal NA in either cocaine-treated or chronic-denervated arteries indicated that the secondary sensitisation was mediated by inhibition of intraneuronal MAO and not by extraneuronal MAO, despite the high concentration of the latter detected histochemically. Other evidence for the presence of neuronal MAO in this artery was obtained using the Falck histochemical fluorescence procedure. Arteries from rabbits previously treated with reserpine failed to display monoamine fluorescence at the medial-

adventitial border, in accord with the well known ability of reserpine to deplete endogenous NA stores. Fluorescence was restored by extraluminal application of NA but only if an inhibitor of MAO (nialamide) was also present, implying that deamination by MAO in the nerve terminals had prevented the accumulation of a sufficient concentration of NA to be detected histochemically (de la Lande et al, 1974).

Both the pharmacological and histochemical studies emphasised the importance of intraneuronal mechanisms in the control of sensitivity to NA, as represented in Fig. I.2. However, these studies also provided evidence that the penetration of intraluminal NA to the nerve terminals was restricted compared with that of extraluminal NA. Thus in contrast to its striking effect on the sensitivity to extraluminal NA, inhibition of MAO had little effect on the sensitivity to intraluminal NA. Secondary sensitisation to intraluminal NA was not apparent when the concentration of the latter was ten times greater than that of extraluminal NA (de la Lande and Jellett, 1972), suggesting that the diffusion of intraluminal NA to the nerve terminals (through the media) was severely limited compared with that of extraluminal NA (through the adventitia). A similar conclusion was drawn from histochemical studies which showed that

intraluminal NA failed to restore monoamine fluorescence at the medial-adventitial border of reserpine-pretreated and MAO-inhibited arteries whereas fluorescence in this region was readily restored by extraluminal NA (de la Lande et al, 1970b; 1974). Comparison of the threshold concentration of extraluminal NA which restored fluorescence ($0.3\mu mol\ l^{-1}$), and the concentration of intraluminal NA which failed to do so ($3.0\mu mol\ l^{-1}$) again suggested that penetration of intraluminal NA to the nerve terminals was extremely limited compared with that of extraluminal NA. Such a difference is not consistent with the concept that the steady state response to NA is associated with a uniform concentration of NA across the artery wall, but instead, implies that there is a considerable gradient of concentration. When NA is applied to the intima, the concentration in the extracellular space of the smooth muscle cells immediately adjacent to the intima is at least ten-fold greater than the concentration achieved at the outermost smooth muscle cells adjacent to the adventitia. The presence of a concentration gradient implies, in turn, that responses to NA are mediated by smooth muscle cells immediately adjacent to the surface of application. This view is favoured by Kalsner (1972) who showed that the steady state response to NA was further increased when an equal concentration of NA was applied to the opposite surface of the artery, thus

supporting the concept of a non-uniform distribution of NA in the artery wall. The findings of de la Lande et al (1974) that fluorescence at the medial-adventitial border was restored by intraluminal NA (to a level of intensity approaching that seen after extraluminal NA) in the presence of an inhibitor of extraneuronal uptake (metanephrine) or an inhibitor of COMT (U0521) suggested that the limited access of intraluminal NA to the nerve terminals was due, at least in part, to extraneuronal uptake and to metabolism by COMT. Thus a more representative model of the artery may be that shown in Fig. 1.3.

The extraneuronal uptake of high concentrations of NA in the smooth muscle of various tissues and species, including the smooth muscle of the rabbit ear artery was well documented in earlier histochemical studies by Gillespie and his associates, and has been discussed above (p.14-17). However, these studies were confined to high concentrations of catecholamines in accord with the high threshold for the uptake process (59μmol l^{-1} NA). Since MAO was distributed at extraneuronal sites throughout the media (de la Lande et al, 1970a), it seemed possible that despite its minor role in the constrictor response to NA (de la Lande and Jellett, 1972), MAO may have inactivated NA when the latter was present in high concentrations and

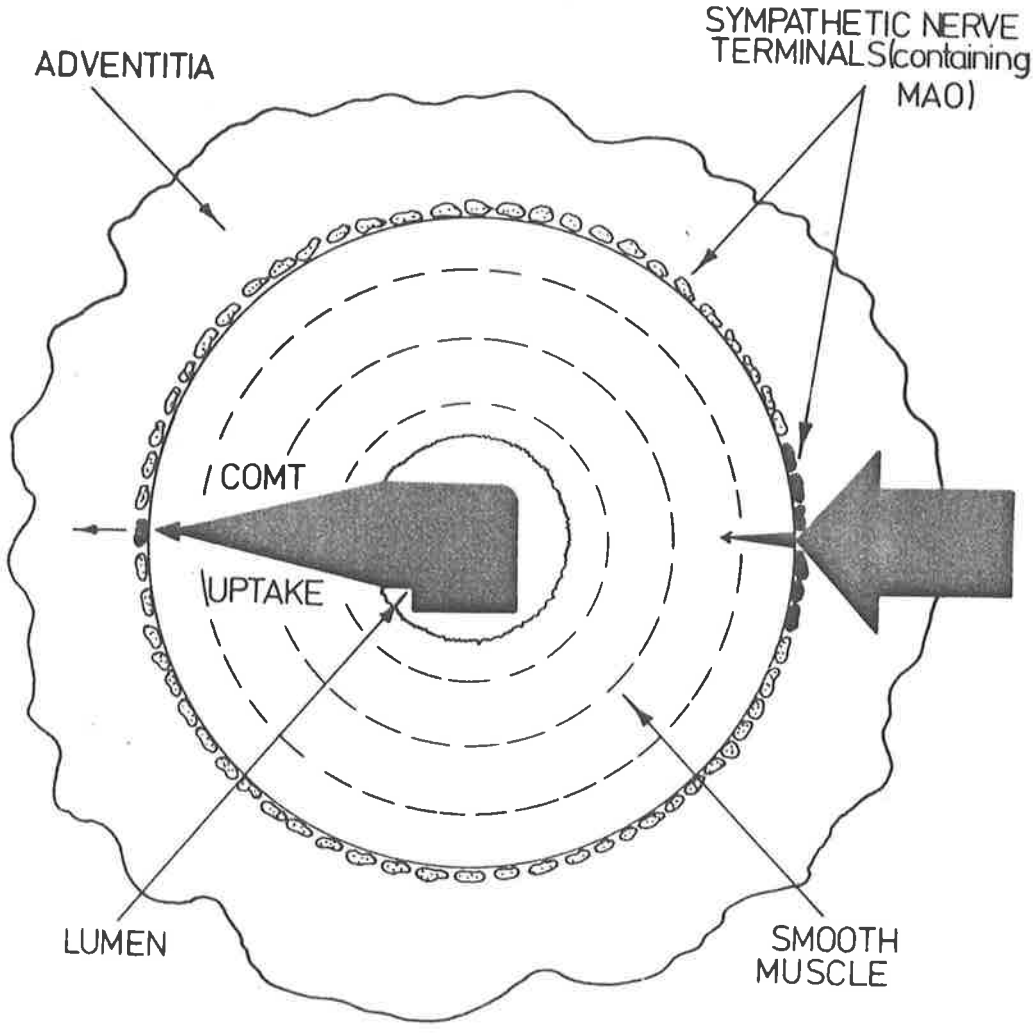


Fig. 1.3 Diagrammatic representation of the influence of neuronal uptake, as indicated in Fig. 1.2. The model differs from that shown in Fig. 1.2 in that it incorporates the concept of restricted passage of NA through the media, partly as a consequence of extraneuronal uptake, and metabolism by COMT.

hence contributed to the high threshold for smooth muscle uptake. This possibility was investigated in the first of the experimental sections of this thesis. The results (reported in Chapter 3) suggested that deamination by MAO represented a major route of inactivation of NA under these conditions. In subsequent histochemical studies, Nicol and Rae (1972) showed that smooth muscle uptake of adrenaline and NA in rabbit ear arteries perfused with high concentrations of these amines (2.7 and 3.0 mmol l^{-1}, respectively) was sensitive to inhibition by steroids. They also found that the prolongation of the constrictor response following removal of these amines from the perfusion medium was markedly reduced if steroids were present during the period of application of the amine, thus supporting the causal relationship between extraneuronal uptake and response prolongation proposed by Gillespie (1968) (see p.16). However, there was no information on the actions of steroids on the sensitivity of the artery to the constrictor or dilator effects of low (sub-maximal) concentrations of catecholamines. There were also few studies on the distribution and functional significance of COMT. Burnstock et al (1972) showed that the total COMT activity in the ear artery was relatively low, although of the same order as that in the rabbit aorta in which Kalsner and Nickerson (1969a) proposed an important role of this enzyme in the inactivation of NA and adrenaline.

More recently, Head et al (1974) showed that COMT activity in the ear artery was not significantly decreased after denervation, suggesting that this enzyme was largely extraneuronal in distribution.

The possibility that both extraneuronal uptake and COMT were pharmacologically important was suggested by the indirect evidence (mentioned above) that an inhibitor of COMT (UO521) and an inhibitor of extraneuronal uptake (metanephrine) altered the diffusion of intraluminal NA in the artery wall. The model of the artery shown in Fig. 1.3 has proved useful in the interpretation of the effects of inhibitors of extraneuronal uptake and of COMT on the sensitivity of the artery to catecholamines, a study of which has formed the major experimental section of this thesis. Pharmacological evidence is presented in Chapter 4 to support the hypothesis that the potentiating action of deoxycorticosterone acetate (DOCA) in the rabbit ear artery is mediated by inhibition of extraneuronal uptake and that the latter represents an important mechanism in the control of sensitivity of the artery to a variety of vasoactive amines. It is further proposed that neuronal and extraneuronal uptake operate interdependently to reduce the concentration of amine at receptors of cells mediating the response to extraluminally applied amine. Additional pharmacological evidence is

presented in Chapter 5 to implicate an important role of COMT and its close association with extraneuronal uptake in controlling vascular sensitivity. More direct evidence for the presence of extraneuronal uptake in the artery and its sensitivity to inhibition by DOCA has been obtained in biochemical studies of the metabolism of low concentrations of ^{3}H-isoprenaline, reported in Chapter 6.

The influence of neuronal and extraneuronal mechanisms in arteries from rabbits with experimental hypertension has also been examined. Although progress with this study was restrained by a high mortality rate in these animals, preliminary findings are reported and treated as a separate entity in Part II of this thesis (Chapter 8).

CHAPTER 2

GENERAL METHODS

The methodology described in this chapter includes:

(i) technique for perfusion of the rabbit ear artery

(ii) preparation of helical strips of the artery

(iii) technique of surgical denervation

All other experimental procedures are described in the appropriate sections of each chapter. A list of the drugs used, and their suppliers, is included in Appendix 2.

(i) ISOLATION AND PERFUSION OF THE EAR ARTERY

Male and female semi-lop-eared rabbits, weighing 1.5-2.5kg were used throughout the course of this study. They were bred at the Central Animal House of the University of Adelaide. The only exception to this arose when 28 New Zealand white-Oxford lop cross rabbits, bred at the Animal Breeding Establishment, John Curtin School of Medical Research, Canberra, were made available from a concurrent study by Dr. E.G. Cleary, Department of Pathology, University of Adelaide, of the effects of experimental hypertension on the collagen and elastin contents of the developing aorta. Ear arteries from these animals were used in part of the study of experimental hypertension, described in Part II of this thesis (Chapter 8). The results obtained were treated separately from those derived

from similar hypertension studies (also described in Chapter 8) in which the normal breed of semi-lop-eared rabbit were used.

Rabbits were stunned and bled and an incision made in the skin at the base of the ear. A 3-4cm segment of artery from the proximal third of the ear (Fig. 2.1) was exposed by blunt dissection and cleared of adhering tissue. Tissues were kept moist with warm Krebs' solution. Polythene cannulae were inserted at both the proximal and distal ends and tied with cotton, leaving a length of approximately 2cm of artery between the tips of the cannulae. The double cannulated artery was transferred to the perfusion apparatus (Fig. 2.2) and set up in the double-jacketed organ bath containing $16cm^3$ Krebs' solution. The preparation was maintained under a longitudinal tension of 1g, and was perfused with Krebs' solution at a constant rate of $4.5cm^3\ min^{-1}$ by means of a roller pump (designed by O. Saxby, Department of Pharmacology, University of Oxford). The perfusate was discarded after being pumped through the distal cannula. Drugs could be added to either the Krebs' solution bathing the adventitia (extraluminal (EL) application) or to the solution perfusing the lumen (intraluminal (IL) application) as described by de la Lande et al (1966). Both these solutions were maintained at 37^oC and were bubbled continuously with a mixture of 95% O_2, 5% CO_2. Leaks due to side branches in the double cannulated

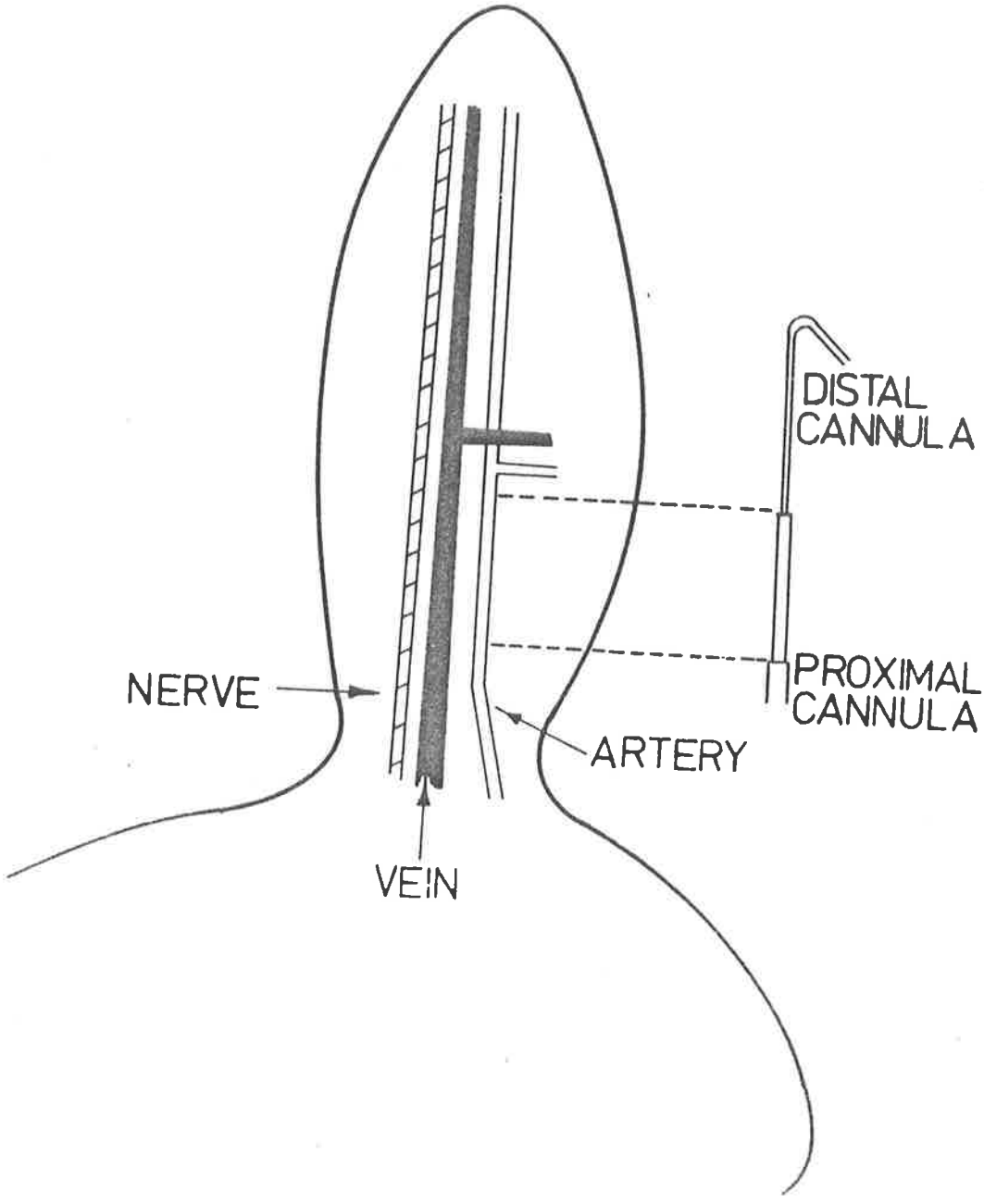


Fig. 2.1 Diagrammatic representation of the convex surface of the left ear of a semi-lop-eared rabbit, indicating the relative positions of the central artery, central vein and auricular nerve. The position of the segment of artery normally double cannulated for subsequent perfusion is shown at right.

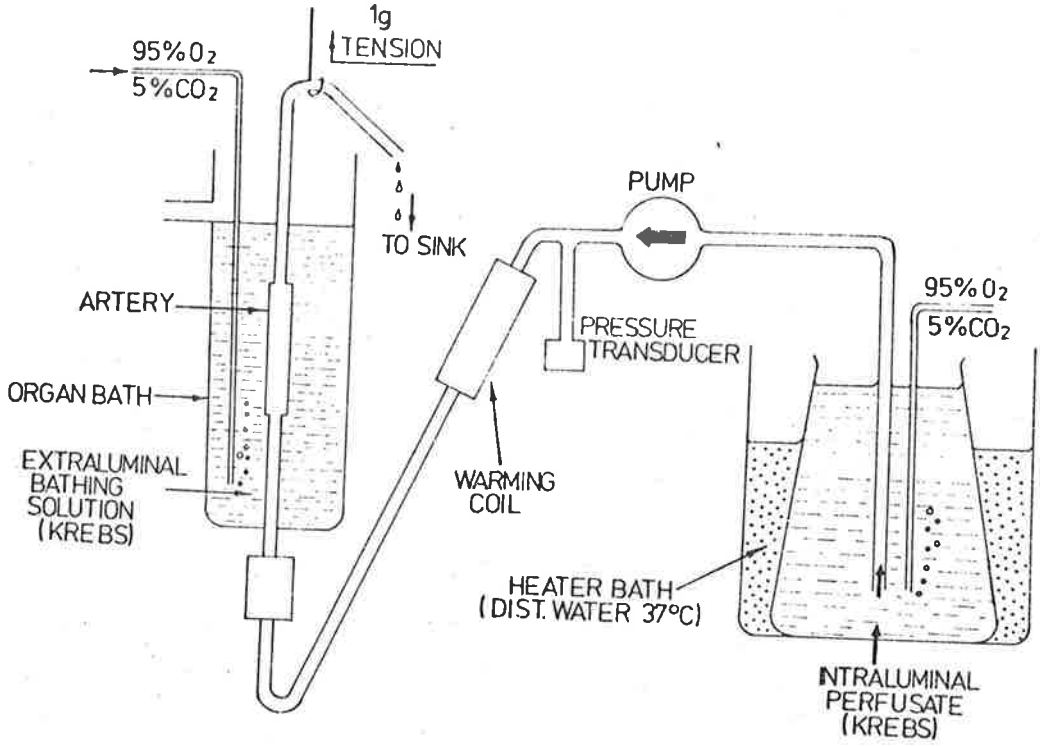


Fig. 2.2 Diagrammatic representation of the apparatus used for perfusion of the double cannulated rabbit ear artery. The constant temperature jacket of the organ bath is not included.

segment were rare and were detected by the increase in the volume of the extraluminal bathing fluid when the overflow tubing of the organ bath was routinely clamped for a short period after commencing perfusion. Since the absence of any mixing of the intraluminal perfusate with the extraluminal bathing solution was essential for most of the experiments to be described in subsequent chapters, other uses were found for any arteries in which leaks were detected.

Responses to constrictor agents

Arteries were perfused with Krebs' solution for at least 60 minutes before any drugs were applied. The perfusion pressure was measured using a Statham P23Ac pressure transducer and Rikadenki chart recorder. Resting pressure under conditions of constant perfusion ($4.5cm^3\ min^{-1}$) was approximately $4 \times 10^3\ Nm^{-2}$ (1mm Hg = $133.32\ Nm^{-2}$). The sensitivities to constrictor amines were estimated in terms of the concentrations eliciting a standard response (i.e. an increase in the perfusion pressure of $8 \times 10^3\ Nm^{-2}$). These concentrations were estimated from cumulative concentration-response curves, the response being the difference between the steady state levels of the perfusion pressure prevailing before and during application of the amine. Changes in sensitivity produced by drugs were measured in two ways:

(a) by measuring (from cumulative concentration-response

curves) the ratio of the concentrations of the amine which were equieffective in eliciting an increase in the perfusion pressure of 8×10^3 Nm^{-2} in the absence and in the presence of the drug (added to both the extraluminal bathing solution and intraluminal perfusate) or,

(b) when the change was small, by adding the drug intraluminally during the steady state of the constrictor response to extraluminally applied amine, and measuring the subsequent increase (or decrease) in the response. The change in the perfusion pressure was termed the incremental response to the drug being tested. The change in sensitivity produced by the drug was then estimated by measuring the ratio of concentrations of amine which were equieffective in eliciting the full response in the absence and in the presence of the drug.

Responses to nerve stimulation

Stimulation of the sympathetic nerve terminals in the artery was achieved by the application of short pulses (1 millisecond duration) delivered transmurally, at a frequency of 0.5 - $5H_Z$ and supramaximal voltage, by platinum electrodes placed in the extraluminal bathing solution on either side of the artery and connected to a Grass Model S_4 stimulator. Arteries were either stimulated for 10 seconds every 1-2 minutes to elicit transient responses, or stimulated continuously to

achieve steady state responses. The effects of drugs on the responses to nerve stimulation were determined by measuring the ratio of the frequencies which were equieffective in eliciting increases in the perfusion pressure of 6.0×10^3 Nm^{-2} in the absence and in the presence of the drug under study.

Dilator responses to isoprenaline

In these experiments, cocaine ($2.9 \mu mol\ l^{-1}$) was present throughout in both the intraluminal perfusate and extraluminal bathing fluid. Dilator effects of isoprenaline were measured in a manner based on that described by Carroll and Glover (1973a), i.e. by the percentage diminution in the height of the transient responses to nerve stimulation during cumulative addition of isoprenaline to the extraluminal bathing fluid. The sensitivity to isoprenaline was estimated in terms of the concentration of isoprenaline which produced 50% diminution of the height of the responses to nerve stimulation (ID_{50}), and in terms of the concentration producing half the maximum diminution in response height (ED_{50}). Changes in sensitivity produced by drugs were expressed as the ratios of the ED_{50} (or ID_{50}) concentrations in the absence and in the presence of the drug (added to both the extraluminal bathing medium and intraluminal perfusate).

(ii) HELICAL STRIP PREPARATIONS

Ac 2-3cm segment of the ear artery was isolated in the manner described above. Cotton ligatures were applied at the proximal and distal ends, the artery excised and placed on gauze soaked with warm ($37^{o}C$) Krebs' solution. The segment was slowly rotated while being cut at an angle of $30\text{-}40^{o}$ with fine iris scissors to form a helical strip about 5mm wide and 2cm in length. The proximal end was attached to a steel holder at the base of a double-jacketed organ bath. A 15cm length of cotton extended from the ligature at the distal end and was attached to the lever of a Harvard Heart/Smooth Muscle transducer. The preparation was maintained under a longitudinal tension of 1g. The organ bath contained $15cm^{3}$ Krebs' solution which was continually bubbled with a mixture of 95% O_2, 5% CO_2, and maintained at a constant temperature of $37^{o}C$. Strips were allowed to equilibrate for 90 minutes before any drugs were applied. Changes in length of the preparation were measured using the Harvard Heart/Smooth Muscle transducer and a Rikadenki chart recorder. The strip shortened when contracted by cumulative addition of adrenaline to the organ bath. Responses were calculated as percentages of the maximum response to adrenaline. The sensitivity of the preparation was estimated in terms of the concentration of adrenaline required to produce 50% of the maximum response (the ED_{50}

of adrenaline). Changes in sensitivity produced by drugs were estimated in terms of the ratio of the ED_{50} concentrations in the absence and in the presence of the drug. The attenuator setting of the Rikadenki recording device was usually 20mv/cm. At the end of each experiment this was adjusted to a 25-fold lower amplication (500mv/cm) and the chart response to a 1cm manual deflection of the transducer lever through the point at which the artery was attached measured. The actual change in length of the strip, corresponding to the maximum response to adrenaline, was then calculated from the following:

$$\text{Maximum length change (cm)} = \frac{\text{Max. chart response to adrenaline (at 20mv/cm)}}{\text{Chart response to 1cm deflection (at 500 mv/cm)}} \times \frac{1}{25}$$

Tests of significance

Since ratios of equieffective concentrations or frequencies of stimulation were used in many cases to assess the effects of drugs, these effects have been expressed as geometric rather than arithmetic means. When comparing the effects of different drugs or of a drug under different experimental conditions, Students' t-tests were performed on geometric rather than arithmetic means. In view of evidence of Fleming et al (1972) that equieffective concentrations of NA

(and of acetylcholine) in various tissues, including the rabbit ear artery, were distributed normally on a logarithmic rather than an arithmetic scale, logarithms of concentrations were used in Students' t-tests to compare sensitivities to amines. The appropriate t-test used, according to whether the samples contained matched pairs or unpaired data, is indicated in each case.

(iii) SYMPATHETIC DENERVATION

Ear arteries were denervated by surgical removal of the homalateral superior cervical ganglion. Rabbits were anaesthetised with pentobarbital (1.5% in sterile saline) injected into the marginal ear vein as described by Murdock (1969). The dose required varied considerably but was usually in the range 30-50mg/Kg. Close attention was directed to respiratory depression which occurred with the use of barbiturate anaesthesia. The injection of pentobarbital was continued slowly until respiration was decreased in rate and increased in depth, and until the expiratory phase became slightly prolonged. At this stage the toe reflex was usually weak. The eye reflex was found to be an unreliable guide to anaesthesia. The neck region was shaved and cleansed with antiseptic (Savlon; 3% in 70% ethanol). A midline incission was made in the neck and muscle layers penetrated by blunt dissection to expose the trachea and the left common carotid

artery. The superior cervical ganglion was readily identifiable posterior to the carotid artery and at a level corresponding to the upper border of the thyroid cartilage. Pre- and postganglionic fibres were sectioned about 1cm either side of the ganglion which was then removed. The wound was closed with silk sutures. Aseptic precautions were taken throughout and no antibiotics were used post-operatively.

For experiments described in Chapter 3, arteries were removed from rabbits 1-6 weeks after denervation, while for those described in Chapter 4, arteries were removed for perfusion 15-20 weeks after denervation. The effectiveness of sympathectomy was confirmed by the failure of arteries from denervated ears to respond to electrical stimulation (described above) in the range of frequencies from 1-10H_z. Small responses were elicited in some arteries stimulated at frequencies as high as 20H_z. In contrast, the threshold frequency in innervated arteries was usually 0.5-1H_z. The effectiveness of sympathectomy was also assessed by fluorescence microscopy examination of denervated and contralateral innervated control arteries as described by Waterson (1968). Sections of arteries approximately 5mm in length were frozen in a mixture of acetone and dry ice and transferred to a freeze dryer (Thermovac model FD_3) at -50^{o}C

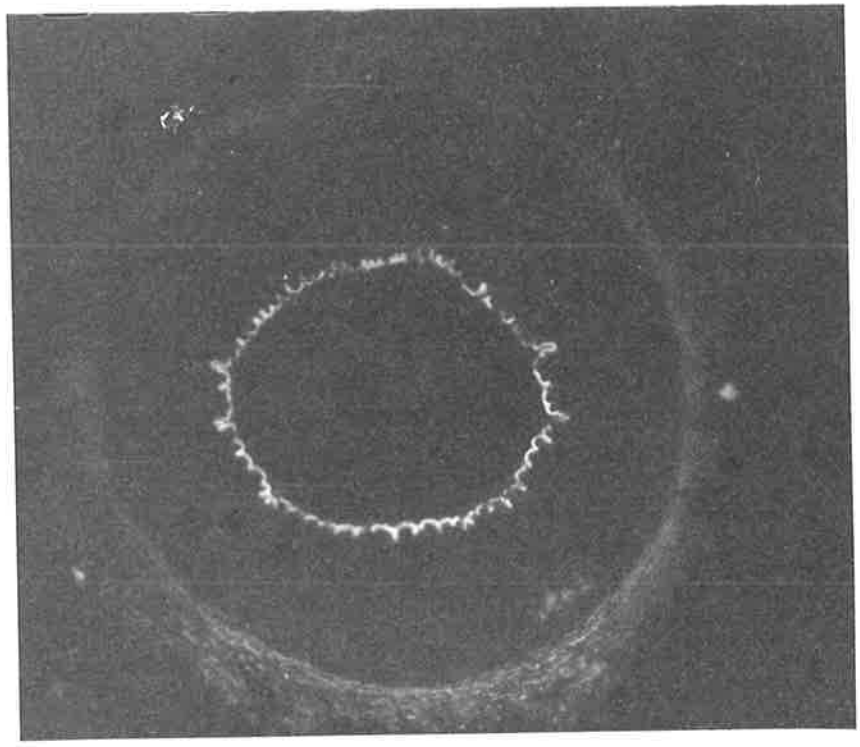
and at a pressure of 2.5-6.5Nm^{-2} for 18 hours. The sections were then placed in a one litre jar containing 5g of paraformaldehyde powder which had been stored over sulphuric acid at a relative humidity of 70% for one week. The jar was sealed and placed in an oven at 80^{o}C for 60 minutes. The formaldehyde-treated specimens were then vacuum infiltrated with paraffin wax at 60^{o}C, blocked in paraffin wax, and cut in transverse sections of 7μm thickness, using a microtome (Leitz model 1212). After mounting in a mixture of Entellan (Merck) and xylol, the sections were examined using a Leitz microscope with a dark field condenser. Fluorescence was produced with an HBO 200 mercury vapour lamp using a 1.5mm Schott BG 12 excitation filter and 530nm barrier filter. Photographs were taken using a Leitz orthomat camera and Kodak photoflure film.

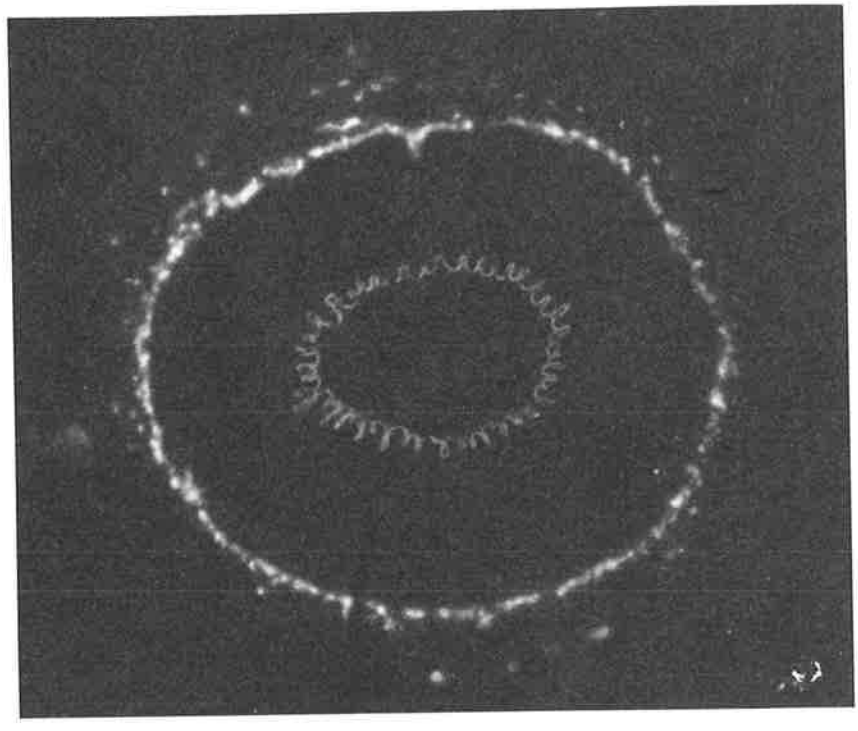
Control sections displayed characteristic dense noradrenergic fluorescence in the region of the sympathetic nerve terminals at the medial-adventitial border (Fig. 2.3a). The success of sympathectomy was indicated by the absence or sparsity of noradrenergic fluorescence in denervated arteries (Fig. 2.3b).

Fig. 2.3 Transverse sections of rabbit ear arteries treated by the histochemical fluorescence method.

Upper panel: section of an innervated artery showing specific noradrenergic fluorescence at the medial-adventitial border and non-specific autofluorescence of the intima.

Lower panel: section of artery from the opposite ear of the same rabbit six weeks after removal of the homolateral superior cervical ganglion. Note sparsity of specific noradrenergic fluorescence at the medial-advential border. Non-specific autofluorescence is still seen.





CHAPTER 3

ROLE OF EXTRANEURONAL MONOAMINE OXIDASE IN THE INACTIVATION OF NORADRENALINE

INTRODUCTION

De la Lande et al (1970a) showed that the only monoamine oxidase (MAO) which could be detected histochemically in the rabbit ear artery was extraneuronal and distributed throughout the media. Subsequent pharmacological studies provided evidence for the presence of intraneuronal MAO and for an important role of this enzyme, but not extraneuronal MAO, in controlling the vasoconstrictor response to NA (de la Lande and Jellett, 1972). The evidence was based on the ability of an inhibitor of MAO (nialamide) to enhance sensitivity to extraluminal NA in innervated but not in denervated arteries. However, the failure of inhibition of extraneuronal MAO to modify the vasoconstrictor response did not exclude the possibility that NA was metabolised by this enzyme. This possibility was tested in the present study by taking advantage of the histochemical findings of Avakian and Gillespie (1968) that the smooth muscle cells of the media rapidly accumulated NA when the latter was present in high concentrations in the Krebs' solution incubating the artery. In one series of experiments the effect of inhibition of MAO on the efflux of NA following its uptake from high concentrations was determined. In a second series, in view of evidence of Avakian and Gillespie (1968) that efflux of NA to the receptors following its accumulation in high concentrations in the smooth muscle prolonged the constrictor response, the

effect of inhibition of extraneuronal MAO on the rate of relaxation of artery segments previously exposed to high concentrations of NA was determined. The rate of relaxation was used as a pharmacological index of the rate of efflux of unchanged amine from its sites of uptake into the region of the receptors.

METHODS

(i) Incubation Experiments

Segments of the central artery of the rabbit ear were incubated at $37^{o}C$ for 15 minutes in $5cm^3$ volumes of Krebs' bicarbonate solution containing NA ($118\mu mol\ l^{-1}$). Following incubation, the segments were washed for one minute in $5cm^3$ volumes of NA-free Krebs' solution in order to remove NA from the adventitial surface. The segments were then incubated in $5cm^3$ volumes of NA-free Krebs' for a further ten minute period during which the cumulative efflux of NA in the incubation media was measured by removing samples at intervals and assaying the NA content of these samples on the sensitised rabbit ear artery as described by de la Lande and Harvey (1965). The Krebs' solutions contained ascorbic acid ($284\mu mol\ l^{-1}$) and EDTA ($9.4\mu mol\ l^{-1}$), and were gassed with 95% O_2, 5% CO_2.

Usually five segments of arteries from one rabbit were

used per experiment. They were treated as follows:

Segment 1: Untreated, i.e., incubated with Krebs' solution alone prior to exposure to NA

Segment 2: Incubated with Krebs' solution containing nialamide (335μmol l^{-1}) for 60 minutes, followed by nialamide-free Krebs' solution for 15 minutes prior to exposure to NA

Segment 3: Treated as for segment 1, except that 3',4'-dihydroxy-2-methyl-propiophenone (UO521, 111μmol l^{-1}) was present throughout the period of incubation with NA and the subsequent incubation in NA-free Krebs' solution

Segment 4: A combination of 2 and 3, i.e. pretreated with nialamide, followed by UO521 as for segment 3.

Segment 5: Treated as for segment 2 except that cocaine (88μmol l^{-1}) was present throughout the period of incubation with NA and for the preceding five minutes. Cocaine was washed out simultaneously with the NA.

The only modification of the above procedure occurred when denervated arteries were employed. The arteries were denervated by removal of the homolateral superior cervical ganglion at operation one to six weeks previously as described in Chapter 2.

In these experiments three segments of arteries were taken from each of the denervated and contralateral innervated ears and treated in an identical fashion to segments 1, 2 and 3 (above).

(ii) Perfusion Experiments

Denervated ear arteries were isolated and perfused with Krebs' solution. The methods of surgical denervation and of perfusion of the arteries have been described in detail in Chapter 2. In this instance both superior cervical ganglia were removed, i.e. both arteries were denervated during the one operation.

One artery from each animal was treated in vitro with nialamide ($335\mu mol\ l^{-1}$), which was present in both the intraluminal perfusate and extraluminal bathing medium. After one hour the nialamide was removed and perfusion with normal Krebs' solution resumed and continued for a further 15 minutes. The second artery was not treated with nialamide but continually perfused with Krebs' solution during this period. NA ($118\mu mol\ l^{-1}$) was then applied extraluminally to each artery for 15 minutes. Perfusion was stopped during the period of application of NA to spare the arteries from the extremely high perfusion pressure ($>25x10^{3}\ Nm^{-2}$) produced by this concentration of NA. After 15 minutes the NA was washed out and thereafter the extraluminal bathing media were

replaced by NA-free Krebs' solutions every 2 minutes for one hour during which the decline in perfusion pressures, and hence the rates of relaxation of both the untreated and MAO-inhibited arteries were determined.

RESULTS

The time course of release of NA under the various conditions of incubation is shown in Fig. 3.1 for innervated and for denervated arteries. In both types of arteries, in the absence of enzyme inhibition, the amount of NA appearing in the bathing solution was relatively small and sometimes not easily detected by bioassay. Treatment with UO521 did not significantly alter the amount released nor did UO521 itself appear to modify the sensitivity of the bioassay preparation to NA. In contrast, prior treatment with nialamide resulted in a marked increase in the amount of NA released. Treatment with both inhibitors did not significantly increase the release of NA above that seen with nialamide alone. The effect of nialamide was not diminished in cocaine-treated innervated arteries nor in chronically denervated arteries (Fig. 3.1).

In perfused denervated arteries, the constrictor response after washout of the extraluminal NA persisted at high levels (approx. 20×10^3 Nm^{-2}) for several minutes and in untreated arteries subsequently declined steadily, reaching the initial

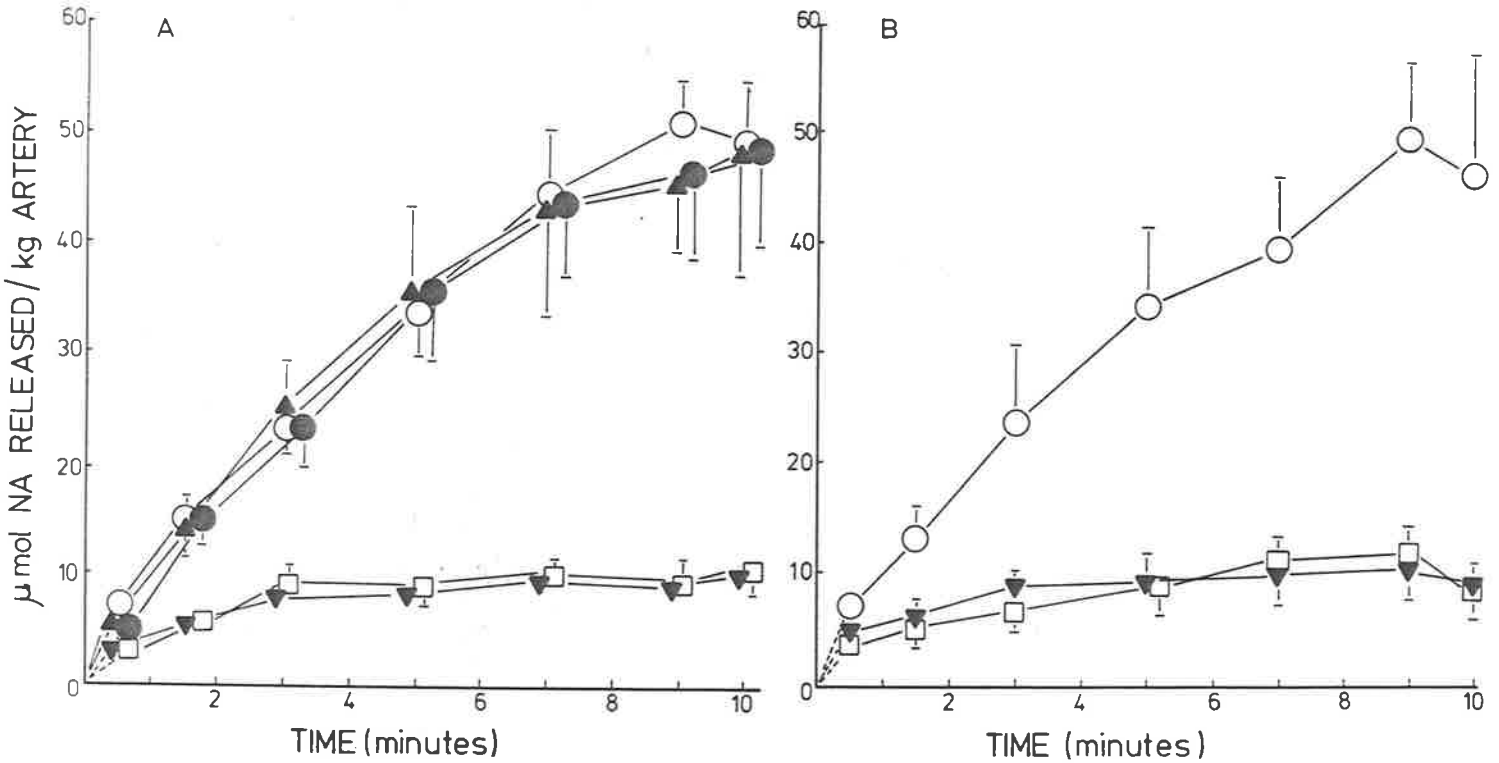


Fig. 3.1 Cumulative efflux of NA into the bathing solution after exposure of the artery to NA (118µmol l^{-1}).
Fig. 3.1A: Normal arteries. Fig. 3.1B: Denervated arteries.
Treatments:

- ▼ nil
- ○ nialamide (335µmol l^{-1})
- □ U0521 (111µmol l^{-1}
- ● nialamide (335µmol l^{-1}) and U0521 (111µmol l^{-1})
- ▲ nialamide (335µmol l^{-1}) and cocaine (88µmol l^{-1})

Standard errors are not shown where these were less than 1.2µmol kg^{-1}. Mean values for 0.5min are approximate since these were usually at the threshold of the bioassay. The number of observations at each point ranged from 6 to 20.

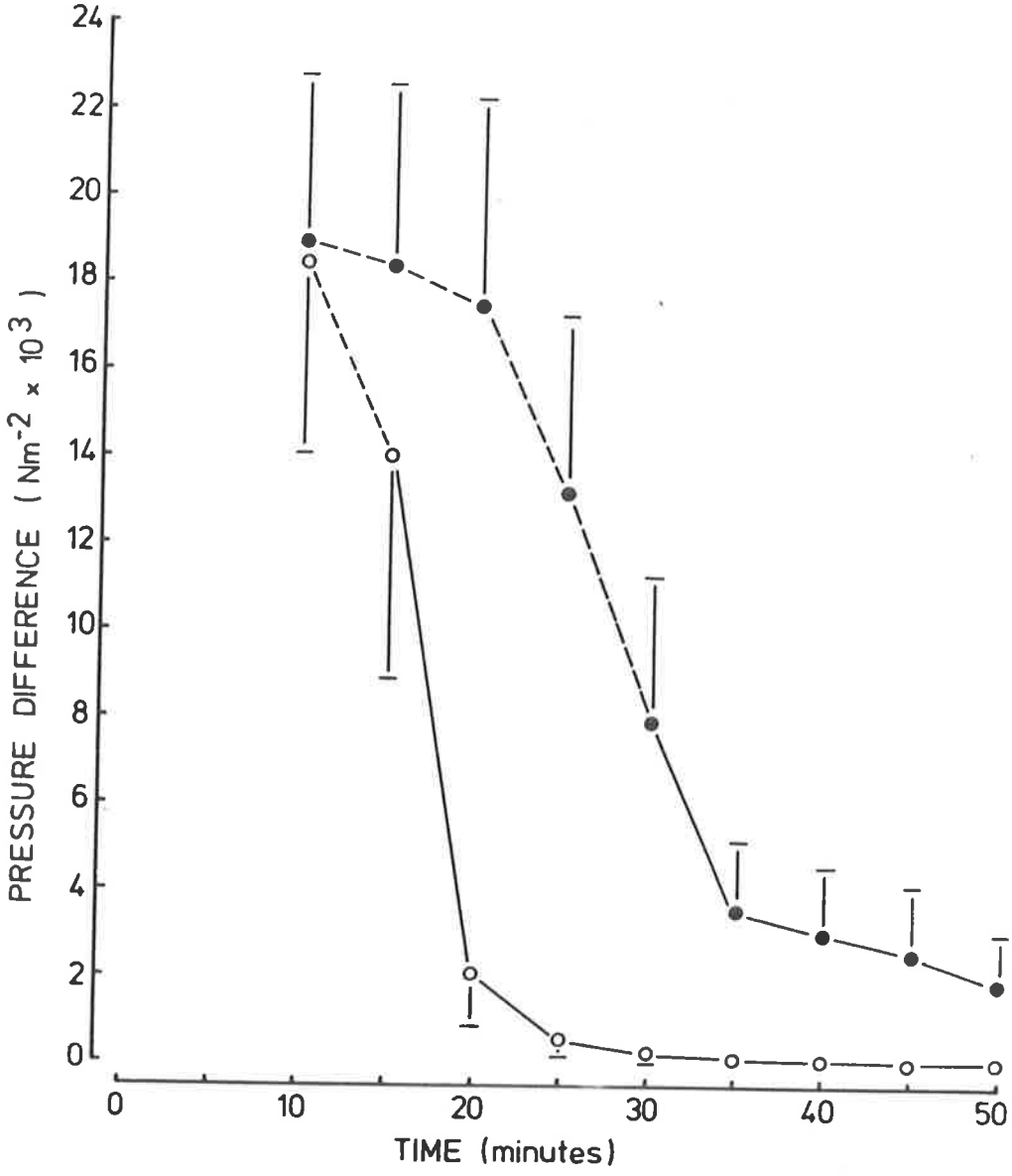


Fig. 3.2 Time courses of recoveries from the constrictor response to extraluminal NA (118μmol l^{-1}) in denervated arteries (o - o) and in nialamide-treated (• - •) denervated arteries. Ordinate: the difference between the actual and the resting perfusion pressure. Abcissa: time after washout of NA. Only pressure differences between 0 and 20 x 10^3 Nm^{-2} were measured. When a pressure difference was greater than 20 x 10^3 Nm^{-2} in one or more of the arteries, the value was taken as 20 x 10^3 Nm^{-2} and the portion of the curve in the corresponding time interval represented by broken lines. Each point represents the mean of 6 observations.

resting level of perfusion pressure (prior to application of NA) within 25 to 35 minutes. In nialamide treated arteries however, the rate of relaxation was markedly slowed and only one of the 6 arteries had fully recovered after 50 minutes (Fig. 3.2).

DISCUSSION

These results indicate (a) that NA which accumulated in the artery wall from high external concentrations was inactivated by MAO and (b) that COMT made no appreciable contribution to this inactivation. The finding that neither cocaine nor chronic denervation significantly reduced the enhanced release in the presence of nialamide excluded the possibility that the effects of nialamide were mediated to any significant extent by inhibition of intraneuronal MAO. The importance of extraneuronal MAO at high concentrations of NA was also shown by the pharmacological experiments. In contrast to the rapid relaxation of denervated arteries exposed to extraluminal NA in a concentration of 3.0μmol l^{-1} (de la Lande and Jellett, 1972), the times required for complete recovery after exposure to higher concentrations of NA (118μmol l^{-1}) were usually of the order of 20 minutes or longer (Fig. 3.2). A similar phenomenon was observed by Avakian and Gillespie (1968) and attributed to smooth muscle uptake of NA at high concentrations and its subsequent efflux to the receptors, thus maintaining a response

for long periods after removal of NA from the perfusion medium. The further increase in the recovery time after MAO inhibition (Fig. 3.2) is consistent with the results of the incubation experiments and suggests that the concentration of NA reaching the receptors after efflux is influenced by extraneuronal deamination. A more remote possibility is that inhibition of MAO resulted in a decrease in the uptake of NA and therefore a higher concentration in the receptor biophase during exposure to NA. This might increase the time required to clear amine from the biophase after removal of the external concentration and hence prolong the response. However, it seems unlikely that this could delay the recovery to the extent observed, particularly since a gradient of concentration from the receptor biophase to the external bathing medium was maintained by replacing the latter every 2 minutes during the recovery period with NA-free Krebs' solution.

Recent biochemical studies have indicated that COMT and not MAO accounts for the major proportion of extraneuronal metabolites in arteries incubated with a concentration of NA 100 times lower than that used in the present study (i.e. 1.18μmol l^{-1}, Head et al, 1975b). The absence of appreciable levels of extraneuronal deaminated metabolites in the latter study accords with the failure of extraneuronal MAO to influence the vasoconstrictor response to NA observed

in a previous pharmacological investigation (de la Lande and Jellett, 1972). Hence the conclusion to be drawn from the present study, and that of Head et al (1975b) is that the relative contributions of MAO and COMT to the inactivation of NA depend on the concentration of NA to which the ear artery is exposed.

The high concentration of NA used here was selected on the basis of histochemical evidence of Avakian and Gillespie (1968) that the smooth muscle cells in the media accumulated NA when the artery was incubated with concentrations of NA exceeding 59μmol l^{-1}. The present results suggest that inactivation by MAO contributed to the high threshold observed by these workers. This proposal is also supported by histochemical evidence of Burnstock et al (1971), published after this investigation was completed. They confirmed the high threshold reported by Avakian and Gillespie (1968), and showed that this was lowered 100-fold (to 0.59μmol l^{-1}) by combined inhibition of MAO and COMT. In spite of the apparently minor role of COMT in the inactivation of the high concentration of NA (Fig. 3.1), it is possible that inhibition of COMT may have contributed to the 100-fold decrease in the threshold for uptake observed by Burnstock et al (1971). This is because inhibition of extraneuronal MAO alone may have decreased the threshold for accumulation of unchanged NA to a level at which inactivation

by COMT predominated.

In studies using the rabbit aortic strip, Kalsner and Nickerson (1969a) concluded that COMT represented the dominant pathway for extraneuronal inactivation of low concentrations of NA ($0.059\mu mol\ l^{-1}$), while MAO was of relatively minor importance. However MAO assumed major importance in strips exposed to higher concentrations of NA ($5.9\mu mol\ l^{-1}$). Kalsner and Nickerson used an indirect method of measuring inactivation, i.e., by measuring the rate of relaxation of the strips in oil following exposure to NA. The rate of relaxation was taken as an index of the elimination of amine from the biophase by uptake and metabolism. Extraneuronal MAO was implicated to explain the slow recovery in oil after MAO inhibition since the effects persisted in strips treated with cocaine. Recently, however, Trendelenburg (1974) showed that prior treatment with cocaine decreased the late phase of slow relaxation after MAO inhibition in aortic strips bathed in aqueous medium. He suggested that the rate of relaxation of the rabbit aortic strip in aqueous medium may be influenced by bidirectional fluxes to and from neuronal and extraneuronal stores adjacent to the receptors. In the experiments of Kalsner and Nickerson (1969a), some filling of neuronal stores may have occurred since cocaine was added only after the steady state response to NA had been established. Although efflux from tissue stores would be less

favoured in strips immersed in oil where diffusion from the biophase is impeded compared with the relatively unimpeded diffusion of NA in strips bathed in aqueous medium, it is conceivable that efflux of NA from neuronal stores may have contributed to the slow relaxation observed after MAO inhibition by Kalsner and Nickerson, who may have therefore overestimated the functional significance of extraneuronal MAO. Despite this, the data of Trendelenburg (1974) confirm that there still remains a significant contribution of extraneuronal MAO.

CHAPTER 4

INFLUENCE OF EXTRANEURONAL UPTAKE ON THE SENSITIVITY OF THE RABBIT EAR ARTERY TO CATECHOLAMINES

INTRODUCTION

Raab (1942) and Raab et al (1950) first showed that cardiovascular responses to NA and adrenaline in humans were enhanced after several days pretreatment with deoxycorticosterone acetate (DOCA), a representative of the naturally occurring mineralocorticoid, deoxycorticosterone. The sensitising action was confirmed by in vivo studies in dogs (Vanatta and Cottle, 1955) and by observations in the rabbit aortic strip (Bohr and Cummings, 1958). However, there was little agreement as to the mechanism underlying the phenomenon. Raab (1952) suggested that potentiation was mediated by altered membrane potential as a consequence of the well-documented action of deoxycorticosterone in elevating intracellular sodium concentration. Bohr and Goulet (1961) showed that adrenaline responses in the rabbit aortic strip were enhanced when the external potassium concentration was doubled, and speculated that deoxycorticosterone exerted similar effects by preventing entry of potassium into the cell and hence reducing the ratio of intracellular to extracellular potassium. Later, however, Bohr (1964) proposed an alternative mechanism, in which deoxycorticosterone facilitated the availability of calcium for excitation-contraction coupling in the rabbit aortic strip. The failure of deoxycorticosterone to potentiate angiotensin responses in the same preparation was then interpreted

by assuming that the magnitude of the angiotensin response was not primarily determined by the process of excitation-contraction coupling but rather by events at the level of the cell membrane. The specificity of the sensitising action of steroids was extended by Besse and Bass (1966) who showed that hydrocortisone potentiated constrictor responses of the rabbit aortic strip to catecholamines and synephrine, but not to other amines such as phenylephrine, metaraminol, serotonin, methoxamine and tyramine, which lacked a catechol nucleus. Specificity in the potentiation of dilator responses was also apparent in the ability of hydrocortisone to enhance isoprenaline β-receptor mediated responses in phenylephrine-contracted strips while having no effect on the relaxation produced by sodium nitrite. The potentiation of isoprenaline responses and the greater effect of hydrocortisone on adrenaline compared with NA distinguished the action of the steroid from that of cocaine which did not potentiate isoprenaline responses and which was equally effective on responses to the latter two amines. Moreover, these authors found no evidence for a relationship between the sensitising action of hydrocortisone and membrane depolarization or availability of extracellular calcium. They therefore proposed that hydrocortisone potentiated responses by inducing conformational changes in the adrenergic receptors, thereby increasing the efficiency of their interaction with phenolic hydroxyl groups of catecholamines. This hypothesis,

however, did not satisfactorily explain the more marked effect of hydrocortisone on adrenaline compared with NA or the potentiation of synephrine. Subsequently Kalsner (1969a,b) found that the synephrine responses were potentiated inconsistently and provided evidence that the sensitising action of a variety of steroids, including deoxycorticosterone and hydrocortisone, was mediated by inhibition of COMT. The evidence was based on the similarities between the effects of a steroid and those of a COMT inhibitor on responses to a number of vasoactive amines in the rabbit aortic strip, and on the failure of either agent to exert these effects in the presence of the other. Kalsner (1969a) confirmed the finding of Besse and Bass (1966) that adrenaline sensitivity was enhanced by hydrocortisone to a greater extent compared with that of NA and explained this in terms of previous evidence (Kalsner and Nickerson, 1969a) that MAO was a more effective alternate pathway for inactivation of NA compared with adrenaline. As an alternative hypothesis, it was proposed that the sensitising action of these steroids might be mediated by preventing access of amines to COMT by impairing amine uptake. Shortly thereafter, Iversen and Salt (1970) investigated the effects of a number of steroids, including deoxycorticosterone, on $uptake_2$ of NA in the rat heart. Although their findings were consistent with the hypothesis favoured by Kalsner (1969a) in so far as the steroids decreased the

formation of NA metabolites, the accumulation of unchanged amine in the heart was also depressed. Furthermore, the accumulation of NA was reduced when metabolism was prevented by combined inhibition of MAO and COMT. Hence these authors concluded that the steroids acted primarily to inhibit $uptake_2$ and hence prevent subsequent metabolism, rather than to inhibit COMT directly. It seemed possible that this was the mechanism underlying the sensitising action of steroids since the pharmacological studies of Kalsner (1969a,b) and of others (Levin and Furchgott, 1970; Kaumann, 1972) showed their actions to be consistent with preventing access of catecholamines to extraneuronal sites of inactivation.

This action of steroids and its physiological implications had not been investigated in muscular arteries. Therefore in the present series of experiments, the effects of DOCA on the sensitivity of the rabbit ear artery to a variety of amines were examined. Its interaction with adrenaline was studied in greatest detail in view of the high affinity of this amine for $uptake_2$ in the rat heart (Iversen, 1967). To assess whether the topographical distribution of nerve and muscle in the artery wall was of importance in the actions of the steroid, the study included its interactions with both intraluminally

and extraluminally applied adrenaline. It had been shown earlier that this distribution was an important factor influencing the differential effects of an inhibitor of neuronal uptake on the sensitivities to extraluminal and intraluminal NA.

METHODS

The methods employed in the experiments described in this chapter are detailed in Chapter 2.

RESULTS

1) COCAINE

The effects of a neuronal uptake inhibitor, cocaine ($2.9\ \mu mol\ l^{-1}$), on responses to extraluminal and intraluminal adrenaline were examined first. It was shown previously that neuronal uptake inhibition exerted a differential effect on extraluminal and intraluminal NA sensitivities, the potentiation of the former being very much greater than that of the latter (de la Lande et al, 1967a). The results for adrenaline are summarised in Fig. 4.1 which also includes data on NA from an earlier study (de la Lande et al, 1970b). Sensitivity to extraluminal adrenaline was potentiated to a significantly greater extent than that to intraluminal adrenaline (4.1 c.f. 1.8). However, the magnitude of the effect on extraluminal adrenaline

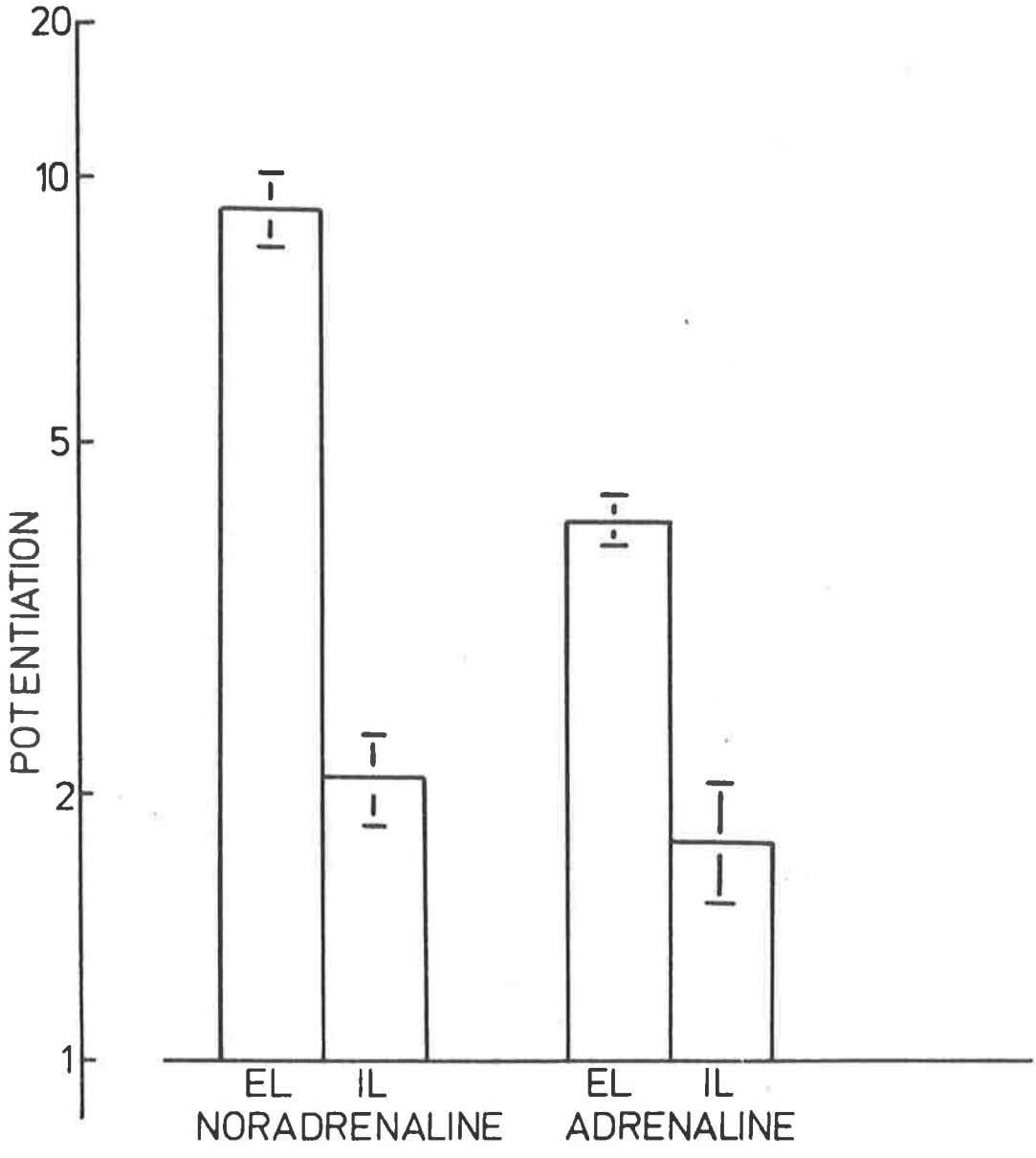


Fig. 4.1 Comparison of the effects of cocaine on the sensitivities to NA and adrenaline. Potentiation refers to the ratio (geom. mean + S.E.) of concentrations of NA and of adrenaline which were equieffective in eliciting increases in the perfusion pressure of 8×10^3 Nm^{-2} in the artery before and during treatment with cocaine (2.9μmol l^{-1}). EL = extraluminal application; IL = intraluminal application. Values for adrenaline shown in Table 4.1a. Data for NA is taken from de la Lande et al (1970b).

was significantly less than that for extraluminal NA. The effects of cocaine on intraluminal adrenaline and NA were both relatively small (1.8 c.f. 2.1), and did not differ significantly. In 4 experiments the concentration of cocaine was increased cumulatively from 2.9 to 29 μmol l^{-1} to determine whether any further sensitisation by cocaine occurred. Although extraluminal adrenaline responses were potentiated a further 1.6-fold, responses to intraluminal adrenaline were also potentiated (1.4-fold) so that the differential effect of cocaine on extraluminal and intraluminal sensitivities was only slightly enhanced at the higher concentration of cocaine. Surprisingly, in another group of arteries in which the sensitivities were estimated in the absence and in the presence of cocaine (29 μ mol l^{-1}), i.e. in which the cocaine concentration was not cumulated, the potentiations of extraluminal and intraluminal adrenaline (4.0 c.f. 1.8) were not significantly different from those previously observed in arteries treated with 2.9 μmol l^{-1} cocaine (Table 4.1a,c,d).

2) DOCA

(i) adrenaline

The effects of DOCA on adrenaline responses under the various conditions discussed below are summarised in Tables 4.2 and 4.3. When applied to the artery in a concentration of

Table 4.1 Effect of cocaine on the sensitivity to adrenaline in the absence and presence of DOCA (27 μmol 1^{-1})

	a	b	c	d
Cocaine (μmol 1^{-1})	2.9	2.9	29	29
Treatment: DOCA	-	+	-	-
Potn. EL adrenaline	4.1(3.8-4.3)	6.6(5.8-7.4)	4.0(3.8-4.3)	1.6(1.5-1.7)
Potn. IL adrenaline	1.8(1.5-2.1)	1.5(1.4-1.6)	1.8(1.6-2.0)	1.4(1.3-1.5)
No. of arteries (EL,IL)	16,8	6,5	17,16	4,4
P	<0.001	<0.001	<0.001	<0.05
EL/IL* Cocaine absent	5.1(4.5-5.8)	4.1(3.6-4.7)	3.1(2.6-3.8)	2.0(1.5-2.6)
EL/IL* Cocaine present	2.5(2.1-3.0)	1.0(1.0-1.1)	1.3(1.2-1.5)	1.7(1.3-2.1)

Footnotes:

(i) Potentiation refers to the ratio (geom. mean ± S.E.) of the concentrations of adrenaline which are equipotent in eliciting increases in the perfusion pressure of 8.0×10^3 Nm^{-2} in the artery before and during treatment with the sensitising agent (See Methods, chapter 2).

(ii) The values in d refer to the effect of increasing the concentration of cocaine cumulatively from 2.9 to 29 μmol 1^{-1}.

(iii) The value P refers to the difference between the potentiations of EL adrenaline and IL adrenaline (paired t-test).

(iv) The difference between the potentiations of EL adrenaline in a and b was also significant (unpaired t-test, $p<0.01$).

(v) *EL/IL refers to the ratios of the concentrations of extraluminal and intraluminal adrenaline (geom. mean ± S.E.) which are equipotent in eliciting increases in the perfusion pressure of 8×10^3 Nm^{-2}.

27 μmol l^{-1}, DOCA itself did not alter the perfusion pressure, but increased the sensitivities to both extraluminal and intraluminal adrenaline. These effects were not associated with changes in the kinetics of the responses, i.e. the times for attaining steady state and for recovery following washout of adrenaline. The magnitude of the potentiation of extraluminal adrenaline (3-fold) was less than that produced by cocaine (4.1-fold), but in contrast to the selective effect of the latter, DOCA caused an equivalent increase in sensitivity to intraluminal adrenaline (Table 4.2a). The increases were manifested both by shifts in the concentration-response curves to the left, and a tendency for the curves to be steeper in the presence of DOCA (Fig. 4.2).

The potentiation of extraluminal adrenaline was significantly enhanced in the presence of a low concentration of cocaine (2.9 μmol l^{-1}, Table 4.2b), and in chronic denervated arteries (Table 4.3). These procedures also enhanced the potentiations of intraluminal adrenaline, but the increases were smaller and were not significant, i.e. the net effect of cocaine and of chronic denervation was to render the potentiating effect of DOCA more selective on extraluminal adrenaline. Although the selective effect of DOCA was also apparent at the higher concentration of cocaine (Table 4.2c), the magnitudes of the potentiations

were significantly lower than those prevailing in chronic denervated arteries and were not, in fact, significantly different from the potentiations observed in untreated arteries.

In the course of the preceding experiments it was established that the potentiating effect of DOCA in cocaine (2.9μmol l^{-1})-treated arteries was dependent on the concentration of DOCA. The threshold for potentiation occurred between 0.27 and 2.7 μmol l^{-1}, and the maximum between 2.7 and 27μmol l^{-1}. The effect of DOCA was not further increased when the concentration was increased to 67μmol l^{-1} (Fig. 4.3).

In all the experiments discussed above the sensitivities were estimated from the magnitude of the steady-state responses to cumulative increases in the concentrations of adrenaline. These responses were usually monophasic, the initial transient phase previously reported (de la Lande et al, 1967a; Bevan et al, 1973a; Steinsland et al, 1973) being rarely observed. In cocaine (2.9μmol l^{-1})-treated arteries in which transient constrictor responses were elicited by intraluminal bolus injections of adrenaline, the potentiation of these responses by DOCA (2.0-fold) was significantly less ($p<0.01$, unpaired t-test) than the potentiation of the steady state response to intraluminal adrenaline (3.6-fold, Table 4.2b).

Table 4.2 Effect of cocaine and of phentolamine on the potentiation by DOCA (27 μmol l^{-1}) of sensitivity to adrenaline

	a	b	c	d
Treatment:				
Cocaine (μmol l^{-1})	-	2.9	29	29
phentolamine (μmol l^{-1})	-	-	-	0.18
Potn. EL adrenaline	3.0(2.8-3.3)	4.7(4.3-5.2)	3.5(3.0-4.0)	1.9(1.6-2.1)
Potn. IL adrenaline	2.7(2.4-3.0)	3.6(3.3-4.0)	2.7(2.4-3.0)	1.5(1.3-1.7)
No. of arteries (EL,IL)	14,13	18,18	10,10	8,8
P	>0.4	<0.01	<0.05	>0.1
EL/IL DOCA absent	5.9(5.1-6.9)	1.7(1.5-1.9)	1.6(1.4-1.8)	1.4(1.2-1.7)
EL/IL DOCA present	5.3(4.7-5.9)	1.3(1.2-1.4)	1.1(1.0-1.2)	1.2(1.0-1.3)

Footnotes:

(i) Potentiations and significance (P) explained in footnotes to Table 4.1.

(ii) Potentiation of EL adrenaline in b was significantly greater than in a (unpaired t-test, $p<0.01$).

(iii) Potentiations of both EL and IL adrenaline in d were significantly less than those in c (unpaired t-test, EL ($p<0.01$), IL ($p<0.001$)).

(iv) Phentolamine decreased the sensitivities to EL and IL adrenaline by factors of 25 and 26 respectively (estimated in terms of dose ratios at a response of $8\times10^{3}Nm^{-2}$).

(v) Concentration ratios (EL/IL) explained in footnote (v) to Table 4.1.

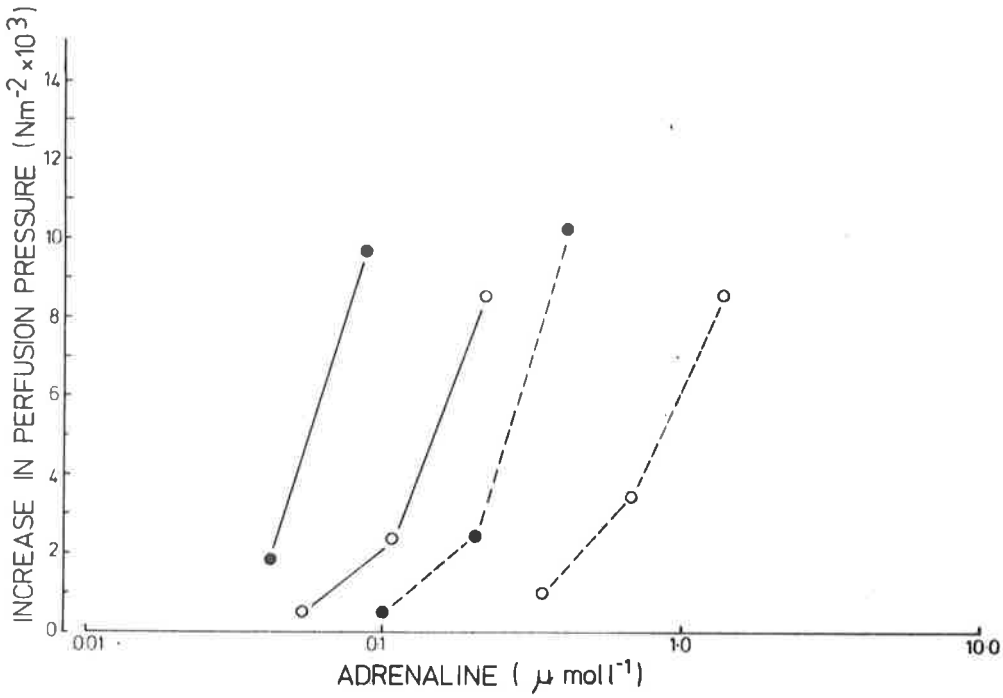


Fig. 4.2 Data from a single experiment showing concentration-response graphs to extraluminal adrenaline (broken lines) and intraluminal adrenaline (solid lines) in the absence (open circles) and in the presence (closed circles) of DOCA (27μmol l^{-1}). DOCA was applied to both the intraluminal perfusate and extraluminal bathing medium. The potentiation by DOCA was estimated as described in Fig. 4.1.

Table 4.3 Effect of chronic denervation on the potentiation by DOCA (27 μmol l^{-1}) of sensitivity to adrenaline

Treatment		Contralateral innervated controls	Chronic denervated arteries
Potn.	EL adrenaline	3.3 (3.1-3.4)	5.8 (5.3-6.2)
	IL adrenaline	3.6 (3.1-4.2)	4.7 (4.3-5.1)
No. of arteries (EL,IL)		7,7	7,7
P		>0.5	<0.05
EL/IL	DOCA absent	5.0 (4.5-5.6)	1.0 (0.7-1.3)
	DOCA present	5.6 (5.0-6.1)	0.8 (0.6-1.0)

Footnotes:

(i) Potentiation and significance (P) explained in footnotes to Table 4.1.

(ii) Potentiation of EL adrenaline but not IL adrenaline was significantly greater in the denervated artery (paired t-test, EL ($p<0.001$); IL ($p>0.1$)).

(iii) Concentration ratios (EL/IL) explained in footnote (v) to Table 4.1.

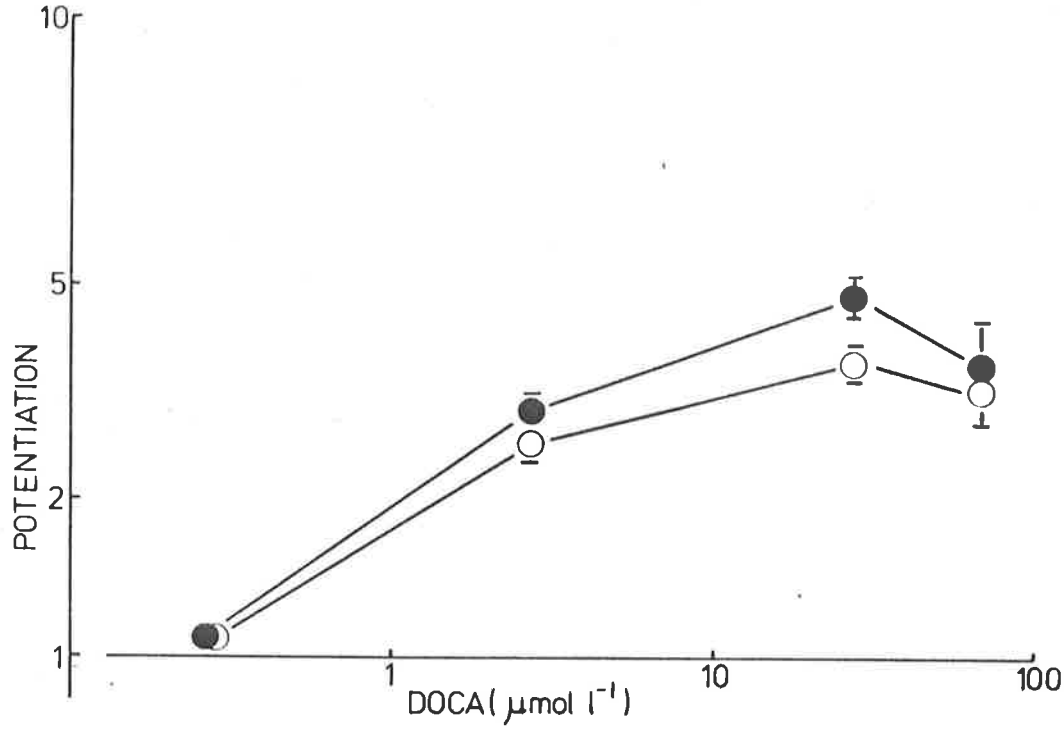


Fig. 4.3 The relationship between the concentration of DOCA and its potentiating effect on adrenaline sensitivity in arteries treated with cocaine (2.9μmol 1^{-1}). Ordinate: potentiation (defined in Fig. 4.1) of extraluminal (•) and intraluminal (o) adrenaline. Each point represents the geometric mean of between 6 and 18 observations. Abcissa: concentration of DOCA (μmol 1^{-1}) in the intraluminal perfusate and extraluminal bathing fluid.

(ii) noradrenaline

In view of the evidence of Iversen (1967) that adrenaline had a higher affinity than NA for uptake$_2$ in the rat heart, it was of interest to determine whether the same order of sensitisation by DOCA occurred in the present experiments. As indicated in Table 4.4a, DOCA increased the sensitivities to both extraluminal and intraluminal NA, but the magnitudes of the potentiations (1.1 and 1.2-fold, respectively) were small compared with those of adrenaline, and were demonstrated unequivocally only by an incremental effect of DOCA (see Methods, Chapter 2). As was the case with adrenaline, the potentiating effect of DOCA on the response to extraluminal NA was significantly increased by cocaine only when the latter was present in a low concentration (2.9μmol l^{-1}) (Table 4.4).

3) NORMETANEPHRINE

It was of interest to determine whether another inhibitor of extraneuronal uptake (normetanephrine; Iversen, 1967) exerted effects which resembled those of DOCA on the sensitivity to adrenaline and NA. As shown in Table 4.5, normetanephrine increased the sensitivities to both extraluminal and intraluminal adrenaline. Sensitivities to NA were also increased, but to a lesser extent. These effects were qualitatively and quantitatively similar to those of DOCA (2.7μmol l^{-1}) (See Table 4.4c

and Fig. 4.3). Furthermore, in arteries treated with DOCA ($27\mu mol\ l^{-1}$), normetanephrine failed to appreciably alter the sensitivities to extraluminal and intraluminal adrenaline (Table 4.5b). It should be noted that normetanephrine, unlike DOCA, is constrictor on the ear artery (de la Lande and Campbell, private communication). For this reason, concentrations of normetanephrine ($1.09\text{-}2.73\mu mol\ l^{-1}$) were selected for each artery to be 50% of that which caused a detectable increase in the perfusion pressure.

4) DOCA and PROPRANOLOL

In view of the extensive use of adrenaline in the preceding experiments, it seemed worthwhile to test whether the steady state constrictor responses to this agent were modified by its concomitant effects on β-receptors. That this was not the case was shown in cocaine-treated arteries in which the sensitivities to extraluminal and intraluminal adrenaline were compared in the presence and absence of propranolol on each artery. Propranolol, at a concentration of either 0.39 or $3.9\mu mol\ l^{-1}$, did not significantly alter these sensitivities (Table 4.6a, c). Propranolol also failed to elicit an incremental response when added during the steady state response to extraluminally applied adrenaline. In other experiments, it was shown that propranolol in the above concentrations significantly reduced the sensitivity

to the dilator effects of isoprenaline in stimulated arteries or in arteries in which the tone had been raised by the extraluminal application of adrenaline. The effect of propranolol on the potentiating action of DOCA in cocaine-treated arteries was also studied. At both concentrations tested propranolol did not significantly alter the potentiation of either extraluminal or intraluminal adrenaline responses by DOCA (Table 4.6b,d).

5) DOCA and ETHANOL

In all experiments, DOCA was prepared as a stock solution in ethanol such that the concentration of ethanol in the Krebs' perfusate and extraluminal bathing medium did not exceed 7.0 mmol l^{-1}. At this concentration, ethanol was without effect on either extraluminal or intraluminal adrenaline responses (Table 4.7a). Furthermore, in 6 cocaine-treated arteries, the potentiating effect of DOCA was determined by first obtaining the sensitivities to adrenaline in ethanol-perfused arteries, then replacing ethanol with a solution of DOCA (27μmol l^{-1}) containing the same concentration of ethanol. The potentiations of extraluminal and intraluminal adrenaline were not significantly different from those determined when ethanol was not present prior to adding the latter solution of DOCA (Table 4.7b).

Table 4.4 Effect of DOCA on the sensitivity to noradrenaline

	a	b	c
DOCA (μmol l^{-1})	27	27	2.7
Treatment: Cocaine (μmol l^{-1})	-	29	2.9
Potn. EL NA	1.1 (1.0-1.2)	1.2 (1.1-1.4)	2.0 (1.9-2.2)
Potn. IL NA	1.2 (1.0-1.4)	-	1.4 (1.2-1.6)
No. of arteries	7,6	7	7,6
P	>0.6	not applicable	>0.05
EL/IL DOCA absent	6.5 (5.0-8.3)	not applicable	1.5 (1.3-1.7)
EL/IL DOCA present	6.7 (4.8-9.3)		1.1 (0.9-1.2)

Footnotes:

(i) Potentiations and significance (P) explained in footnotes to Table 4.1.

(ii) Potentiations of adrenaline sensitivity with treatments identical to a and b are shown in Table 4.2 a and 4.2 c respectively.

(iii) Concentration ratios (EL/IL) explained in footnote (v) to Table 4.1.

(iv) Potentiation of EL NA in b was not significantly different from that in a (unpaired t-test, $p>0.6$), but the potentiation of EL NA in c was significantly greater than that in a (unpaired t-test, $p<0.01$).

Table 4.5 Effect of normetanephrine on the sensitivity to adrenaline and noradrenaline.

	a	b
Treatment:		
Cocaine (μmol l^{-1})	2.9	2.9
DOCA (μmol l^{-1})	-	27
Potn. EL adrenaline	3.1 (3.0-3.1)	1.1 (1.1-1.2)
Potn. IL adrenaline	2.1 (1.6-2.9)	1.1 (1.1-1.2)
Potn. EL NA	1.8 (1.6-2.0)	-
Potn. IL NA	1.5 (1.3-1.8)	-
No. of arteries (EL,IL)	A (4,4); NA (5,5)	4,4

Footnotes:

(i) Potentiations measured as in Table 4.1

(ii) Concentration of normetanephrine (1.09-2.73 μmol l^{-1}) was selected in each artery to be 50% of that which caused a detectable increase in perfusion pressure.

Table 4.6 Influence of propranolol on sensitivity to adrenaline and on the potentiating action of DOCA in arteries treated with cocaine (2.9 μmol l^{-1}).

	a	b	c	d
Treatment:				
Propranolol (μmol l^{-1})	0.39	0.39	3.9	3.9
DOCA (μmol l^{-1})	-	27	-	27
Potn. EL adrenaline	1.1(1.0-1.2)	6.3(5.7-7.1)	1.1(1.1-1.2)	4.2(3.7-4.8)
Potn. IL adrenaline	1.0(1.0-1.1)	3.0(2.6-3.5)	1.1(1.1-1.2)	3.3(3.0-3.5)
No. of arteries (EL,IL)	4,4	4,4	10,9	10,10
P	-	<0.02	-	<0.01
EL/IL DOCA absent	-	2.6(2.4-2.8)	-	1.4(1.2-1.7)
EL/IL DOCA present	-	1.2(1.1-1.3)	-	1.1(0.9-1.3)

Footnotes:

(i) Potentiation and significance (P) explained in footnotes to Table 4.1.

(ii) Values in a and c indicate that propranolol did not significantly alter the sensitivities to EL and IL adrenaline.

(iii) P value for comparisons of potentiating effects of DOCA in b and d were EL v EL, $p>0.05$; IL v IL $p>0.5$ (unpaired t-tests).

(iv) Potentiations in (b) and (d) did not differ significantly (unpaired t-tests) from those in the absence of propranolol (see Table 4.2 b).

(v) Concentration ratios (EL/IL) explained in footnote (v) to Table 4.1.

Table 4.7 Influence of ethanol on the sensitivity to adrenaline and on the potentiating action of DOCA in arteries treated with cocaine (2.9 μmol l^{-1}).

	a	b
Treatment:		
Ethanol (mmol l^{-1})	7.0	7.0
DOCA (μmol l^{-1})	-	27
Potn. EL adrenaline	1.0 (1.0-1.0)	5.1 (4.2-6.1)
Potn. IL adrenaline	1.1 (1.0-1.1)	3.3 (2.7-4.1)
No. of arteries	7,6	6,6
P	Not applicable	<0.05

Footnotes:

(i) Potentiations and significance (P) explained in footnote to Table 4.1.

(ii) Values in a indicate that ethanol alone did not significantly alter the sensitivities to EL and IL adrenaline.

(iii) In b, arteries were pretreated with ethanol for 30 minutes. The ethanol was then replaced by DOCA in ethanol such that the concentration of the latter was not altered. The potentiations of adrenaline sensitivities by DOCA were not significantly different from those observed in the absence of ethanol pretreatment (see Table 4.2 b; unpaired t-tests; EL v EL, $p>0.7$; IL v IL, $p>0.7$).

RELATIONSHIP BETWEEN CONCENTRATION AND POTENTIATING EFFECT OF DOCA

To test for a possible relationship between the concentration of adrenaline and the potentiation by DOCA, the effect of DOCA was examined in arteries treated with phentolamine ($0.18\mu mol\ l^{-1}$) to enable high concentrations of adrenaline to be employed. As shown in Table 4.2d the potentiation by DOCA was markedly reduced under conditions where equipotent concentrations of adrenaline had been increased by phentolamine, suggesting that the influence of the DOCA-sensitive mechanism diminished with increasing concentrations of adrenaline. In Figure 4.4 is shown a plot of the magnitude of the potentiating effect of DOCA as a function of the concentration of adrenaline in the absence of DOCA, under the variety of experimental conditions discussed above. A significant negative correlation between potentiation and concentration was apparent for both extraluminal ($r=-0.8320$, $p<0.02$) and intraluminal ($r=-0.8360$, $p<0.01$) applications of adrenaline. However, within the much smaller ranges of concentrations which occurred within each of the experimental conditions summarised in Table 4.8, significant negative correlations between the concentrations of adrenaline and the potentiating effects of DOCA were not apparent. In fact, at low concentrations of adrenaline in arteries treated with cocaine ($2.9\mu mol\ l^{-1}$), a highly significant correlation in the opposite (positive) direction was observed, i.e. the potentiating

Table 4.8 Relationship between the potentiating effect of DOCA and the concentration of adrenaline

Treatment:	NIL		Chronic Denervation		Contra-lateral Innervated Controls		Cocaine (2.9 μmol l^{-1})		Cocaine (29 μmol l^{-1})		Cocaine (29 μmol l^{-1}) + Phentolamine (0.18 μmol l^{-1})		Cocaine (2.9 μmol l^{-1}) + Propranolol (0.39 μmol l^{-1})		Cocaine (2.9 μmol l^{-1}) + Propranolol (3.9 μmol l^{-1})	
Concentration of adrenaline* (μmol l^{-1})	1.01 ±0.21	0.18 ±0.04	0.09 ±0.02	0.11 ±0.04	0.68 ±0.26	0.19 ±0.05	0.13 ±0.02	0.08 ±0.01	0.07 ±0.01	0.05 ±0.01	3.99 ±0.91	2.77 ±0.63	0.15 ±0.03	0.06 ±0.01	0.20 ±0.02	0.16 ±0.03
Potn. by DOCA (27 μmol l^{-1})	3.0	2.7	5.8	4.7	3.3	3.6	4.7	3.6	3.5	2.7	1.9	1.5	6.3	3.0	4.2	3.3
No. of arteries	14	13	7	7	7	7	18	18	10	9	8	8	4	4	10	10
r	-0.259	-0.063	0.223	0.485	0.722	0.260	0.512	0.743	0.429	0.389	-0.319	-0.473	0.404	0.888	0.052	-0.262
p	>0.1	>0.1	>0.1	>0.1	>0.05	>0.1	<0.05	<0.001	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1

Footnotes:

(i) * refers to the mean concentration of adrenaline required to elicit an increase in the perfusion pressure of $8.0 \times 10^{3} Nm^{-2}$ prior to application of DOCA. Data on the left-hand side of each column refer to EL applications of adrenaline; those on the right-hand sides to IL applications.

(ii) Potentiation measured as in Table 4.1.

(iii) r is the coefficient of linear correlation between potentiation and concentration and P is the corresponding level of significance. The significant correlations in the 4th column refer to increasing potentiations with increasing concentrations of adrenaline.

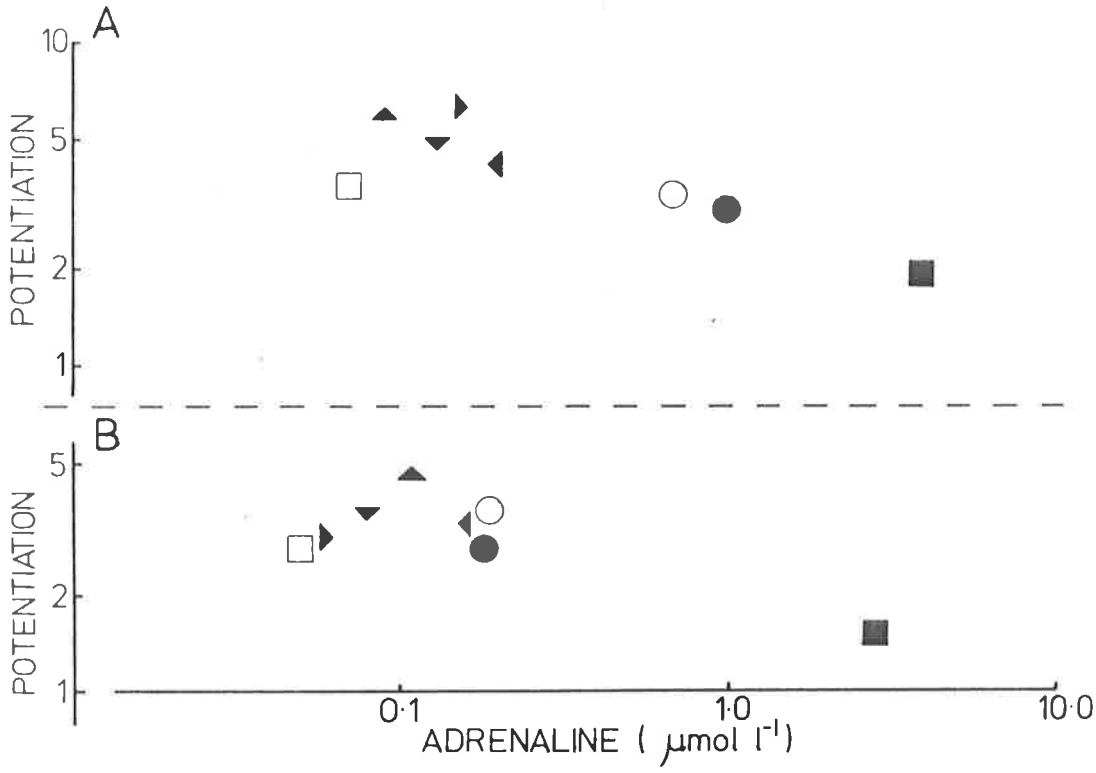


Fig. 4.4 Relationship between the potentiating effect of DOCA and the concentration of adrenaline. Ordinates: potentiation (defined in Fig. 4.1) of adrenaline sensitivity by DOCA ($27\mu mol\ l^{-1}$). Upper panel (A) refers to extraluminal adrenaline; lower panel (B) refers to intraluminal adrenaline. Mean values (± S.E.) are shown in the tables indicated below. Abcissa: Concentrations of adrenaline required to elicit increases in the perfusion pressure of $8 \times 10^3\ Nm^{-2}$ in the absence of DOCA. Mean values (± S.E.) are shown in Table 4.8.

Treatments	Table
● : Nil	4.2a
▼ : Cocaine ($2.9\mu mol\ l^{-1}$)	4.2b
▲ : Denervation	4.3
○ : Contralateral innervated controls	
□ : Cocaine ($29\mu mol\ l^{-1}$)	4.2c
■ : Cocaine ($29\mu mol\ l^{-1}$) + Phentolamine ($0.18\mu mol\ l^{-1}$)	4.2d
▶ : Cocaine ($2.9\mu mol\ l^{-1}$) + Propranolol ($0.39\mu mol\ l^{-1}$)	4.6b
◀ : Cocaine ($2.9\mu mol\ l^{-1}$) + Propranolol ($3.9\mu mol\ l^{-1}$)	4.6d

effect of DOCA increased with increasing concentrations of adrenaline (Fig. 4.5).

In many of the preceding experiments, DOCA ($27\mu mol\ l^{-1}$) was first applied to the artery by perfusing it intraluminally after the response to extraluminal adrenaline had reached steady state. This procedure permitted the kinetics of the action of DOCA to be determined. The onset proved to be rapid, commencing within 30 seconds after contact of DOCA with the artery and the new steady state level in the presence of DOCA was attained within a further 4-10 minutes (Fig. 4.6). In contrast, after washout of DOCA following periods of exposure for from 1 to 3 hours, the offset was extremely slow so that even after periods of two hours, most arteries had not returned to the levels of sensitivity which prevailed prior to application of DOCA. However, in many of the arteries exposed to lower concentrations of DOCA ($2.7\mu mol\ l^{-1}$), the potentiating effect was completely reversible after washout.

EFFECT OF DOCA ON THE MAXIMUM RESPONSE TO ADRENALINE

One of the problems of the perfused segment preparation is that it is difficult to achieve a well defined maximum steady state response to NA (de la Lande, 1975). The same difficulty was encountered with adrenaline, i.e., as the concentration of adrenaline was progressively increased to levels producing

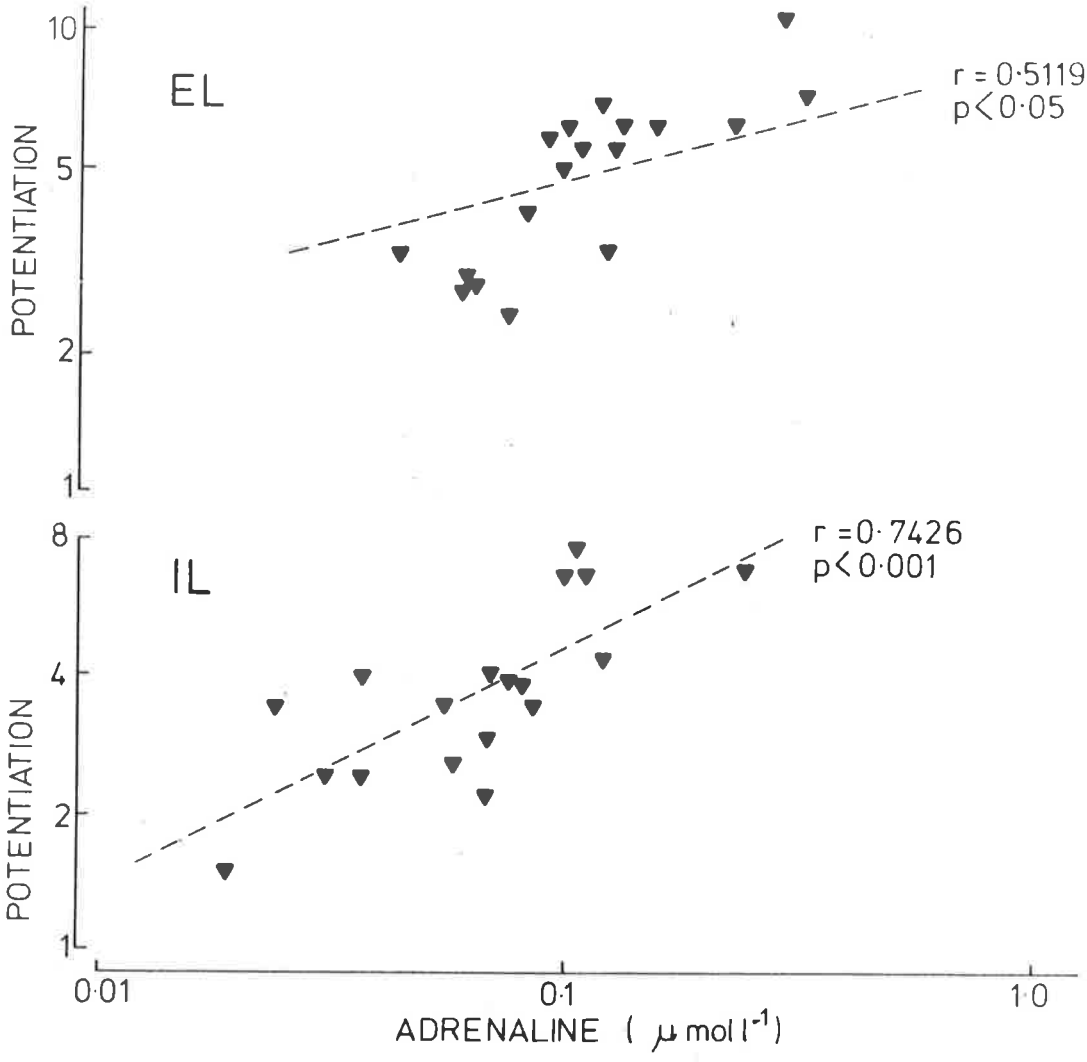


Fig. 4.5 Relationship between the potentiating effect of DOCA and the concentration of adrenaline in arteries treated with cocaine ($2.9\mu mol\ l^{-1}$). Ordinates: potentiation (defined in Fig. 4.1) of the sensitivity to extraluminal (EL) and intraluminal (IL) adrenaline by DOCA ($27\mu mol\ l^{-1}$). Abcissa: Concentrations of adrenaline which were equieffective in eliciting increases in the perfusion pressure of $8 \times 10^3\ Nm^{-2}$ in the absence of DOCA. r refers to the coefficient of linear correlation and p to the corresponding level of significance.

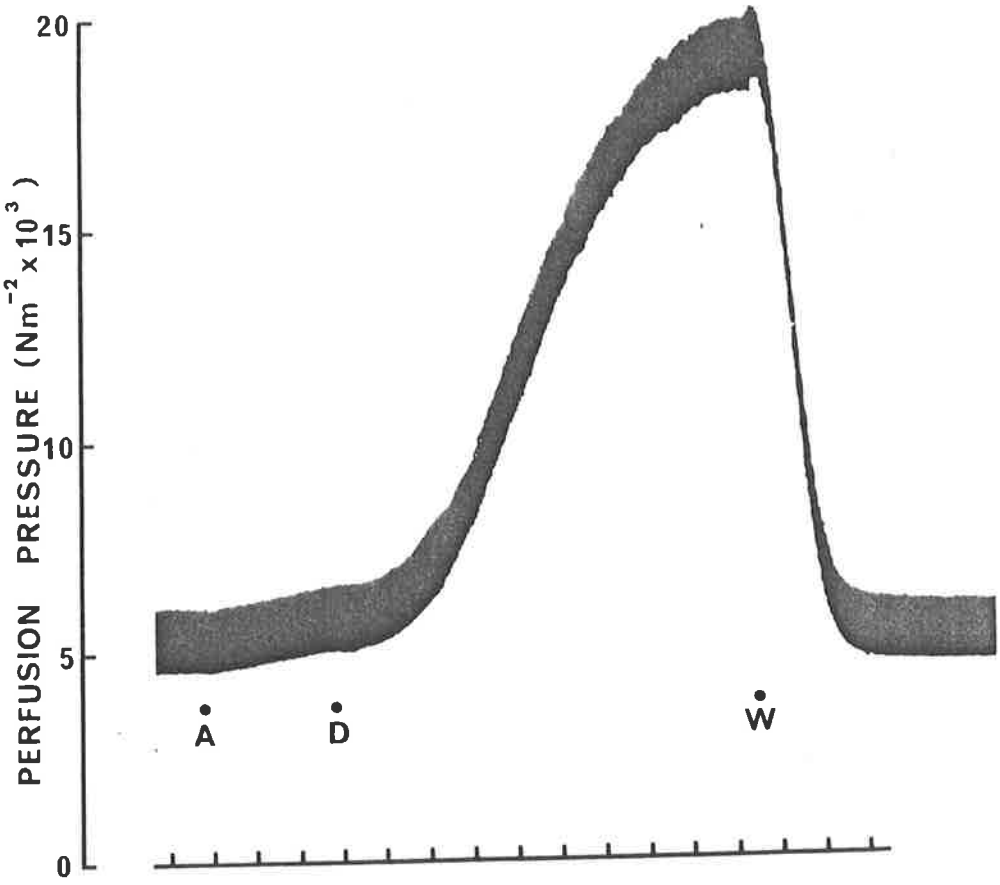


Fig. 4.6 Perfused ear artery illustrating response to extraluminal application of adrenaline (0.017μmol l^{-1}) at A in the presence of cocaine (29μmol l^{-1}). DOCA (27μmol l^{-1}), applied to the intraluminal perfusate, reached the artery at D (calculated from fluid space of apparatus and from flow rate). Adrenaline was washed out (W) when the new steady state response had been reached. Time scale in minutes.

large but still sub-maximal steady state responses of $2.0x10^{4}Nm^{-2}$ or greater, the perfusion pressure declined abruptly and subsequently fluctuated erratically. Although estimates were made in many experiments of the peak perfusion pressures elicited by further increases in the concentration of adrenaline, it was apparent that this did not provide a reliable estimate of the true maximum response. However, well defined and sustained maximum responses were readily elicited in helical strip preparations of the artery (Fig. 4.7b). In the strip preparations, DOCA proved to be without significant effect on the maximum response to adrenaline. The potentiating effect of DOCA estimated at the ED_{50} concentration of adrenaline was 2.5-fold, which was somewhat less than that estimated at the $8x10^{3}Nm^{-2}$ level of response to intraluminal adrenaline in the perfused segment. At the ED_{10} to ED_{20} levels, where concentrations of adrenaline (0.05-0.10μmol l^{-1}) corresponded to the mean concentration of intraluminal adrenaline required to elicit an increase in perfusion pressure of $8x10^{3}$ Nm^{-2} in segments, the potentiating effect of DOCA was also less than that in segments.

THE SLOPES OF THE CONCENTRATION-RESPONSE CURVES

The slopes of the concentration-response curves to adrenaline in the perfused segment were measured in a manner similar to

that described by Langer and Trendelenburg (1969). Concentrations of adrenaline required to produce increases in the perfusion pressure of 5.3×10^3 Nm^{-2} ($[A]_{5.3}$) and 9.3×10^3 Nm^{-2} ($[A]_{9.3}$) were determined and the slopes calculated as:

$$\frac{9.3 - 5.3}{\log [A]_{9.3} - \log [A]_{5.3}}$$

In untreated arteries the slopes of concentration-response curves to extraluminal and to intraluminal adrenaline did not differ significantly and were also unaltered by treatment with cocaine at a concentration (29μmol l^{-1}) which selectively potentiated extraluminal responses. DOCA caused increases in the slopes of the concentration-response curves to both extraluminal and intraluminal adrenaline. However, the increases were more marked for extraluminal adrenaline so that, in the presence of DOCA, concentration-response curves to extraluminal adrenaline were steeper than those to intraluminal adrenaline (Fig. 4.7a). The only exception to this pattern occurred in arteries in which the sensitivity to adrenaline had been reduced by treatment with phentolamine. Under these conditions the slopes of the curves were not significantly increased and in fact those to intraluminal adrenaline tended to be less steep in the presence of DOCA. It should be noted that these measurements reflect changes in slopes

over a narrow range of responses (Fig. 4.7a). The effect of DOCA on the slope of the complete concentration-response curve to adrenaline was revealed in the helical strip preparation (Fig. 4.7b). Although DOCA caused an essentially parallel shift to the left in the lower region, the predominant effect was to decrease the slope of the curve.

OTHER CONSTRICTOR AGENTS AND STIMULI

DOCA also potentiated the responses to nerve stimulation (both brief and sustained applications of pulses), to histamine (steady state responses to extraluminal applications and transient responses to intraluminal injections), to serotonin and to methoxamine (steady state responses to extraluminal applications). Of the latter three agents, histamine was potentiated to the greatest extent(2.2-fold for steady state and 3.8-fold for transient responses), while the potentiation of methoxamine was extremely small (<1.05) and was detected only by a small incremental effect of DOCA on the steady state response in 4 of 6 arteries (Table 4.9).

The effect of DOCA on transient responses to brief applications of pulses was reversed within 15 minutes after washout of DOCA when the latter had been applied for periods of 6 to 10 minutes. Although this observation is apparently at variance with the irreversibility of the sensitising action of DOCA on steady state

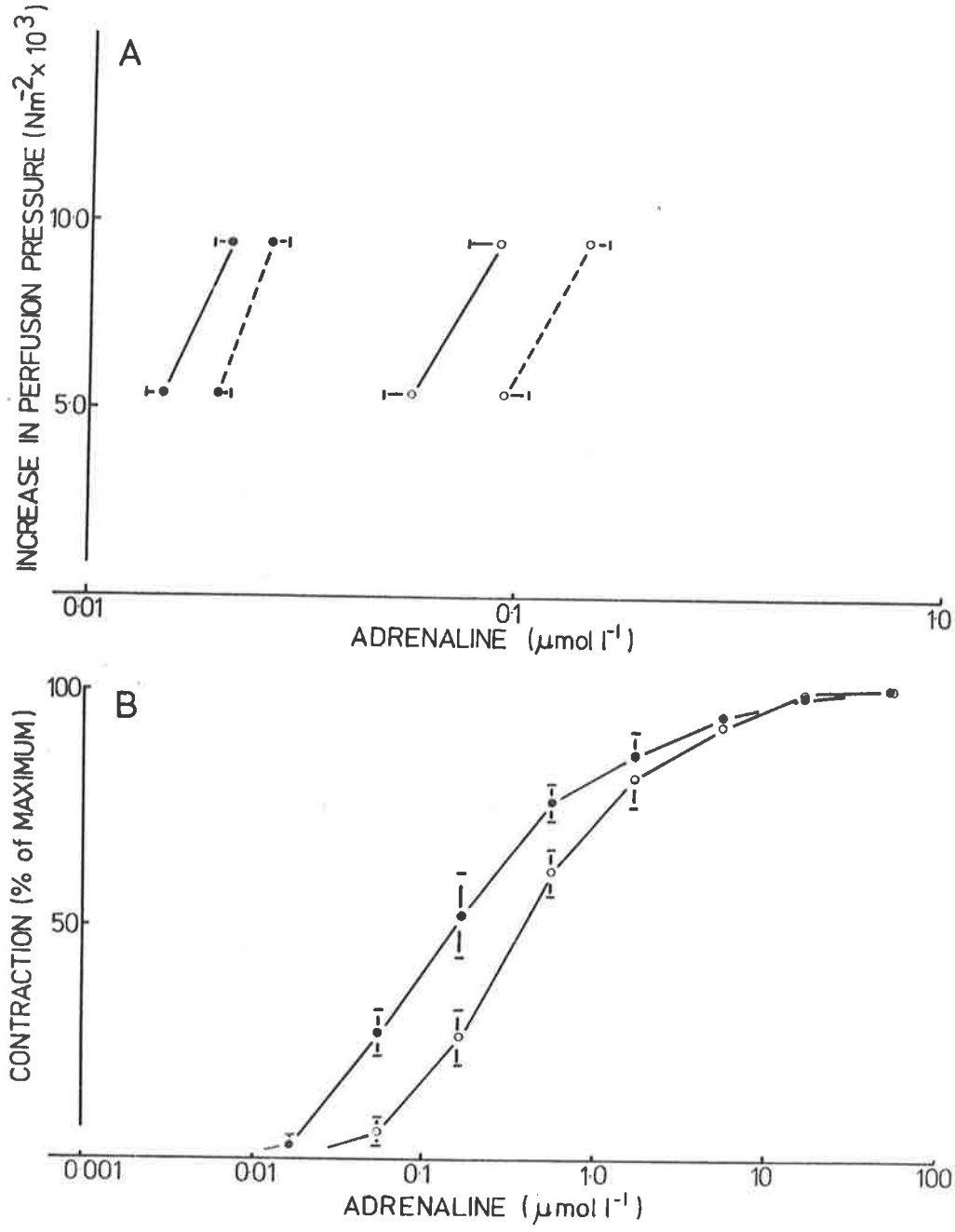

A
INCREASE IN PERFUSION PRESSURE ($Nm^{-2} \times 10^3$)
10·0
5·0
0·01
0·1
1·0
ADRENALINE (μmol l^{-1})
B
CONTRACTION (% of MAXIMUM)
100
50
0·001
0·01
0·1
1·0
10
100
ADRENALINE (μmol l^{-1})

Table 4.9 Effect of DOCA (27 μmol l^{-1}) on constrictor responses to methoxamine, sympathetic nerve stimulation, serotonin and histamine.

	Methoxamine	Sympathetic nerve stimulation		Serotonin	Histamine	
	a	b	c	d	e	f
Treatment: Cocaine μmol l^{-1}	-	2.9	2.9	-	-	2.9
Potn.	1.0 (1.0-1.0)	1.3 (1.3-1.3)	1.3 (1.2-1.3)	1.4 (1.2-1.5)	2.2 (2.0-2.4)	3.8 (3.4-4.3)
No. of arteries	6	2	9	4	7	7

Footnotes:

(i) Potentiations measured as in Table 4.1, or in b and c, in terms of ratios of equieffective frequencies of stimulation.

(ii) Responses were either *steady state* (extraluminal applications in a, d, e, or sustained applications of pulses in b) or *transient* (brief (10 sec.) applications of pulses in c or intraluminal bolus injections in f).

responses to extraluminal and intraluminal adrenaline, it should be noted that in the latter case, arteries were exposed to DOCA for periods of at least 60 minutes.

DOCA AND ISOPRENALINE

Dilator responses to isoprenaline were measured as described in Chapter 2. The ED_{50} and ID_{50} concentrations of isoprenaline were 0.16±0.03 and 0.23±0.04μmol l^{-1} respectively, and the maximum decrease in responses to stimulation ranged from 65% to 100%, i.e. to complete abolition of this response. Even in maximally effective concentrations (0.81-5.3μmol l^{-1}), isoprenaline did not alter the resting perfusion pressure. That the dilator effect of isoprenaline was mediated by stimulation of beta receptors was indicated by the ability of propranolol (0.39 and 3.9μmol l^{-1}) to decrease the effect by factors of 2 to 20 in each case. DOCA (27μmol l^{-1}) potentiated the dilator responses to isoprenaline by factors of 2.5 and 2.9 (estimated at the ID_{50} and ED_{50} levels, respectively; Table 4.10b). Ethanol (7.0 mmol l^{-1}) did not significantly alter the responses to isoprenaline (Table 4.10a). However, in arteries in which the control sensitivity to isoprenaline was examined first in the presence of ethanol, the subsequent potentiating effect of DOCA tended to be reduced (1.6 c.f. 2.5, Table 4.10b,c), although this decrease was not statistically significant. In contrast to its potentiating effect on dilator

Table 4.10 Effect of DOCA on isoprenaline dilator responses in arteries treated with cocaine (2.9 μmol l^{-1})

	a	b	c
Treatment:			
Ethanol (mmol l^{-1})	7.0	7.0	7.0
DOCA (μmol l^{-1})	-	27	27
Potn. ED_{50}	1.0 (1.0-1.0)	2.9 (2.4-3.4)	1.6 (1.3-1.9)
Potn. ID_{50}	1.0 (1.0-1.0)	2.5 (2.0-3.1)	1.6 (1.3-1.9)
Maximum response	91±6	83±6	90±6
No. of arteries	4	6	4

Footnotes:

(i) Mean ED_{50} and ID_{50} concentrations of isoprenaline were 0.16±0.03 and 0.23±0.04 μmol l-1 respectively.

(ii) Potentiation refers to the ratio of the ED_{50} (or ID_{50}) concentrations of isoprenaline in the absence and presence of DOCA (in b and c) and in the absence and presence of ethanol (in a).

(iii) In c, arteries were pretreated with ethanol for 30 minutes. The ethanol was then replaced by DOCA in ethanol such that the concentration of the latter was not altered.

(iv) The maximum responses (% decreases in the heights of the constrictor responses to nerve stimulation) in a, b and c did not differ significantly (unpaired t-tests).

Table 4.11 Effect of DOCA (27 μmol l^{-1}) on isoprenaline constrictor responses.

	a	b	c	d
Treatment:				
DOCA	-	+	+	+
Ethanol (mmol l^{-1})	7.0	7.0	7.0	7.0
Cocaine (μmol l^{-1})	2.9	2.9	29	2.9
Propranolol (μmol l^{-1})	-	-	-	3.9
Potn. of EL ISO	1.0(1.0-1.0)	0.8(0.7-0.9)	1.0(1.0-1.0)	0.7(0.6-0.8)
No. of arteries	2	3	3	3

Footnotes:

(i) Potentiation measured as in Table 4.1; values in b and d thus indicate a decrease in the sensitivity to isoprenaline after DOCA.

(ii) The value in a refers to the ratio of sensitivities to isoprenaline in the absence and in the presence of ethanol.

responses to isoprenaline, DOCA did not potentiate, but rather tended to depress constrictor responses elicited by higher concentrations (>6.1μmol l^{-1}) of extraluminally applied isoprenaline (Table 4.11b,c). Ethanol alone did not alter the constrictor response. In case potentiation of beta-receptor sensitivity had in some way masked a potentiating effect on the constrictor response, the influence of DOCA was also examined in arteries treated with propranolol (3.9μmol l^{-1}) to diminish beta-receptor mediated effects. However, DOCA still failed to enhance the constrictor responses, and, in fact, as was observed in the absence of propranolol, the sensitivity tended to decline after the application of DOCA (Table 4.11d).

DISCUSSION

The action of adrenaline on this artery was qualitatively similar to that of NA (de la Lande et al, 1967a) in that it was much less potent by the extraluminal than by the intraluminal route of application, and the extraluminal sensitivity was selectively enhanced by chronic denervation and by cocaine. The net effect was that the sensitivity to extraluminal adrenaline approached that to intraluminal adrenaline. It is significant that the major quantitative differences were that the ratio of intraluminal to extraluminal sensitivities and the potentiating

effect of chronic denervation and of cocaine on extraluminal sensitivities were significantly less for adrenaline compared with NA. These differences are entirely consistent with the concept that uptake by the sympathetic nerve terminals is responsible for the low sensitivities to the extraluminally applied amines, if it is assumed that the uptake of adrenaline by the nerves is less than that of NA. Although not tested directly on this preparation, this assumption is consistent with the evidence of Iversen (1967) that the affinity of adrenaline for $uptake_1$ in the rat heart is less than that of NA.

The data also indicates that the influence of neuronal uptake on intraluminal adrenaline sensitivity is minor as was shown in earlier studies for NA (de la Lande et al, 1967a). It is of interest that the slopes of the concentration-response curves for extraluminal and intraluminal adrenaline were approximately parallel. This is also consistent with the concept that neuronal uptake is largely responsible for the lower sensitivity to extraluminal compared with intraluminal adrenaline if it is assumed that the proportional uptake of extraluminal and intraluminal adrenaline each remain constant over the concentration ranges used to establish the respective curves. Langer and Trendelenburg (1969) have provided theoretical and experimental evidence for this assumption, using, however, amines of differing

potencies rather than using the same amine applied by different routes as in the present study.

The potentiating action of DOCA on this preparation is explicable in terms of inhibition by this steroid, of the access of adrenaline to extraneuronal sites of inactivation. Kalsner (1969a,b) first proposed this as a possible mechanism to explain the action of steroids on the rabbit aortic strip, and subsequently Iversen and Salt (1970) demonstrated that a number of steroids including deoxycorticosterone, prevented the extraneuronal accumulation of NA in the rat heart. Some characteristics of the action of DOCA on the ear artery which are in accord with Kalsner's explanation are: (a) the extraneuronal nature of the action, since it was not decreased by cocaine or by chronic denervation; (b) DOCA did not alter the maximum responses to adrenaline (demonstrated in the helical strip preparation), in accord with a mechanism involving "deviation" of amine concentration from its receptor sites (Fleming, 1975); (c) the potentiating effect of normetanephrine, which is also an inhibitor of extraneuronal uptake (Iversen, 1967), was abolished by DOCA, suggesting that the two agents have a common pharmacological mechanism of action. It seems unnecessary to invoke alternative mechanisms such as that proposed by Besse and Bass (1966), that steroids sensitise adrenergic receptors by inducing conformational changes in the latter (discussed in the Introduction to this

chapter). The work of Besse and Bass was carried out before the physiological significance of extraneuronal uptake was appreciated and before there was evidence that steroids inhibited extraneuronal uptake. While the present study does not exclude their hypothesis, it does show that the actions of the steroid can be explained in terms of uptake inhibition. The problem with the hypothesis of Besse and Bass is that there is as yet no simple way of measuring conformational changes in receptors. In view of the above considerations, it is reasonable to interpret data on the interactions between DOCA and cocaine in terms of the functional relationship between extraneuronal uptake and inactivation when adrenaline is applied to the intima and to the adventitia of the artery.

In the presence of neuronal uptake, the action of DOCA was non-selective in that it sensitised extraluminal and intraluminal adrenaline responses to about the same extent. When neuronal uptake was impaired (by low concentrations of cocaine or by surgical denervation) there was a selective increase in the potentiation of extraluminal adrenaline sensitivity by DOCA. Conversely, in arteries treated with DOCA, the potentiation of extraluminal adrenaline by cocaine was more marked, while that of intraluminal adrenaline remained unaltered or slightly reduced. If it is assumed that the magnitude of the potentiating

effects of cocaine and DOCA reflect the respective influences of the neuronal and extraneuronal uptake systems in decreasing the extracellular concentration of adrenaline, the interaction between the two agents implies that the two uptake processes operate in parallel such that inhibition of one enhances the influence of the other. The fact that the functional relationship was more evident for extraluminal than for intraluminal adrenaline sensitivity accords with earlier evidence that neuronal uptake has relatively little influence on the latter sensitivity (de la Lande et al, 1967a). The concept that neuronal and extraneuronal mechanisms operate in parallel is not new, and was proposed by Hughes (1972) on the basis of evidence that the influence of extraneuronal inactivation on the stimulation-induced overflow of NA in the rabbit vas deferens was augmented when neuronal uptake was eliminated at a site of removal of NA. Similarly, the influence of neuronal uptake was enhanced in tissues in which extraneuronal inactivation was prevented by corticosterone. Osswald and Branco (1973) also drew attention to the interactive roles of neuronal and extraneuronal uptakes on the removal of NA from the circulation of the hindlimb of the dog. They found that deoxycorticosterone influenced removal only when neuronal uptake was impaired by cocaine. The selective effect of DOCA on adrenaline sensitivity in the ear artery in the absence of neuronal uptake suggests that the extraneuronal uptake system is more active in removing adrenaline

from receptor sites associated with effector cells mediating the responses to extraluminal adrenaline. These cells may be those in the outer region of the media, since there is pharmacological (de la Lande and Jellett, 1972; Kalsner, 1972) and histochemical (de la Lande et al, 1970b, 1974) evidence that the distribution of NA in the artery wall is probably non-uniform, the cells exposed to the highest concentration of amine being those closest to the surface of application. It should be noted that the interactions between DOCA and cocaine on intraluminal adrenaline depended on the order of application of these agents. Thus the effect of DOCA was enhanced by cocaine (although to a lesser extent than its effect on extraluminal adrenaline), whereas the relatively small potentiating effect of cocaine was not increased, but instead tended to be slightly reduced in the presence of DOCA. It is conceivable that the latter is a reflection of a small extraneuronal effect of cocaine, evidence for which has been proposed by Kalsner and Nickerson (1969b). If this is so, it is difficult to explain why the potentiating effect of DOCA on intraluminal adrenaline was also not decreased when the agents were added in the reverse order. Nevertheless, an extraneuronal component to the action of cocaine could account for the decrease in the effect of DOCA when the concentration of cocaine was increased from 2.9 to 29μmol l^{-1} (compare values in Table 4.2b and 4.2c).

RELATIONSHIP TO CONCENTRATION

When the potentiations from each group of experiments were plotted against concentration of adrenaline, significant negative correlations between the two parameters were apparent for both extraluminal and intraluminal applications of adrenaline. Thus the potentiating effect of DOCA was less marked in arteries in which equieffective concentrations were increased by phentolamine. Furthermore, while DOCA potentiated β-mimetic effects elicited by low concentrations of isoprenaline (approx. 0.01μmol l^{-1}), potentiation of the α-mimetic effects, elicited at much higher concentrations of isoprenaline (>6.0μmol l^{-1}) was not observed. These findings accord well with recent studies (published after the present investigation was completed) by Graefe and Trendelenburg (1974), of the action of hydrocortisone in the cat nictitating membrane, and are consistent with the presence of a steroid-sensitive extraneuronal uptake mechanism, which, unlike that described in previous histochemical studies (Avakian and Gillespie, 1968; Gillespie and Towart, 1973; Nicol and Rae, 1972), is readily saturable. Saturation was evident at amine concentrations of the order of 5μmol l^{-1}, i.e., approximately 1/100th of the Km for extraneuronal uptake estimated by Gillespie and Towart (1973). The concept of a readily saturable extraneuronal uptake mechanism is supported by the failure of DOCA to enhance the sensitivity to catecholamines when COMT is inhibited (described in subsequent chapters). Other recent studies have provided detailed evidence for the presence of a high affinity, low capacity extraneuronal

uptake system in other tissues, namely cat heart (Kaumann, 1972) and rat and guinea pig hearts (Bonisch and Trendelenburt, 1974; Bonisch et al, 1974; Uhlig et al, 1974; discussed in more detail in Chapter 7). However, although the present findings agree with the concept of a readily saturable steroid-sensitive uptake mechanism, a tendency for the potentiation by DOCA to increase with the concentration of adrenaline was also apparent for *low* concentrations of intraluminal adrenaline in Fig. 4.4. Inspection of Fig. 4.4 suggests that a single point, widely separated from the remainder which are closely clustered, has biased the correlation coefficient in favour of a linear negative relationship. A significant positive correlation between the potentiating effect of DOCA and the concentration of adrenaline was also observed in a group of arteries treated with cocaine (2.9μmol l^{-1}, see Table 4.8 and Fig. 4.5). While these observations do not challenge the concept of a readily saturable uptake mechanism, they do suggest that the true relationship between the concentration of adrenaline and the potentiation by DOCA is more complex than a simple inverse one. A study of the interaction between DOCA and serotonin, an agent which exerts a powerful and non-specific sensitising action in this artery (de la Lande et al, 1966, 1967b) would be of interest. This might permit the effect of DOCA on the vasoconstrictor response to very low concentrations of adrenaline (0.01 - 0.05μmol l^{-1}) to be examined and hence provide further insight into the true relationship between these parameters.

Langer and Trendelenburg (1969) provided theoretical and experimental evidence that inhibition of a saturable uptake mechanism resulted in a shift to the left and in a decrease in slope of the concentration-response curve to an amine which caused progressive saturation of uptake within the range of amine concentrations required to establish this curve in the absence of the inhibitor. Hence a complex relationship between the concentration of adrenaline and the potentiation by DOCA is suggested by the present findings that the slopes of concentration-response curves to adrenaline in artery segments were *increased* in the presence of DOCA. The change in slope correlated with the magnitude of the potentiation, thus being most marked for extraluminal adrenaline in denervated and in cocaine-treated arteries (i.e., for low concentrations of adrenaline, Fig. 4.7a), and was not significant in arteries treated with phentolamine (i.e., for high concentrations of adrenaline).

In view of the negative correlation between the concentration of extraluminal adrenaline and the potentiating effect of DOCA, it is possible that the smaller effect of DOCA on extraluminal adrenaline in arteries possessing an intact neuronal uptake mechanism may be related to the higher concentrations of adrenaline required to elicit responses under these conditions, rather than to an interaction between the neuronal and extraneuronal

uptake systems in the outer media as discussed above. In the experiments of Graefe and Trendelenburg (1974), hydrocortisone failed to potentiate the sensitivity of the innervated cat nictitating membrane to NA. However, in denervated preparations, in which the sensitivity to NA was increased approximately 10-fold, hydrocortisone caused marked potentiation (approx. 5-fold). Since the potentiating effect of hydrocortisone was greatly reduced when the sensitivity of the denervated muscle was reduced (by phentolamine) to about the same level as that of the innervated muscle, they concluded that the sensitivity of the preparation was a very important factor in determining the magnitude of the hydrocortisone potentiation. A similar experiment has not been carried out in the rabbit ear artery since in the present study the concentration of phentolamine ($0.18\mu mol\ l^{-1}$) was selected to ensure a much larger decrease in sensitivity to adrenaline so that a relationship between the potentiating effect of DOCA and the concentration of adrenaline could be demonstrated unequivocally (Fig. 4.4). At present it is only possible to say that the magnitude of the potentiating effect of DOCA may be influenced both by the sensitivity of the artery and by an interaction between the neuronal and extraneuronal uptake mechanisms. However, it should be noted that although Graefe and Trendelenburg (1974) stressed the importance of the sensitivity of the cat nictitating membrane in determining the

magnitude of hydrocortisone potentiation, their data does not exclude an interaction between neuronal and extraneuronal uptakes. Thus when the ED_{50} of NA in the denervated nictitating membrane (neuronal uptake absent) was increased to approximately the same level as that of the innervated muscle (neuronal uptake intact), significant hydrocortisone potentiation (approx. 2-fold) was observed in the denervated but not in the innervated muscle, suggesting that neuronal uptake had masked a small influence of the hydrocortisone-sensitive mechanism in the innervated muscle (see Table 1 and Fig. 6b of Graefe and Trendelenburg, 1974).

The finding that the effects of DOCA were not significantly altered by propranolol excluded the possibility that β-effects of adrenaline had introduced artefacts into assessment of the pharmacological actions of DOCA. In fact, surprisingly, no potentiation of the steady state responses to adrenaline by propranolol was observed when the latter was present in concentrations (0.39 and 3.9 mol l^{-1}) which antagonised isoprenaline dilator responses. This contrasts to the ability of propranolol to potentiate the transient constrictor response to adrenaline when both these agents are administered by intraluminal injection (Carroll and Glover, 1973b). It is possible that β-receptors are of little importance in influencing the steady state compared with the transient constrictor response to adrenaline.

If the DOCA-sensitive mechanism primarily involves transport into smooth muscle cells, one might postulate that transient responses following very brief exposures to amine would be little affected by DOCA. To test this hypothesis, the effects of DOCA on the steady state response were compared with its effects on transient responses elicited by intraluminal bolus injections of adrenaline. The results supported the hypothesis inasmuch as the potentiation of the transient response was significantly less than that of the steady state response. However, significant potentiation of the transient response was observed, suggesting that the DOCA-sensitive mechanism equilibrates rapidly with the concentration of adrenaline in the region of the receptors.

The potentiating action of DOCA was only partly specific in that while it was restricted to certain sympathomimetic amines, it also extended to histamine and serotonin. The ability of DOCA to sensitise the artery to the constrictor effects of histamine and to a lesser extent, serotonin, suggests that these amines may also have affinities for extraneuronal uptake. Non-specific sensitisation appears excluded by the failure of DOCA to potentiate the constrictor effect of methoxamine and isoprenaline. A steroid (oestradiol)-sensitive accumulation of serotonin in this artery has been described, for high concentrations of amine at least (Buchan et al, 1974). Recent biochemical studies have provided evidence for a DOCA-sensitive accumulation

of histamine following incubation of the rabbit ear artery with low concentrations of ^{3}H-histamine (de la Lande and Foldes, private communication). Kalsner (1970, 1975) observed modification in responses to histamine and serotonin by steroids in the rabbit aortic strip, and attributed these effects to inhibition of extraneuronal uptake. The effects of DOCA on constrictor responses to histamine in the ear artery are of particular interest, since in contrast to its effects on adrenaline, DOCA potentiated transient responses to intraluminal bolus injections of histamine to a greater extent than the steady state responses to extraluminal applications. It is conceivable that a major mechanism of control of sensitivity to histamine is located at or near the intimal surface of the artery.

CHAPTER 5

INFLUENCE OF CATECHOL-O-METHYL TRANSFERASE ON THE SENSITIVITY OF THE RABBIT EAR ARTERY TO CATECHOLAMINES

INTRODUCTION

The distribution of COMT in sympathetically innervated tissues appears to be predominantly extraneuronal, with a smaller and more variable distribution in the sympathetic nerves, depending on the species and tissue (See Chapter 1, p.10-13). The present study was undertaken to determine whether COMT played a significant role in the sensitivity of the rabbit ear artery to catecholamines, and if so, whether these actions were consistent with a neuronal or extraneuronal distribution of the enzyme. One of the major differences between the effects of a neuronal and extraneuronal uptake inhibitor which was revealed in the experiments described in the preceding chapter, was that the sensitisation produced by the latter (DOCA) did not depend on the surface of entry of the amine into the artery wall. Hence as part of this study, the effects of a COMT inhibitor (UO521), on the sensitivities of the artery to both intraluminally and extraluminally applied amines were examined. Furthermore, in view of pharmacological evidence in the rabbit aorta (Kalsner 1969a, b; Levin and Furchgott, 1970) and cat heart (Kaumann, 1972) that the potentiating effects of COMT inhibitors were decreased by treatment with steroids, and of biochemical evidence that the levels of O-methylated metabolites were increased in the absence of neuronal uptake (Iversen et al, 1966; Eisenfeld et al; 1967a, b), the interactions between UO521

and DOCA, and between UO521 and cocaine were also examined. The experiments were also intended to supplement biochemical studies on the activity and distribution of COMT in the ear artery being carried out concurrently in this laboratory by R. J. Head.

METHODS

The methods employed in these experiments were identical to those on the preceding chapter and have been described in detail in Chapter 2.

RESULTS

i) adrenaline

The effects of UO521 on the sensitivity of the artery to adrenaline are summarised in Table 5.1. When applied to the artery in a concentration of 11μmol l^{-1}, UO521 did not alter the perfusion pressure. However, the sensitivities to both intraluminal and extraluminal adrenaline were significantly increased. This sensitising action resembled that of DOCA previously described (Chapter 4) in that both intraluminal and extraluminal adrenaline responses were enhanced to the same extent (Table 5.1a). The potentiating effects of UO521 were not significantly altered in arteries treated with cocaine (Table 5.1b), but were markedly reduced in the presence of DOCA, irrespective of whether cocaine was also present or not (Table 5.1c,d). Conversely, the potentiating effects of DOCA on the sensitivity to adrenaline in

cocaine-treated arteries were markedly reduced or abolished in the presence of U0521 (Table 5.2). The relationship between the effects of U0521 and DOCA was further investigated in the following experiments. In cocaine-treated arteries, the potentiation of adrenaline sensitivity by U0521 was estimated between 45 and 60 minutes after removal of DOCA. At this time, the arteries had only partially recovered from the potentiating effects of the latter and the magnitude of the potentiation by U0521 was less than in arteries not previously treated with DOCA. The potentiation by U0521 was rapidly reversed after washout, after which a second treatment with DOCA (27μmol l^{-1}) resulted in a further increase in sensitivity to adrenaline to approximately the same level as that which prevailed during the first application of DOCA. A second application of U0521 now had little or no potentiating effect.

The sensitising action of U0521 differed from that of DOCA described earlier (Chapter 4) in that it was associated with an alteration in the shapes of the responses to adrenaline, the time requirement for the attainment of the steady state level being considerably increased. However the increase in the time course of the response was much less prominent in the presence of both U0521 and DOCA (Fig. 5.1).

Table 5.1 Effect of UO521 (11 μmol l^{-1}) on the sensitivity to adrenaline

	a	b	c	d
Treatment				
Cocaine (μmol l^{-1})	-	2.9	2.9	-
DOCA (μmol l^{-1})	-	-	27	27
Potn. EL adrenaline	3.8(3.7-4.0)	4.0(3.5-4.7)	1.3(1.2-1.3)	1.3(1.2-1.3)
Potn. IL adrenaline	3.8(3.4-4.2)	4.2(3.4-5.2)	1.1(1.0-1.2)	-
No. of arteries (EL,IL)	8,6	8,8	9,7	5
P	>0.7	>0.8	>0.4	Not applicable

Footnotes:

(i) Potentiation refers to the ratios of the concentrations of adrenaline which are equipotent in eliciting increases in the perfusion pressure of 8.0×10^3 Nm^{-2} in the artery before and during treatment with the sensitising agent. (See Methods, chapter 2).

(ii) The value P refers to the difference between the potentiations of EL and IL adrenaline (paired t-test).

(iii) The potentiations of EL and IL adrenaline in a did not differ significantly from the respective potentiations in b (unpaired t-tests, $p>0.7$(EL); $p>0.6$(IL), but those in c were significantly less than those in b (unpaired t-tests, $p<0.001$(EL); $p<0.001$(IL)). The potentiation of EL adrenaline in d was not significantly different from that in c (unpaired t-test, $p>0.5$) but was significantly less than that in a (unpaired t-test, $p<0.001$).

Table 5.2 Influence of U0521 (11 μmol l^{-1}) on the potentiating effect of DOCA (27 μmol l^{-1}) in arteries treated with cocaine (2.9 μmol l^{-1}).

	a	b
Treatment U0521	--	+
Potn. EL adrenaline	4.7 (4.3-5.2)	1.0 (0.9-1.1)
Potn. IL adrenaline	3.6 (3.3-4.0)	0.9 (0.7-1.1)
No. of arteries (EL,IL)	18,18	6,2

Footnotes:

(i) Potentiation measured as in footnote (i), Table 5.1.

(ii) Values in a are taken from Table 4.2b (p.68)

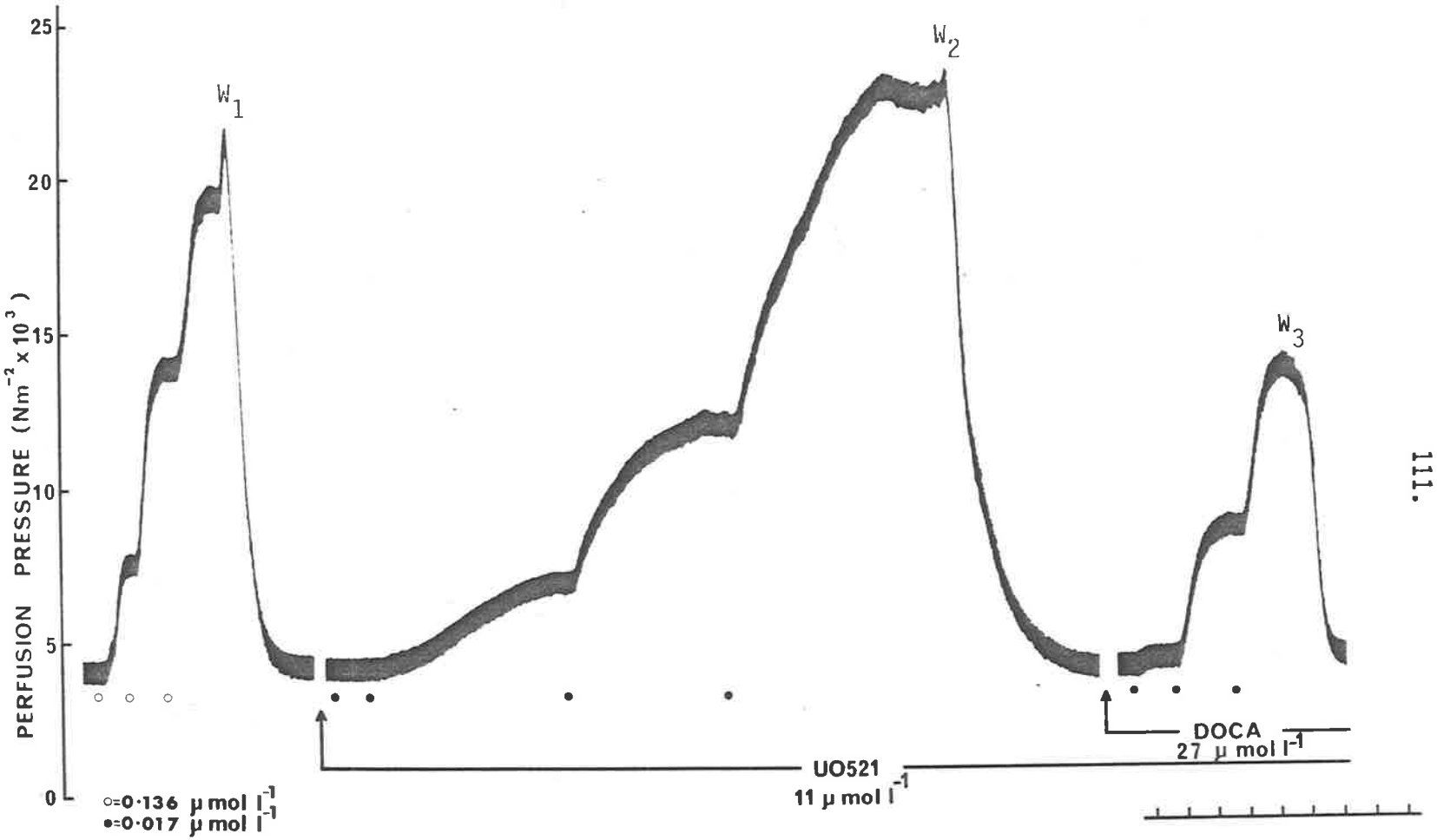


Fig. 5.1 Constrictor responses to adrenaline in the perfused ear artery treated with cocaine ($2.9\mu mol\ l^{-1}$). Adrenaline was added cumulatively to the extraluminal bathing medium in the concentrations and at the points indicated. Adrenaline was washed out at W_1, and a second series of responses obtained 15 minutes after addition of UO521. After washout of adrenaline (W_2), DOCA was applied, and a third series of adrenaline responses obtained 15 minutes later. UO521 and DOCA were applied to both the intraluminal perfusate and extraluminal bathing medium. Time scale in minutes.

(ii) noradrenaline

The potentiating effects of U0521 on the sensitivities to NA were small compared with those on the sensitivities to adrenaline, and were estimated from the increases in the steady-state responses to NA when U0521 was applied during these responses. U0521 increased the sensitivities to both extraluminal and intraluminal NA. The increases in untreated and cocaine-treated arteries did not differ significantly, but were markedly reduced in the presence of DOCA (Table 5.3). The time course of action of U0521 was rapid, potentiation commencing within 15-20 seconds after its application and reaching a steady state level within 5-10 minutes.

(iii) isoprenaline

The constrictor potency of isoprenaline was approximately 20-fold lower than that of adrenaline or NA. The potentiating effects of U0521 on the constrictor responses to isoprenaline, both in the presence and absence of propranolol, were much less prominent compared with its effects on the sensitivities to adrenaline and NA (Table 5.4).

(iv) Sympathetic Nerve Stimulation

U0521 potentiated the transient responses to brief applications of pulses to approximately the same extent in untreated and cocaine-treated arteries (Table 5.5), an observation

Table 5.3 Effect of UO521 (11 μmol l^{-1}) on the sensitivity to noradrenaline.

	a	b	c
Treatment			
Cocaine (μmol l^{-1})	-	29	29
DOCA (μmol l^{-1})	-	-	27
Potn. EL NA	1.6 (1.4-1.7)	1.6 (1.5-1.7)	1.2 (1.2-1.2)
Potn. IL NA	2.2 (1.9-2.5)	-	-
No. of arteries (EL,IL)	7,6	11	7
P	>0.05	not applicable	not applicable

Footnotes:

(i) Potentiation of EL NA was measured from the increases in perfusion pressure when UO521 was added during the steady state of the response to NA. (See Methods, Chapter 2) Mean steady state perfusion pressures ($Nm^{-2}x10^3$) in the presence of UO521 were: 7.9±2.3 in a; 8.4±1.7 in b; 4.7±0.7 in c.

(ii) Significance (P) explained in footnote (ii), Table 5.1. The difference in the potentiation of EL and IL NA in a was due mainly to a relatively marked (4-fold) potentiation of IL NA observed in one of these 6 arteries.

(iii) The potentiation of EL NA in a did not differ significantly from that in b (unpaired t-test, $p>0.6$) but that in c was significantly less than that in b (unpaired t-test, $p<0.001$).

Table 5.4 Effect on U0521 on the sensitivity to isoprenaline

	a	b	c
U0521 (μmol l^{-1})	11	11	55
Treatment:			
Cocaine (μmol l^{-1})	2.9	2.9	-
Propranolol (μmol l^{-1})	-	3.9	3.9
Potn. EL ISO	1.2 (1.1-1.3)	1.3 (1.1-1.5)	1.4 (1.1-1.7)
No. of arteries	3	3	3

Footnote:

(i) Potentiation measured as in footnote (i), Table 5.1.

Table 5.5 Effect of U0521 (11 μmol l^{-1}) on transient responses to sympathetic nerve stimulation.

	a	b	c
Treatment:			
Cocaine (μmol l^{-1})	-	29	29
DOCA (μmol l^{-1})	-	-	27
Potn.	1.4 (1.3-1.6)	1.3 (1.3-1.4)	1.5 (1.4-1.6)
No. of arteries	8	12	7

Footnotes:

(i) Potentiation refers to the ratios of equieffective frequencies of stimulation in the absence and in the presence of U0521. (See Methods, Chapter 2) Differences in the potentiations in a, b and c were not statistically significant (unpaired t-tests, $p>0.3$).

consistent with the failure of cocaine to significantly alter the potentiating effects of UO521 on steady-state responses to adrenaline and NA noted above (Tables 5.1 and 5.3). However, in contrast to their interaction on the latter responses, the potentiation of the transient response to nerve stimulation by UO521 was not significantly altered in the presence of DOCA (Table 5.5b,c). UO521 also potentiated the steady-state response to nerve stimulation but the effect was less marked compared with transient responses and was not observed in 6 of 13 arteries in which UO521 was applied during the steady-state phase of the response.

DISCUSSION

The potentiating action of UO521 on the sensitivities to adrenaline and NA provides indirect evidence that O-methylation by COMT is an important mechanism of inactivation of adrenaline, and to a lesser extent, of NA, in this artery. That O-methylation is equivalent to inactivation is indicated by evidence from another study in this laboratory that the constrictor potencies of the O-methylated metabolites of adrenaline and NA, namely metanephrine and normetanephrine, are at least 20-fold less than those of the respective parent amines (de la Lande and Campbell, unpublished private communication).

The sensitising action of U0521 resembled that of DOCA described earlier in that intraluminal and extraluminal responses were potentiated to the same extent and the effects were more marked for adrenaline compared with NA. These findings, as well as the observation that the effects of U0521 on the sensitivities to both adrenaline and NA were not significantly altered by cocaine but markedly reduced in the presence of DOCA suggests that COMT activity is located mainly at extraneuronal sites, possibly associated with the smooth muscle cells of the media. The relative failure of U0521 to potentiate in the presence of DOCA is then explicable on the basis that the latter impedes access of the amine to intracellular sites of inactivation by COMT. This explanation is consistent with observations and conclusions of others using a variety of tissues (Kalsner, 1969a,b; Levin and Furchgott, 1970 (rabbit aorta); Kaumann, 1972 (cat heart); Graefe and Trendelenburg, 1974 (cat nictitating membrane)). The reverse interaction, namely the failure of DOCA to exert its potentiating effect in the presence of U0521, suggests that the DOCA-sensitive mechanism influences the concentration of adrenaline at the receptors only when COMT is also functionally intact. This implies that the physiologically important component of extraneuronal uptake is readily saturable (i.e. has little capacity for storing unchanged amine).

Unexpected findings were that the potentiating effects of U0521, unlike those of DOCA, were not increased in the presence of cocaine

(Tables 5.1a,b and 5.3a,b). This applied to both intraluminal and extraluminal adrenaline so that a further distinction between UO521 and DOCA was that there was no difference between the effects of UO521 on the sensitivities to extraluminal and intraluminal adrenaline in cocaine-treated arteries (compare values in Tables 5.1b and 5.2a). The failure of cocaine to enhance the sensitising action of UO521 is at variance with the findings of Kaumann (1970) in cat heart and of Trendelenburg et al (1971) in the cat nictitating membrane and other tissues. In both cases the sensitivity to NA was increased after inhibition of COMT only if cocaine was also present. However, it should be noted that Levin and Furchgott (1970) made qualitatively similar observations on the rabbit aortic strip to those reported here, in that cocaine and an inhibitor of COMT (4-tropolone acetamide) appeared to act independently in enhancing the sensitivity of the preparation to catecholamines. These workers made the reasonable suggestion that the apparent lack of interaction between neuronal uptake and extraneuronal O-methylation was due to the asymetry of the adrenergic innervation in blood vessels. As a consequence, inhibition of neuronal uptake enhanced the rate of O-methylation only in smooth muscles cells near the adventitial surface, and since these represented only a small proportion of the medial muscle, O-methylation estimated in the tissue as a

whole was not appreciably altered. While this explanation is appropriate to results obtained using a strip preparation, it would not apply to the perfused segment, where there is considerable evidence (outlined in Chapter 1, p.27-30), suggesting that responses to extraluminal application of amine primarily reflect events which occur near the adventitial surface. Thus the present findings also appear at variance with those of Levin and Furchgott (1970).

The apparent discrepancy could be explained if part of the potentiating effect of UO521 was mediated by inhibition of neuronal COMT. Loss of COMT activity resulting from inhibition of neuronal uptake by cocaine might then nullify the enhanced contribution of extraneuronal O-methylation expected under these conditions. Some support for this possibility is offered by the findings of Head et al (1975b), that the formation of ^{3}H-normetanephrine in arteries incubated with ^{3}H-NA (1.18 µmol l^{-1}) was significantly increased following denervation but not by treatment with cocaine. This result could be explained if cocaine prevented access of ^{3}H-NA to neuronal COMT. However, it may also reflect an inhibitory action of cocaine on extraneuronal uptake. Unfortunately the results of a recent biochemical analysis of the COMT activities in homogenates of innervated and denervated arteries by Head et al (1974), while clearly

indicating the presence of extraneuronal COMT, were sufficiently variable to prevent assertions as to the presence of a small amount of neuronal COMT. However there is other evidence from the present study against the possibility that the effects of UO521 were mediated in part by inhibition of neuronal COMT. This was the finding that DOCA reduced the potentiating effect of UO521 to the same extent in cocaine-treated arteries and in arteries in which neuronal uptake was intact (Table 5.1c,d). Another recent study has shown that DOCA ($27\mu mol\ l^{-1}$) itself is without effect on neuronal uptake of ^{3}H-NA (Head and de la Lande, private communication).

The interpretation of the failure of cocaine to enhance the potentiating effects of UO521 would be assisted by a closer understanding of the events leading to an increased concentration of amine at the receptors after COMT inhibition. While it appears that COMT inhibition leads to a decrease in the net uptake to an extent where the latter has little effect on the concentration at the receptors, it is not known whether this is due mainly to a decrease in the gross influx or to an increase in the gross efflux of amine. However, the increase in the time course of the response to adrenaline after COMT inhibition (Fig. 5.1) is consistent with the latter view. The prolonged response may reflect the continued efflux from the cell until steady state is achieved. In that

case, inhibitors of extraneuronal uptake (DOCA) and of COMT (UO521) would be expected to differ in their interactions with an inhibitor of neuronal uptake (cocaine). It has been proposed that interaction between DOCA and cocaine is a reflection of the two uptake systems operating in parallel to decrease the extracellular concentration of extraluminally applied adrenaline (Chapter 4). The deviation of adrenaline concentration to the two sites presumably occurs as amine in the extracellular space approaches the receptors. Thus neuronal uptake can exert appreciable influence on the apparent ability of the DOCA-sensitive mechanism to reduce this concentration. If, however, after COMT inhibition, unchanged amine approaches from within the cell to increase the concentration at the receptors, the influence of neuronal uptake in reducing this concentration would be significantly impaired as amine reaches the receptors prior to efflux into the extracellular space.

UO521 also potentiated transient responses to sympathetic nerve stimulation, suggesting that COMT may be functionally important in the inactivation of released transmitter. The failure of cocaine to modify this effect of UO521 is consistent with the interaction of these agents in the steady state response to extraluminal applications of NA and adrenaline. Surprisingly, however, the potentiating action of UO521 was

also not diminished by combined treatment with cocaine and DOCA, but rather, was slightly enhanced, although the latter effect was not statistically significant. In view of evidence presented in Chapter 4, it is conceivable that the failure of DOCA to prevent the effect of UO521 on nerve stimulation is a consequence of a saturation of the DOCA-sensitive uptake mechanism by the very high concentrations of NA released (Bevan and Su, 1973b), in a highly localised area of smooth muscle after stimulation (Gillespie and Rae, 1972). An alternate possibility is that NA released during nerve stimulation is susceptible to inactivation at a site, access to which is not impaired by cocaine and DOCA. The experiments of Levin (1974) and of Blakely et al (1974) are of interest in this connection. Levin (1974) measured O-methylated and deaminated metabolites formed in isolated pieces of adventitia and media of the rabbit aorta during incubation with ^{3}H-NA. He concluded that O-methylation occurred in the media and that O-methylated products present in the adventitia (approx. 20% of the total) were associated with fibroblasts or a few smooth muscle cells (stripped off with the adventitia during separation) rather than sympathetic nerves, since their formation was not prevented by cocaine. Blakely et al (1974) attempted to mimic transmitter liberation in the perfused cat spleen by injecting ^{3}H-NA as a series of rapid injections, and measured the amount of tritiated material bound in the

tissue after each injection. The uptake was relatively insensitive to inhibitors of neuronal and extraneuronal uptake (desipramine and 17-β-oestradiol, respectively), but was markedly reduced by denervation or by combined treatment with desipramine, 17-β-oestradiol and phenoxybenzamine. They postulated the existence of an uptake process which was dependent on sympathetic nerves but which was distinct from classical neuronal uptake ("uptake$_1$"). The uptake process was also impaired by continuous nerve stimulation or infusion of NA. Interestingly, in the present experiments, U0521 consistently enhanced the transient responses to nerve stimulation but its effect on the steady state response to continuous application of pulses could not be demonstrated unequivocally. Although there may be other explanations to account for the failure of combined treatment with cocaine and DOCA to prevent the potentiating effect of U0521 on transient responses to nerve stimulation, the possibility that an uptake process similar to that described by Blakely et al may be important in these responses, warrants further investigation.

CHAPTER 6

UPTAKE AND O-METHYLATION OF ^{3}H-ISOPRENALINE

INTRODUCTION

The experiments described in preceding chapters showed that DOCA sensitised the ear artery to both the constrictor and dilator effects of low concentrations of catecholamines. The potentiating action of DOCA was independent of the route of application of amine to the artery, was more marked for adrenaline compared with NA, and was not decreased but rather enhanced in cocaine-treated or chronic-denervated arteries. In addition, the action of DOCA was closely linked with that of a COMT inhibitor, as indicated by the failure of either of these agents to potentiate in the presence of the other. These effects were interpreted as indirect evidence that DOCA inhibited the access of catecholamines to extraneuronal sites of inactivation by COMT. In the present study the disposition of ^{3}H-isoprenaline in the artery was examined to determine, by more direct means, whether the artery possessed a mechanism for extraneuronal uptake and O-methylation of low concentrations of catecholamines, and if so, whether this mechanism was modified by DOCA and U0521. The catecholamine isoprenaline was selected on the basis of its low affinity for neuronal uptake, high affinity for extraneuronal uptake, and because it was not a substrate for MAO (Hertting, 1964; Iversen, 1967). Thus in terms of disposition and metabolism it offered a relatively selective measure of extraneuronal uptake and O-methylation by COMT.

METHODS

Introductory Note

The experiments described in this chapter were undertaken in conjunction with R.J. Head and R.J. Irvine, who devised the technique of separation of isoprenaline and its O-methylated product, 3-methoxyisoprenaline (MeOISO), during a much broader investigation of the uptake and metabolism of catecholamines in the ear artery and other tissues. The technique achieves a highly discreet separation of the two amines by thin layer chromatography on silica gel plates impregnated with sodium borate. Isoprenaline, selectively chelated by the borate, is firmly bound at the origin while MeOISO migrates with the organic solvent front. Details of the method are outlined below. A more extensive description of the method, including confirmation of its validity, and of other relevant procedures, such as assay and purification of ^{3}H-isoprenaline, is currently being prepared for publication by Head and Irvine. The constant advice and assistance of these workers during the present experiments is gratefully acknowledged.

Semi-lop-eared rabbits were stunned and bled. Segments of ear arteries approximately 2cm in length (usually 3 segments from each ear) were rapidly excised and allowed to equilibrate

for 60 minutes in $1cm^3$ aliquots of Krebs' solution. The arteries were then incubated for 30 minutes in $1cm^3$ aliquots of Krebs' solution containing purified d,l-^{3}H-isoprenaline ($0.81\mu mol\ l^{-1}$), ethylene diamine tetra-acetic acid (EDTA, $10.8\mu mol\ l^{-1}$) and ascorbic acid ($290\mu mol\ l^{-1}$). This was followed by a one minute wash in ^{3}H-isoprenaline-free Krebs' solution. All Krebs' solutions were maintained at 37°C and bubbled continuously with 95% O_2, 5% CO_2. After washing, each artery was extracted without homogenisation for 24 hours at 4°C in a $1cm^3$ aliquot of 0.1N HCl containing EDTA ($21.5\mu mol\ l^{-1}$). After a further 30 minute extraction with a fresh $1cm^3$ aliquot of 0.1N HCl, the extracts were pooled. Small volumes ($0.01cm^3$) of stock solutions of non-radioactive isoprenaline and MeOISO were added to the pooled extract to give final concentrations of 0.47 and $0.44mmol\ l^{-1}$, respectively. $0.5cm^3$ of the tissue extract was taken for estimation of the tritium content by scintillation spectrometry (see Appendix 2). The remainder was concentrated by lyophilization and reconstitution in $0.2cm^3$ 0.1N HCl in ethanol. The Krebs' incubating medium was adjusted to pH5.5 with HCl. $0.2cm^3$ of the incubate was taken and non-radioactive isoprenaline and MeOISO added to give final concentrations of $7.9mmol\ l^{-1}$ and $7.4mmol\ l^{-1}$, respectively.

Thin Layer Chromatography

Silica gel plates were subjected to ascending chromatography in freshly prepared sodium borate solution (200mmol l^{-1}) and the solvent allowed to migrate for approximately 7cm. The plates were oven dried (100^{o}C) and stored at room temperature. In preliminary experiments it was shown that when isoprenaline and MeOISO were applied to silica gel plates and chromatographed in toluene:ethanol (1:1), no appreciable separation of the amines occurred. If however, the plates were previously impregnated with borate, a compound which selectively chelated catechols, isoprenaline was firmly bound at the origin while MeOISO migrated with the organic solvent and became concentrated at the region of the borate front (Fig. 6.1). When ^{3}H-isoprenaline, purified by alumina chromatography, was applied to the plate, the amount of ^{3}H-isoprenaline which became localised at the borate front was approximately 0.01% of that applied at the origin.

0.02cm^{3} of the Krebs' incubate and 0.1cm^{3} of the concentrated tissue extract (both containing the non-radioactive carrier amines) were applied at the origins of the borate-impregnated plate and dried in a stream of cold air. The plates were then subjected to ascending chromatography with a mixture of toluene:ethanol (1:1) for 90 minutes and air dried. The non-radioactive carrier amines were located under uv light and the regions of the plate corresponding to MeOISO removed and

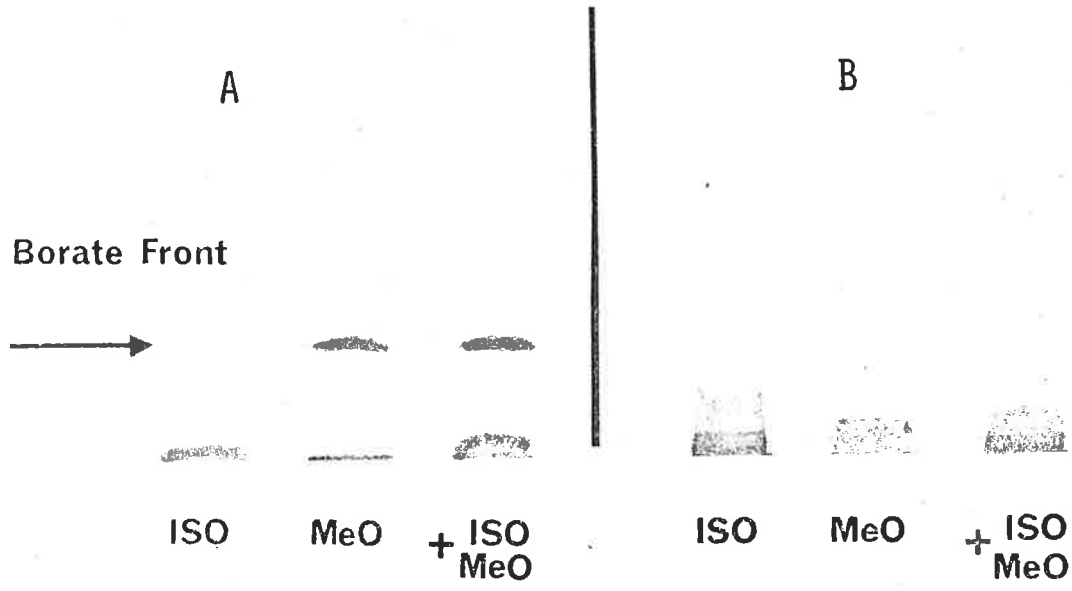


Fig. 6.1 Separation of isoprenaline (ISO) and 3-methoxy-isoprenaline (MeO) by thin layer chromatography. On the borate-impregnated silica gel plate (A) 3-methoxyisoprenaline migrates with the solvent (toluene:ethanol (1:1)) and concentrates at the borate front (indicated by the arrow). No separation occurs if the silica gel plate is not impregnated with borate (B). The phenolic compounds were detected after the plate was sprayed with a solution of 0.05% potassium ferricyanide and 0.5% ferric chloride.

added to tubes containing $2cm^3$ of 0.3N HCl. The tubes were agitated for 6 hours, centrifuged briefly and $1.0cm^3$ of the supernatants taken for estimation of tritium levels, from which the MeOISO contents in the Krebs' incubate and tissue extract were determined.

The remainder of each supernatant was assayed for content of non-radioactive carrier MeOISO to measure the efficiency of recovery. The paranitroaniline method of visualising phenols and methoxyphenols was adapted for automated photometric assay on a Technicon Autoanalyser. The reagent concentrations and module arrangement is shown in Fig. 6.2. The sample of 0.3N HCl was introduced into the manifold, made alkaline by addition of K_2CO_3, and the diazonium derivitive formed by the addition of freshly mixed paranitroaniline and sodium nitrite. Samples were passed through the flow cuvette of the colourimeter and the OD_{505} recorded. For each experiment the OD_{505} was plotted as a function of standard concentrations of MeOISO and from this the amounts of non-radioactive MeOISO in the extracts determined. The recoveries of MeOISO from samples of both Krebs' incubates and tissue extracts ranged from 80-90%. The MeOISO contents previously estimated from the tritium levels in these samples were appropriately corrected for losses of MeOISO during extraction. The amount of unchanged isoprenaline in the tissue

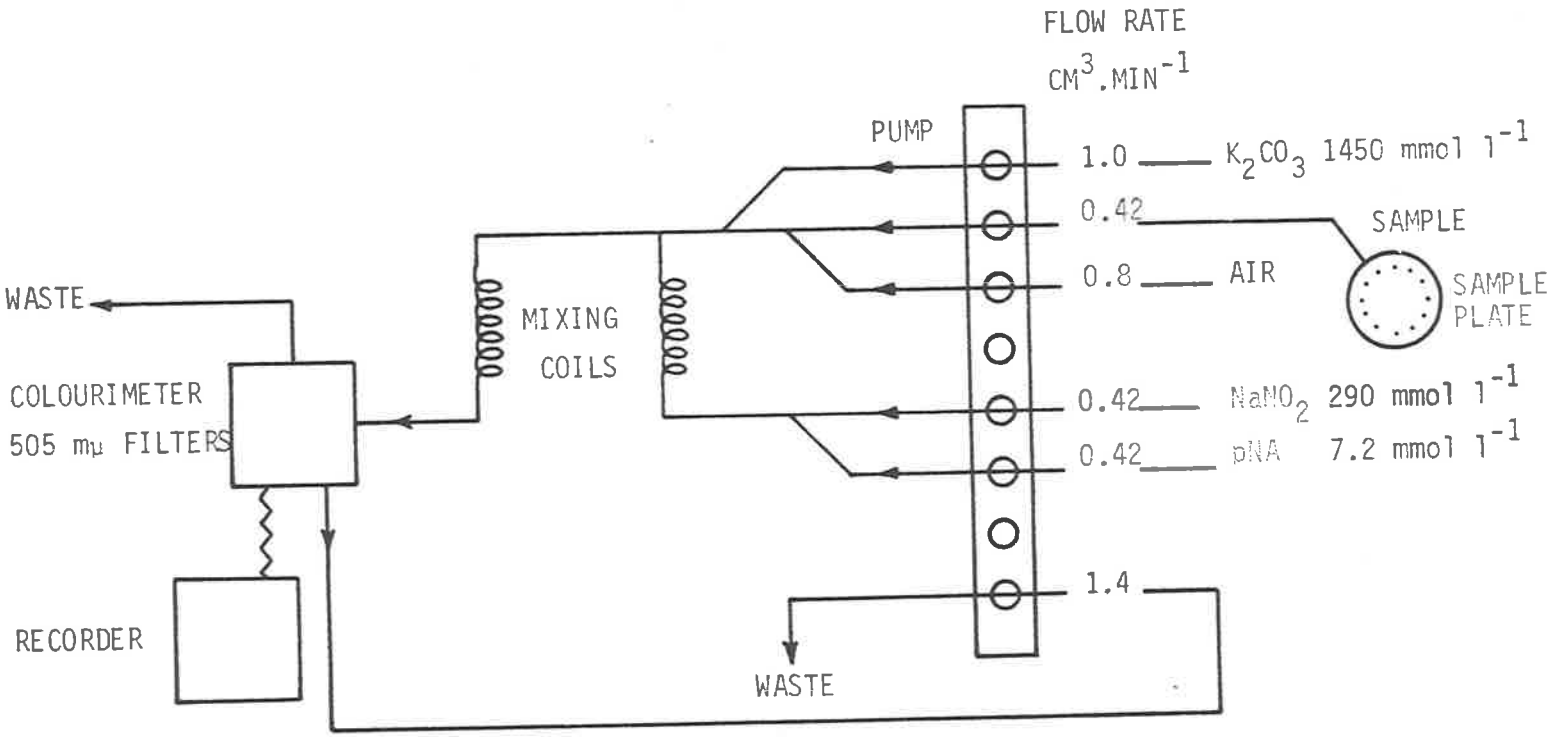


Fig. 6.2 Schematic representation of the colourimetric assay of 3-methoxyisoprenaline

extract was determined from the difference between the total tritium content and the corrected MeOISO value.

RESULTS

The levels of ^{3}H-isoprenaline and ^{3}H-MeOISO in the artery and the level of ^{3}H-MeOISO in the Krebs' incubate are shown in Fig. 6.3 which also summarises the effects of DOCA and UO521 when the latter agents were added singly or in combination 10 minutes prior to and during incubation with ^{3}H-isoprenaline. In untreated arteries both ^{3}H-MeOISO and unchanged ^{3}H-isoprenaline were present in the tissue. ^{3}H-MeOISO was also present in the Krebs' incubating medium at a level approximately 4 times higher than that in the tissue.

The mean extracellular space, estimated in the present study by measuring the amount of ^{14}C-sorbitol in the tissue after a 30 minute incubation and one minute wash, was 0.09cm^{3} g^{-1}. The artery contents of ^{3}H-isoprenaline and ^{3}H-MeOISO were considerably greater than the ^{3}H-isoprenaline equivalent of this extracellular space (0.07μmol kg^{-1}). They were also greater than the ^{3}H-isoprenaline equivalent of the extracellular space estimate of 0.61cm^{3} g^{-1} by Bevan and Waterson (1971) who incubated ear arteries for one hour in ^{14}C-inulin without a subsequent wash (Fig. 6.3).

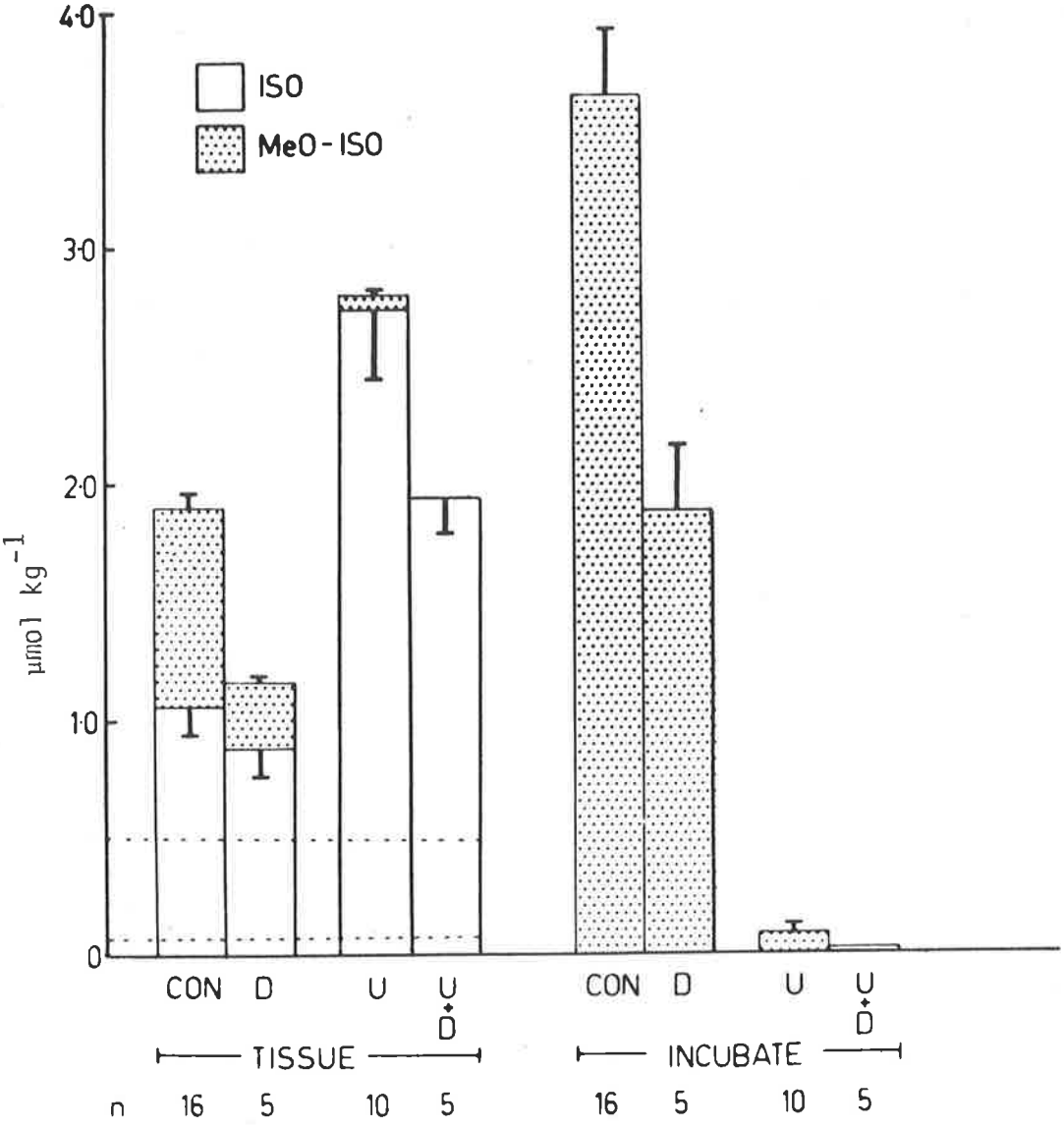


Fig. 6.3 Levels of ^{3}H-isoprenaline and ^{3}H-MeOISO (µmol per kg artery) in the tissue and Krebs' incubate. Arteries were incubated for 30 minutes with ^{3}H-isoprenaline (0.81µmol l^{-1}) and washed for one minute in ^{3}H-isoprenaline-free Krebs'.
Treatments:

CON = Untreated
D = DOCA (27µmol l^{-1})
U = UO521 (55µmol l^{-1})

n refers to the number of arteries.
The ^{3}H-isoprenaline equivalents of the extracellular space estimates of Bevan and Waterson (1971) and of the present study are indicated by the upper broken line (0.49µmol kg^{-1}) and lower broken line (0.07µmol kg^{-1}), respectively.

The effect of DOCA was significant ($p<0.05$) in all comparisons except on unchanged ^{3}H-isoprenaline accumulation in the absence of UO521.

The formation of ^{3}H-MeOISO was strikingly reduced in arteries treated with the COMT inhibitor (UO521, 55μmol l^{-1}). However the accumulation of unchanged ^{3}H-isoprenaline was increased to a level which was significantly greater than that expected on the basis of a stoichiometric replacement of ^{3}H-MeOISO by unchanged amine after COMT inhibition. In arteries treated with DOCA (27μmol l^{-1}), the amounts of ^{3}H-MeOISO in the tissue and incubating medium were decreased compared with untreated arteries, while the accumulation of unchanged ^{3}H-isoprenaline was not appreciably altered. However the accumulation of unchanged ^{3}H-isoprenaline in the UO521-treated arteries was significantly decreased in the presence of DOCA.

DISCUSSION

The present findings indicate that the artery accumulates and O-methylates ^{3}H-isoprenaline when the latter is present in low concentrations. Since the levels of unchanged amine and of ^{3}H-MeOISO were greater than could be accounted for by the presence of these amines in the extracellular space, the uptake may be attributed to binding at intracellular sites or other tissue structures. Another recent study has shown that the total tritium content of the tissue at the end of incubation with ^{3}H-isoprenaline was only slightly and not significantly decreased in arteries treated with an inhibitor

of neuronal uptake (cocaine) (Head et al, 1975a). The latter finding accords with the very low affinity of isoprenaline for neuronal uptake (Hertting, 1964; Iversen, 1967), and suggests that the binding and O-methylation of ^{3}H-isoprenaline occurred largely at extraneuronal sites.

The presence of relatively large amounts of ^{3}H-MeOISO in the Krebs' incubating medium is consistent with binding of ^{3}H-isoprenaline followed by O-methylation and subsequent efflux of the ^{3}H-MeOISO back into the bathing medium. The accumulation of unchanged amine was limited by COMT activity or the product of this activity (MeOISO), as indicated by the significant increase in accumulation of ^{3}H-isoprenaline after COMT inhibition. The marked decrease in the formation of ^{3}H-MeOISO in the presence of DOCA may have been due to direct inhibition of COMT by DOCA. However this seems unlikely in view of evidence that steroids are without effect on COMT activity in tissue homegenates (Hapke and Green, 1970). Furthermore, the effect of DOCA differed strikingly from that of a known COMT inhibitor (U0521) which enhanced the accumulation of ^{3}H-isoprenaline. The decreased content of ^{3}H-MeOISO in the Krebs' incubate could also be explained in terms of inhibition of efflux of ^{3}H-MeOISO from the tissue by DOCA. However, in that case the tissue levels of ^{3}H-MeOISO should have been increased rather than decreased. Thus the simplest explanation for the

effects of DOCA is that the latter prevented access of ^{3}H-isoprenaline to sites of O-methylation by COMT. This proposal is supported by the inhibitory effect of DOCA on the accumulation of ^{3}H-isoprenaline in COMT-inhibited arteries and is consistent with the findings of Iversen and Salt (1970) that steroids inhibited the extraneuronal uptake of NA in the rat heart.

It is not possible, from the limited information obtained here, to deduce whether the ear artery possesses more than one compartment for the extraneuronal uptake and O-methylation of ^{3}H-isoprenaline as has been described in rat and guinea pig hearts and in cat nictitating membrane by Trendelenburg and his associates (discussed in more detail in Chapter 7). However in the present experiments an appreciable formation of ^{3}H-MeOISO occurred in arteries treated with DOCA despite the significant inhibitory effect of the latter. The residual ^{3}H-MeOISO may have been formed in a compartment into which access of ^{3}H-isoprenaline was not impaired by DOCA. Alternatively it may reflect only partial inhibition of access of ^{3}H-isoprenaline into a single steroid-sensitive compartment. Kinetic analyses of the uptake and metabolism of ^{3}H-isoprenaline and of the nature of the inhibitory effect of DOCA may help to resolve these questions.

A possible relationship between the present findings and the previously observed sensitising actions of UO521 and DOCA is represented in Fig. 6.4. It is assumed that extraneuronal uptake and subsequent O-methylation of the catecholamine decreases the concentration of the latter at the receptors in the smooth muscle. By interrupting uptake and hence O-methylation, DOCA diverts amine concentration to the receptors and thus enhances the response to the amine. The potentiating effect of UO521 on the response to a low concentration of catecholamine can be explained as follows; in the absence of COMT activity, the unchanged amine accumulates in its storage sites to an extent which results in a considerable increase in the efflux of the amine back into the region of the receptors. This explanation assumes (a) that the amine which accumulates in the absence of COMT activity has little, if any, influence on the concentration of amine which reaches the receptors and (b) that the physiologically important component of extraneuronal uptake has very little capacity for storing unchanged amine. The second assumption (b) is necessary to explain the failure of DOCA to sensitise the artery in the presence of a COMT inhibitor. If unchanged amine which accumulated after its uptake was of physiological importance (i.e. in increasing the concentration of amine at the receptors), then inhibition of this uptake should have enhanced the response to the

amine. The fact that this did not occur in the COMT-inhibited arteries is readily explained if, under these conditions, DOCA caused a decrease in efflux of unchanged amine from low capacity (readily saturable) storage sites as a secondary consequence of inhibiting uptake.

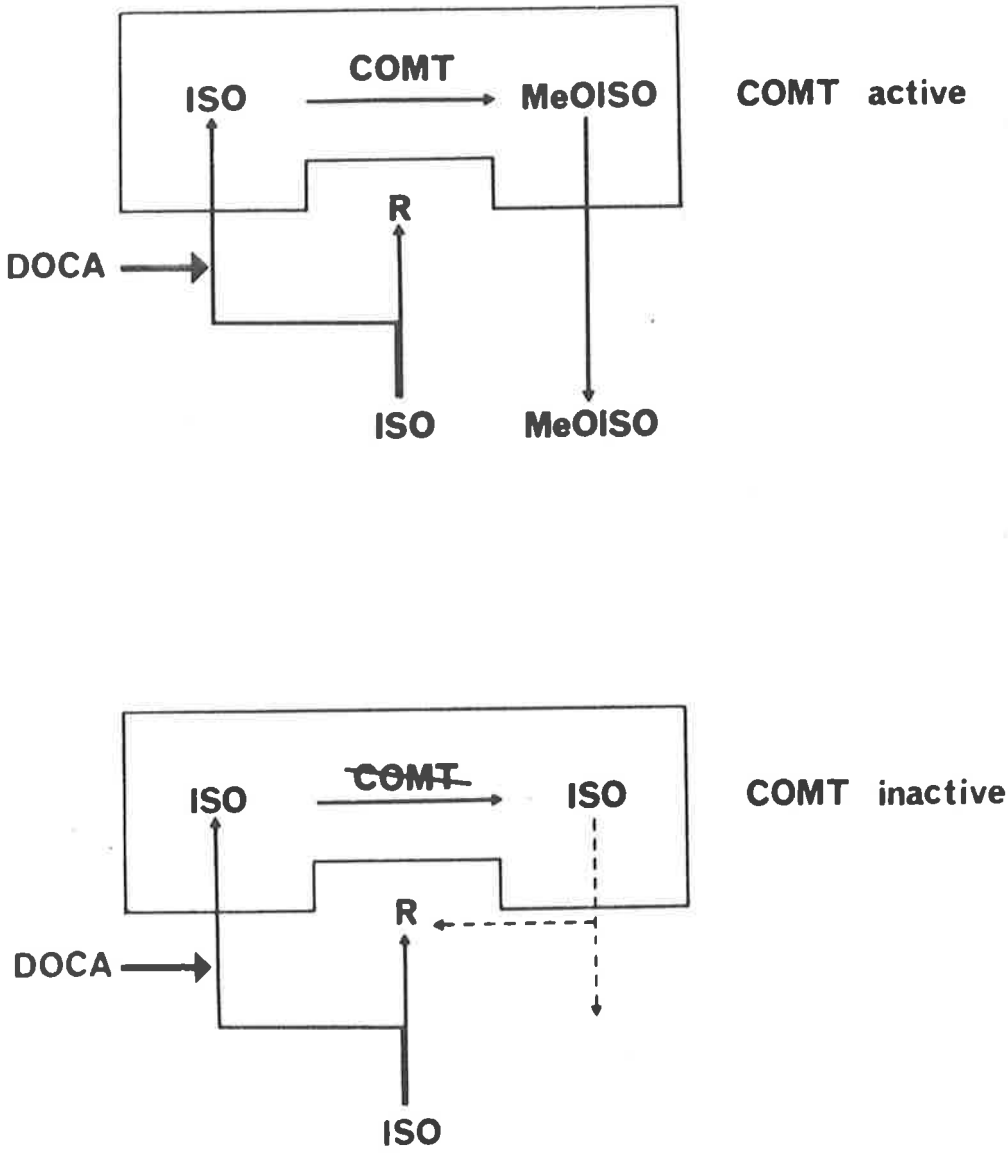


Fig. 6.4 Diagrammatic representation of the influence of uptake and O-methylation on the concentration of isoprenaline at the receptors in the smooth muscle of the rabbit ear artery. The directions of the arrows indicate the directions of isoprenaline fluxes.

CHAPTER 7

GENERAL DISCUSSION

GENERAL DISCUSSION

This investigation stemmed from a morphological concept of the influence of neuronal uptake on the sensitivity of the ear artery to NA. The essential features (discussed in the Introduction) were that the site of entry of the amine into the artery wall was of major importance in determining the quantitative influence of neuronal uptake and inactivation by MAO on the sensitivity to NA, and that this reflected the distribution of sympathetic nerves and smooth muscle, i.e., the location of the nerves at the medial-adventitial border and their failure to penetrate the media. The major question examined was whether the effects of inhibition of extraneuronal uptake and enzymatic inactivation differed from those of neuronal uptake and enzymatic inactivation. The results have permitted a part answer to this question.

Extraneuronal MAO

An earlier pharmacological study failed to show an influence of extraneuronal MAO on the sensitivity to NA (de la Lande and Jellett, 1972). However, the experiments described in Chapter 3 showed that under conditions of high substrate concentration (118μmol l^{-1}), extraneuronal MAO did represent a major pathway for inactivating NA. Head et al (1975b) have recently extended this study to show in arteries

incubated with concentrations of ^{3}H-NA comparable with those used in the present pharmacological experiments (1.18μmol l^{-1}), that acid metabolites are derived from neuronal and not extraneuronal MAO. Hence their data strongly support the pharmacological studies of de la Lande and Jellett (1972), suggesting that extraneuronal MAO was unimportant in the control of sensitivity to low concentrations of NA in the ear artery. This contrasts with the important role of extraneuronal MAO in the response to tyramine (de la Lande et al, 1970b) and gives rise to the possibility that the extraneuronal enzyme in this artery is Type B MAO described by Neff and Goridis (1972). It should also be mentioned here that both normetanephrine (a substrate for Type A MAO) and metanephrine were not significantly potentiated by nialamide (de la Lande and Campbell, private communication), suggesting that extraneuronal MAO is also of little importance in the inactivation of the O-methylated products of NA and adrenaline in this artery. In view of the above considerations, the discussion which follows places little emphasis on extraneuronal MAO as a factor which may modify interpretation of the effects of inhibitors of extraneuronal uptake and COMT on the sensitivity to low concentrations of catecholamines.

Extraneuronal COMT

The pharmacological experiments described in Chapter 5

showed that in contrast to the effects of inhibition of neuronal uptake or neuronal MAO, inhibition of COMT increased the sensitivities to intraluminal and extraluminal amines to the same extent. The effects were not decreased in cocaine-treated arteries. These observations suggested that COMT activity was extraneuronal in distribution and that there was little difference in the COMT inactivating system in different parts of the artery wall. In subsequent biochemical studies, Head et al (1974) showed significant COMT activity in the artery which was not modified by sympathetic denervation and which was hence largely extraneuronal. The extraneuronal activity was also indicated by Head et al (1975b) who observed that the formation of normetanephrine was not decreased but increased after denervation when arteries were incubated with ^{3}H-NA.

After COMT inhibition the kinetics of the response to adrenaline were altered so that the time required for attainment of the steady state response was increased. Similar effects after COMT inhibition in the cat nictitating membrane were described by Trendelenburg et al (1971), and have formed part of the evidence for a readily saturable uptake compartment which is normally limited by COMT and which removes low concentrations of amine from the biophase of the receptors.

Extraneuronal uptake

Since DOCA was used as an extraneuronal uptake inhibitor, it is appropriate to first summarize the evidence generated in this study that its effects in the rabbit ear artery were also mediated by inhibition of extraneuronal uptake. This evidence is summarised in Table 7.1. DOCA decreased the O-methylation of ^{3}H-isoprenaline and when COMT was inhibited, decreased the uptake of unchanged amine. Since part of these effects may have been mediated by inhibition of neuronal uptake, it should be noted that it also decreased the total tissue tritium content in arteries incubated with ^{3}H-isoprenaline in the presence of cocaine (Head et al, 1975a). More recently it has been shown that DOCA (27μmol l^{-1}) is without effect on neuronal uptake on NA in this tissue (Head and de la Lande, private communication). In another recent study from these laboratories, Parker and de la Lande (1975) measured the rate of diffusion of extraluminal ^{3}H-NA across the artery wall and showed that the tritiated products reaching the lumen comprised approximately equal proportions of ^{3}H-NA, ^{3}H-normetanephrine and tritiated deaminated metabolites. The proportion of the deaminated metabolites was greatly decreased in the presence of cocaine (29μmol l^{-1}). In the presence of both cocaine and DOCA (27μmol l^{-1}), the proportion of ^{3}H-normetanephrine was also markedly reduced. These effects are also consistent with an extraneuronal site of action of DOCA, leading to inhibition of O-methylation.

The actions of DOCA and of the COMT inhibitor (UO521) in the present study were similar in that both these agents potentiated intraluminal and extraluminal sensitivities to the same extent and each failed to potentiate in the presence of the other (Chapters 4,5). Although these observations are consistent with a direct inhibitory effect of DOCA on COMT activity, it should be emphasised that the inhibition of O-methylation by steroids in intact tissues is explicable in terms of the steroids impeding access of catecholamines to intracellular COMT (Iversen and Salt, 1970; present study, Chapter 6). For instance, although hydrocortisone enhanced the sensitivity of the rabbit aortic strip to catecholamines by preventing O-methylation (Kalsner 1969a), it had no direct inhibitory effect on COMT activity in homogenates of the aorta (Hapke and Green, 1970). Additional evidence against a direct inhibitory effect of steroids on COMT activity was obtained by Uhlig et al (1974) who showed that corticosterone prevented efflux of isoprenaline from extraneuronal stores in the rat heart previously filled with this amine. Under these conditions, corticosterone did not decrease, but rather enhanced the rate of O-methylation. Although not tested in the present study, the suggestion that DOCA is without direct effect on COMT activity is supported by the observation that whereas DOCA did not appreciably alter the accumulation of unchanged ^{3}H-isoprenaline in the ear artery, an inhibitor of COMT (UO521) markedly increased this accumulation.

The results of pharmacological experiments (summarised in Table 7.1) showed as a major feature, a sensitising action of DOCA which was independent of the route of application of adrenaline in untreated arteries but which was maximal for extraluminal adrenaline in cocaine-treated arteries (Chapter 4). This result was interpreted as evidence that there was interaction between the neuronal and extraneuronal uptake systems in reducing the concentration of adrenaline at the receptors. Another feature was that the sensitising action (tested mainly for extraluminally applied amines) conformed to that described in concurrent studies by Kaumann (1972) and by Graefe and Trendelenburg (1974), i.e., it was dependent on low substrate concentration and on intact COMT activity (Chapters 4,5).

Summaries of pharmacological and biochemical evidence relating to the concept of a readily saturable, COMT-containing component of extraneuronal uptake in various tissues, including the rabbit ear artery, are shown in Tables 7.2 and 7.3 In a study of the potentiating effects of hydrocortisone on the sensitivity of cat heart muscle to NA and isoprenaline, Kaumann (1972) found that whenever the tissue was sensitive to low concentrations of these amines, hydrocortisone caused potentiation. However when the concentrations were increased to about 1μmol l^{-1}, the potentiating effect of hydrocortisone

Table 7.1 Actions of DOCA in the rabbit ear artery

I. Pharmacological

(a) Potentiating effect was *extraneuronal*

(i) independent of route of application of amine

(ii) not decreased by cocaine or denervation

(b) Potentiation was mediated by inhibition of *extraneuronal uptake*

(i) selective (A>ISO>NA>methoxamine), according to relative affinities for uptake

(ii) DOCA prevented sensitisation by normetanephrine, an inhibitor of extraneuronal uptake

(iii) DOCA prevented sensitisation by a COMT inhibitor

(c) Potentiation was mediated by inhibition of a *readily saturable mechanism*

(i) effect of DOCA diminished with increasing concentrations of adrenaline and isoprenaline

(ii) effect of DOCA abolished by inhibition of COMT

II. Biochemical

(a) DOCA inhibited uptake and O-methylation of ^{3}H-isoprenaline

on the sensitivity to isoprenaline was greatly reduced and its effect on the sensitivity to NA was abolished. Kaumann postulated the presence of a functionally significant extraneuronal mechanism which, unlike that described previously, was readily saturable. More recently, similar effects of hydrocortisone on the sensitivity of the cat nictitating membrane to catecholamines have been described by Graefe and Trendelenburg (1974).

Detailed biochemical evidence for a high affinity, low capacity component of extraneuronal uptake has been provided in a recent analysis of the uptake and metabolism of ^{3}H-isoprenaline in rat and guinea pig hearts by Trendelenburg and his associates. When hearts were perfused at a constant rate with low concentrations of ^{3}H-isoprenaline (0.95μmol l^{-1}), a steady state rate of removal of ^{3}H-isoprenaline from the perfusing medium and a steady state rate of accumulation of ^{3}H-isoprenaline in the tissue was reached only slowly, in about 25 to 30 minutes. In contrast, the rate of O-methylation of ^{3}H-isoprenaline was constant virtually from the beginning of perfusion; the initial rate of O-methylation was equal to the steady state rate. The absence of any parallelism between the ^{3}H-isoprenaline content of the heart and the rate of O-methylation suggested that these two processes occurred in different compartments within the tissue (Bonisch and

Trendelenburg, 1974). Both the removal of ^{3}H-isoprenaline from the perfusion fluid and the rate of appearance of the O-methylated product (^{3}H-3-methoxyisoprenaline (^{3}H-MeOISO)) in the venous effluent were mediated by saturable processes. The removal process was kinetically very similar (Km = 20.7μmol l^{-1}, Vmax = 38.4nmol g^{-1} m^{-1}) to the "uptake$_2$" of isoprenaline described earlier by Callingham and Burgen (1966). On the other hand, the rapidly equilibrating COMT-containing compartment was characterised by its high affinity but low capacity for O-methylation (Km = 2.87μmol l^{-1}, Vmax = 1.68nmol g^{-1} m^{-1}). Both the low affinity amine storage compartment and the readily saturable COMT compartment were sensitive to inhibition by corticosterone (Bonisch et al, 1974). Further evidence for a two compartment extraneuronal uptake system was obtained by Bonisch et al (1974) in experiments in which rat hearts were perfused for 30 minutes with low concentrations of ^{3}H-isoprenaline (0.95μmol l^{-1}) in the presence of a COMT inhibitor, and the efflux of isoprenaline measured during subsequent perfusion with an amine-free medium. ^{3}H-isoprenaline effluxed from at least two compartments in the heart, one associated with a short half time (10.1 minutes) and the other with a longer half time of efflux (22.6 minutes). In similar experiments, but in which COMT was not inhibited, Uhlig et al (1974) measured the efflux of both ^{3}H-isoprenaline and ^{3}H-MeOISO and

concluded that there was little or no COMT activity in the compartment with the long half time of efflux (the amine storage compartment). The cumulative efflux of ^{3}H-MeOISO was 3 to 4 times higher than the level of ^{3}H-MeOISO present in the heart at the end of perfusion with ^{3}H-isoprenaline, suggesting that most of the O-methylation occurred during washout. The latter observation must have complicated any interpretation of the origin of ^{3}H-MeOISO which effluxed from the heart. Nevertheless, the convexity of the efflux curve was consistent with efflux from a partially saturated compartment, such as the high affinity COMT-containing compartment (Km = 2.87μmol l^{-1}) of Bonisch et al (1974). This hypothesis was supported by the very marked convexity of the ^{3}H-MeOISO efflux curve after perfusion of the heart with a 30-fold higher concentration of ^{3}H-isoprenaline.

Although the amine storage compartment seemed to possess little or no COMT activity, the COMT compartment was able to store unchanged amine. This was suggested by the failure of corticosterone, when present during washout only, to decrease the efflux of ^{3}H-MeOISO. Since most of the O-methylation occurred during washout, its lack of dependence on the steroid-sensitive fluxes suggested that it proceeded via a store of unchanged amine already present in the COMT compartment at the end of perfusion with ^{3}H-isoprenaline (Uhlig et al, 1974).

The morphological arrangement of the compartments postulated in these studies was not determined. However, it is known that at least two structures in the heart (cardiac and smooth muscle) are able to accumulate catecholamines. Interestingly, the accumulation of ^{3}H-isoprenaline and the formation of ^{3}H-MeOISO in the isolated ventricular strips of the rat heart were 1/4 and 1/50 respectively, of the corresponding values for the intact perfused heart. Therefore Bonisch et al (1974) suggested that the O-methylating compartment may be largely confined to the vascular smooth muscle of the rat heart. This may also provide an explanation for the rapid equilibration of the COMT compartment with the concentration of ^{3}H-isoprenaline in the perfusing medium. On the other hand, their evidence does not exclude the possibility that ^{3}H-isoprenaline was distributed to COMT sites or stored as unchanged amine after transport to a unique extraneuronal structure.

Comparable information on vascular smooth muscle is by no means as detailed as that now available from the experiments just described. However, in an earlier pharmacological study, Kalsner (1969a) showed that both hydrocortisone and a COMT inhibitor (UO521) enhanced the sensitivity of the rabbit aortic strip to catecholamines and that each agent failed to potentiate in the presence of the other. Levin and Furchgott (1970)

confirmed the potentiating action of hydrocortisone and the failure of COMT inhibition to sensitise the rabbit aortic strip in the presence of the steroid. These effects were similar to those of DOCA on the sensitivity of the rabbit ear artery to catecholamines described in the present study. However, neither Kalsner nor Levin and Furchgott examined the relationship between amine concentration and the potentiation by the steroid. The present results indicated a decline in the potentiation by DOCA when the concentration of amine was increased to approximately 3-5μmol l^{-1}. The latter observation resembled those of hydrocortisone in the cat heart (Kaumann, 1972) and cat nictitating membrane (Graefe and Trendelenburg, 1974). The interactions between steroids and COMT inhibitors and the dependence of the sensitising action of the steroids on relatively low concentrations of amines (Table 7.2) are consistent with the biochemical studies in rat heart (discussed above) and suggest the presence of a steroid-sensitive extraneuronal uptake mechanism which is readily saturable and which can reduce the concentration of catecholamines in the region of the receptors only when COMT is functionally intact. It should be noted that the evidence for this mechanism in the rabbit ear artery is based largely on experiments with adrenaline. Whether the concentration of NA is also important in the potentiating action of DOCA and whether COMT activity is essential for the DOCA-sensitive

mechanism to influence the concentration of NA and isoprenaline in the biophase remain to be determined. Nevertheless the pharmacological evidence in the ear artery and other tissues (Table 7.2) presents a reasonably coherent pattern in support of the concept of a readily saturable COMT-dependent extraneuronal inactivating mechanism. However, it is unwise to generalise too far since, as is evident from the summary in Table 7.3, there are important differences between tissues which still require explanation. For instance, the cat nictitating membrane appears to possess two O-methylating compartments, only one of which is sensitive to hydrocortisone (Graefe and Trendelenburg, 1974). It is possible that the hydrocortisone-resistant COMT compartment contributed to some of the unexpected properties of the nictitating membrane, since whereas hydrocortisone potentiation was ranked NA>A>1SO, the reverse order was observed for the effects of a COMT inhibitor. Furthermore, the sensitivity to isoprenaline was enhanced by hydrocortisone only when the concentration of the latter was ten-fold greater than that which potentiated the sensitivity to NA and adrenaline. The biochemical evidence in the present study showed that the conditions under which DOCA maximally sensitised the ear artery were those where it decreased O-methylation of ^{3}H-isoprenaline but did not appreciably alter the accumulation of unchanged amine. This emphasises the probability that COMT activity

Table 7.2 Effects of steroids on the sensitivity to catecholamines

Reference	Tissue	Steroid	Potentiating Effect	Effect on COMT inhibitor	Dependence on COMT activity	Relation to amine concentration
Kaumann (1972)	cat heart muscle	hydrocortisone (HC) 25 μmol l^{-1}	potentiated ISO and NA (in absence of neuronal uptake). ISO>NA	reduced potentiating effect of COMT inhibitor (U0521)	not determined	potentiation diminished when amine concentration increased to 1μmol l^{-1}
Graefe and Trendelenburg (1974)	cat nictitating membrane	hydrocortisone (HC) 28 μmol l^{-1}	potentiated NA and A (in absence of neuronal uptake) and ISO NA>A>ISO	not determined	potentiation abolished in presence of COMT inhibitor (U0521)	potentiation diminished when amine concentration increased to 1-10μmol l^{-1}
Kalsner (1969a)	rabbit aortic strip	hydrocortisone (HC) 25 μmol l^{-1}	potentiated A and NA (in presence and absence of neuronal uptake) and ISO ISO≥A>NA	abolished potentiating effect of COMT inhibitor (U0521)	potentiation abolished in presence of COMT inhibitor (U0521)	not determined
Present study	rabbit ear artery	DOCA 27 μmol l^{-1}	potentiated sensitivity to A and NA (in presence and absence of neuronal uptake) and to ISO A>ISO>NA	reduced or abolished potentiating effect of COMT inhibitor (U0521) - *except effect of U0521 on sympathetic nerve stimulation	potentiation abolished in presence of COMT inhibitor (U0521)	potentiation diminished when amine concentration increased to 3-5 μmol l^{-1}.

Table 7.3 Multicompartmental extraneuronal uptake and O-methylation

Reference	Tissue	Compartment	Steroid Sensitivity	Compartment	Steroid Sensitivity
Bonisch and Trendelenburg (1974); Bonisch et al (1974); Uhlig et al (1974) *Graefe et al (1975)	perfused rat heart	rapidly equilibrating short $t_{½}$ of efflux of ISO high affinity, low capacity for O-methylation of ISO: Km = 2.87 μmol l^{-1} Vmax = 1.68 nmol $g^{-1}m^{-1}$ storage of unchanged amine restricted by COMT activity	sensitive to corticosterone (competitive, *Ki = 2.0 μmol l^{-1})	slowly equilibrating long $t_{½}$ of efflux of ISO low affinity, high capacity for storage of ISO: Km = 20.7 μmol l^{-1} Vmax = 38.4 nmol $g^{-1}m^{-1}$ little or no COMT activity	sensitive to corticosterone (competitive, *Ki = 4.0 μmol l^{-1})
as above	perfused guinea pig heart	qualitatively similar to rat heart by approx. 1/10th O-methylating activity	sensitive to corticosterone	qualitatively similar to rat heart but approx. 1/10th amine storage capacity	sensitive to corticosterone
Graefe and Trendelenburg (1974)	cat nictitating membrane	rapidly equilibrating high affinity for O-methylation: NA : Km = 7.5 μmol l^{-1} Vmax = 0.75 nmol $g^{-1}m^{-1}$ ISO : Km = 12.8 μmol l^{-1} Vmax = 3.3 nmol $g^{-1}m^{-1}$ storage of unchanged amine restricted by COMT activity	sensitive to hydrocortisone (non-competitive, HC depressed Vmax (ISO) by 36%; Vmax (NA) by 62%)	slowly equilibrating low affinity for O-methylation: NA : Km = 131 μmol l^{-1} Vmax = 8.5 nmol $g^{-1}m^{-1}$ ISO : Km = 201 μmol l^{-1} Vmax = 2.9 nmol $g^{-1}m^{-1}$ storage of unchanged amine restricted by COMT activity	resistant to hydrocortisone
Trendelenburg (1973)	rabbit aorta	COMT compartment storage of unchanged amine (NA) restricted by COMT activity short $t_{½}$ of efflux of NA (when COMT inhibited).	sensitive to corticosterone	little or no COMT activity long $t_{½}$ of efflux of NA	resistant to corticosterone
Present study	rabbit ear artery	Compartments? Pharmacological evidence (Table 7.1,2) consistent with presence of high affinity, low capacity DOCA-sensitive COMT compartment c.f. low affinity, high capacity steroid-sensitive extraneuronal uptake of catecholamines described in earlier histochemical studies (Gillespie and Towart, 1973; Nicol and Rae, 1972)			

is important to the steroid-sensitive process which removes catecholamines from the biophase. The fact that DOCA still decreased uptake of ^{3}H-isoprenaline under conditions where it did not sensitise the artery (i.e., in the presence of a COMT inhibitor) suggests that in this artery the uptake of unchanged amine is not, per se, a process which reduces the concentration of catecholamines at the receptors. This is explicable if, in association with accumulation of amine there is an increased efflux of amine back into the region of the receptors. This concept accords with the findings of Bonisch et al (1974) in the rat heart, and has also been advanced by Trendelenburg (1974) in a recent analysis of the effects of a COMT inhibitor on the kinetics of relaxation of the rabbit aortic strip after exposure to catecholamines. These considerations make no assumptions about compartments in the rabbit ear artery other than a compartment represented by the biophase and a compartment in which ^{3}H-isoprenaline accumulates and in which accumulation is normally limited by COMT.

Whether the concept, described earlier in this discussion, of more than one extraneuronal uptake compartment, also applies to the ear artery, remains to be determined. However, it should be noted that the high affinity, low capacity DOCA-sensitive mechanism appears at variance with the low affinity, high

capacity steroid-sensitive system described in earlier histochemical studies (Gillespie and Towart, 1973; Nicol and Rae, 1972). The high threshold for accumulation of unchanged amine in the latter studies may be due in part to inactivation by MAO and COMT (Burnstock et al, 1971; present study, Chapter 3) and to the relative insensitivity of the Falck histochemical procedure. Nevertheless, the evidence of Gillespie and Towart (1973) for an extraneuronal mechanism of high capacity for accumulation of unchanged amine in the rabbit ear artery is indisputable. This may be reconciled with the low capacity system, suggested by the results of the present study, in the following way. The evidence indicates that the extraneuronal transport system is inhibited by steroids at all concentrations of extracellular amine. Hence it may be assumed that the steroid-sensitive transport system is capable of removing amine from the region of the receptors at all extracellular amine concentrations. At low concentrations, the subsequent O-methylation ensures that the removal results in a net decrease in the amine concentration at the receptors. At high concentrations, amine which escapes metabolism by MAO and COMT accumulates in the unchanged form to a level which results in an increased rate of efflux back into the region of the receptors. Under these conditions, inhibition of extraneuronal uptake by the steroid, by preventing unchanged amine from accumulating, will automatically

eliminate the efflux process which tends to augment the concentration of the amine in the receptor biophase. This explanation assumes that the amine accumulates to a level which saturates the low capacity, COMT-containing compartment before it is accessible to deamination by extraneuronal MAO. In this sense a two compartment system for storing unchanged amine is implied, according the scheme outlined in Fig. 7.1. The experiments described in Chapter 3 indicated that inhibition of extraneuronal MAO resulted in delayed recovery of arteries from the constrictor response to high concentrations of extraluminal NA (118μmol l^{-1}). One possible explanation for this observation is that following washout of the NA from the extraluminal bathing medium, the NA which had accumulated in the MAO containing compartment (2) diffused into the COMT compartment (1) and thus indirectly maintained a high rate of efflux of unchanged amine from the latter compartment to the receptors.

Interaction between neuronal and extraneuronal uptake

The nature of the relationship between neuronal and extraneuronal uptakes is best considered in terms of the morphological concept advanced by de la Lande et al (1970b;1974) to explain many of the features of neuronal control in the artery. This model, the evidence for which was presented in the Introduction (p.24-30), is reproduced in Fig. 7.2. Its

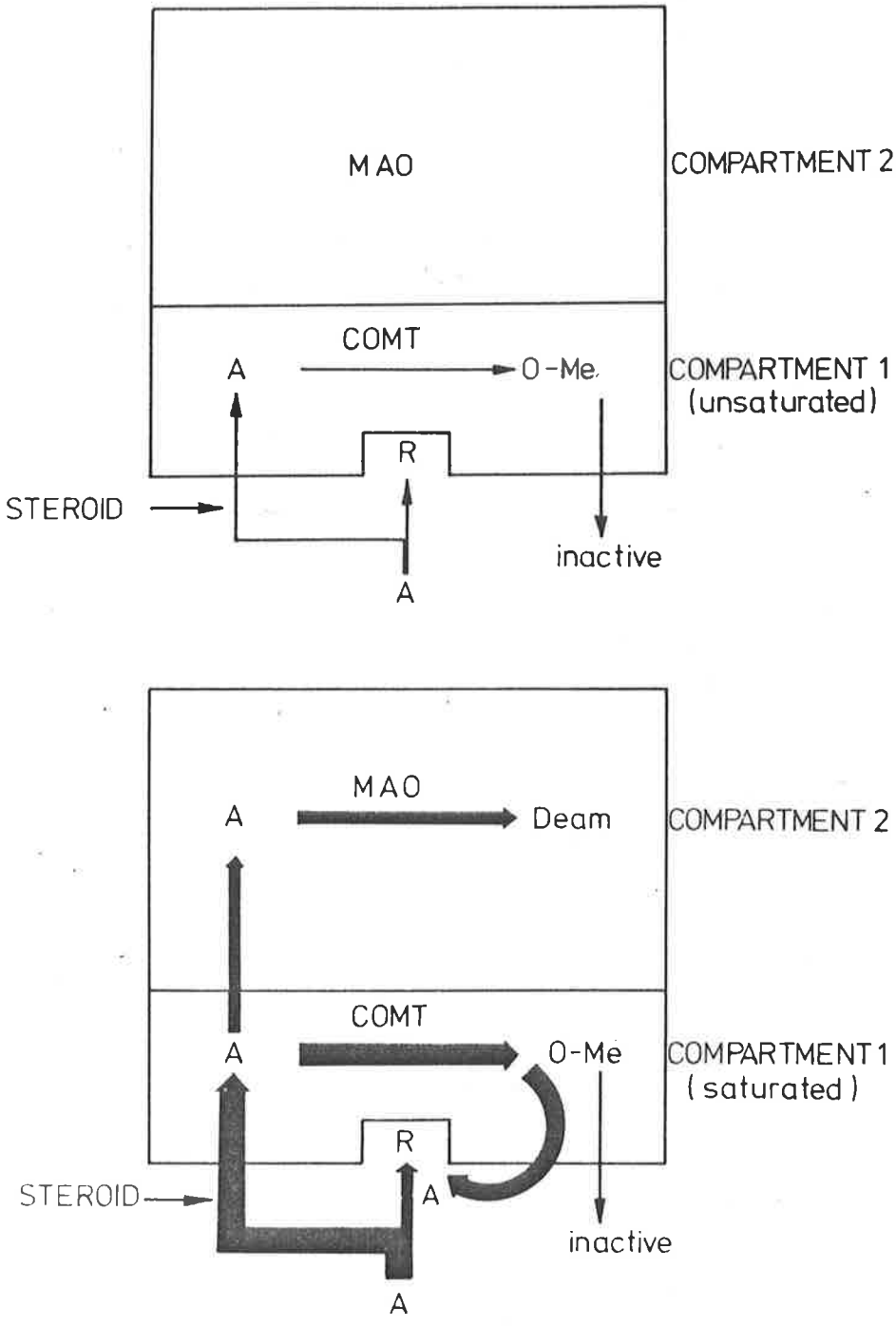


Fig. 7.1 Possible arrangement of a two compartment extraneuronal system in the rabbit ear artery. Concentrations of adrenaline (A) are represented by the thickness of the arrows, the directions of which indicate fluxes into and out of the smooth muscle cell. A = adrenaline; R = receptor; O-Me = inactive O-methylated product; Deam = inactive deaminated product.

essential feature is that there is a diffusion gradient of amine across the artery wall, due in part to uptake and O-methylation of amine in the media, which implies that responses to intraluminally and extraluminally applied catecholamines are mediated largely by smooth muscle cells in different regions of the artery wall, i.e. by those cells closest to the surface to which the amine is applied. The actions of DOCA described in this thesis appeared to be compatible with this model. These actions included the tendencies of DOCA (i) to selectively enhance the sensitivity to extraluminal adrenaline once neuronal uptake was decreased, and (ii) to reduce the difference between sensitivities to extraluminal and intraluminal adrenaline which persisted after neuronal uptake was decreased. The selective effect of DOCA on extraluminal adrenaline in the absence of neuronal uptake was explicable in terms of the greater activity of the uptake system in the outer cells, an activity which was masked when neuronal uptake was functionally intact. The tendency of DOCA to decrease the residual difference between the sensitivities to extraluminal and intraluminal amines in the absence of neuronal uptake is illustrated in the case of adrenaline in Fig. 7.3 In the untreated artery, equieffective concentrations of extraluminal adrenaline were approximately 4.5-fold greater than those to intraluminal adrenaline. Treatment with cocaine reduced this ratio to approximately

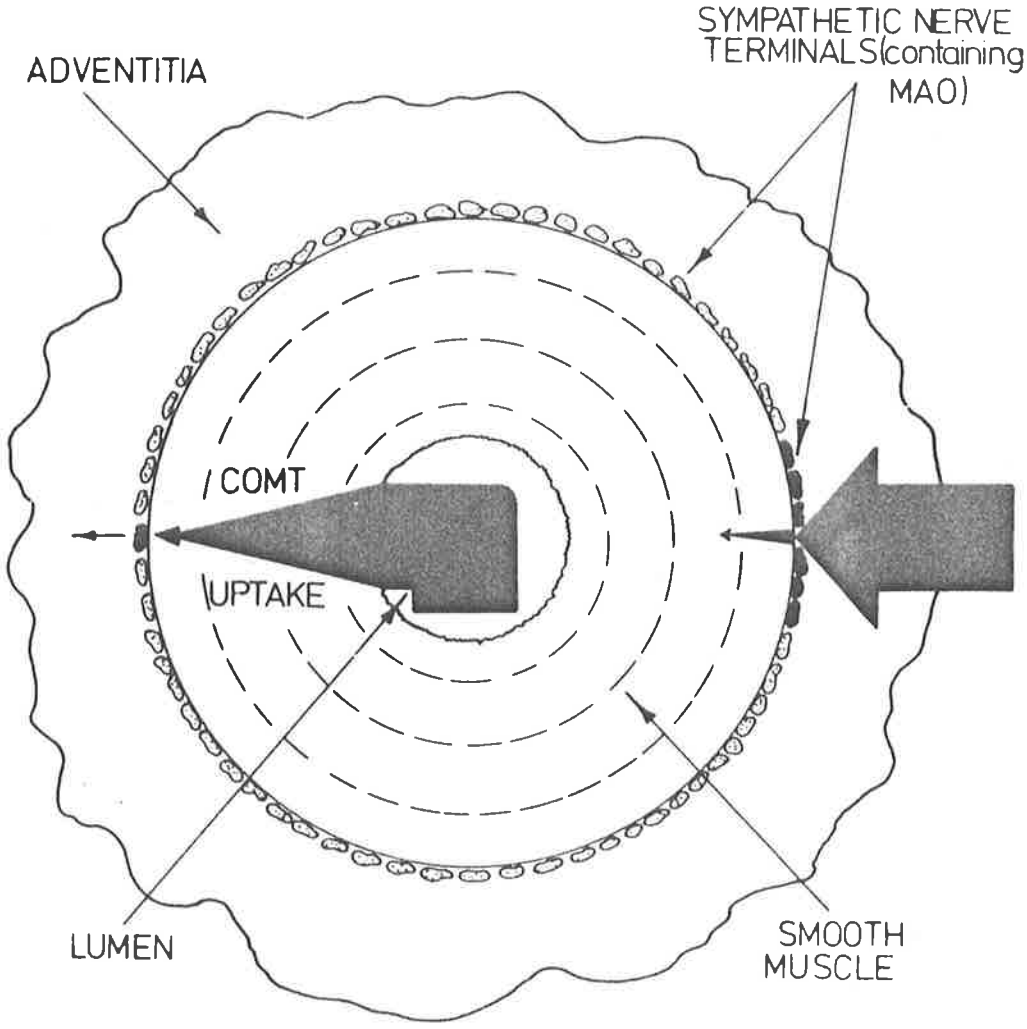


Fig. 7.2 Diagrammatic representation of the factors which may influence the concentration at receptor sites in the smooth muscle of the artery. The directions of arrows indicate the directions of diffusion of NA. Thickness of arrows represent concentrations of NA. Uptake by the sympathetic nerve terminals and subsequent deamination results in a loss of concentration of extraluminal NA. There is a gradient of concentration of NA across the artery wall due in part to extraneuronal uptake and O-methylation in the media.

1.6-fold. The residual difference was further reduced to 1.2-fold after treatment with DOCA. Thus the difference between the sensitivities of the receptors on smooth muscle cells mediating the responses to extraluminal and intraluminal adrenaline became minimal when the influence of extraneuronal, as well as neuronal, uptake was taken into account. Although fewer observations were made, a similar trend was evident for adrenaline in denervated arteries and for NA in cocaine-treated arteries. In each case the ratio of equieffective concentrations of extraluminal and intraluminal amine was not significantly different from 1.0 after treatment with DOCA (see Table 4.3 (p.70) and 4.4c (p.75)). These effects are difficult to interpret in terms of an earlier model (see Introduction, p.23 and Fig. 1.2) which assumed that the responses to intraluminally and extraluminally applied amines were mediated by the entire smooth muscle mass, i.e., by the same population of target calls. Accordingly, regional differences in the activity of an extraneuronal uptake system should not be reflected in different effects of an inhibitor on the sensitivities to an amine according to the site of entry of the latter into the artery wall.

During the course of this study, the neuronal uptake concept of the control of sensitivity depicted in Fig. 7.2 has been criticised by Kalsner (1972) and by Yong and Chen (1975). Kalsner suggested, probably correctly, that the steady state

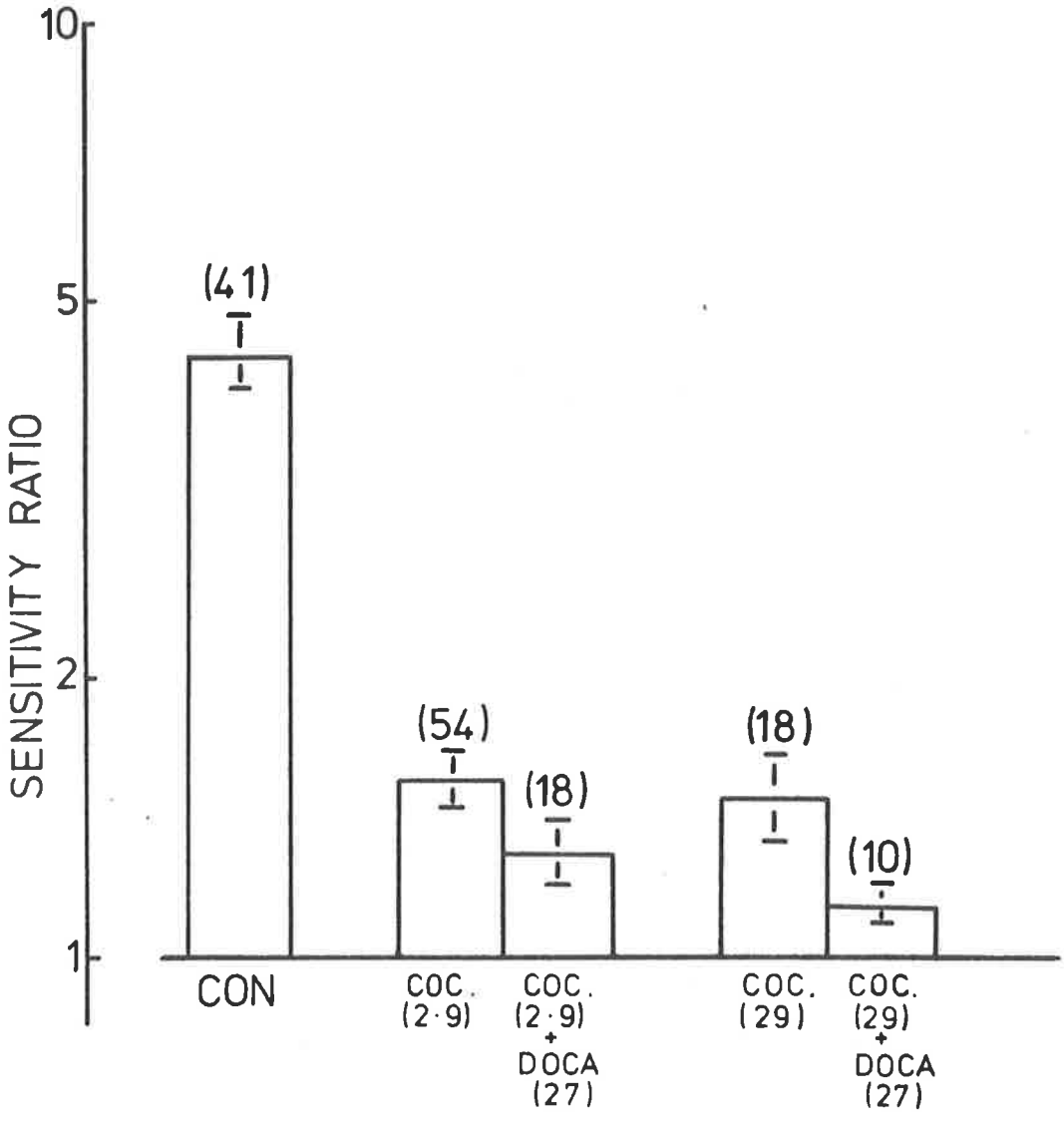


Fig. 7.3 Effect of cocaine and of combined treatment with cocaine and DOCA on the ratio (geom. mean + S.E.) of the concentrations of extraluminal and intraluminal adrenaline which were equieffective in eliciting increases in the perfusion pressure of 8×10^3 Nm^{-2}. CON = untreated control arteries. Cocaine (coc) and DOCA were applied to both the intraluminal perfusate and extraluminal bathing fluid in the concentrations shown ($\mu mol\ l^{-1}$). The number in parentheses above each column refers to the number of observations.

distribution of NA in the artery wall was not uniform. He further proposed that the high sensitivity to intraluminal NA might be due, at least in part, to supersensitivity arising from chronic deprivation of a sympathetic innervation (termed "postsynaptic" by Trendelenburg (1966) but more recently defined as postjunctional non-deviation supersensitivity by Fleming (1975)). Implicit in the latter proposal is that the sensitivity of the inner cells is normally considerably greater than that of the outer cells. However, as has been noted above, the present results suggest that for NA and adrenaline at least, the sensitivities of the outer and inner cells are nearly equal when both neuronal and extraneuronal inactivations are taken into account. Yong and Chen (1975) found that although the artery was more sensitive to intraluminal compared with extraluminal NA, this did not apply to several other sympathomimetic amines, namely metaraminol, phenylephrine, methoxamine, metanephrine and isoprenaline, which were 1.5 to 3-fold more potent when applied extraluminally. Agents such as cocaine, reserpine and 6-hydroxydopamine, which impaired neuronal inactivation, selectively potentiated the sensitivity to extraluminal NA but not to the other amines. However treatment with 6-hydroxydopamine caused small (1.5 to 2-fold) potentiations when several of the amines were applied intraluminally. Yong and Chen therefore suggested that uptake by the sympathetic nerve terminals at the medial-adventitial

border was not a major factor contributing to the differences in the sensitivities to extraluminal and intraluminal applications of each of these amines. However, with the exception of metaraminol, the amines have low affinities for neuronal uptake (Iversen, 1967) and therefore the failure of removal of neuronal uptake to selectively potentiate the sensitivities to extraluminal applications is not unexpected. Thus although the failure of cocaine to potentiate extraluminal metaraminol warrants further investigation, their data does not provide convincing evidence against the functional importance of neuronal uptake. It is difficult to assess the significance of their findings that the artery was more sensitive to extraluminal compared with intraluminal applications of these amines, since they differ quantitatively from observations of several investigators at different times in this laboratory. For instance, de la Lande et al (1970b) showed that the artery was equally sensitive to extraluminal and intraluminal methoxamine. Similarly the constrictor potencies of isoprenaline did not differ significantly when this amine was applied by different routes (de la Lande and Johnson, unpublished observations). Different experimental conditions may account for the discrepancies, e.g. the use of slower rates of perfusion by Yong and Chen ($2cm^3 min^{-1}$ compared with $4.5\text{-}6cm^3 min^{-1}$ in this laboratory) and possibly of a different type of rabbit (semi-lop-eared in the present study

but not specified in that of Yong and Chen). Unfortunately they did not examine the influence of extraneuronal uptake. The apparent discrepancy may reflect quantitative variations in the regional distribution of extraneuronal uptake with different types of rabbit and perhaps also with different rates of perfusion. Here it is relevant to note that de la Lande et al (1971) found a tendency for a COMT inhibitor (UO521) to exert a more marked effect on the sensitivity to intraluminal NA in nialamide-treated ear arteries when the perfusion rate was decreased from 6.5 to $1.5 cm^3 \ min^{-1}$.

The simplest way of interpreting the present findings appears to be in terms of the model shown in Fig. 7.2, in which a major functional role is ascribed to neuronal uptake. The final question to be discussed is the functional significance of extraneuronal uptake. The maximum potentiation of NA by DOCA was approximately 2-fold when the influence neuronal uptake was removed by treatment with cocaine, suggesting that, at most, extraneuronal uptake can reduce the concentration of NA at its receptors by 50%. In the case of adrenaline where the maximum potentiation by DOCA was about 6-fold in denervated arteries, the corresponding influence of extraneuronal uptake is 83%. These estimates are based on the assumption that in cocaine-treated or

denervated arteries, extraneuronal uptake was the only mechanism which exerted any appreciable influence in reducing the concentration of amine at the receptors. It therefore seems possible that extraneuronal inactivation is directed to adrenaline after the latter diffuses into the artery wall from the circulation. Although extraneuronal uptake seems quantitatively less important for NA compared with adrenaline, the evidence that the activity of this system is greater in the outer smooth muscle cells raises the possibility that extraneuronal uptake may serve a minor role in removing the neurotransmitter from the extracellular space of the outer cells.

In conclusion, it is evident that much further investigation is required before the precise roles of neuronal and extraneuronal uptakes and their interrelationship may be defined. The present study points to some of the directions in which such investigations should proceed. For instance, the possibility that binding to collagen and elastin may also influence the concentration of amines at their receptors needs further consideration. Powis (1973) proposed this mechanism on the basis of the potentiating effect of an inhibitor (oxytetracycline) on the sensitivity of the ear artery to exogenous NA and to sympathetic nerve stimulation. However other experiments in this laboratory have failed to show an appreciable effect of

oxytetracycline on the steady state response of the artery to NA (de la Lande, private communication). Perhaps the most important requirement is for detailed kinetic studies on the properties of the extraneuronal uptake system, which may help to relate pharmacological evidence indicating that the functionally important component is represented by a high affinity, low capacity system, and the histochemical evidence that the artery possesses a low affinity, high capacity system for accumulating catecholamines. In this respect the detailed studies of Trendelenburg and his associates on other tissues (discussed above) offer a valuable guide to further experiments on the rabbit ear artery.

PART II

CHAPTER 8

PRELIMINARY STUDIES IN EXPERIMENTAL HYPERTENSION

INTRODUCTION

It is generally agreed that increased resistance in the peripheral vessels is a dominant factor in most forms of hypertension. The increased resistance is probably a consequence of a decrease in the internal diameter of such vessels (Lundgren, 1974). However, the mechanisms responsible for the arteriolar narrowing remain obscure. Considerable effort, using a variety of experimental models (see below), has been directed to the question of vascular hyper-responsiveness, since this is an often observed phenomenon in human hypertension (Doyle, 1968; Mendlowitz, 1967). However, while enhanced vascular reactivity occurs frequently, but not inevitably, in experimental hypertension, the underlying mechanisms are elusive or controversial. There are at least three dominant views. Firstly, the vasculature may respond in a normal way to elevated levels of constrictor stimuli. Secondly, the vascular smooth muscle cells may be hypersensitive to normal levels of constrictor stimuli. Thirdly, structural alterations in the vascular wall could alone account for the phenomenon.

An extensive theoretical and experimental analysis of the consequences of structural alterations of resistance vessels has been provided (Folkow et al, 1958; Conway, 1958; Folkow et al, 1973; Sivertsson, 1970; Lundgren, 1974). If medial thickening occurs,

resulting in an increased wall to lumen ratio in the hypertensive vessel, enhanced vascular reactivity will ensue as a direct consequence. The term "vascular reactivity" in this sense, should be applied to the vessel as a whole, since it is entirely structurally based and does not necessitate any increase in the reactivity of individual smooth muscle cells. This concept has been derived in part from studies of systemic pressor responses in the Okamoto strain of genetically hypertensive rat. The evidence for the importance of structural changes in this form of hypertension is strengthened by observations that, whereas systemic pressor responses are consistently enhanced, isolated strip preparations from similar animals often do not display heightened sensitivity to constrictor agents (Hallback et al., 1971; Shibita et al, 1973; Spector et al, 1969). Folkow et al (1973) have proposed that intermittent pressure and cardiac output arises elicited by the central nervous system might be factors involved in triggering such structural alterations in genetically predisposed individuals.

While alterations in the wall to lumen ratios in resistance vessels may be of fundamental importance in the spontaneously hypertensive rat, other explanations have been invoked to account for the increase in vascular responsiveness observed in other studies. Enhanced cellular excitability was postulated by

Bandick and Sparks (1970) to explain the lower threshold to NA and the increase in frequency of spontaneous contractions in strips of femoral arteries from rats with renal hypertension. In the isolated ventral artery of rats with DOCA/NaCl hypertension, Hinke (1966) observed enhanced reactivity to KCl and to NA. The contractions were more difficult to abolish in Ca-free solution in the hypertensive compared with normotensive arteries, and could be re-established with lower Ca concentration in the hypertensive arteries. These findings suggested an increased vascular smooth muscle permeability to Ca, a view supported by Holloway and Bohr (1973) in studies of femoral artery strips of rats with renal and DOCA/NaCl hypertension. The increased incidence of spontaneous rhythmic activity observed by Holloway and Bohr provided further evidence of an alteration in the functional properties of the cell membrane in vascular smooth muscle in these forms of hypertension. Finch and Haeusler (1974) studied the vascular reactivity in the perfused whole animal preparation and in isolated perfused hindquarters of renal, DOCA/NaCl and spontaneously hypertensive rats. In each case vascular reactivity was enhanced compared with normotensive control rats. In the genetically hypertensive animals this was manifest by steeper slopes and increased maxima, without change in the threshold of concentration response curves to NA. These changes were in full agreement with the

concepts of Folkow et al (1973), relating the enhanced reactivity to an increase in the wall to lumen ratio of the resistance vessels. However, in renal and DOCA/NaCl hypertensive rats, similar changes in slopes and maxima were also accompanied by a lower threshold to NA. This finding led Finch and Hauesler to postulate that enhanced vascular reactivity in these forms of experimental hypertension was the consequence not only of structural alterations but also of enhanced cellular reactivity, possibly reflecting an alteration in excitation-contraction coupling in smooth muscle cells.

Hypertension and the Sympathetic Nervous System

Reed et al (1944) first suggested that the importance of the renal pressor system might be superceded in the chronic phase of renal hypertension by a neurogenic component mediated by the sympathetic nervous system. McQueen (1956) noted that increased vascular reactivity to NA in the perfused hindlimb was a factor common to both renal and renoprival hypertension in the rat. The importance of a neurogenic component to the maintenance of increased peripheral resistance in rats with renal and spontaneous hypertension was further suggested by Laverty and Smirk (1961), on the basis of the ability of hexamethonium to abolish the difference in peripheral resistance between hypertensive and normotensive control animals.

McCubbin and Page (1963) demonstrated that small amounts of angiotensin enhanced pressor responses to tyramine in the dog and postulated that this action could account for the neurogenic component in chronic renal hypertension. This view was supported by observations that pressor responses to tyramine were strikingly enhanced in dogs with both acute and chronic renal hypertension, whereas those to exogenous NA were little affected (Page et al, 1966). These observations suggested that hypertension might be maintained by a defective storage mechanism of endogenous NA in peripheral sympathetic nerves.

Advances in the understanding of adrenergic mechanisms permitted a more direct analysis of whether experimental hypertension was associated with a disturbance in amine metabolism which might lead to elevated concentrations of NA at receptor sites in sympathetically innervated tissues. The findings, particularly pertaining to DOCA/NaCl hypertension in rats, have been extensively reviewed by de Champlain (1972). In the hearts of these animals, although the initial uptake of exogenous NA appeared normal, the endogenous NA levels and the ability to retain exogenous NA were severely impaired. In addition, the turnover of NA was significantly increased. It was proposed that these abnormalities might lead to elevated concentrations of NA in the region of the receptors. The increased levels of NA and its associated metabolites in the

urine of hypertensive rats following intravenous injection of NA were in agreement with this view. On the basis that the increased rate of turnover appeared to precede the development of hypertension, it was further implied that such abnormalities could contribute directly to the development of elevated blood pressure. An increased turnover of NA has also been demonstrated in the hearts of rats with renal hypertension (Volicer et al 1968).

Following the discovery that 6-hydroxydopamine (6OHDA) selectively destroyed sympathetic nerve terminals (Thoenen and Tranzer, 1968), this agent has been employed in several attempts to assess the contribution of the sympathetic nervous system to the development and maintenance of various forms of experimental hypertension. The results of these studies are somewhat conflicting. Whereas de Champlain (1972) and de Champlain and van Ameringen (1972) reported the failure of DOCA hypertension to develop in rats injected with 6-OHDA, Finch and Leach (1970a,b), in similar studies, reached opposite conclusions. However in the former studies rats were treated repeatedly from birth with 6-OHDA to ensure sustained sympathectomy. In contrast, Finch and Leach administered treatment for a shorter period and in older animals. The discrepancy may be due in part to the rapid regeneration of sympathetic terminals

following a brief treatment and to the relative resistance of older animals to chemical sympathectomy (Finch et al, 1973a,b). It is of interest that Grewal and Kaul (1971) reported that cardiac catecholamine stores of rats injected as weanlings with 6-OHDA remained severely depleted 10 weeks after cessation of treatment whereas those of adult rats were significantly replenished after 8 weeks. In these studies 6-OHDA prevented the development of renal hypertension in weanling rats but failed to appreciably alter the development in adult rats. In accord with the findings of Grewal and Kaul (1971), sustained renal hypertension was not produced in "total" immunosympathectomised rats (Ayitey-Smith and Varma, 1970). Significant retardation in the rate of development of renal hypertension was also apparent in the studies of Finch and Leach (1970a). More recently, intravenous injections of 6-OHDA have also been reported to prevent the development of spontaneous hypertension in rats (Vapaatolo et al, 1974). 6-OHDA, however, did not reverse well established hypertension. These observations were interpreted in terms of a centrally mediated action of 6-OHDA, since they were in accord with the studies of Hauesler et al (1972), in which 6-OHDA, injected directly into the left lateral brain ventricle of rats, impaired developing, but not well established renal, DOCA, and spontaneous hypertension. Hauesler et al postulated that central adrenergic neurones served to "trigger" changes leading to hypertension which was maintained independent

of central mechanisms. In contrast, Chalmers et al (1974) provided evidence that central adrenergic neurones participated in both the development and maintenance of renal hypertension in rabbits. The evidence for the participation of the sympathetic nervous system in experimental hypertension is supported by ultrastructural studies, indicating proliferation of sympathetic neurones supplying the renal and pancreatic arterioles of hypertensive animals (Burnstock et al, 1970; Graham et al, 1970).

In view of the probable importance of the sympathetic nervous system in the pathogenesis of experimental hypertension, other studies have examined vascular reactivity with reference to the relative influences of mechanisms responsible for the control of NA sensitivity in hypertensive and normotensive animals. However, while vascular reactivity to NA and other amines is frequently enhanced, in few instances has it been possible to associate this with a specific abnormality in metabolism. An exception is the study of Page et al (1966), in which pressor responses to tyramine were specifically enhanced, suggesting abnormal storage of NA in chronic renal hypertension in dogs. However, in the pithed preparation of DOCA hypertensive rats, Finch (1971) observed a generalised increase in cardio-vascular reactivity. A specific cause for the increase was not apparent, but it was suggested to be largely independent of the

influence of neuronal uptake. In the same study, responses to NA, but not nerve stimulation were enhanced in the isolated perfused mesentery of renal hypertensive rats. The latter findings were in agreement with those of Hauesler and Haefely (1970) who suggested arterial wall thickening rather than impairment of neuronal uptake as the significant factor contributing to the increase in reactivity. Kalsner et al (1971) were unable to explain enhanced responses to NA in the rabbit aortic strip on the basis of an alteration in the activity of catechol-O-methyl transferase (COMT), which constitutes a major mechanism of control of noradrenaline sensitivity in this preparation. It is nevertheless of interest that Trajkov et al (1974) and Creveling et al (1969) measured increased COMT activity in homogenates of various tissues in rats with renal and spontaneous hypertension.

The difficulty in interpreting results from hypertension studies is due in part to the multifactorial basis of the disease (Page, 1974). As such, the contribution of a particular system to vascular reactivity may be distorted by numerous other facets. In the present study, an attempt was made to analyse the effects of experimental hypertension in rabbits on the sensitivities of the ear arteries to intraluminally and extraluminally applied NA and adrenaline, and on the changes produced in these sensitivities by cocaine and by DOCA. In

this way it was hoped to assess whether the properties of neuronal and extraneuronal uptake systems were modified by the presence of a sustained increase in perfusion pressure in vivo. Unfortunately the investigation was limited by the high mortality rate in these rabbits. However the data are presented here as they point to some trends which may form the basis of future experiments.

METHODS

The most widely used models of experimental hypertension are: (a) strains of genetically hypertensive rats (Smirk and Hall, 1958; Okamoto and Aoki, 1963), (b) rats treated with deoxycorticosterone acetate (DOCA) and salt (Seyle, 1942) and (c) animals with renal hypertension, induced either by partial occlusion of the renal artery (Goldblatt, 1960; Pickering and Prinzmetal, 1938) or by the formation of a fibroblastic perinephritis (Page, 1939; Page et al, 1955). The method of Page et al (1955) by which perinephric hypertension is induced by cellophane wrapping of the kidneys was employed in this investigation for two series of rabbits. In the first series, hypertension was induced in weanling (5-10 week-old) rabbits of New Zealand White-Oxford lop cross breeding, obtained from the Animal Breeding Establishment, John Curtin School of Medical Research, Canberra. Ear arteries from these animals were made available by Dr. E.G. Cleary,

Department of Pathology, University of Adelaide, during a study of the effects of hypertension on the collagen and elastin contents of the developing aorta. The method of inducing hypertension was preferred partly on the basis of its ease of application in young rabbits, whereas the method of Pickering and Prinzmetal (1938) required a critical degree of constriction of the renal artery and was found less suitable for developing animals (Cleary, private communication). In a second series of experiments, the same method was employed using adult (approximately 1 year-old) semi-lop-eared rabbits, obtained from the Central Animal House, University of Adelaide (i.e. of the same breeding as those used in all preceding studies described in this thesis).

Surgical Procedures

The operative procedures were similar except that in the case of New Zealand White-Oxford lop (NZOL) rabbits each kidney was wrapped in cellophane in separate operations at two week intervals, whereas both kidneys of semi-lop-eared rabbits were wrapped in single operations lasting 30-45 minutes.

Rabbits were anaesthetised with pentobarbital (1.5% in sterile 0.9% saline) injected intravenously (30-50 mg/Kg). Anaesthesia was assessed as discussed on Page 43. The

kidney was approached retroperitoneally through a longitudinal incision in the costo-vertebral angle approximately 0.5cm lateral to the paravertebral muscles. The muscle layers were penetrated by blunt dissection to expose the kidney which was freed of adhering adipose tissue and delivered to the exterior. The hilar region was carefully stripped of remaining adipose and fibrous tissue to expose the renal artery, renal vein and ureter. A 20cm x 20cm sheet of cellophane (Dupont no. 215 PD) was loosely wrapped around the surface of the kidney, leaving the hilar region free. A ligature was passed around the cellophane at the hilus and secured so that an opening about 1cm in diameter was left for the hilar structures. The cellophane was trimmed off below the ligature and folded back over the wrapped kidney which was then returned to its normal position and covered with adipose tissue. Muscle layers were closed with chromic (3/0) and the skin sutured with silk (2/0). In sham operations, surgical procedures were identical, except that, having been wrapped in cellophane and relocated, the kidney was delivered again to the exterior and the cellophane removed. The kidney was then replaced in the perinephric fat and the wound closed. Aseptic precautions were taken during the operations. No antibiotics were used postoperatively. Cellophane was washed in Zephiran chloride solution (1/2500) for 30 minutes and rinsed in sterile saline before use.

Measurement of Blood Pressure

Systolic blood pressures were measured with the aid of an ear capsule device. In later experiments, direct measurements of systolic blood pressures were also obtained by transcutaneous puncture of the central ear artery. Details of each of these procedures are described in Appendix 1. Although Standard International units of pressure (Nm^{-2}) have been used throughout this thesis, the units "mm Hg" have been specifically used for expression of blood pressure in this chapter (1mm Hg = 133.32 Nm^{-2}).

Perfusion of Ear Arteries

The methods of perfusion of ear arteries, and of measuring sensitivities to constrictor agents and changes in these sensitivities produced by drugs were identical to those used in preceding studies, and have been detailed in Chapter 2.

RESULTS

I. First Series: New Zealand White-Oxford lop rabbits

(i) Blood Pressures

The systolic blood pressures of 5-7 week old rabbits, measured using the ear capsule, were approximately 60-80mm Hg. In sham-operated animals these increased over the next 3 months

and reached stable levels of 80-95mm Hg for the remainder of the experiment (Table 8.1). In each of 15 rabbits with both kidneys wrapped in cellophane, blood pressure increased compared with sham-operated controls, but the time course of these increases varied considerably. A "high" blood pressure was arbitrarily defined as one which was greater than 115mm Hg. Table 8.2 shows that whereas hypertension developed within 4 to 6 weeks after the second operation in most rabbits, in others (e.g. rabbits H6, H7, H11) the onset was more gradual. Furthermore, 5 of 15 treated rabbits developed hypertension which subsequently tended to reverse. Since the blood pressures of these rabbits (Table 8.2b) were lower than 115mm Hg for several weeks prior to death, this group was classified as "treated, not hypertensive". The remaining 10 rabbits (Table 8.2a), with blood pressures greater than 115mm Hg for at least 5 weeks prior to death were considered as hypertensive. The 2 groups were compared separately with sham-operated controls.

(ii) Sensitivities to noradrenaline and cocaine

Preliminary comparison of 10 hypertensive and 8 sham-operated control rabbits indicated a small but significant increase in the sensitivity to extraluminal NA and a decrease in the potentiating effect of cocaine on this sensitivity in ear arteries from hypertensive rabbits (de la Lande et al, 1973).

However, inspection of these preliminary data suggested that an age difference between control and hypertensive rabbits may have been a factor which contributed to the difference in extraluminal NA sensitivities. The sham controls were generally older than the hypertensive rabbits when the ear arteries were removed and perfused. Therefore additional information was obtained from a further 5 sham-operated controls, the ear arteries of which were removed at appropriate ages so that the difference between the mean age of the total number of 13 sham controls and the mean age of the 10 hypertensive animals was minimised (31 $\pm$ 2 and 28 $\pm$ 3 weeks, respectively). Fig. 8.1 shows a tendency for equieffective concentrations of both extraluminal and intraluminal NA to increase with increasing ages of the 13 control rabbits. A similar trend was evident for intraluminal NA in hypertensive arteries (Fig. 8.2). However similar relationships between age and the potentiating effect of cocaine on extraluminal NA sensitivity were not evident for either sham control ($r= 0.0614$) or hypertensive rabbits ($r= -0.1085$).

With the age difference between controls and hypertensive animals minimised, the sensitivity of hypertensive arteries to extraluminal NA was only slightly and not significantly increased compared with control arteries. Sensitivities of hypertensive and control arteries to intraluminal NA did not differ significantly. Despite this, the ratio of equieffective concentrations of

Table 8.1 Systolic blood pressures of sham-operated New Zealand White-Oxford lop rabbits

Rabbit No.	AGE (weeks)																		
	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42
HS14	o	•90	90	99	102	98													
HS7	o	•74	90	88	90	80	78	84	80										
HS17	o	•	88	100	100	100	104	107	103										
HS5	67	o•91	116	99	102	110	107	112	101	99	93								
HS8	o	•78	86	92	92	90	84	80	86	88	82	82							
HS9	o72	•78	70	86	86	84	86	90	84	86	80	84							
HS10	o82	•84	90	82	86	94	90	90	92	82	86	80	86						
HS11	o68	•82	84	78	86	92	94	86	92	86	80	84	80						
HS1	o74	•68	77	80	87	84	89	87	87	79	84	84	80	85	83	82	86		
HS2	o76	•61	70	72	75	77	82	86	87	78	91	82	88	89	90	92	91		
HS3	o	•74	78	79	78	93	89	88	87	90	92	91	90	92	91	94	91	92	
HS4	o	•68	69	74	75	74	78	78	82	79	88	92	84	83	84	79	83	83	
HS6	o	•79	82	84	87	96	97	99	97	95	91	84	83	83	84	81	89	87	87

Footnotes:

(i) Blood pressures measured indirectly with the aid of an ear capsule (see Appendix 1)

(ii) Each value represents the mean of 2-5 readings during the preceding 2 weeks

(iii) o and • indicates the times of the 1st and 2nd operation, respectively

Table 8.2 Systolic blood pressures of treated New Zealand White-Oxford lop rabbits

(a)

Rabbit No.	AGE (weeks)																		
	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42
H13	○	●	110	128	137	125													
H9	77	○77	●100	130	120	132	130	129	127	127									
H10	○82	●82	99	121	120	137	132	122	123	123									
H3	○56	●56	88	120	126	129	130	121	119	116	115								
H12	○63	●63	74	96	97	111	127	118	125	125	125								
H5	84	90	95	○● 96	99	100	118	122	107	114	118	133	126						
H6	○81	●92	98	101	101	106	104	118	117	114	124	123	126	126					
H7 .	○80	●92	97	101	104	101	105	111	113	109	121	121	121	121					
H11	○82	●82	96	103	99	100	105	104	108	113	112	130	130	130	130	135			
H16			○● 108	117	121	121	126	135	145	143	115	125	127	139	136	136			
(b)																			
H15	○	●	96	109	117	117	127	130	143	109	109	106	106						
H20	○	●103	98	105	115	122	130	130	117	107	102	104	106	109	105	96			
H19	○	●	96	108	104	126	124	133	128	118	110	115	107	103	100	100	102		
H22	○	●	103	118	121	119	115	119	119	119	123	125	113	108	108	100	100		
H21	○	●	104	109	112	123	117	125	120	118	101	114	115	108	110	102	100	98	

* See footnotes to Table 8.1

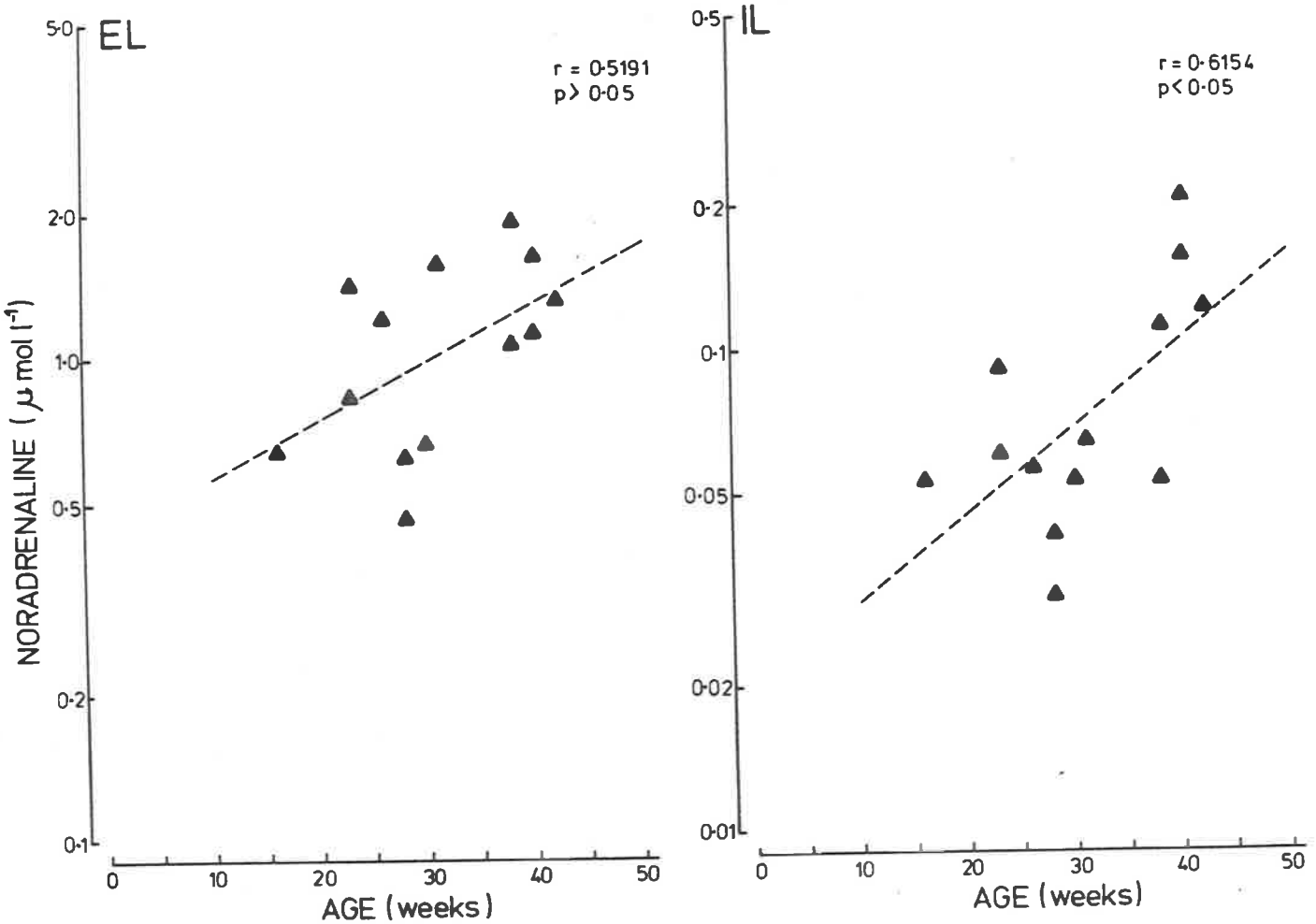


Fig. 8.1 Sham operated NZOL rabbits. Relationship between sensitivities to NA and the ages of rabbits when ear arteries were removed. Ordinates: concentrations of extraluminal (EL) and intraluminal (IL) NA which were equieffective in eliciting increases in the perfusion pressure of 8.0×10^3 Nm^{-2}. Abcissae: ages of rabbits. r refers to the coefficient of linear correlation and p to the corresponding level of significance.

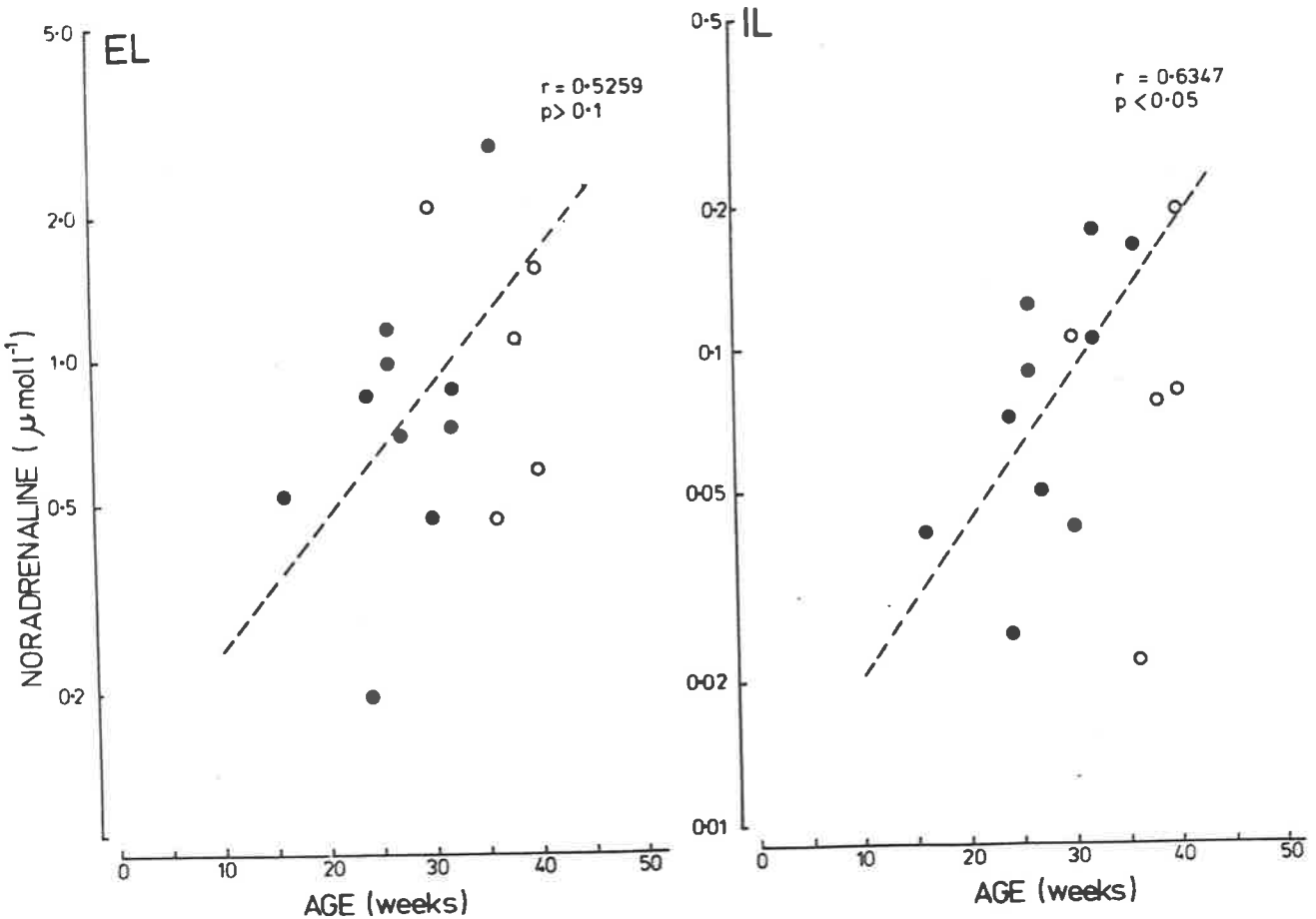


Fig. 8.2 Treated NZOL rabbits. Relationship between sensitivities to NA and the ages of rabbits when ear arteries were removed. Ordinates: concentrations of extraluminal (EL) and intraluminal (IL) NA which were equieffective in eliciting increases in the perfusion pressure of 8.0×10^3 Nm^{-2}. Abcissae: ages of rabbits. ● = treated, hypertensive; o = treated, not hypertensive. r refers to the coefficient of linear correlation (in arteries from treated, hypertensive rabbits) and p to the corresponding level of significance.

extraluminal and intraluminal NA in hypertensive arteries was less than that in control arteries. This difference was statistically significant. The potentiation of extraluminal NA by cocaine was also reduced. On the other hand, the potentiation of intraluminal NA by cocaine was significantly enhanced. These changes were not evident in arteries from treated rabbits which were not hypertensive (Table 8.3).

II. Second Series: semi-lop-eared rabbits

These experiments were undertaken subsequent to the investigation of the role of extraneuronal factors in the control of sensitivity of the ear artery described in earlier chapters. Therefore part of their aim was to compare the influence of DOCA in arteries from hypertensive and normotensive animals. However, a limited amount of data was obtained due to the high mortality rate in the treated animals (discussed further in Appendix 1).

(i) Blood Pressures

Systolic blood pressures of sham operated and treated rabbits are shown in Table 8.4a,b. Mean systolic blood pressures, measured using the ear capsule just prior to removal of arteries, were 81 $\pm$ 2mm Hg in controls and 116 $\pm$ 6mm Hg in treated rabbits. In view of the high mortality rate in the treated rabbits, ear arteries were removed soon after increased blood pressures were evident. Thus the duration of hypertension was

Table 8.3 Sensitivities to NA and cocaine : New Zealand white-Oxford lop rabbits

	a	b	c	
	sham operated	treated, not hypertensive	treated, hypertensive	P
B.P. (mm Hg)	88 ± 2	100 ± 2	126 ± 2	
Sensitivity (μmol l^{-1})				
EL NA	1.094 ± 0.122	1.147 ± 0.300	0.916 ± 0.226	>0.4
IL NA	0.084 ± 0.010	0.096 ± 0.026	0.089 ± 0.014	>0.8
Potn. by cocaine (2.9 μmol l^{-1})				
EL NA	15.4(14.1-16.9)	17.4(15.8-19.1)	11.5(8.1-13.0)	<0.1
IL NA	1.6 (1.5- 1.7)	1.5 (1.3- 1.7)	2.2(2.0- 2.5)	<0.02
EL/IL NA				
coc. absent	13.9(12.6-15.5)	13.4(11.1-16.1)	10.0(8.9-11.2)	<0.05
coc. present	1.5 (1.3- 1.7)	1.2 (1.0- 1.4)	2.0(1.7- 2.4)	=0.2
No. of arteries	13	5	10	

Footnotes:

(i) B.P. - represents the mean of the systolic blood pressures (measured using the ear capsule) during the 2 week period prior to removal of ear arteries (see Table 8.1 and 8.2)

(ii) Sensitivity refers to the mean concentration of NA required to elicit an increase in the perfusion pressure of $8 \times 10^{3} Nm^{-2}$

(iii) Potentiation refers to the ratio (geom. mean ± S.E.) of the concentrations of NA which are equipotent in eliciting increases in the perfusion pressure in the artery before and during treatment with the sensitising agent (see Methods, Chapter 2)

(iv) EL/IL NA refers to the ratios of the concentrations of extraluminal and intraluminal NA which are equipotent in eliciting increases in the perfusion pressure of $8 \times 10^{3} Nm^{-2}$

(v) P refers to the differences between values in a and c. None of the values in b was significantly different from the corresponding values in a (unpaired t-tests)

Table 8.4 Systolic blood pressures of semi-lop-eared rabbits

(a) treated

Rabbit No.	WEEKS after operation											
	0	1	2	3	4	5	6	7	8	9	10	11
H7	86	94	94	105	140							
H18	94	98	104	115	110	100						
H12	78	75	80	102	102	120	128					
H10	82	87	106	100	90	100	104	100				
H3	92	86	84	94	102	118	115	116	116	114		
H6	82	92	100	100	112	120	127	116	107	116	116	

(b) sham operated

HS6	96	92	96	88	90							
HS2	86	70	84	82	84	84	80	76	80			
HS3	83	86	86	85	88	86	84	70	74			
HS4	68	94	88	88	90	82	78	76	78			
HS5	68	86	80	82	83	80	74	76	86			
HS1	80	84	84	75	75	76	76	80	80	70	70	80

considerably shorter than that in the first series of experiments. Arteries from all treated rabbits which survived were included for comparison with control arteries, despite the moderate increases in blood pressure of rabbits 10 and 18 (Table 8.4a).

(ii) Sensitivities to noradrenaline

The sensitivities of hypertensive arteries to both extraluminal and intraluminal NA were increased compared with those of control arteries. However, the difference was statistically significant only for extraluminally applied NA (Table 8.5).

Table 8.5 Sensitivity to noradrenaline; semi-lop-eared rabbits

	sham operated	treated	P (unpaired t-tests)
Sensitivity ($\mu mol\ l^{-1}$)			
EL NA	1.101 ± 0.087	0.681 ± 0.169	<0.05
IL NA	0.130 ± 0.024	0.071 ± 0.022	<0.1
No. of arteries	6	6	

Footnotes:

(i) Sensitivity measured as in footnote (ii), Table 8.3

(iii) Cocaine

The effects of cocaine in control and hypertensive arteries are summarised in Table 8.6. In control arteries, cocaine (2.9μmol l^{-1}) selectively potentiated the sensitivity to extraluminal NA as had been noted in earlier studies by de la Lande et al (1967a). When the concentration of cocaine was increased cumulatively from 2.9 to 29μmol l^{-1}, the sensitivity to extraluminal NA was further enhanced while that to intraluminal NA was little affected. In hypertensive arteries, the potentiations of extraluminal NA sensitivities by cocaine (2.9 and 29μmol l^{-1}) were less than those in control arteries. However these differences were not statistically significant. In contrast to the significant increase in the potentiation of intraluminal NA sensitivity by cocaine observed in arteries from hypertensive NZOL rabbits (Table 8.3), this effect of cocaine in arteries from hypertensive semi lop-eared rabbits was not increased, and in fact tended to be less marked.

(iv) DOCA

The influence of DOCA (27μmol l^{-1}) on the sensitivity to adrenaline in cocaine-treated control arteries conformed to the pattern noted earlier (Chapter 4) in that extraluminal adrenaline was potentiated to a greater extent than intraluminal adrenaline and the magnitude of the potentiations

Table 8.6 Effect of cocaine on the sensitivities to NA; semi-lop-eared rabbits

	sham operated	treated	P (unpaired t-tests)
cocaine concn.			
a) 2.9 μmol l^{-1}			
Potn. EL NA	14.5(14.0-15.1)	11.5(9.3-14.1)	>0.2
Potn. IL NA	2.0 (1.8- 2.3)	1.5(1.3- 1.7)	>0.1
EL/IL NA			
coc. absent	10.0 (7.7-13.0)	11.3(9.3-13.6)	>0.7
coc. present	1.4 (1.1- 1.7)	1.4(1.2- 1.7)	>0.9
b) 29 μmol l^{-1}			
Potn. EL NA	19.6(17.6-21.8)	17.1(12.7-23.1)	>0.7
Potn. IL NA	2.4 (1.9- 3.1)	1.6(1.4- 1.9)	>0.1
No. of arteries (EL,IL)	5,5	6,6	

Footnote:

(i) Potentiation explained in footnote (iii), Table 8.3

Table 8.7 Potentiation of the sensitivity to adrenaline by DOCA (27 μmol l^{-1}); semi-lop-eared rabbits

	sham operated	treated	P	sham operated	treated	P
Treatment: cocaine	2.9 μmol l^{-1}			29 μmol l^{-1}		
*sensitivity to adrenaline (μmol l-1)						
EL A	0.133±0.017	0.089±0.022		0.086±0.007	0.070±0.022	
IL A	0.075±0.008	0.071±0.028		0.050±0.007	0.052±0.013	
Potentiation						
EL A	5.3(4.6-6.0)	3.8(2.8-5.1)	>0.2	4.7(4.2-5.4)	2.5(2.2-2.8)	<0.02
IL A	3.8(3.3-4.3)	2.9(2.1-3.9)	>0.3	2.9(2.6-3.2)	2.0(1.6-2.4)	>0.1
No. of arteries (EL,IL)	6,6	4,4		5,5	6,6	

Footnotes:

(i) *Sensitivity refers to the concentration of adrenaline (mean ± S.E.) required to elicit an increase in the perfusion pressure of $8x10^3 Nm^{-2}$ prior to application of DOCA.

(ii) Potentiation explained in footnote (iii), Table 8.3

(iii) P refers to the differences between sham operated and treated rabbits (unpaired t-tests).

were decreased when a higher concentration of cocaine ($29\mu mol\ l^{-1}$) was employed (Table 8.7). In hypertensive arteries the potentiations of both extraluminal and intraluminal adrenaline by DOCA were decreased although these decreases were not statistically significant, except for extraluminal adrenaline in the presence of $29\mu mol\ l^{-1}$ cocaine. As a result, the selective effect of DOCA on the extraluminal adrenaline tended to be less marked (Table 8.7).

DISCUSSION

In order to assess the integrity of the neuronal uptake mechanism in hypertensive arteries, advantage was taken of earlier evidence that the sensitivities to extraluminal and intraluminal NA differed markedly and that this difference provided a measure of the contribution which neuronal uptake made to the vasoconstrictor response. A further measure of the influence of neuronal uptake was provided by cocaine, by its ability to enhance the sensitivity to extraluminal NA to a level approaching that to intraluminal NA (de la Lande et al 1967a). In ear arteries from hypertensive NZOL rabbits, the ratio of sensitivities to extraluminal and intraluminal NA and the potentiation of extraluminal NA by cocaine were both decreased compared with arteries from normotensive animals. Although the latter decrease was not statistically

significant ($0.05<p<0.1$), these changes point to a disturbance in neuronal uptake in the hypertensive arteries. Since the sympathetic nerve terminals are located at the media-adventitia junction, neuronal uptake normally reduces the concentration of extraluminally applied NA reaching the underlying smooth muscle. A decrease in the efficiency of this process would enhance the concentration of NA reaching the receptors and consequently increase the sensitivity of the artery to extraluminal NA. However the sensitivity of hypertensive arteries to extraluminal NA was only slightly and not significantly increased. Furthermore, in contrast to the decrease in the potentiating effect of cocaine on extraluminal NA, the potentiation of intraluminal NA by cocaine was not decreased but significantly increased. It is possible that the small potentiating effect of cocaine on intraluminal NA is not mediated by inhibition of neuronal uptake, but rather by inhibition of an extraneuronal mechanism which is enhanced in hypertensive arteries. Alternatively, the effect of cocaine on intraluminal NA may be mediated by neuronal uptake inhibition but is normally small because relatively little of the NA applied to the lumen reaches the sympathetic nerve terminals. Evidence for the failure of intraluminal NA to penetrate the artery wall was obtained in earlier pharmacological (de la Lande and Jellett, 1972) and histochemical studies (de la Lande et al, 1970b; 1974). A non-uniform distribution

of amine in the artery wall was also suggested by experiments described earlier in this thesis (Chapter 4). It is possible that in hypertensive arteries there is an alteration in medial processes so that more NA penetrates to the nerve terminals and results in a greater influence of neuronal uptake. More direct measurements of the neuronal and extraneuronal uptake systems using ^{3}H-NA may help to resolve some of these problems and permit a more cohesive interpretation of changes in arteries from these hypertensive animals.

Interpretation of results from the second series of experiments, using semi-lop-eared rabbits, is limited by the relatively small number of observations. Nevertheless some trends were evident which may provide the basis for further investigation. For instance, the significant increase in the sensitivity to extraluminal NA and the tendency for the potentiation of this sensitivity by cocaine ($2.9\mu mol\ l^{-1}$) to be reduced in arteries from hypertensive animals suggests an abnormality in neuronal inactivation in these vessels. However, the sensitivity to intraluminal NA was also enhanced so that the ratio of extraluminal to intraluminal sensitivities was not altered. It is possible that an alteration in extraneuronal inactivation contributed to the generalised increase in sensitivity of the hypertensive arteries, since

the potentiations of both extraluminal and intraluminal adrenaline sensitivities by DOCA were decreased. Despite this, other factors may be responsible for the enhanced sensitivities. Hauesler and Haefely (1970) found that isolated perfused mesenteric arteries from rats with genetic hypertension were 3 times more sensitive to NA compared with arteries from normotensive control rats. Cocaine caused a 3-fold potentiation of NA sensitivity in normal arteries but was without effect on this sensitivity in hypertensive arteries. Thus the enhanced sensitivity of hypertensive vessels was explicable in terms of a defect in neuronal uptake. However, the sensitivity to KCl was also enhanced to the same extent as that to NA. Furthermore, the unchanged thresholds, increased slopes and increased maxima of concentration-response curves to NA and KCl were consistent with the concept of Folkow et al (1973), ascribing enhanced reactivity to structural changes, namely an increase in the wall to lumen ratio in the hypertensive vessels. Hauesler and Haefely supported a structurally-based increase in reactivity and attributed the failure of cocaine to potentiate NA sensitivity in the hypertensive vessels to hindered diffusion of NA through the thickened artery wall. In the present experiments the enhanced reactivity of hypertensive arteries was associated with a decreased threshold and an essentially parallel shift to the left in the concentration-response curve to NA (Fig. 8.3). It is therefore

unlikely that an alteration in wall to lumen ratio could account for the phenomenon. However the evidence obtained does not exclude enhanced sensitivity which is unrelated to cocaine and DOCA-sensitive mechanisms. Kalsner et al (1971) observed increased sensitivity to NA in aortic strips from rabbits made hypertensive by a combination of unilateral nephrectomy and DOCA/NaCl treatment, but were unable to explain this in terms of an alteration in O-methylation by COMT, which is a major mechanism of inactivation of NA in the rabbit aorta.

In many forms of experimental hypertension, the sensitivity of the aorta to catecholamines is decreased (see de Champlain, 1972). However the present results point to an increase rather than a decrease in the sensitivity of the muscular rabbit ear artery, suggesting that changes in vascular sensitivity in hypertension differ with different types of arteries.

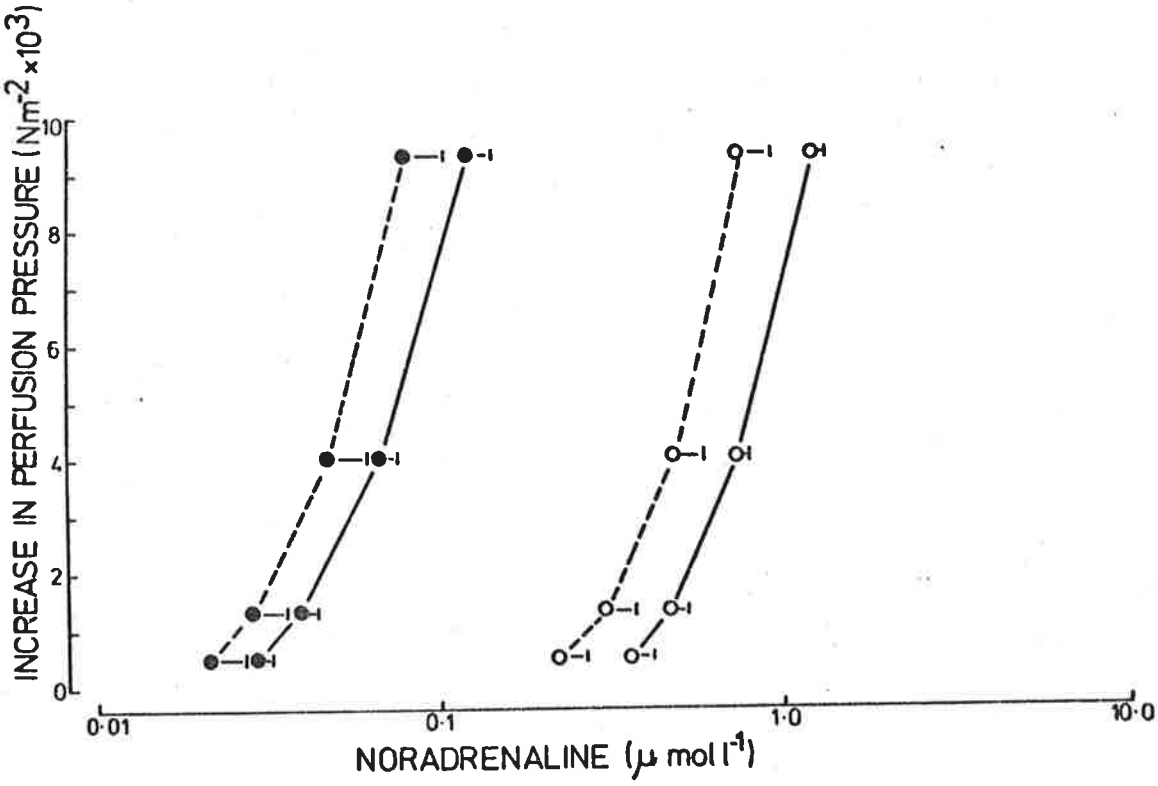


Fig. 8.3 Concentration-response curves to extraluminal (o - o) and intraluminal (• - •) NA in ear arteries from sham-operated (solid lines) and treated (broken lines) semi-lop-eared rabbits. Each point represents the mean concentration (+ S.E.) of 6 arteries.

APPENDICES

APPENDIX 1

Measurement of Blood Pressure

Systolic blood pressures of rabbits used in the experiments described in Chapter 8 were measured indirectly in the central ear artery with the aid of a capsule device based on that described by Grant and Rothschild (1934) and similar in design to that used by Moont (1963). The capsule (Fig. A.1, A.2a) consisted of a pressure chamber (A), positioned on the lower arm of a transparent perspex frame (B), and covered by an inflatable latex rubber membrane (C). Pressure could be applied to the chamber (A) with the aid of a syphygmomanometer (Fig. A.2b,c). To use the capsule, the rabbit's left ear was placed between the two arms of the perspex frame so that the centre of the pressure chamber was located approximately 2cm distal to the bifurcation in the ear vein (Fig. A.2c,d). The artery was positioned above the centre of the chamber and was viewed through the upper arm of the perspex frame. As pressure was applied in the chamber, the latex membrane inflated, compressing the ear and the central artery. The device could be used in two ways to measure blood pressure:

(a) The pressure in the chamber was increased to 120mm Hg or more which blanched the ear, causing the pulsating artery to disappear (Fig. A.2c,d). The pressure was then slowly lowered until the pulsations reappeared and the broken column of blood was finally rejoined. The pressure in the

chamber was then taken as systolic blood pressure in the artery.

(b) An alternate method was to apply just sufficient pressure to interrupt the arterial column. As the pressure was gradually increased the pulsations of the artery became more marked and finally the column was discontinued. This method also provided an estimate of the systolic blood pressure in the artery, and was preferred as it was found easier to judge when the arterial column became discontinuous rather than rejoined.

In the absence of any applied pressure the ear usually fitted comfortably, but without being compressed, in the 4mm gap between the upper perspex arm and the pressure chamber. A loose fit could be corrected by the application of an appropriate pressure which was then subtracted from the final reading of systolic blood pressure measured as described above. The problem of fluctuating calibre of the ear artery pointed out by Grant and Rothschild (1934) and by Goldblatt (1960) was also encountered in the present experiments. For instance, when the artery was constricted and therefore difficult to see, measurements were either difficult to obtain or gave fasely low estimates of blood pressure. However fluctuations in calibre were minimised by vasodilation produced by palpation of the artery and the warmth provided by a light

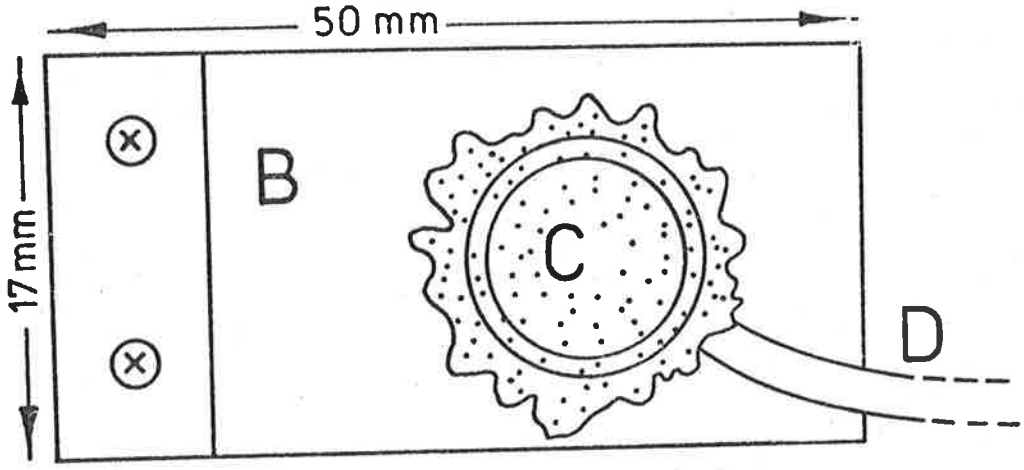


Fig. A.1 Diagram (not to scale) of capsule used in measuring systolic blood pressure in the rabbit ear artery.

A = pressure chamber. Internal diameter = 15mm; external diameter = 19mm
B = transparent perspex frame
C = inflatable latex membrane

Pressure applied to A with the aid of a syphygmomanometer connected through tubing at D.

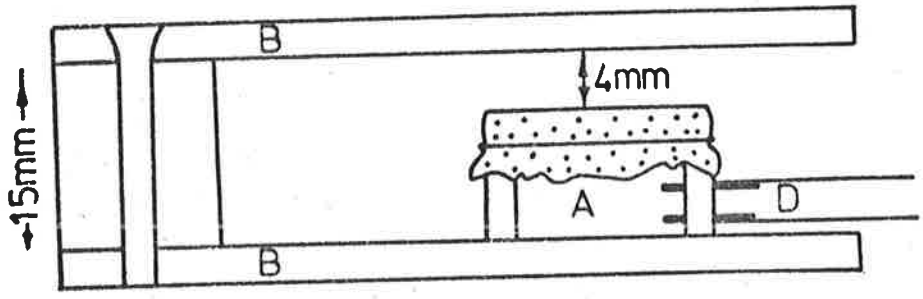


Fig. A2a-d. Application of the capsule device in measuring systolic blood pressure in the ear artery of the rabbit.

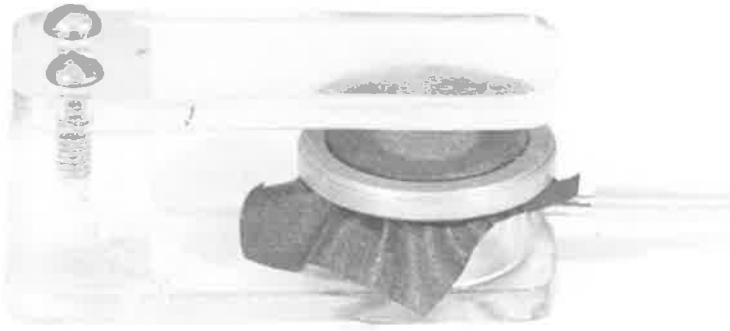


Fig. A.2a

Fig. A.2b

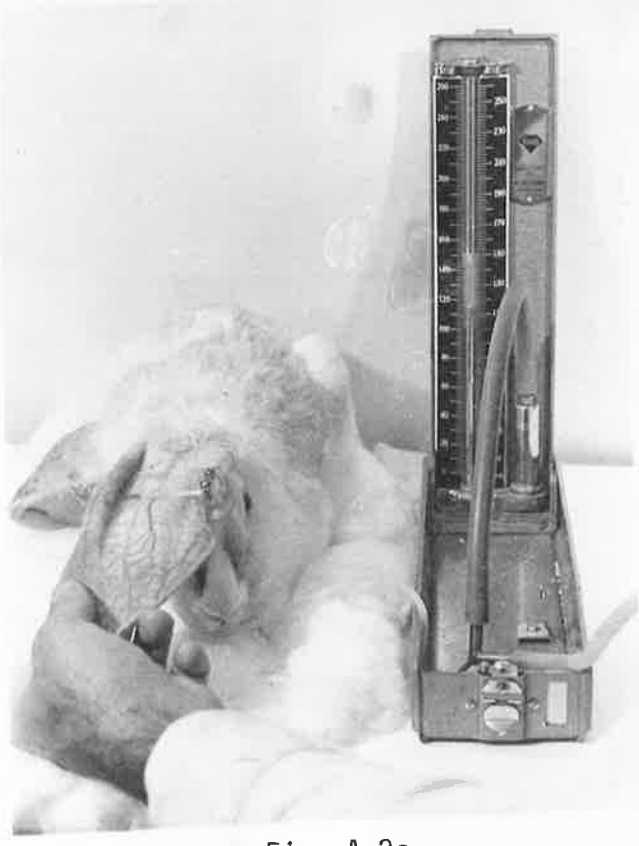


Fig. A.2c

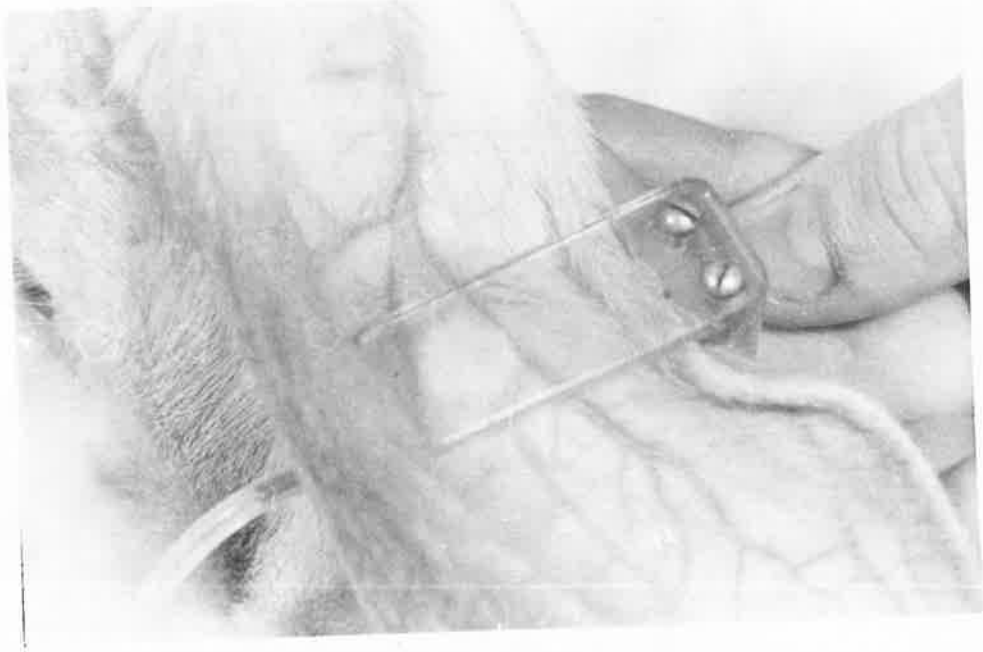


Fig. A.2d

placed about 20cm above the ear. Constant readings could then be obtained from several measurements repeated over 15 minutes.

The accuracy of the ear capsule

In preliminary experiments, systolic blood pressures measured in one ear using the capsule were compared with those measured directly in the opposite ear artery in each of two rabbits anaesthetised with 50mg/Kg diallylbarbituric acid (1.5% in propylene glycol) injected into an ear vein. This anaesthetic was selected since it had previously been shown to produce long-lasting anaesthesia without serious depressant effects (Kerr, private communication). The central artery of one ear was exposed and cleared of adhering tissue before heparin (1000 units/Kg) was injected intravenously. A polythene catheter, filled with saline containing heparin (100 units/cm^3) was inserted into the artery and blood pressure monitored using a Statham P23 Ac pressure transducer and Rikadenki chart recorder. Systolic blood pressure was measured indirectly in the opposite ear artery using the capsule and compared with that measured directly. Comparisons were also made when the blood pressure was increased by injections of adrenaline (0.5 - 10µg) into the marginal ear vein. A number of different membranes were tested on the pressure chamber of the ear capsule and several blood pressure

measurements made using each membrane. The reliability of the capsule in measuring increases in blood pressure varied depending on the membrane used. Membranes made from various types of surgical gloves generally proved unsatisfactory. Latex rubber (0.177 mm thickness) gave best results and was used as the membrane for all subsequent determinations of blood pressure. Comparison between indirect and direct measurements of blood pressure is shown in Fig. A.3. Measurements made using the capsule were between 15 and 30mm Hg lower than those made directly. The discrepancy between the readings tended to be more marked when the true systolic blood pressure in the ear artery was relatively high.

In later experiments systolic blood pressure was also measured directly by transcutaneous puncture of the ear artery in unanaesthetised rabbits using a 21-gauge needle connected to a Statham P23Ac pressure transducer and Rikadenki chart recorder. The technique was practised in several 12-16 week-old semi-lop-eared rabbits before being applied to 4 of the 6 hypertensive semi-lop-eared rabbits used in the second series of experiments described in Chapter 8 (i.e. rabbits H_3, H_6, H_7 and H_{12}; see Table 8.4). Immediately prior to removal of the ear arteries of these animals, systolic blood was estimated firstly using the ear capsule and then by transcutaneous puncture of the ear artery at the same point

at which the capsule measurement had been made i.e. approximately 2cm distal to the bifurcation of the ear vein, where the artery lies close to the surface. The segment of artery used for subsequent perfusion was always taken from nearer the base of the ear (see Fig. 2.1). Comparison of indirect (ear capsule) and direct (artery puncture) systolic blood pressure measurements is shown in Fig. A.4. The systolic blood pressures of untreated normotensive rabbits were between 75 and 85mm Hg when measured directly. Discrepancies between these measurements and those made using the ear capsule were observed, but there was no obvious pattern in the inaccuracy of the capsule. However, as was noted above in measuring relatively high systolic blood pressures in anaesthetised rabbits, the capsule estimates of systolic blood pressure in the 4 hypertensive rabbits were consistently lower than those measured directly, and the error in using the capsule was more marked for rabbits with the highest blood pressures. For instance, the capsule measurements of systolic blood pressure in rabbits H_7 and H_{12} were 30 to 35mm Hg lower than direct estimations (Fig. A.4). The above observations indicate that the ear capsule may not be reliable for accurate quantitative determination of high blood pressure. However they justify the use of the capsule in providing a qualitative index of hypertension in rabbits.

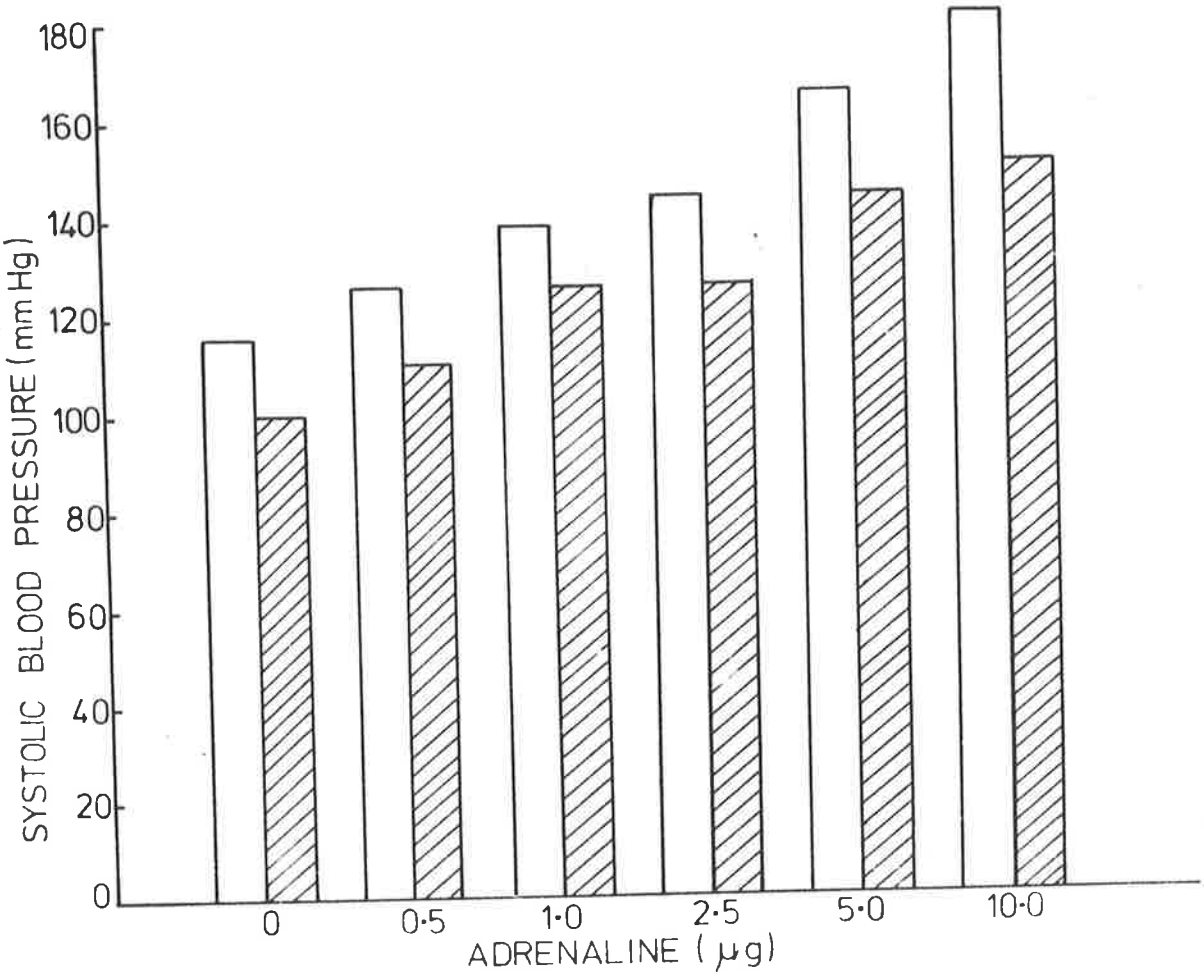


Fig. A.3 Comparison of direct and indirect measurements of systolic blood pressure in an anaesthetised semi-lop-eared rabbit. Direct measurements (unshaded bars) were made using a catheter inserted into the left ear artery. Indirect measurements (shaded bars) were made in the right ear artery using the ear capsule. Blood pressure was increased by intravenous injections of adrenaline in the doses indicated.

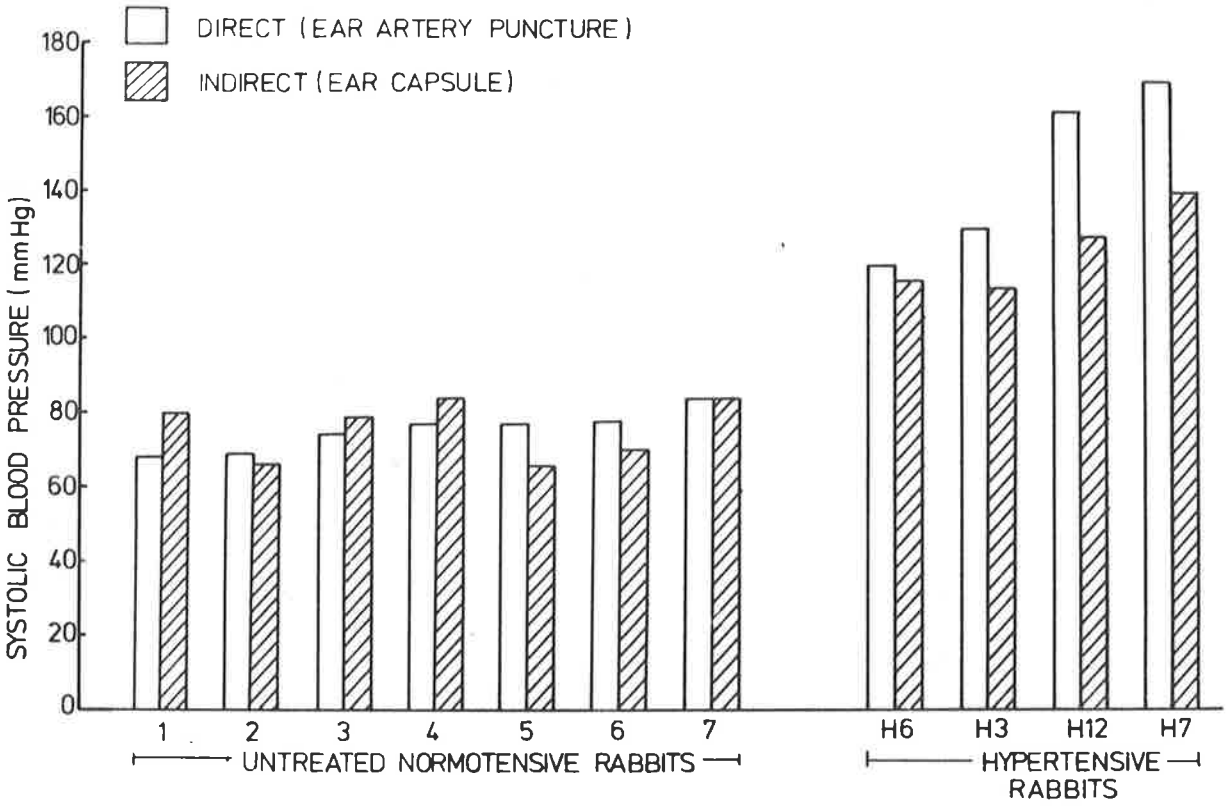


Fig. A.4. Comparison of direct and indirect measurements of systolic blood pressure in ear arteries of unanaesthetised normotensive and hypertensive semi-lop-eared rabbits. Direct measurements were made using transcutaneous puncture of the artery (unshaded bars). Indirect measurements were made using the ear capsule (shaded bars).

Mortality Rate in Rabbits with Cellophane Perinephritis

A limited amount of information was obtained from the study of experimental hypertension in semi-lop-eared rabbits (Chapter 8, Series II), due largely to the high mortality rate in these animals. 16 of 22 rabbits died in the early stages of hypertension usually 3-5 weeks after bilateral cellophane wrapping of the kidneys. Necropsy examination revealed renal infarction in some cases with or without prolapse of one of the renal papillae through the hilar opening in the cellophane capsule. However, in the majority of cases no cause for death was found. In many instances the cellophane capsule was distended with dark blood which compressed the underlying kidney and presumably the renal artery at the hilus. The bleeding was possibly the result of some local inflammatory response to the cellophane. While this response was prevalent in this series, it was not seen in the earlier series. In some cases the cellophane capsule contained a cream floculent sterile debris which was amorphous on histological examination. Neither organisms nor inflammatory cells were demonstrated in this material, which was therefore probably not due to infection, but presumably the result of some reaction to the cellophane. In all treated rabbits, including those which survived, perinephric fibrosis was present. None of the above effects was observed in sham operated rabbits which were examined after removal of the ear arteries.

Since this series of experiments was undertaken in the latter stages of preparation of this thesis, an extensive investigation of possible causes of the high mortality rate in treated animals was not possible. However the following basic modifications in the surgical procedures were made:

(a) the use of Zephiran antiseptic solution was omitted, and the cellophane sterilized by soaking in absolute ethanol for 24 hours prior to use, as described by Chalmers et al (1974). The two rabbits in which this procedure was followed died within 5 weeks of the operation.

(b) in another 2 rabbits, the cellophane was applied much more loosely than normal. These animals also died within 5 weeks of the operation, before any appreciable rise in blood pressure was observed.

(c) in 2 other rabbits, each kidney was wrapped in cellophane in separate operations at 2 week intervals, i.e., by the same protocol as that used for weanling rabbits in the first series of experiments. These animals died within 6 weeks of the second operation.

(d) A different type of capsule material (Technicon cuprophan No. 105-1058) was tried in one rabbit, which did not subsequently develop hypertension, and which died 6 weeks after operation.

The mortality rate was conceivably related to the type of rabbit used since it was not encountered in the first study of experimental hypertension in which rabbits of the New Zealand white-Oxford lop cross breed were used. However a high mortality rate has previously been observed in the latter breed in studies of perinephric hypertension by Cleary (private communication).

APPENDIX 2

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

$CaCl_2$ and $MgCl_2$ were added from standardised 10% stock solutions. The Krebs' was filtered before use.

Liquid Scintillation Spectrometry

For the experiments described in Chapter 6, radioactivity was counted using a Packard Model 3310 Liquid Scintillation Spectrometer. The scintillant contained Triton x-100 (Packard) and Toluene scintillant (1:2). The latter consisted of:

PPO (2,5-diphenyloxazole)	8.25g
POPOP (1,4-di[2-(5-phenyloxazolyl)]benzene)	0.25g
Toluene	to 1 litre

Radioactivity in samples was corrected for efficiency of counting which was determined by internal standardisation using ^{3}H-toluene (2.51×10^{6}dpm/cm^{3}) (New England Nuclear).

Drugs

The following drugs were used:

l-adrenaline bitartrate	Koch Light Laboratories
cocaine hydrochloride	MacFarlane-Smith
diallybarbituric acid (DIAL)	Ciba
DOCA (4-pregnen-21-ol-3,20-dione acetate)	Steraloids
histamine dichloride	Koch Light Laboratories
d,l-isoprenaline hydrochloride	Sigma
D,L-[7-^{3}H] isoprenaline hydrochloride (Specific Activity = 10Ci/mmol)	Amersham
methoxamine hydrochloride	Burroughs-Wellcome
3-methoxyisoprenaline	Boehringer
nialamide	Pfizer
l-noradrenaline bitartrate	Koch Light Laboratories
d,l-normetanephrine	Sigma
pentobarbital (Sagatal)	May & Baker
phentolamine methane sulphonate	Ciba
d,l-propranolol hydrochloride	Sigma
serotonin creatinine phosphate	Koch Light Laboratories
U0521 (3',4'-dihydroxy-2-methyl-propiophenone)	Upjohn

Preparation of Drugs

(i) The catecholamines and U0521 were prepared in 0.9% saline containing ascorbic acid (0.57mmol l^{-1})

(ii) DOCA was prepared as a stock solution of 67mmol l^{-1} in ethanol

(iii) Nialamide solution was prepared by dissolving the required amount of nialamide in 20cm^3 of 0.9% saline with the aid of gentle heat. This solution was then made to the appropriate volume with Krebs' solution immediately prior to use.

(iv) For intravenous injection, pentobarbital was prepared as a 1.5% solution in sterile 0.9% saline. Diallylbarbituric acid was prepared as a 1.5% solution in propylene glycol

(v) All other drugs were prepared in 0.9% saline.

Concentrations of adrenaline, noradrenaline, isoprenaline, serotonin and phentolamine refer to the bases. Concentrations of all other drugs refer to the salts.

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