THE DIFFUSION AND METABOLISM OF NORADRENALINE IN THE ARTERY WALL

A THESIS SUBMITTED FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

by

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To

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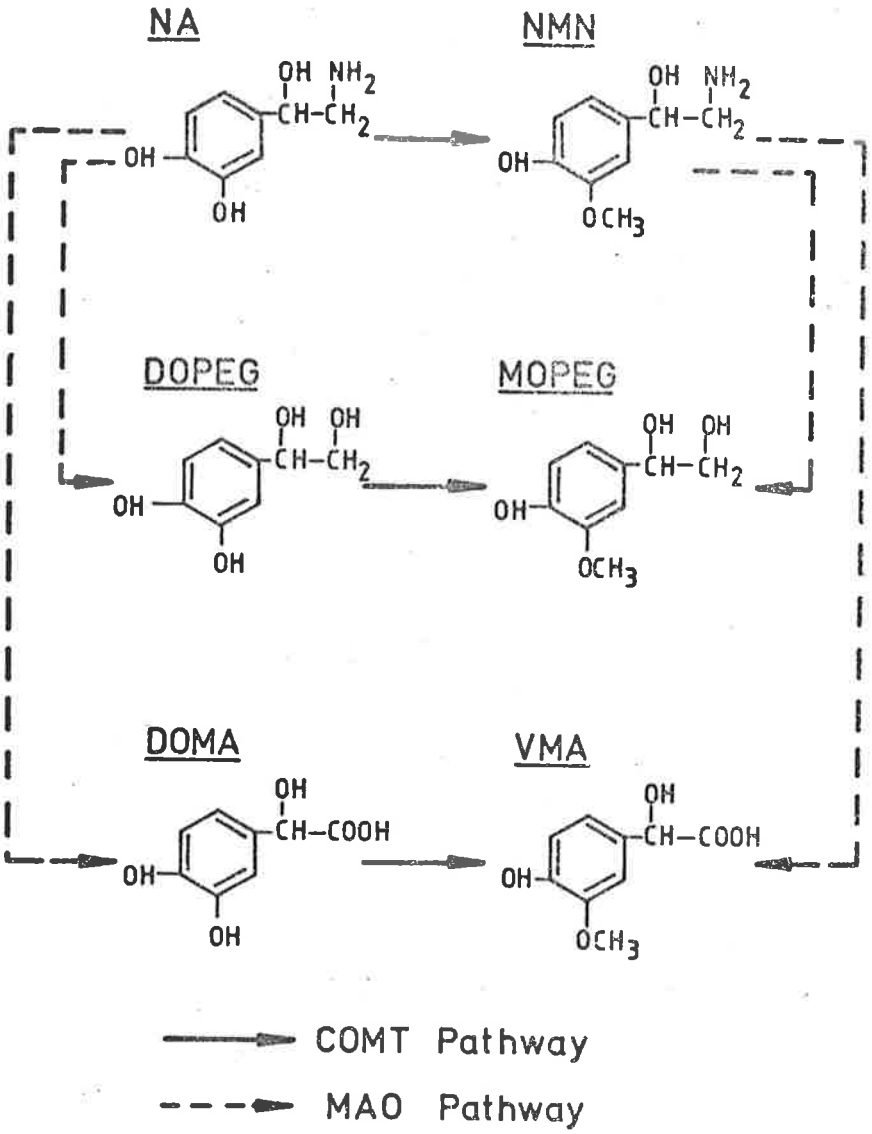

MAJOR PATHWAYS OF NORADRENALINE METABOLISM
IN PERIPHERAL TISSUES AND
MOLECULAR STRUCTURES

SUMMARY

(1) The major study of this thesis deals with the relationship between the metabolism of NA and its surface of entry into the artery wall. It was prompted by earlier evidence that the surface of entry of NA exerted a profound influence on its pharmacological actions on the isolated artery. This influence has been attributed to the location of the nerves at the medial-adventitial border, and the non-uniform gradient of concentration of NA within the artery wall when the amine is applied to one surface only.

(2) To study the influence of the surface of entry on NA metabolism, ^{3}H.NA was applied separately to the adventitia and to the intima (referred to as EXT and INT ^{3}H.NA, respectively) of the isolated perfused rabbit ear artery. The associated effluxes of metabolites into the solutions bathing the two surfaces were measured. Experimental conditions included reserpine pretreated rabbits to minimise retention of unchanged ^{3}H.NA in the nerves and to enhance the formation of metabolites of neuronal origin. The relative rates of formation of one of these metabolites (dihydroxyphenylethylene glycol, DOPEG) from EXT and from INT ^{3}H.NA were used as an indirect measure of the relative concentrations which NA achieved in the region of the nerve terminals, and hence the magnitude of the gradient of concentration of the amine across the vessel wall. In a number of experiments Ca^{++} free bathing medium containing prazosin was used to prevent constriction of the vessel during incubation with the ^{3}H.NA.

(3) The results showed that the surface of entry exerted a profound effect on the metabolism of ^{3}H.NA, in that EXT ^{3}H.NA was metabolised primarily by neuronal monoamine oxidase (MAO), with DOPEG as the principal metabolite, whereas INT ^{3}H.NA formed considerably less DOPEG and was

metabolised primarily by extraneuronal catechol-O-methyl transferase (COMT) to ^{3}H.normetanephrine (NMN). The difference between these metabolite patterns is explained in terms of the gradient of concentration of INT ^{3}H.NA across the wall, so that the concentration achieved in the region of the nerves is less than that achieved by EXT ^{3}H.NA.

(4) The surface of entry of NA affected the efflux of its metabolites in different ways, in that DOPEG, and the minor neuronal metabolite dihydroxymandelic acid (DOMA), effluxed preferentially from the adventitial surface irrespective of the surface of application of NA. In contrast, NMN effluxed preferentially from the surface of entry of the NA. The difference is explained in terms of the media, but not the adventitia, representing the major barrier to the diffusion of NA and its metabolites within the vessel wall. This was confirmed in another study where the efflux of methoxy-isoprenaline (MeOISO) showed the same trend as NMN when ^{3}H.isoprenaline was applied to either surface of the vessel.

(5) Constriction of the vessel, in response to the ^{3}H.NA, modified its metabolism in several ways. As deduced from their rates of efflux, the ratio of formation of ^{3}H.DOPEG from EXT ^{3}H.NA, compared with that from INT ^{3}H.NA (termed the DOPEG formation ratio) increased from a mean value of 4.4 in relaxed arteries, to 10 in arteries which constricted to INT ^{3}H.NA only, and to 24 in vessels which constricted to INT and EXT ^{3}H.NA. These increases imply that there is a much steeper gradient of concentration of INT ^{3}H.NA in the constricted vessel, and are interpreted as evidence that, as the vessel wall thickens, there is decreased access of INT ^{3}H.NA, although not EXT ^{3}H.NA, to the region of the nerves. In contrast to ^{3}H.DOPEG formation, ^{3}H.NMN formation from both INT and EXT ^{3}H.NA tended to decrease with increased constrictor response.

This finding is compatible with the concept of restricted diffusion within the media as it constricts as well as evidence that the sites of O-methylation are distributed uniformly within the media.

(6) The preceding findings accord, for the most part, with the roles of neuronal and extraneuronal uptake and inactivation in controlling the concentration of NA in the vessel wall as deduced indirectly from pharmacological studies. In the General Discussion, attention is drawn to some quantitative discrepancies between the metabolic and pharmacological data, principally the small effect of cocaine on the flux of EXT NA across the vessel wall, compared with the effect predicted from pharmacological data.

(7) The results of the study also shed some further light on the possible origins of the O-methylated-deaminated metabolites (OMDA). Irrespective of the surface of entry of NA, approximately equal proportions of the NA were metabolised to OMDA. This fraction was not further separated into its constituents (MOPEG and VMA) in the present study. However, it is assumed to be mainly MOPEG (3-methoxy, 4-hydroxyphenylethylene glycol) both in view of its high medium to tissue ratio, and in view of earlier evidence in non-reserpinised ear arteries where MOPEG was demonstrated to be the major constituent when analysed by thin layer chromatography.

(8) Unlike ^{3}H.NMN, the formation of ^{3}H.OMDA was insensitive to corticosteroid. It is suggested that OMDA is formed by different mechanisms in different regions of the artery wall. The evidence is based on the ability of a neuronal uptake inhibitor (cocaine) to partially inhibit OMDA formation from EXT ^{3}H.NA, but not from INT ^{3}H.NA.

(9) In a separate study on artery strips, it was shown that ^{3}H.DOPEG is O-methylated to ^{3}H.MOPEG by the artery by a corticosteroid-insensitive mechanism. Accordingly the sensitivity of ^{3}H.OMDA formation from EXT ^{3}H.NA to cocaine is attributed to the ability of highly lipid soluble ^{3}H.DOPEG, after its release from sympathetic nerves at the medial-adventitial border, to diffuse directly into the COMT-containing compartment. It is proposed that this mechanism is important only in the outer region of the vessel wall. The second pathway of OMDA formation, which it insensitive to both cocaine and corticosteroid, is inhibited by phenoxybenzamine (PBZ) and appears to operate throughout the vessel wall.

(10) A pharmacological study on the effect of preventing efflux of INT NA and its metabolites from the adventitia on its vasoconstrictor activity is also described. To do this, the EXT aqueous bathing medium was replaced by paraffin oil during a steady-state vasoconstrictor response to INT NA. The results confirmed both the non-uniform distribution of INT NA within the vessel wall, and pointed to the presence of a cocaine and corticosteroid insensitive mechanism of NA inactivation within the vessel wall, in accord with the metabolic findings.

(11) The last study deals with the metabolism of NA, and isoprenaline (ISO), in tail arteries of normotensive and DOCA-salt hypertensive rats. Although tangential in aim to the remainder of the studies presented in the thesis, it is included to provide some comparative data from another species in a similar type of artery to that of the rabbit ear. It also provided an opportunity to study the chronic treatment with a corticosteroid on the metabolism of ^{3}H.NA and ^{3}H.ISO. This study failed to reveal any consistent differences between the normotensive and hypertensive tissues.

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.

RAYMOND GREGORY MORRIS,

January, 1982.

PUBLICATIONS

Some of the material presented in this thesis has been published in the following books and journals:-

in: "Vascular Neuroeffector Mechanisms", ed. J.A. Bevan et al, Raven Press (NY). 1980, p. 148-160.

Proc. Eighth. Intern. Congr. Pharmacol. Tokyo, 1981. abstract 1892.

Blood Vessels 18: 217. 1981.

Aust. Physiol. Pharmacol. Soc. Proc. 8: 159P. 1977.

Aust. Physiol. Pharmacol. Soc. Proc. 10: 207P. 1979.

Clin. Exp. Pharmacol. Physiol. 6: 645 (abstract 15).

Clin. Exp. Pharmacol. Physiol. (Dec., 1981 meeting ASCEP, in press).

Clin. Exp. Pharmacol. Physiol. (Dec., 1981 meeting ASCEP, in press).

Proc. Fourth. Meeting on Adrenergic Mechanisms. Porto, 1981. p. 75-91.

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CHAPTER 1

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

The general theme of the study, described in the first and major part of this thesis, is the influence of inactivation of noradrenaline (NA) on its concentration at α-receptors on vascular smooth muscle cells. Specifically, the study considers, in the rabbit ear artery, the influence of the surface of entry of NA into the vessel wall on its subsequent diffusion and metabolism. From the metabolic changes, inferences are drawn about the gradient of concentration of NA within the artery wall. To assist in the interpretation of the data on NA metabolism, the metabolism of another catecholamine, isoprenaline (ISO) has also been studied.

The second part of the thesis describes the accumulation and metabolism of NA and of ISO in the rat tail artery. Originally it was intended to ascertain whether the relationships between the surface of entry of NA and its metabolism, as established in the rabbit ear artery, applied to the rat tail artery and then to determine whether the relationships were modified during experimental hypertension. Time did not allow this second study to be completed; however, sufficient data was obtained on the origins of the metabolites of NA and ISO in this vessel in normotensive and in DOCA-salt hypertensive rats to justify its presentation in the thesis.

The following introduction describes pharmacological, histochemical and biochemical evidence dealing with the relationships between the constrictor response to NA and its inactivation in the artery wall. The evidence refers mainly to the rabbit ear artery.

1. PHARMACOLOGY AND HISTOLOGY

It was shown in 1967 by de la Lande and Waterson (1967) and by Waterson and Smale (1967) that the sympathetic nerves innervating the central artery of the rabbit ear, terminated in a dense sheath at the border of the media and the adventitia. This was later confirmed by Burnstock et al. (1970) and by Bevan et al.(1972b). De la Lande et al. (1967) also showed that the sensitivity of the artery to NA was markedly influenced by the surface of entry of the NA into the vessel wall, such that NA applied to the adventitia was 10-20 times less potent in producing vasoconstriction than NA applied to the intima. (Note:- Throughout this thesis, NA applied to the adventitia, i.e. the extraluminal surface of the vessel, will be abbreviated to EXT NA; and NA applied to the intima, i.e. the intraluminal surface of the vessel, will be abbreviated to INT NA.) The difference in sensitivity was greatly reduced by either chronic homolateral sympathetic denervation or by pretreatment with cocaine as a result of a marked increase in sensitivity of the vessel to EXT NA. The potentiation of the response to INT NA was relatively minor (approximately 1.5 fold) and was attributed to the failure of neuronal uptake to influence the concentration of NA in the media when the amine entered via the intimal surface. They proposed a simple model (Fig. 1.1) to explain the result. It illustrates that when NA enters the vessel via the intima, it reaches the nerve terminal region only after diffusing through the smooth muscle layer. Hence the concentration of NA at receptors on most of the smooth muscle cells in the media would not be greatly influenced by neuronal uptake. In contrast, NA entering via the adventitial surface must first negotiate the neuronal uptake barrier before diffusing to the receptors on the underlying smooth muscle cells. Their model assumed that the concentration of NA was uniform throughout the media (except in the immediate environment of the nerves where it was decreased by neuronal uptake). However, it was

soon realised that this model was oversimplified; de la Lande et al. (1970a) found that the loss of noradrenergic fluorescence in the nerve terminals of monoamine oxidase (MAO) inhibited ear arteries from rabbits pretreated with reserpine could be restored by exposing the vessel to EXT NA. However, when the same concentration of amine was applied to the INT surface restoration of fluorescence was not detected. They suggested that enzymic inactivation limited the penetration of INT NA, but not EXT NA, to the region of the nerve terminals. This was supported by evidence that partial restoration of fluorescence occurred when UO521, an inhibitor of catechol-O-methyltransferase (COMT), or metanephrine, an inhibitor of uptake into smooth muscle cells, was present (de la Lande et al., 1974). Hence it appeared that uptake and metabolism of the amine into the smooth muscle cells may play a significant role in determining the concentration which INT NA achieves in the region of the nerve terminals. Other indirect evidence of limited penetration of INT NA to the region of the nerve terminals was provided by de la Lande and Jellett (1972) in the course of studies examining the effects of MAO inhibitors on the constrictor response of the rabbit ear artery to NA. They showed that nialamide sensitised the artery to EXT NA, but not to INT NA. The mechanism of the sensitisation appeared to be the same as in the guinea pig atria (Furchgott and Sanchez Garcia, 1968) and in the cat nictitating membrane (Trendelenburg, 1971). It involves uptake of NA by the sympathetic nerves. Because intraneuronal MAO is inhibited the NA accumulates in the cytoplasm of the nerve until its rate of efflux equals its rate of uptake, i.e. net uptake is zero. Hence the failure of nialamide to increase the sensitivity to INT NA indicated a failure of INT NA to penetrate to the region of the nerve terminals. De la Lande and Jellett also pointed out that their results constituted pharmacological evidence for the presence of intraneuronal MAO, and that the failure of nialamide to modify the magnitude of the response to INT NA indicated that extraneuronal MAO was of little functional importance in the inactivation of NA in the rabbit ear artery.

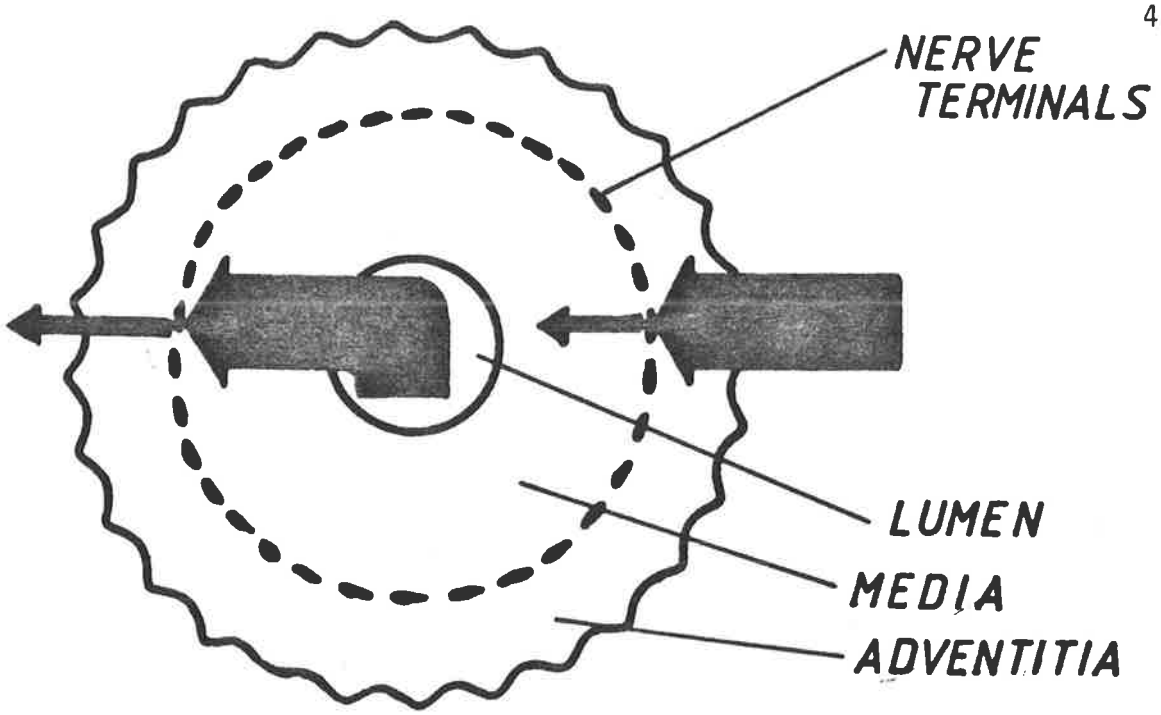

Fig. 1.1 A diagrammatic representation of the influence of uptake of NA by the sympathetic nerve terminals on the concentration of NA in the smooth muscle of the rabbit ear artery. The arrows indicate the diffusion of NA and their thickness indicates the relative concentration of NA in the vessel wall.

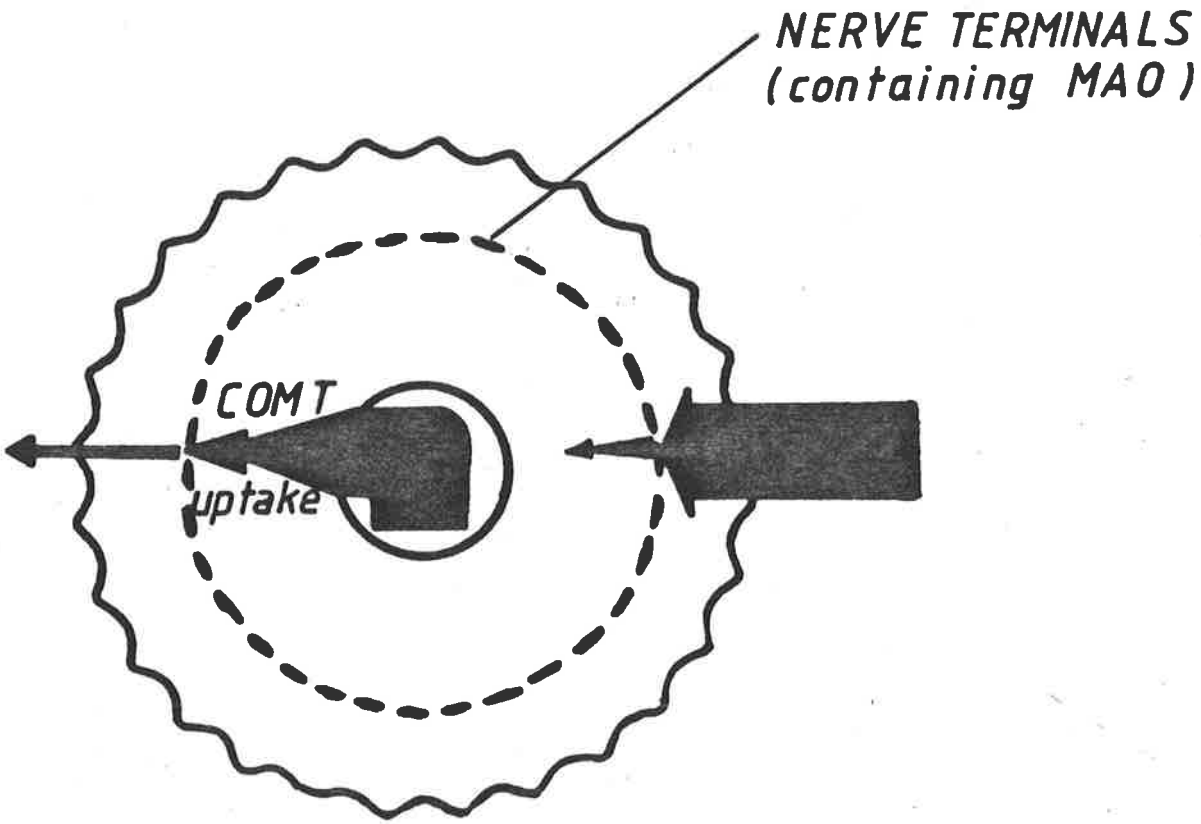

Fig. 1.2 A diagrammatic representation of the influence of neuronal uptake. Unlike Fig. 1.1, this model incorporates the the influence of restricted diffusion of NA through the media, partly as a consequence of extraneuronal uptake and metabolism by COMT.

Independent evidence of a non uniform (i.e. declining) gradient of concentration of agonists across the vessel wall when they entered via one surface only was provided by Kalsner (1972a) He showed that when an agonist was applied to both surfaces of the rabbit ear artery simultaneously, the sensitivity to the agonist was 2-3 fold greater than when it was applied to one surface only. He suggested that this indicated that the receptors on the smooth muscle cells were not uniformly occupied by the agonist at increasing depths of the media. He suggested also that those cells nearest to the surface of application were exposed to the greatest concentration of amine and therefore contributed more to the constrictor response than did more distant cells. Since histamine and K^+ behaved like NA, it was evident that enzymic inactivation was not the only factor limiting the penetration of INT NA to the region of the nerve terminals. An obvious factor to be considered was the purely physical one, namely the decline in concentration within the vessel wall imposed by the diffusivity of the agonist. This factor is considered later in this General Introduction (p.23). There were thus three separate lines of evidence suggesting that the concentration which INT NA achieved in the region of the nerve terminals was less than that achieved by EXT NA in the same region. For this reason, the model shown in Fig. 1.1 has subsequently been modified, as shown in Fig. 1.2, to take into account the possibility that the minor role of neuronal uptake in the response to INT NA may have reflected a declining concentration of INT NA between the intima and the nerve terminal region.

The pharmacological evidence in two of the above studies (de la Lande and Jellett, 1972, and Kalsner, 1972) was based on the assumption that the sensitivity of the outer and inner smooth muscle cells of the artery media were identical, i.e. when the concentration of NA at the receptors on a smooth muscle cell was the same, the cell contracted to the same extent

irrespective of its position in the media. In the case of the rabbit ear artery, evidence in support of this assumption is based on the observation that, when neuronal uptake is blocked, the difference in sensitivities is small, amounting only to about a 1.5 fold greater sensitivity to INT NA. (It should be noted that sensitivity refers to comparisons of steady state responses to sustained application of NA.) However, Graham and Keatinge (1972) suggested that this assumption is open to question. They showed that the inner smooth muscle cells of the sheep carotid artery responded to a lower concentration of NA than did the outer smooth muscle cells. They suggested that the greater sensitivity of the inner cells was analogous to denervation supersensitivity since the greater distance of these cells from the nerve terminals meant that they were exposed to lower concentrations of transmitter than were the outer cells (i.e. cells close to the nerves). They suggested that this "supersensitivity" of the inner cells was a useful compensatory mechanism which ensured that they could respond to the lower concentrations of transmitter. The magnitude of the difference in sensitivity was 15 fold and was more marked for NA than for the other agonists tested (i.e. histamine, angiotensin and serotonin) and persisted in the presence of neuronal uptake inhibition by desimipramine.

Evidence which suggested that the kinetics of the responses of the smooth muscle cells to NA differed in different regions of the artery wall was presented by Pascual and Bevan (1979). These workers studied the contractile responses of rabbit aortic strips when the entry of drugs from one or other surface of the vessel was blocked by a coating of silicone grease. They showed that when NA entered the cocaine-treated vessel via the intima, the contractile response reached a higher steady state level and possessed a shorter latency and with a higher initial velocity when the

amine entered via the intimal surface than when it entered via the adventitial surface. They showed that when the amine entered via both surfaces simultaneously, the response of the uncoated strip was the same as when it entered via the intimal surface only, implying that the response of the uncoated strip was determined largely by the NA which had entered via the intima. Despite the above evidence of inhomogeneity of the responses of vascular smooth muscle cells to NA, the following qualifications should be noted. Keatinge's Graham and results were obtained on a vessel (the sheep carotid artery) whose wall thickness (3.6mm) is approximately 36 fold greater than that of the rabbit ear artery (0.1mm). If the increase in sensitivity between the inner and outer surfaces was uniformly distributed in the wall of the sheep carotid artery and was applicable to other vessels, then in the case of the rabbit ear artery and the rabbit aorta (wall thickness 0.3mm) then the predicted differences in sensitivity would be 1.4 and 2.2 fold respectively. These are close to the sensitivity differences of 1.5 fold (reported by de la Lande et al., 1967) and 2 fold (reported by Pascual and Bevan, 1979) in the rabbit ear artery and rabbit aorta respectively. Hence the different dimensions of the vessels under investigation may explain the apparently contradictory nature of the findings on the rabbit ear artery and other vessels. This possibility is further supported by observations in the rat tail artery (wall thickness 0.07mm). In this vessel Venning and de la Lande (1981) were unable to detect significant differences in the sensitivities to INT and to EXT NA, based on steady state responses of cocaine treated vessels.

Another assumption which is central to the model of vascular sensitivity proposed by de la Lande (Fig. 1.2) is that the enhanced sensitivity to EXT NA produced by cocaine is primarily due to the selective inhibitory action of the drug on neuronal uptake of NA. It has been proposed by

Kalsner and Nickerson (1969a) that in the rabbit aorta the action of cocaine was mainly extraneuronal. Their evidence was based on the assumption that the rate of relaxation of aortic strips immersed in oil, after previously contracting to NA, was a measure of the rate at which NA was removed from the biophase of the postsynaptic α-receptors. They observed that cocaine delayed relaxation of the reserpine pretreated preparation in which MAO and COMT were inhibited; that is, it exerted its effect under conditions where neuronal uptake, neuronal vesicular storage and neuronal and extra-neuronal metabolism were not operative. However, Trendelenburg (1974) has suggested that these puzzling findings can be explained if part of the delayed relaxation observed in Kalsner and Nickerson's study was caused by efflux of NA which had accumulated in nerve terminals prior to cocaine treatment; Kalsner and Nickerson had applied cocaine only during the response to NA prior to immersing the strip in oil. He verified this by showing that pretreatment with cocaine 10 minutes before a contraction induced by NA appreciably reduced, rather than increased, the slow relaxation phase of the vessel.

With respect to the rabbit ear artery the simplest conclusion drawn from the controversy about cocaine's action is that, if an extraneuronal action does contribute to the sensitising effect of the drug on the rabbit ear artery, the contribution is only a small one when NA is applied to the EXT surface. This is based on the assumption that the small (1.5 fold) sensitisation of responses to INT NA in the rabbit ear artery must represent the maximum contribution which such an extraneuronal action makes to NA sensitivity. Such a contribution is small compared with the 10-20 fold sensitisation to EXT NA produced by cocaine. However, the extraneuronal component of cocaine's action is probably even less than the estimate of 1.5 fold suggests. This is because cocaine does not sensitise the rabbit

ear artery to the sympathomimetic amine, methoxamine, which unlike NA is not a substrate for neuronal uptake (Iversen, 1967). It should be noted that the conclusion of de la Lande et al.(1970a) was criticised by Yong and Chen (1975) on the basis that only two comparisons were made between methoxamine and NA. However, the failure of cocaine to influence the sensitivity to both INT and EXT methoxamine has been subsequently confirmed in a further eight rabbit ear arteries (de la Lande, private communication).

From the above considerations there seems little doubt that the modified model of vascular sensitivity (Fig. 1.2) proposed by de la Lande is consistent with the known features of the pharmacological interaction between cocaine and NA on the rabbit ear artery.

2. METABOLISM

Before considering the background evidence on the metabolism of NA in the blood vessel wall, it is important to address the basic inter-related questions that these studies are attempting to resolve; firstly, the specific morphological regions where the two primary enzymatic metabolising systems operate; secondly, the influence of the surface of entry of amines on the metabolic pathway followed; and thirdly, the physiological importance of inactivation of biogenic amines in relation to their source (i.e., neuronal or circulating). Despite the fact that metabolic studies on the rabbit ear artery generally confirm the interpretation of the roles of neuronal and extraneuronal uptake and the metabolising enzymes, the evidence is incomplete in one major respect. The pharmacological studies emphasise that the functional roles of neuronal and extraneuronal uptake and of enzymatic inactivation depends on the surface of entry. However, this factor has not been analysed in biochemical studies since these usually

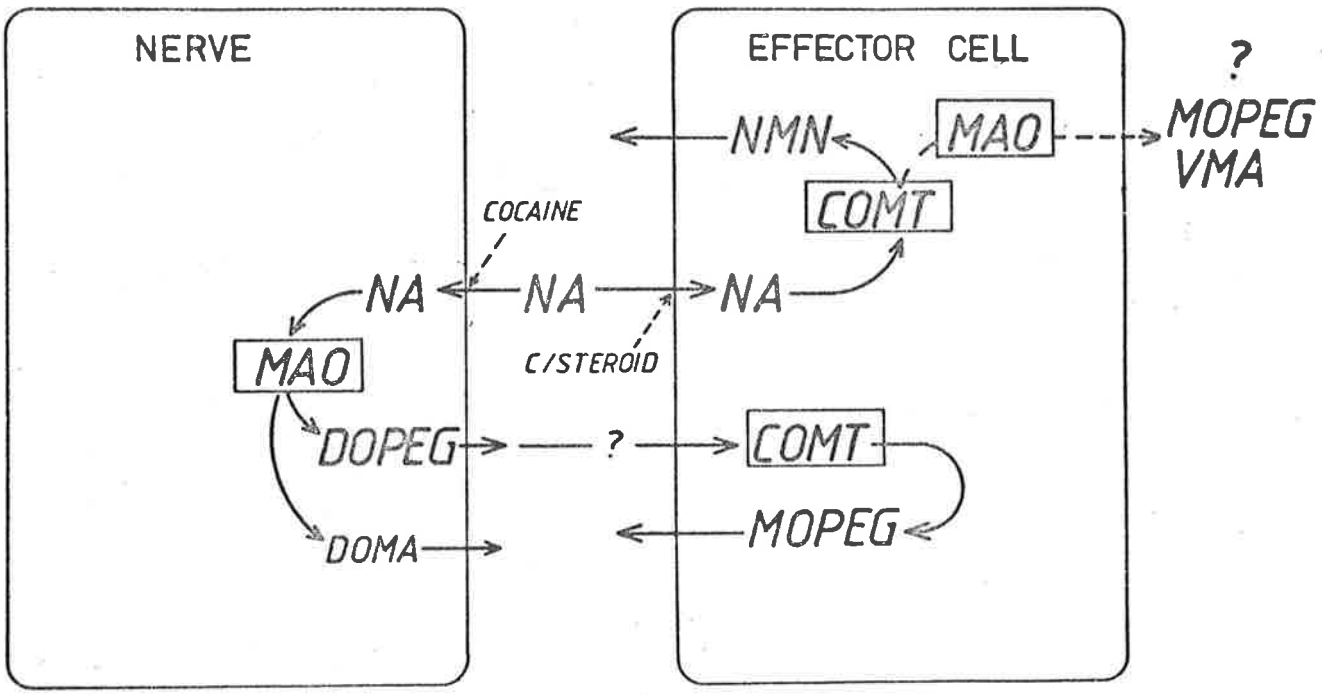

Fig. 1.3 A diagrammatic representation of the inactivation pathways of NA in nerve terminal and effector cell. This shows NA removed from the synaptic cleft by two processes,

(a) by the cocaine-sensitive neuronal uptake process, followed by deamination by monoamine oxidase (MAO) to form DOPEG and DOMA, and

(b) by the corticosteroid-sensitive extraneuronal uptake process, followed by O-methylation by catechol-O-methyl transferase (COMT) to form NMN.

Two conceivable mechanisms for the formation of the O-methylated-deaminated metabolites (OMDA, ie, MOPEG and VMA) are shown.

employed artery segments or strips where ^{3}H.NA penetrated from both surfaces. One might argue, for example, from the biochemical studies (discussed later), that neuronal deamination was the major metabolic pathway of NA inactivation. However, the pharmacological studies of de la Lande and Jellett (1972) demonstrated that the deaminating pathway did not significantly influence the response of the rabbit ear artery to NA entering via the INT surface, and therefore could not be considered to be of physiological importance in the inactivation of circulating NA and adrenaline which act only on cells near to the lumen. As a background to the experimental section the following introduction will consider the evidence of the relationship between the morphology and the functional importance of the NA metabolising enzymes, monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT), in the rabbit ear artery and other vascular tissues. These enzyme pathways are illustrated in Fig. 1.3.

(a) Monoamine Oxidase

The presence of MAO activity in the rabbit ear artery was first demonstrated histochemically by Koelle and Valk (1954), who associated this activity with the media using tyramine and tryptamine as substrates. This was later confirmed by de la Lande and Waterson (1968). Using tyramine as a substrate, these latter workers showed that the MAO activity was distributed throughout the media and extended to the intima, but could not demonstrate any MAO activity to be associated with the sympathetic nerves at the medial-adventitial border. To date, the only histochemical evidence for the presence of MAO activity in these nerves is indirect and based on the findings of de la Lande et al. (1970, 1974). These workers showed that in reserpinised vessels where the monoamine fluorescence characteristic of nerves was absent, the application of NA to the adventitial

surface could restore the fluorescence, but only in those arteries where MAO activity was blocked with nialamide. However, in a biochemical study Head et al. (1974) demonstrated a small proportion of the total MAO activity was associated with nerve terminals. This was based on a significant 15% reduction in tyramine oxidase activity in ear artery homogenates following homolateral chronic sympathetic denervation of the vessel for 14 days. The significance of this small proportion of MAO activity associated with nerves was indicated in a subsequent study by Head (1976). He provided biochemical evidence that it was this neuronal MAO activity which was of major importance in the metabolism of ^{3}H.NA. The evidence was derived from studies on intact ear artery segments. When incubated with ^{3}H.NA (1.2μM) for 30 minutes, the major deaminated metabolite was 3,4-dihydroxyphenylethylene glycol (DOPEG). Treatment with cocaine (29μM) or by prior chronic denervation reduced DOPEG formation by 79% and 87% respectively, suggesting that a large proportion of the deamination was associated with neuronal structures, despite the fact that the extra-neuronal MAO activity accounted for 85% of the total MAO activity when tyramine was used as the substrate. Hence, he concluded that the extra-neuronal MAO activity was of little quantitative importance in the metabolism of the transmitter amine, NA. Earlier, de la Lande and Jellett (1972) had provided pharmacological evidence that inhibition of extraneuronal MAO had little influence on the rate of inactivation of NA in ear artery segments when the concentration of amine was low (less than 3μM). Their evidence was that the sensitising effect of nialamide on the vasoconstrictor response to EXT NA was completely prevented by cocaine, or by prior chronic denervation of the artery. In contrast, when the amine concentration was greatly increased to 120μM, de la Lande and Johnson (1972) detected a 5 to 6 fold increase in NA released into the bathing solution when MAO was inhibited.

This effect could be equally demonstrated in cocaine treated, or in chronically denervated vessels, suggesting that the increased NA outflow was derived from extraneuronal structures. There is also pharmacological evidence that the extraneuronal MAO activity may have a functional role in the inactivation of tyramine. This stems from the sensitising effects of nialamide on the indirect neuronally-mediated vasoconstrictor response to tyramine. De la Lande et al. (1970a) showed that the indirect response occurred when tyramine was applied to the EXT surface, but not to the INT surface, implying that INT tyramine does not penetrate to the nerve terminals. However, nialamide treatment sensitised the indirect response to INT tyramine much more than to EXT tyramine. These workers suggested that the inactivation of tyramine by extraneuronal MAO activity in the media of this vessel limited the penetration of INT tyramine to the region of the nerve terminals.

The relative insignificance of the extraneuronal MAO pathway in deaminating NA was demonstrated biochemically by Head (1976), who showed that in segments of artery that were chronically denervated, or cocaine treated, deamination of either ^{3}H.NA or ^{3}H.NMN proceded at a very slow rate compared with arteries with neuronal inactivating systems intact.

In summary, the biochemical and pharmacological studies are in agreement with respect to the substrate specificity of neuronal and extra-neuronal MAO activities. These indicated that tyramine was a substrate for both neuronal and extraneuronal MAO pathways, whereas NA in low concentrations was only a substrate for the small proportion of MAO located in neuronal structures. This substrate specificity difference for tyramine and NA has been reported in other tissues and will be considered again in a later section (page 20).

(b) Catechol-O-methyl transferase

Early investigation into the localisation of COMT was hampered by the lack of a histochemical method to demonstrate its localisation within tissues. This early disadvantage may now have been rectified by the recent immunohistochemical techniques of Lowe and Creveling (1978), who used an antibody to COMT to demonstrate its presence in aortic and capillary endothelial cells and in myocardial cells of the rat. Surprisingly, the smooth muscle cells of the aorta and coronary vessels did not display COMT activity. (This apparent discrepancy is discussed further in Chapter 4.) In the rabbit ear artery, the presence of COMT was demonstrated biochemically by Head et al. (1974), who showed that artery homogenates O-methylated dihydroxy benzoic acid and that this O-methylation was prevented by 3,4-dihydroxy-2-methyl propiophenone (UO521), an inhibitor of COMT. The location of the enzyme was shown to be extraneuronal as indicated by the failure of prior chronic homolateral sympathetic denervation to influence the activity of this enzyme. This was later supported by the studies of Head (1976) on the metabolism of ^{3}H.NA in rabbit ear artery segments. The evidence was that ^{3}H.normetanephrine (^{3}H.NMN) formation in the intact vessel was either unaffected or increased by cocaine treatment in concentrations which largely eliminated the formation of the deaminated catechol metabolites (DOPEG and DOMA). This result, together with those in artery homogenates mentioned above, did not completely exclude the possibility, however, that a small proportion of the total COMT activity may have been associated with nerves. Evidence against this possibility was obtained by Head (1976). He preloaded the sympathetic nerves in the artery by incubating segments in ^{3}H.NA (0.9μM) for 60 minutes. At the end of this incubation the vessels were immediately immersed in ^{3}H-free Krebs solution containing phenoxybenzamine (PBZ) in a concentration known to inhibit both the neuronal and extraneuronal

uptake of NA. During the second 30 minutes of washing, when the efflux was largely ^{3}H.NA which was present in the nerves, only ^{3}H.NA and ^{3}H.DOPEG were detected in the efflux (i.e. no O-methylated metabolites). In the presence of nialamide, this efflux comprised only unchanged ^{3}H.NA. The failure to detect ^{3}H.NMN or other O-methylated metabolites implied that if COMT were present in the nerves, it played no role in the metabolism of NA both when the intraneuronal deaminating pathway was active, and when it was not active. The pharmacological studies of Johnson, 1975 and de la Lande et al., 1978) also supported the evidence of a purely extraneuronal activity of COMT. They showed that COMT inhibition by U0521 markedly enhanced the sensitivity of the ear artery to adrenaline. This action was independent of the surface of entry of adrenaline. This sensitisation by U0521 was not affected by cocaine treatment, but was completely abolished by inhibition of extraneuronal uptake with the corticosteroid, DOCA. These results indicated that O-methylation normally decreases the concentration of adrenaline at its receptors and that this O-methylation occurs only after its extraneuronal uptake into effector cells. The question of the relative importance of the extraneuronal COMT pathway in inactivating catecholamines when compared with the neuronal deaminating pathway is hence not entirely resolved, but the above results suggest that the surface of entry of a substrate into the vessel wall would be of prime importance in determining which of the major enzymatic pathways predominates. The biochemical evidence of Head (1976) suggested that the COMT pathway was of minor importance as evidenced by the low rate of NMN formation (representing only 10% of the total metabolite formation) when compared with DOPEG formation (representing approximately 70% of the total metabolite formation) when the intact artery segment was incubated with ^{3}H.NA (1.2μM). However, in his study, both surfaces were equally exposed to

the NA, whereas the pharmacological evidence suggested that inactivation by COMT might be more important than by MAO when NA entered via the intima (de la Lande, 1975). For this reason, in the present study, parallel incubations using two preparations (representing proximal and distal portions of the same vessel) were carried out; one piece was doubly cannulated and the ^{3}H.substrate applied to one or other surface; the other piece was carefully cut longitudinally and incubated under identical conditions to provide comparative metabolic data on the simultaneous entry of substrate into both surfaces of the vessel.

A particular problem has been the origin of the metabolites which are both O-methylated and deaminated (OMDA; i.e., MOPEG and VMA). The biochemical evidence of Head (1976) suggested that OMDA was probably extraneuronal in origin since cocaine failed to modify its formation. However, the question of OMDA formation will be considered again in this introduction (p.19) and in chapters 4 and 6.

Isoprenaline has proved to be a valuable tool for investigation of extraneuronal metabolism, since it has little affinity for neuronal uptake, but a high affinity for extraneuronal uptake (compared with NA or adrenaline) (Iversen, 1967). It was shown by Hertting (1964) that ISO was a good substrate for COMT but was not a substrate for MAO. The only metabolite observed in rat urine following ISO administration was 3-methoxy-isoprenaline (MeOISO). Head et al. (1980) described the factors which influenced the inactivation of (±)^{3}H.ISO in the rabbit ear artery. They demonstrated that:-

(i) the O-methylation pathway was saturable and limited the accumulation of ^{3}H.ISO in low concentrations (Km = 2.7μM),

(ii) the access of ^{3}H.ISO to the one O-methylating compartment was sensitive to steroid treatment (in this case, DOCA), however,

(iii) ^{3}H.ISO itself accumulated in 2 separate compartments, other than extracellular space,

(iv) chronic denervation had a small significant inhibitory effect on ^{3}H.ISO accumulation, but failed to influence the metabolism, suggesting that a minor proportion of ^{3}H.ISO accumulated in neuronal structures but was not metabolised therein. Subsequently, there have been suggestions that chronic denervation may influence extraneuronal enzyme activity (Branco et al., 1981b).

(c) MAO and COMT, Other Tissues

This section will consider further evidence, primarily from studies in the rabbit thoracic aorta, of the morphological origins of the metabolites of NA. The accumulation and metabolism of (-)^{3}H.NA (0.3μM) was compared in the intact rabbit aorta, its isolated media and its isolated adventitia by Levin (1974). He showed that the metabolism of ^{3}H.NA in the intact aorta was characterised by high rates of formation of ^{3}H.DOPEG and ^{3}H.NMN representing 43% and 40% of the total metabolite formation respectively. The other metabolites, DOMA, MOPEG and VMA were present in only small amounts (3%, 12% and 2% respectively). The metabolite formation in the isolated adventitia was consistent with the localisation of the neuronal uptake and deaminating systems in this tissue. The evidence was that the isolated adventitia had a high rate of ^{3}H.DOPEG formation which represented 77% of the total metabolites formed and this was 10 fold greater than ^{3}H.NMN formation in this tissue. In contrast, the isolated media formed mainly O-methylated metabolites, ^{3}H.NMN represented 74% of the total metabolites formed and was 9 fold greater than the ^{3}H.DOPEG formed in this region of the tissue. Comparing the isolated adventitia with the isolated media, he showed that the former accumulated 5 fold more unchanged ^{3}H.NA, formed 10 fold more ^{3}H.DOPEG and 9 fold less ^{3}H.NMN than the latter tissue.

Hence Levin concluded that the accumulation and deamination of ^{3}H.NA and specifically ^{3}H.DOPEG formation was primarily associated with the adventitia and that O-methylation of ^{3}H.NA and specifically ^{3}H.NMN formation was primarily associated with the media. The origins of the O-methylated-deaminate metabolites was less clear. The overall rate of ^{3}H.OMDA formation (i.e., MOPEG plus VMA) was approximately 2 fold greater in the isolated media than the isolated adventitia, mainly as a result of the significantly greater formation of ^{3}H.MOPEG in the media. The proportion of ^{3}H.OMDA formed was nevertheless small representing only 8%, 16% and 14% of the total metabolite formation in the isolated adventitia, isolated media and intact aorta respectively.

Subsequently, Schrold and Nedergaard (1981) investigated the metabolites in the spontaneous efflux and electrically stimulated efflux of ^{3}H.NA from either the isolated adventitia or the intact rabbit aorta, following preloading of the tissues with (-)^{3}H.NA. Their results in the intact aorta were consistent with those of earlier workers (e.g. Henseling et al., 1978 a,b ; Su and Bevan, 1970). These workers all showed that the resting efflux consisted mainly of deaminated ^{3}H.metabolites. Schrold and Nedergaard showed that electrical stimulation increased the proportion of unchanged amine in the efflux, as well as the total ^{3}H efflux. The stimulated efflux from the isolated adventitia showed the same pattern of unchanged ^{3}H.NA and ^{3}H.deaminated metabolites as the intact aorta, suggesting that these deaminated metabolites were formed independently of the media. The neuronal origin of DOPEG was indicated by the marked reduction in its formation in electrically stimulated vessels treated with cocaine. However, the source of the NMN was less clear in this study since only minor amounts were formed (2-3% of the total ^{3}H efflux). This low

rate of ^{3}H.NMN formation is consistent with the poor penetration of NA into the media, compared with the adventitia, reported by Török and Bevan (1971). Hence, it was difficult for Schrold and Nedergaard to demonstrate a steroid sensitivity of this minor amount of O-methylation. However, they did observe a significant increase in NMN formation when the intact aorta was treated with cocaine, and this enhanced formation was abolished by the additional treatment with corticosterone, suggesting that NMN was formed in an extraneuronal, corticosterone-sensitive compartment. Further, this result could not be demonstrated in the isolated adventitia which suggested that this corticosterone-sensitive extraneuronal compartment was located in the media. With respect to OMDA formation, their results suggested that OMDA was formed independently of cocaine-sensitive (i.e. neuronal) and steroid-sensitive extraneuronal pathways. They attributed this to the O-methylation and deamination of released ^{3}H.NA in extraneuronal adventitial cells where corticosteroid-sensitive uptake was not a pre-requisite. This possibility is compatible with the findings of Jacobowitz (1972), who showed that fibroblast cells, grown from guinea pig ventricle in tissue culture, contained MAO and COMT. These fibroblast cells have also been demonstrated in the adventitia of the aorta (Branco et al., 1981a) and hence could possibly represent the sites of extraneuronal metabolism (in particular, OMDA formation) referred to by Schrold and Nedergaard.

The conclusions from the above studies in rabbit aorta are consistent with DOPEG and DOMA being formed by a cocaine-sensitive neuronal deaminating pathway, that NMN formation proceded by an extraneuronal corticosteroid-sensitive O-methylating pathway located in the media, and that OMDA formation proceded by an extraneuronal steroid-insensitive pathway located in either the media or the adventitia.

Recently, Branco et al.(1981a) described the uptake and metabolism of ^{3}H.ISO in the rabbit aorta. Their results will be considered in greater detail in the discussion of Chapter 5; but essentially they showed by autoradiography that the smooth muscle cells were the primary site of O-methylation in that tissue, with some O-methylation associated with other structures in the adventitia.

(4) Subtypes of MAO

The first direct evidence of two subtypes of MAO activity was provided by Johnston (1968), who showed that in rat brain and liver, one variety of MAO (type A) deaminated tyramine and 5-hydroxytryptamine (5-HT) and was very sensitive to clorgyline. The other variety (type B) deaminated tyramine, but not 5-HT, and was less sensitive to clorgyline. Subsequent workers confirmed the probable existence of two subtypes of MAO, each with different specificities for substrates and inhibitors (Goridis and Neff, 1971; Jarrott, 1971; Coquil et al., 1973; Knoll and Magyar, 1972). Substrates for 'type A' MAO included tyramine, NA and 5-HT. Type A was sensitive to clorgyline inhibition and its presence in sympathetic nerves indicated by depletion from chemically denervated blood vessels (Goridis and Neff, 1971). Type B MAO only deaminated tyramine, was inhibited by deprenyl and appeared to be located extraneuronally, since its activity was not influenced by chemical denervation of the rat mesenteric artery (Goridis and Neff, 1973). Levin and Wilson (1977) associated type A MAO activity with the isolated adventitia of the rabbit aorta, since low doses of clorgyline inhibited deamination of NA more than low doses of deprenyl; in contrast, they showed in the isolated media, again with NA as substrate, that low doses of diprenyl inhibited the low rate of deamination more than low doses of clorgyline, suggesting that type B MAO activity was associated with the smooth muscle cells in this tissue. However, in the rat heart

there is evidence that type A MAO is the major extraneuronal as well as neuronal enzyme variety (Fowler et al., 1978). Although the subtypes of MAO have not been reported in the rabbit ear artery, there is indirect evidence that the extraneuronal MAO may be of the type B variety. The histochemical evidence of de la Lande et al. (1970b) showed that tyramine entering via the INT surface was a substrate for deamination by extraneuronal MAO, whereas the pharmacological results of de la Lande and Jellett (1972) showed that NA entering via the INT surface was insensitive to MAO inhibition by nialamide, suggesting, therefore, that the two amines had different affinities for the extraneuronal MAO, and the low sensitivity of NA would suggest the presence of type B MAO activity.

3. COMPARTMENTALISATION

(a) MAO.

In a series of studies which examined the inactivation of ^{3}H.NA (1.2μM) in rabbit aortic strips, Henseling et al., 1978a,b; Henseling and Trendelenburg, 1978; Henseling et al., 1973; Henseling et al. 1976a,b and Eckert et al., 1976a,b, considered the sites of metabolic inactivation by dividing the ^{3}H distribution in the tissue into five compartments according to their half-times of ^{3}H efflux. These were generally, but not exclusively associated with specific morphological sites. The first two (I and II) were extracellular space; compartment III was entirely an extraneuronal site from which ^{3}H.NA effluxed rapidly (half time of 3 minutes), the fourth compartment (IV) was distributed between the extraneuronal cytoplasmic and the neuronal axoplasmic accumulation (half-time of 11 minutes). The compartment with the largest half-time of efflux (95 minutes) was found to be the neuronal vesicles (called compartment V). The origins of these compartments were indicated, in part, by the actions of cocaine which

did not influence the filling of compartment III, but partly and completely inhibited the filling of compartment IV and V respectively during the preceding incubation with ^{3}H.NA. Corticosteroid, on the other hand, only inhibited the filling of compartment III. From the composition of the ^{3}H.metabolites in the efflux, these workers considered that the metabolism of ^{3}HNA effluxing from compartment V was primarily via intraneuronal MAO, and from compartment III was primarily via extraneuronal COMT. The long half-time of efflux from compartment V (95 minutes) was due to the slow release of unchanged amine from the neuronal vesicles into the axoplasm where it was rapidly deaminated (mainly to ^{3}H.DOPEG). The presence of a small proportion of ^{3}H.NMN in this late efflux was due to extraneuronal O-methylation of the ^{3}H.NA after its release from the nerves. In a later study (Mack and Bönisch, 1979) the rate at which metabolites effluxed from the tissue was shown to be consistent with their relative lipid solubilities as indicated by their partition coefficients between octanol and water. This test ranked the metabolites in decreasing order of lipid solubility; MOPEG > DOPEG > NMN >> DOMA > VMA, which compared favourably with the observed rates of efflux from the tissues, i.e. DOPEG > MOPEG = NMN >> DOMA > VMA. They pointed out that this rate of appearance in the effluent does not necessarily reflect the rate of formation; since those metabolites with a high lipid solubility (i.e. DOPEG and MOPEG) will appear in the effluent at a rate determined by the metabolism of ^{3}H.NA, whereas those with a low lipid solubility (i.e. DOMA and VMA) will appear in the effluent at a rate determined by the passage across the cell membrane. This ranking order was in good agreement with Levin (1974) who arrived at a ranking order of appearance of metabolites in the bathing solution of DOPEG > MOPEG > NMN >> VMA > DOMA in the same tissue.

(b) COMT

A detailed analysis of the kinetic properties of extraneuronal inactivation (i.e. compartment III and part of IV, described previously) in the rat heart was described by Bönisch and Trendelenburg (1974) and Trendelenburg(1978) using ^{3}H.ISO (0.95μM) as a substrate. Compartment III accumulated and O-methylated ^{3}H.ISO; both processes were saturable and had a high affinity for ^{3}H.ISO (Km for O-methylation of ISO was 3.0μM). Similar results have been reported in other tissues, e.g. the cat nictitating membrane (Graefe and Trendelenburg, 1974), the rat submaxillary gland (Major et al., 1978), the rabbit aorta (Henseling, 1980a) and the rabbit ear artery (Head et al., 1980). Each of these studies describe an extraneuronal O-methylating system characterised by a low Km (between 1.7 and 12μM) and sensitivity to corticosteroids. In the case of the rabbit ear artery, the efflux of ^{3}H.ISO suggested it was derived from two compartments (other than extracellular space), but its O-methylation proceded in a single compartment (Km = 2.7μM). The relevance of the latter result to the present study lies in the implication that in the rabbit ear artery, O-methylation of NA probably also occurs within a single compartment, hence, multiple sources of this metabolite are unlikely. A single O-methylating compartment is not a universal feature of the extraneuronal system, for example, in the nictitating membrane there is evidence of a second O-methylating system of low affinity for catecholamines (Graefe and Trendelenburg, 1974).

4. DIFFUSION

As indicated previously, there is pharmacological evidence that the concentration which NA, entering the rabbit ear artery via the intimal surface, achieves in the region of the nerve terminals is low compared with NA entering via the adventitial surface (de la Lande et al.,1970b).

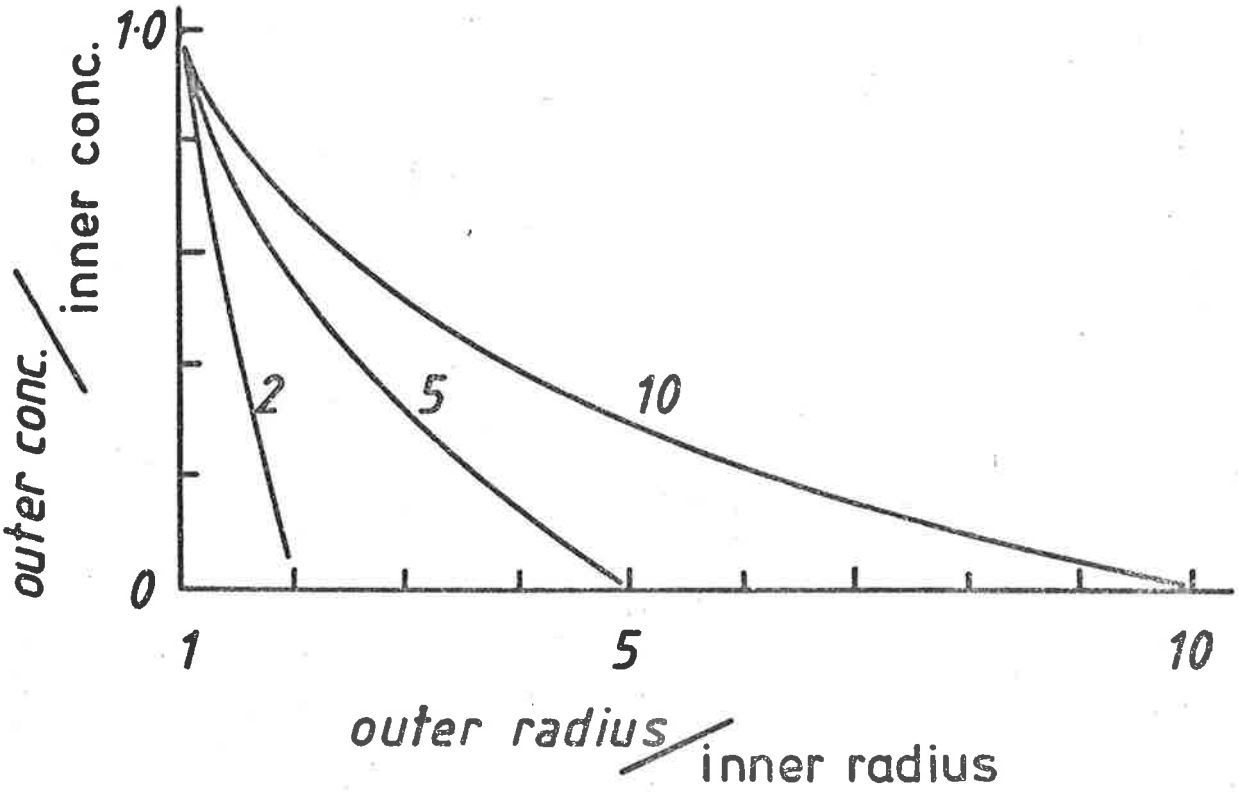

Fig. 1.4 This shows the steady-state concentration distribution of a substance diffusing through the wall of a hollow cylinder. Numbers on the curves are values of the outer radius (b) divided by the inner radius (a). As the wall thickness decreases (ie, b/a approaches one), then the concentration distribution becomes more linear. In the case of the rabbit ear artery, where b/a is usually less than 2, the distribution approximates to a linear gradient. This figure is derived from Crank (1956),Fig. 5.1, p 63.

Subsequent pharmacological evidence suggests that the difference in concentration may be as much as 10 fold (de la Lande, 1975). Some of the possible factors which are responsible for this difference will be discussed. According to Fick's first law of diffusion, a gradient of concentration must exist between the surface to which the amine is applied and the opposite surface. In theory, if the blood vessel was a perfect cylinder, and the wall were homogeneous with respect to diffusivity of NA, this gradient of concentration would be of the form shown in Fig. 1.4 (from Crank, 1956; Fig. 5.1, p. 63). As indicated, the gradient becomes more linear when the difference between the inner and outer radii is small, i.e. the ratio of external radius (b) to internal radius (a) approaches unity, until it approximates to the linear gradient existing across the wall of a plane sheet. Hence the relative distance of the nerves from the two surfaces will be one factor in determining the relative concentration achieved by NA in the region of the nerve terminals. These distances, estimated in a group of histological sections of ear arteries which were relaxed at the time of fixation was approximately 0.11 $\pm$ 0.01 mm from the intima to the outer media, and 0.08 $\pm$ 0.01 mm from the outer media to the adventitial surface (Jellett, 1971). These estimates are only approximate since the intima was convoluted and the outer surface of the adventitia was irregular in shape. Nevertheless, they suggest that the location of the nerves at the medial-adventitial border was not the only determinant of the concentration of NA in this region. A second factor influencing the concentration of NA across the artery wall may be the regional differences in diffusivity of NA in the media and adventitia. The evidence from a number of laboratories (reviewed by de la Lande,1975) suggests that in the rabbit ear artery 80-90% of the ^{3}H.NA which diffused away from the nerve terminals appeared in the EXT

bathing solution, i.e. diffused through the adventitia. This suggested that the media represented a far greater barrier to the diffusion of amine than did the adventitia. This was supported by similar evidence by Török and Bevan, 1971; Allen et al., 1973; Steinsland et al. 1973, and reviewed by de la Lande, 1975. Török and Bevan (1971) showed that in the rabbit aorta the faster diffusion through the adventitia resulted partially from its more "open fabric", since they demonstrated a 59% inulin space in the adventitia, compared with only 39% in the media. Hence the main diffusional barrier in the artery wall was shown to be the media. A third factor determining the amine concentration in the biophase is its removal by the extraneuronal O-methylating pathway of the media. The earlier evidence of de la Lande et al. (1974), who studied the uptake of NA into nerves of MAO-inhibited reserpinised ear arteries, included the demonstration that the extraneuronal O-methylating system was able to influence the concentration NA achieved in the region of the nerve terminals when the amine entered through the intimal surface. The evidence was that INT NA only restored fluorescence in the nerve terminals when either an inhibitor of extraneuronal uptake, or an inhibitor of COMT (i.e., metanephrine or UO521, respectively) was also present. This was supported by the pharmacological evidence of Johnson and de la Lande (1978) that inhibition of COMT or extraneuronal uptake (by UO521 or DOCA respectively) caused a two fold increase in sensitivity of the ear artery to INT NA. This suggested that the extra-neuronal O-methylating system caused a reduction of approximately 50% in the NA in the biophase and hence might account for some portion of the apparent decline in concentration of NA, i.e. it would steepen the gradient. A fourth factor which has no experimental support to date is the specific binding of NA to receptors which presumably could also account for a portion of the removal of amine from the extracellular solution and hence increase the gradient of concentration of NA across the artery wall.

An understanding of these factors is important since the nature of the gradient will determine the concentration of NA in the extracellular environment of the cell, and hence its availability to the receptors. Further, in view of the evidence that NA is inactivated by different mechanisms in the media and the adventitia, one might expect that the gradient of concentration will bear an intimate relationship to metabolism, i.e., metabolism may influence the gradient across the wall, and hence the gradient itself determine the relative contribution of the media and the adventitia to inactivation. Perhaps the most important reason for seeking information about this gradient is that in the physiological situation, the concentration of NA in the vessel wall is probably always distributed non-uniformly; i.e. when released from the nerve terminals the concentration of NA will decline as the amine diffuses from this region. When circulating adrenaline and NA enter via the intimal surface, the situation is more complex. The extent to which the concentration declines across the media towards the adventitia will be dependent on the extent of associated release of NA from the nerves.

5. SPECIFIC AIMS

The present study was undertaken in the expectation that, since ^{3}H.DOPEG was neuronal in origin (Head, 1976), then the relative rates of ^{3}H.DOPEG formation when ^{3}H.NA was applied separately to the adventitial or the intimal surface would indicate the relative concentration which NA achieved in the region of the nerve terminals. By defining the magnitude of the decrease in concentration between the intima and the nerve terminals, the results would provide a test of the model proposed by de la Lande et al.(1970b) (Fig. 1.2) to account for the differences in

sensitivity of the artery to INT and to EXT NA. The comparison of the metabolism of INT and of EXT NA was also undertaken to define more precisely the relative contributions of medial and adventitial processes in the inactivation of NA. In this respect the study can be compared with those of Levin (1974) in the isolated media and isolated adventitia of the rabbit aorta. However, the study of metabolism when the amine is applied to only one surface has an advantage in that it does not eliminate interactions which may occur between the medial and adventitial inactivating systems. One example which illustrates such an interaction is the mechanism of formation of the O-methylated-deaminated metabolites (OMDA) as discussed in Chapter 6 and also in the Section 2 of this introduction (page 19).

As the study progressed it became apparent that the approach adopted was leading to new insights into the factors influencing the metabolism of NA in the artery wall. One of these factors was vasoconstriction in response to the applied amine. Hence it became important to compare the diffusion and metabolism of INT and of EXT ^{3}H.NA in unconstricted (i.e., relaxed) and constricted vessels. Initially Ca^{++} was omitted from the Krebs solution to minimise constriction; subsequently it was found necessary to include the α_1-antagonist prazosin into the bathing solution to ensure that vasoconstriction did not occur. These and other considerations, which are explained in the individual chapters, led the study to compare;

(1) the kinetics of metabolite formation,

(2) the effects of reserpine pretreatment and of Ca^{++} on metabolite formation,

(3) the effects of inhibition of neuronal and extraneuronal uptake, and of α-receptor binding on the metabolite formation,

(4) the effects of surface of entry on the metabolism of a catecholamine (ISO) which was inactivated solely by extraneuronal O-methylation in order to elucidate the influence of surface of entry of NA on the formation and efflux of its O-methylated metabolites.

The study of the effects of vasoconstriction were an integral part of the effects of Ca^{++} and of α-receptor blockade. Of the above studies (1) and (2) are summarised in Chapter 3; (3) in Chapter 4 and (4) in Chapter 5. The mechanism of formation of the O-methylated-deaminated metabolites (OMDA) and in particular MOPEG formation from DOPEG are dealt with in Chapter 6. Chapter 7 describes some pharmacological studies where the aim was to provide further information on the influence of the gradient of concentration of NA on its pharmacological response. Chapter 8 describes the metabolism of ^{3}H.NA in a different vessel, the rat tail artery. As indicated earlier, it was hoped this study would include similar studies to those in the rabbit ear artery to provide a comparison of the interaction of the diffusion gradient and metabolism in a vessel with a thinner wall (i.e. the rat tail artery) and then to extend this part of the study to a pathological state of vascular hypertrophy such as has been described in vessels from DOCA-salt hypertensive rats. Unfortunately, time did not allow completion of this study.

CHAPTER 2

GENERAL METHODS

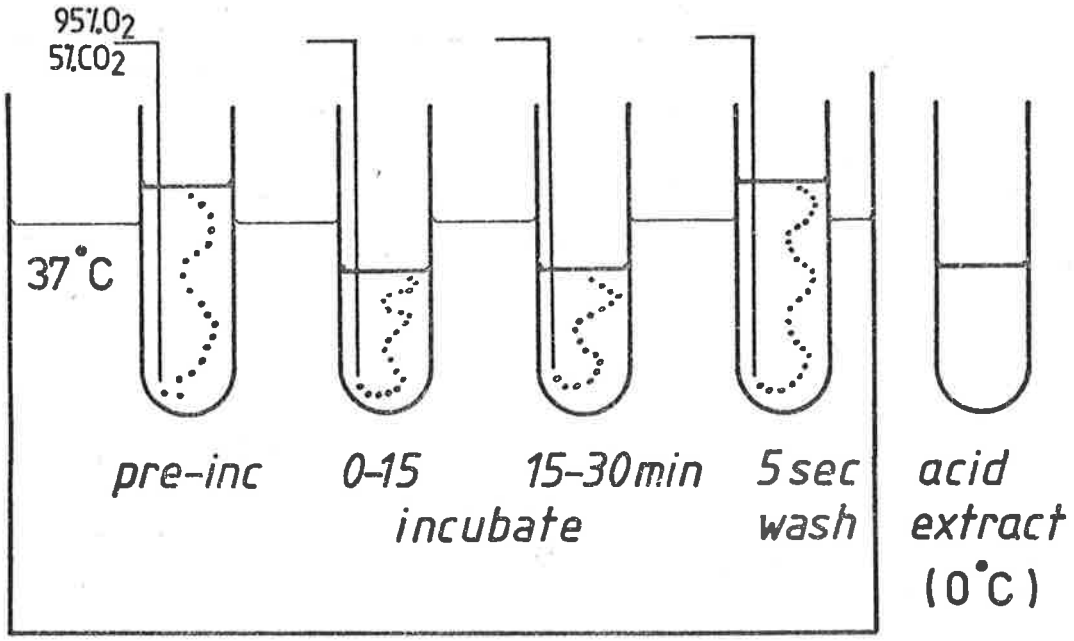

Fig. 2.1 A diagrammatic representation of the procedure used for incubating rabbit ear artery strips. Any drug treatments were applied for 30 minutes in the "pre-inc" tube as well as during the incubation with ^{3}H.labelled catecholamines (shown here as two successive 15 minute incubations, but in many experiments comprising one 30 minute incubation). This was followed by a 5 second wash and acid (0.4M $HClO_4$) extraction.

CHAPTER 2.

GENERAL METHODS

1. INCUBATION STUDIES

(a) Isolated artery strips

Ear arteries were removed from semi-lop-eared rabbits of a strain developed at the Central Animal House, the University of Adelaide. Unless otherwise stated the rabbits (2.5-3.5Kg) had been pretreated with reserpine, 1.0mg.Kg^{-1} at 24 hours and again with 0.5mg.Kg^{-1} at 3 hours prior to stunning and bleeding. The central artery of each ear was isolated and a 20 to 25mm segment cut longitudinally with iridectomy scissors taking care to minimise trauma to the vessel. This artery strip was then placed in a glass vial containing Krebs solution at 37°C and bubbled with a mixture of 95% O_2, 5% CO_2 (Fig. 2.1). In many experiments $CaCl_2$ was omitted from the Krebs solution (as specified in the text) to minimise constriction when the vessel was exposed to NA.

Artery strips were blotted on moist filter paper, weighed and then placed in Krebs solution for 30-60 minutes prior to adding ^{3}H.catecholamine (or ^{3}H.metabolite). The incubation was continued for a further 30 minutes in Krebs solution containing the ^{3}H.catecholamine. Where the effects of drugs were studied, these were added 30 minutes prior to and during incubation with the ^{3}H. catecholamine. At the end of the incubation the tissues were rapidly removed, rinsed for 5 seconds in 2.0ml of ^{3}H.free Krebs solution and placed in 0.4M perchloric acid (containing 3mM EDTA and 10mM Na_2SO_3) at 4°C and kept for assay the following day. The incubating medium was immediately acidified with 0.2ml of 0.1M HCl and 0.02ml of 0.6M ascorbic acid and placed on ice. Before assaying the acidified incubating medium, or the acid extract of ^{3}H remaining in the tissue for ^{3}H.NA and ^{3}H.metabolites, 0.1ml of each solution was sampled and the radioactivity determined.

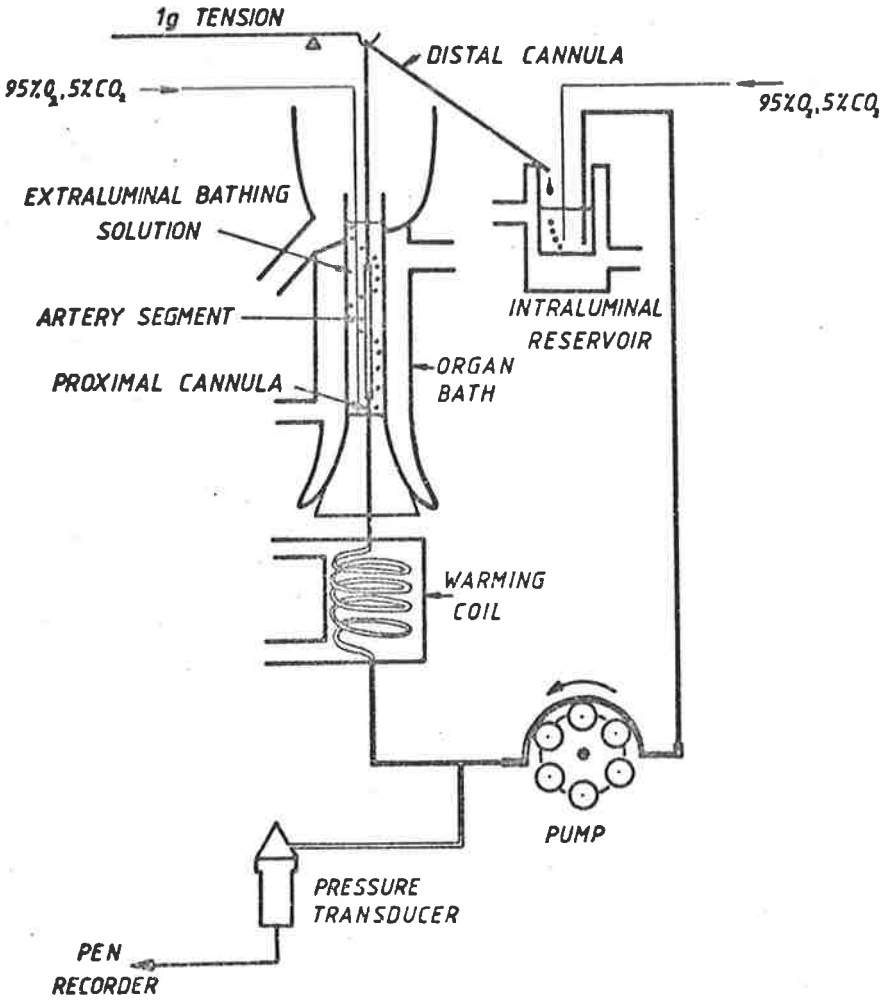

Fig. 2.2 The perfusion system used for incubating rabbit ear artery segments. Any drug treatments were applied to both surfaces of the vessel 30 minutes before incubating with ^{3}H.labelled catecholamines, which were added either to the extraluminal bathing solution, or to the intraluminal reservoir. Note that the INT perfusate was recirculated during the 30 minute incubations, following warming and gassing in the reservoir.

(b) Perfused segments

Segments of ear arteries (15 to 25mm) from rabbits (as described in (a) above) were cannulated at both ends and placed in organ baths containing Krebs solution bubbled with 95% O_2 and 5% CO_2 at 37°C. The vessels were then perfused intraluminally with Krebs solution, and the longitudinal tension adjusted to 1g. This technique is that of de la Lande et al (1966); it enables the two surfaces of the vessel (adventitia and intima) to be bathed separately with Krebs solution. Intraluminal peristaltic flow was maintained at a constant rate of 0.5ml . min^{-1} by means of a Desaga pump (model 772388). The extraluminal (EXT) bath volume was 1 or 2ml. All segments were checked for leakage (via side branches) of the INT perfusate into the EXT bathing solution by assessing whether the EXT bath volume remained constant, vessels suspected of leaking were discarded.

Vasoconstriction was measured by the increased resistance to flow as indicated by an increase in perfusion pressure. The latter was measured via a Statham pressure transducer (model P23AC) lineated between the pump and the vessel, and recorded on a Rikidenki double-channel pen recorder (model B24). As shown in Fig. 2.2, a small reservoir collected the effluent from the top cannula of the artery so that the INT perfusate was again warmed, gassed and recirculated through the artery during incubations. After 30-60 minutes perfusion, ^3H labelled catecholamine was added either (a) to the intraluminal perfusing solution only (referred to as INT), (b) to the extraluminal bathing solution only (referred to as EXT), or (c) to both the INT and EXT solutions simultaneously. Incubation was for a 30 minute period unless specified otherwise. As in the case of artery strips, drugs were

added (to both solutions simultaneously) 30 minutes prior to commencing incubation with the labelled amine and were present throughout the incubation period. After recording the volumes of the INT and EXT bathing solutions, these solutions were acidified with 0.2ml of 0.1M HCl and 0.02ml of 0.6M ascorbic acid. The ^{3}H content of 0.1ml was measured and the remainder stored on ice until assayed for unchanged ^{3}H. catecholamine and the ^{3}H.metabolite(s).

Notes:- (a) The volumes of the INT and EXT bathing solutions were kept small (1.0 to 2.0ml) to enable the small amounts of ^{3}H material which diffused across the vessel wall to be analysed. For this reason, narrow bore tubing was used in the perfusion lines (silastic pump tubing, 1.0mm i.d. and 2.0mm o.d. and polythene delivery tubing and cannulae, 0.5mm i.d. and 1.0mm o.d.).

(b) To enable diffusion coefficients to be measured, the length of the segment and its diameter were routinely measured with a Zeiss binocular dissecting microscope. When measuring diameter, a stainless steel wire of known diameter (placed beside the vessel in the organ bath) was used for reference. When measuring length, a graduated polythene rule placed beside the vessel was used for reference. At the end of the experiment, the segment between the cannulae ties was blotted on moist filter paper and weighed. The diffusion equation is shown on page 42a

(c) In most experiments more than one incubation was carried out on each segment. Following the first incubation with ^{3}H.catecholamine the intimal surface was continuously perfused, and the adventitial surface washed 8-10 times,

Table 2.1

Abbreviations

NA	noradrenaline
DOPEG	3,4-dihydroxyphenylethylene glycol
MOPEG	3-methoxy,4-hydroxyphenylethylene glycol (ie,the methoxy derivative of DOPEG)
DOMA	3,4-dihydroxymandelic acid
VMA	vanillyl mandelic acid, or 3-methoxy,4-hydroxy mandelic acid (ie, the methoxy derivative of DOMA)
NMN	normetanephrine, (ie, the methoxy derivative of NA)
OMDA	O-methylated-deaminated metabolites (ie, MOPEG + VMA)
ISO	isoprenaline
MeOISO	3-methoxy isoprenaline
^{3}H.NA	Tritiated noradrenaline

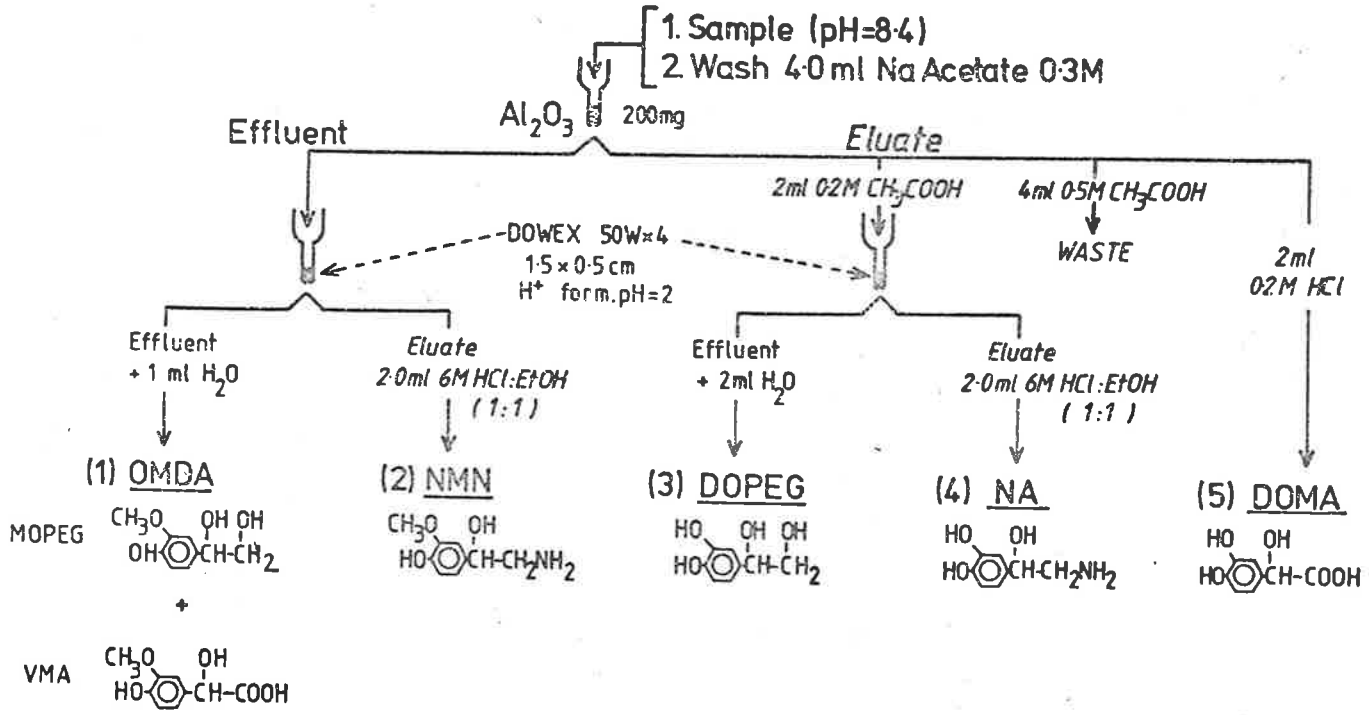

Fig. 2.3 A flow diagram of the cascade column chromatographic assay for separating NA and its metabolites. The sample, either 1.0ml of acidified Krebs solution (incubating medium) or 1.0ml perchloric acid (tissue extract), were added to 0.1ml EDTA (0.3M), 0.1ml Na_2SO_3 (1.0M), 0.01ml ascorbic acid (0.6M), 0.01ml carrier solution (each 0.6M) and 1.0ml (medium) or 1.5ml (tissue) of TRIS buffer (1M, pH=8.4), before loading the alumina column. Note that MOPEG and VMA both appear in fraction (1).

with ^{3}H-free Krebs solution at 37^{o}C over at least a 60 minute period. When required, drugs were added to the INT and EXT solutions 30 minutes prior to the start of the second incubation.

(d) In most of the studies with perfused segments, artery strips from the same ear were incubated under otherwise identical conditions to provide information on the metabolism of the ^{3}H.catecholamine when applied to both surfaces simultaneously.

2. ASSAY OF ^{3}H.NA AND ^{3}H.METABOLITES

To facilitate presentation, the abbreviations used for metabolites are represented in Table 2.1. The assay for separating unchanged ^{3}H.NA and the ^{3}H.metabolites was essentially the same as that of Graefe et al (1973). The principal of this cascade column chromatographic method, as shown in Fig. 2.3 involves separation of the catechols from the phenolic metabolites by adsorption onto alumina at pH = 8.4 and separation into acids, bases and neutral compounds by means of adsorption onto DOWEX 50. When tissue extracts were analysed by this method, an additional 0.5ml of 1M TRIS buffer (pH = 8.4) was added to the mixture before loading the alumina column. At the completion of the separation procedure, 1.0ml of each fraction was sampled for its ^{3}H content.

The efficiency of separation and recovery of NA and metabolites was measured by subjecting unlabelled NA and each of the metabolites to the above chromatographic procedure. The NA and various metabolites in the fractions were then assayed by their native fluorescence. The results are shown in Table 2.2.

Table 2.2

The recovery of unlabelled NA and metabolites and their crossover into other fractions using the cascade column chromatogrphic method (Fig. 2.3)

Fraction	MOPEG	VMA	NMN	DOPEG	NA	DOMA
1.	96.8	92.3	0	0	0.3 ±0.0	0
2.	2.0	0.7	100	0	0.1 ±0.0	0
3.	0	1.2	0	95.8	0.03±0.0	0
4.	1.1	0	0	0	77.0 ±4.0	2.3
5.	0	0	0	1.9	2.5 ±0.1	56.3
4ml Wash	0	4.8	0	2.3	0.4 ±0.0	41.4
n	1	1	1	1	7	1

Table 2.3

The recovery of unlabelled ISO and the metabolite and their crossover into other fractions using the cascade column chromatographic method (Fig. 2.4). The appearance in fraction 1 was not determined.

Fraction	MeOISO	ISO
1.	?	?
2.	100 ±0	0
3.	0	91 ±1
n	2	2

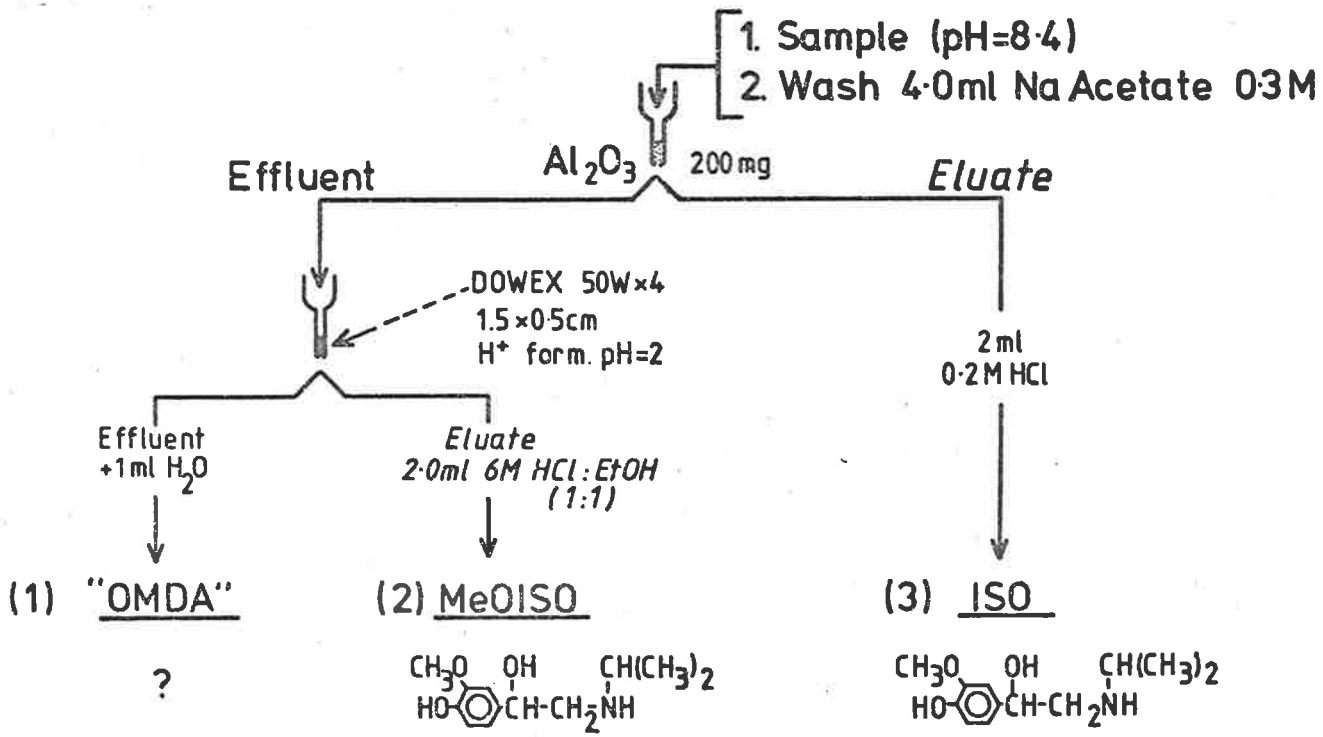

Fig. 2.4 A flow diagram of the cascade column chromatographic assay for separating ISO and its metabolite, MeOISO. Samples were prepared as described in Fig. 2.3, before being loaded onto the alumina column. The "metabolite(s)" appearing in the OMDA fraction were not identified as they could not be demonstrated using TLC.

The assay was adapted to separate unchanged ^{3}H.ISO and its metabolite as shown in Fig. 2.4. The recovery of unlabelled ISO and MeOISO was measured by assaying the fractions for their native fluorescence. The results are shown in Table 2.3. This shows that recovery of ISO and MeOISO was high, and that no crossover of ISO into fraction 2, or MeOISO into fraction 3, was detected. The crossover of either compound into fraction 1 was not measured.

3. SCINTILLATION SPECTROMETRY

The radioactivity of samples was determined by liquid scintillation spectrometry using either a Packard Scintillation Spectrometer (model 3310) or a Beckman Scintillation Spectrometer (model LS 7500). The scintillation cocktail was prepared in this laboratory and contained toluene and triton-X 100 (2 to 1 ratio) and the spectrofluors PPO (2,5-diphenyloxazole) and dimethyl-POPOP (1,4-bis(2(4-methyl-5-phenyloxazolyl))benzene) (5.5 $g.l^{-1}$ and 0.17 $g.l^{-1}$ respectively). In either spectrometer, quenching was determined by reference of the ratio of counts in 2 appropriate tritium windows to a quench curve constructed for each counter using a commercially prepared set of quenched standards. Hence counts per minute (CPM) were converted to disintegrations per minute (DPM) and this latter figure used in all calculations. In the former spectrometer, samples were counted for 10 minutes, or until 900,000 counts had accumulated (whichever occurred first); and in the latter spectrometer counting proceded until a CPM accuracy of $\pm$2% was achieved, or for 10 minutes (whichever occurred first). Quenching (ranging from 50 to 80%) was determined for every sample counted in either spectrometer by reference to a quench curve constructed using a commercial set of quenched tritium standards.

4. RADIOCHEMICALS

(a) (-)^{3}H.(7-C) NA (Radiochemical Centre, Amersham, Batch 54), specific activity 15 Ci. mmol^{-1}, was used in early experiments. When its supply was discontinued, New England Nuclear (NEN) (-)^{3}H.NA was used. However, it was ascertained that several batches used (NEN 1271-115, 1293-077 and 1217-039) were partly labelled on the 8-C position (Starke et al, 1980; confirmed in personal communication with NEN). Data using these suspect batches was rejected. As NEN would not then guarantee that 7-C labelled material was not also labelled on the 8-C atom, ring (2,5,6-C) labelled (-)^{3}H.NA (Batch number 1271-144, specific activity 46.5(Ci.mmol^{-1}) was subsequently used in all experiments.

Note:- The reason for rejecting the partly 8-C labelled material was, as shown by Starke et al (1980), (a) that ^{3}H released by MAO deaminating an 8-C labelled NA molecule would appear as ^{3}H-water and contaminate the OMDA fraction, and (b) the evidence that the 8-C labelled molecule is less actively metabolised by MAO.

Stock solutions of (-)^{3}H.NA were a 9:1 mixture 0.2M acetic acid and ethanol. In the case of the 7-C (-)^{3}H.NA, an appropriate volume was freeze-dried and reconstituted in gassed ascorbic Krebs solution at 37^{o}C immediately prior to use. The 2,5,6-C (-)^{3}H.NA, because of its higher specific activity, was diluted directly with unlabelled (-)NA dissolved in normal saline containing 0.6mM ascorbic acid and added to gassed ascorbic Krebs solution at 37^{o}C (dilution factor approximately 1 to 500). The final specific activity of the (-)^{3}H.NA in the incubating medium was of the order of 4 Ci.mmol^{-1}. The purity of the ^{3}H.NA was routinely determined by its recovery in the NA fraction (fraction 4)

from column chromatography. The purity in experiments with 7-C (-)^{3}H.NA was greater than 80%. In the case of 2,5,6-C (-)^{3}H.NA, the purity was greater than 95%. In some of the earlier experiments using the 7-C (-)^{3}H.NA, the purity of the stock label was less than 80%. In these cases, the stock was first purified by adsorption onto alumina, as described by Head et al (1978).

(b) (±)^{3}H.ISOPRENALINE (Radiochemical Centre, Amersham, batch numbers 12, 15 and 18) was used. The appropriate volumes were freeze-dried, or vortex-evaporated to dryness, and reconstituted in gassed Krebs solution at 37^{o}C containing ascorbic acid (0.6mM) together with unlabelled (±)ISO in the appropriate concentration. Experiments where the final concentration of ISO was 0.18μM, a 1:1 dilution of labelled to unlabelled ISO was used; whereas in experiments using 0.8μM, a 1:9 ratio was used. Again, radioactive purity was determined by the recovery of ^{3}H in the ISO fraction of the column chromatographic assay and was found in all experiments to be greater than 90%.

(c) (-)^{3}H.DOPEG. In one series of experiments the metabolism of (-)^{3}H.DOPEG was examined. Since ^{3}H.DOPEG is not commercially available, it was prepared by the author. The method of preparation and purity is described in detail in Chapter 6.

5. CALCULATION OF DIFFUSION COEFFICIENT

The internal radius, and hence the wall thickness of the artery segment, is estimated from the following formula:

$$a = \sqrt{(b^2 - \frac{w}{\pi l})}$$

where a = internal radius (cm), b = external radius (cm),

w = weight (gm), and l = length (cm).

It is assumed that the specific gravity of the artery is 1.0. The diffusion coefficient is then estimated from the formula for the diffusion of a substance across the wall of a cylinder (Crank, 1956).

$$D = \frac{Q_t \ln \frac{b}{a}}{2\pi t (C_1 - C_2) l}$$

where D = diffusion coefficient ($cm^2.sec^{-1}$),

t = time (sec),

C_1 = concentration of substance maintained at one surface,

C_2 = concentration of substance at the opposite surface,

Q_t = flux of substance across the wall, i.e., the quantity appearing in the solution bathing the opposite surface, in time (t).

CHAPTER 3

UPTAKE AND METABOLISM OF ^{3}H.NORADRENALINE IN ISOLATED ARTERY STRIPS

CHAPTER 3.

UPTAKE AND METABOLISM OF ^{3}H.NORADRENALINE IN ISOLATED ARTERY STRIPS

INTRODUCTION

Although the major studies in this thesis concern the metabolism of NA in perfused segments of rabbit ear arteries, the metabolism of ^{3}H.NA in non-perfused artery strip preparations will be described first as these results are essential to the interpretation of much of the data on the perfused segments. In the studies in this chapter, isolated artery strips were incubated with ^{3}H.NA in a fixed volume of Krebs solution (1 or 2ml). Under these conditions it is assumed that the amine entered both surfaces of the vessel simultaneously. This is the usual method of studying catecholamine metabolism in isolated tissues and was used in the present study primarily to provide kinetic data which would have proved difficult and time consuming to derive from perfused segments. The kinetic data emphasises the relationship between the substrate (^{3}H.NA) concentration and ^{3}H.DOPEG formation. Other data presented in this chapter refer to the effects, on NA metabolism in artery strips, of the various treatments used in the studies on perfused segments. These treatments compared, (a) the use of arteries from reserpine pre-treated rabbits (to minimise retention of unchanged amine in the tissue), (b) the use of Ca^{++}-free medium (to minimise constriction), (c) the use of prazosin (to abolish the constrictor response to NA), (d) the effect of cutting the segment to form a strip, and (e) the use of cocaine and hydrocortisone to minimise neuronal and extraneuronal uptake, respectively, of ^{3}H.NA.

Table 3.1

The accumulation and metabolism of (-)^{3}H.NA (0.18 μM) in rabbit ear artery strips
Values shown are means ± SEM together with their percentage distributions.
* Data for reserpine plus prazosin indicates efflux into the bathing medium only.

Pretreatment	Medium	Preparation	n	Tissue NA	Total Metabolism nmol. $g^{-1}.30min^{-1}$ DOMA	DOPEG	NMN	OMDA
Untreated	Ca^{++}	Segment	4	2.85 ±0.24 (66%)	0.03 ±0.01 (1%)	0.55 ±0.07 (13%)	0.39 ±0.09 (9%)	0.49 ±0.04 (11%)
	Ca^{++}	Strip	4	2.25 ±0.22 (60%)	0.02 ±0.01 (15)	0.52 ±0.09 (14%)	0.51 ±0.04 (14%)	0.45 ±0.07 (12%)
Reserpine	Ca^{++}	Strip	6	0.55 ±0.17 (13%)	0.46 ±0.11 (11%)	2.08 ±0.22 (48%)	0.37 ±0.08 (8%)	0.90 ±0.17 (21%)
	Ca^{++}free	Strip	4	0.39 ±0.14 (10%)	0.72 ±0.08 (18%)	1.84 ±0.17 (47%)	0.43 ±0.05 (11%)	0.57 ±0.10 (14%)
Reserpine plus Prazosin *	Ca^{++}	Strip	21	-	0.46 ±0.05	2.07 ±0.12	0.49 ±0.04	0.71 ±0.06

METHODS

Rabbit ear artery strips or segments were incubated with (-)^{3}H.NA as described in the General Methods (Chapter 2). The principle of the method was that freshly excised central ear arteries were incubated for 30 minutes with ^{3}H.NA in Krebs solution at 37^{o}C and bubbled with 95% O_2, 5% CO_2. Both segments, and segments slit longitudinally to form strips were used. Treatments included reserpine pretreatment of rabbits, as described in General Methods, and the presence or absence of Ca^{++} in the bathing medium. Drugs were present 30 minutes prior to, and during, incubation with the ^{3}H.NA. The amount of unchanged ^{3}H.NA and ^{3}H.metabolites present in the incubating medium or tissue extract were assayed by cascade column chromatography (described in the General Methods).

In the kinetic studies where two 15 minute incubations were carried out, the procedures were identical to the above except that the tissue was transferred to a second tube, containing the same incubating medium as the first, after 15 minutes (Fig. 2.1).

Many of the experiments on strips were carried out at the same time as those on perfused segments, the strip being removed from a more distal part of the ear. These experiments included those involving drug treatments (other than the effect of reserpine pretreatment).

RESULTS

(1) Reserpine:-

As shown in Table 3.1, the effect of reserpine pretreatment, with or without Ca^{++} in the bathing solution, was to markedly reduce (by 75%) the retention of unchanged ^{3}H.NA and markedly increase the formation of the deaminated metabolites, ^{3}H.DOPEG and ^{3}H.DOMA (by 3.5 fold and

Table 3.2

Efflux of metabolites from reserpinised rabbit ear artery strips incubated with $(-)^{3}$H.NA (0.18 μM) in two successive 15 minute incubations, compared with one 30 minute incubation.
Values shown are means ± SEM.

Incubation time (mins)	n	Metabolite Efflux nmol.g^{-1}			
		DOMA	DOPEG	NMN	OMDA
0 - 15	11	0.19 ±0.03	0.86 ±0.08	0.22 ±0.02	0.24 ±0.04
15 - 30	11	0.23 ±0.03	1.03 ±0.10	0.29 ±0.03	0.39 ±0.05
0 -30	6	0.59 ±0.07	1.71 ±0.15	0.39 ±0.04	0.48 ±0.08

Table 3.3

MEDIUM TO TISSUE RATIOS

The relative amounts of ^{3}H.metabolites effluxing into the incubating medium, to that retained by the tissue, at three substrate concentrations for Ca^{++} free media, and one concentration for Ca^{++} media, for rabbit ear artery strips incubated with $(-)^{3}H$.NA.

Treatment	^{3}H.NA (μM)	n	Tissue NA ($nmol.g^{-1}$)	Medium/Tissue Ratios			
				DOMA	DOPEG	NMN	OMDA
Ca^{++}free	0.02	4	0.01 ±0.00	3	18	8	2
	0.05	4	0.06 ±0.01	5	14	11	6
	0.18	6	0.39 ±0.14	5	16	10	5
Ca^{++}	0.18	6	0.55 ±0.17	4	20	2	19

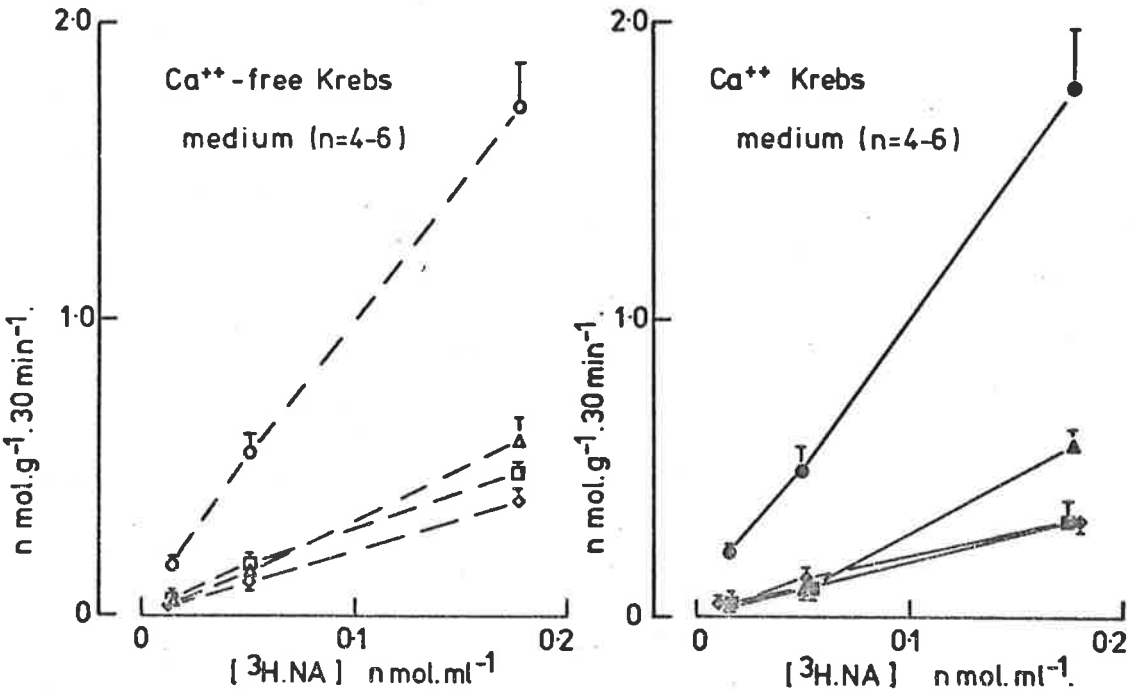

Fig. 3.1 The relationship between ^{3}H.metabolite efflux (nmol.g^{-1}.30min^{-1}) and the concentration of ^{3}H.NA (μM) in reserpinised rabbit ear artery strips incubated in Ca^{++} free and normal Ca^{++} Krebs solution. Circles indicate DOPEG, triangles indicate DOMA, squares indicate OMDA and diamonds indicate NMN. This shows that DOPEG efflux predominates, that efflux of each of the metabolites approximates to a straight-line over the concentration range used and that the omission of Ca^{++} from the bathing medium had little effect on metabolite efflux.

36 fold, respectively). These changes are consistent with impaired vesicular storage, and increased availability, of ^{3}H.NA to the intraneuronal MAO pathway, following reserpine pretreatment.

(2) Kinetics:-

The kinetics of ^{3}H.NA metabolism with respect to substrate concentration and time is presented in Fig. 3.1 and Table 3.2. The results in Fig. 3.1 shows that when reserpinised artery strips were incubated with graded concentrations of ^{3}H.NA for 30 minutes in either Ca^{++} or Ca^{++} free media, ^{3}H.DOPEG was the principal metabolite. The relative proportions of the total metabolites formed at 0.18μM ^{3}H.NA(Ca^{++}-free) comprised ^{3}H.DOPEG (52%), ^{3}H.DOMA (20%), ^{3}H.OMDA (20%) and ^{3}H.NMN (12%). The proportions of each of the metabolites retained in the tissue was small compared with those which effluxed into the bathing medium during the 30 minute incubation period. In Table 3.3, this distribution is presented in terms of the ratio of the amount of metabolite in the incubating medium to that retained by the tissue at each of the substrate concentrations in Ca^{++} free media, and at 0.18μM ^{3}H.NA in Ca^{++} media. In the case of the Ca^{++} free media incubated with 0.18μM ^{3}H.NA, the proportions retained in the tissue were ^{3}H.DOPEG (6%), ^{3}H.NMN (9%), ^{3}H.OMDA (22%) and ^{3}H.DOMA (17%). Unchanged ^{3}H.NA represented 47% of the total ^{3}H retained in the tissue. Of the ^{3}H.NA removed from the bathing medium, 10% was retained in the tissue and 90% was metabolised. The results in Fig. 3.1 also shows that within the range of concentrations of ^{3}H.NA examined (0.018 to 0.18μM), the amounts of the metabolites which effluxed into the bathing medium were directly proportional to the substrate concentration in both Ca^{++} and Ca^{++} free media. The rate of formation of the metabolites was unaffected by the omission of Ca^{++} from the bathing solution. There was a tendancy for the Ca^{++} free tissues to retain less unchanged ^{3}H.NA, but this difference was not significant at the 5% level.

The time course of efflux was examined by comparing the amount of the metabolites which effluxed into the bathing medium during two successive 15 minute incubations with ^{3}H.NA. As shown in Table 3.2 the efflux of each of the metabolites increased in the second 15 minute period. However, with the exception of ^{3}H.OMDA, this increase was sufficiently small (20-30%) to suggest that there was little error involved in using the total efflux during the 30 minutes as a measure of their respective rates of formation. In the case of ^{3}H.OMDA a significant 60% increase in the second 15 minutes was observed. The formation of this fraction is considered in greater detail in Chapter 6.

(3) Segments:-

The effect o the metabolism of ^{3}H.NA (0.18μM) of cutting an artery segment longitudinally to form a strip is shown in Table 3.1. There was a non-significant tendency for the strip to form more ^{3}H.NMN, consistent with the probability that the intimal surface was more accessible to the substrate in the artery strip than in the segment . The artery strip also tended to retain less unchanged ^{3}H.NA; this may have reflected the trauma or injury to the nerves in preparing the artery strip.

(4) Prazosin:-

The effect of prazosin (0.2μM) on the metabolism of ^{3}H.NA in artery strips is shown in Table 3.4. Although only data on the efflux of metabolites into the incubating medium is shown, the results suggest that the prazosin treatment was without a significant effect on metabolite formation in isolated strips.

Table 3.4

The efflux of metabolites into the incubating medium of reserpinised rabbit ear artery strips incubated with (-)^{3}H.NA (0.18 μM) in Ca^{++} free medium.
Values shown are means ±SEM.
* indicates significance ($p < 0.05$); unpaired t-test.

Treatment (n)	Metabolite Efflux nmol.g^{-1}.30min^{-1}			
	DOMA	DOPEG	NMN	OMDA
Untreated (n=9)	0.06 ±0.07	2.32 ±0.20	0.48 ±0.03	0.97 ±0.24
Prazosin (n=21)	0.46 ±0.05	2.07 ±0.12	0.49 ±0.04	0.71 ±0.06
Cocaine (n=7)	0.08 * ±0.01	0.12 * ±0.02	0.78 * ±0.07	0.33 * ±0.09
Hydrocortisone (n=6)	0.50 ±0.21	1.53 ±0.49	0.06 * ±0.01	0.77 ±0.11

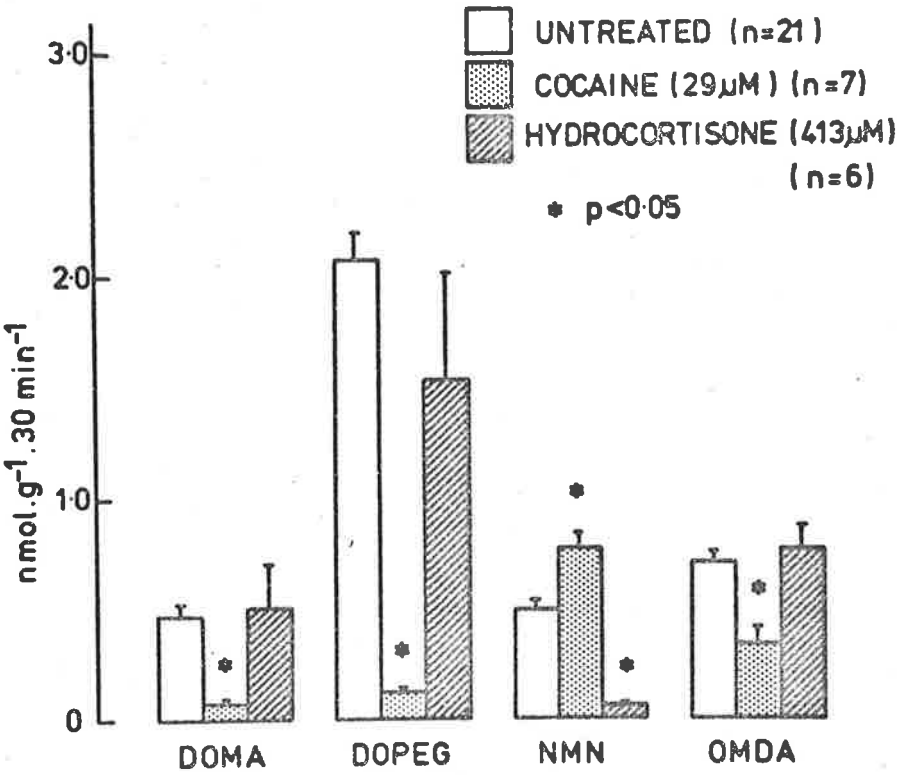

Fig. 3.2 The efflux of ^{3}H.metabolites in nmol.g^{-1}.30min^{-1} from rabbit ear artery strips incubated with (-)^{3}H.NA (0.18 μM) in Ca^{++} free media with prazosin (0.2 μM). The effects of cocaine (29 μM) and of hydrocortisone (413 μM) are also shown. These artery strips represent the distal portion of the same vessels used in studies on perfused segments presented in Chapter 4.
* indicates significance ($p<0.05$); unpaired t-test.

(5) Cocaine and hydrocortisone:-

The effect of cocaine (29μM), in the prazosin treated artery strip, on the metabolism of ^{3}H.NA (0.18μM) is shown in Table 3.4 and also in Fig. 3.2. It shows that the efflux of the deaminated metabolites, ^{3}H.DOPEG and ^{3}H.DOMA, was strongly inhibited by 94% and 83% respectively in the presence of cocaine. The efflux of ^{3}H.NMN was significantly increased by 1.6 fold. The effect of cocaine on ^{3}H.OMDA efflux was to reduce it by 54%.

The effect of hydrocortisone (413μM), in the prazosin treated preparation, on the metabolism of ^{3}H.NA (0.18μM), is also shown in Table 3.4 and Fig. 3.2. The efflux of ^{3}H.NMN into the bathing medium was reduced by 88%. The tendancy to also reduce ^{3}H.DOPEG efflux was not significant at the 5% level.

DISCUSSION

The pattern of metabolites of ^{3}H.NA in untreated artery strips incubated in Ca^{++} Krebs indicated that the major proportion (60%) of the amine removed from the incubating medium was accumulated unchanged in the tissue. In accord with earlier findings (Head et al, 1975; de la Lande et al, 1978; Head, 1976), DOPEG was the principal metabolite. In contrast, the reserpine pretreated artery, the major proportion was metabolised (87%), although ^{3}H.DOPEG remained as the principal metabolite. These results accord with the well documented ability of reserpine to inhibit retention of NA by neuronal vesicles. The actual retention of unchanged amine in the reserpine-pretreated artery was probably less than the 0.5 $nmol.g^{-1}.30\ min^{-1}$ shown in Table 3.1, since the tissues were only washed for 5 seconds at the end of the incubation period. The extracellular compartment in this tissue is approximately $0.6ml.g^{-1}$ (de la Lande et al, 1980). Hence, approximately

0.13 nmol.g^{-1} of the ^{3}H.NA remaining in the tissue would have resulted from the distribution of the amine into the extracellular compartment. Reserpine pretreatment was also associated with a considerable increase in ^{3}H.DOMA formation; however, it still represented only a minor proportion of the total metabolites (11%) compared with ^{3}H.DOPEG (48%).

The marked increase in deaminated metabolite formation with reserpine pretreatment accords with the drugs inhibitory action on vesicular binding of NA, since after its uptake into the axoplasm of the nerve, the NA is exposed to intraneuronal deamination by MAO. The marked predominance of DOPEG over DOMA indicates that deamination to the intermediate aldehyde is associated with subsequent metabolism via the aldehyde reductase pathway, rather than via the aldehyde oxidase pathway. In this respect the rabbit ear artery resembles a number of other peripheral tissues; e.g., the cat nictitating membrane (Langer, 1970), the rat heart (Fiebig and Trendelenburg, 1978 a,b), the rat vas deferens (Graefe et al, 1973), the rabbit aorta adventitia (Levin, 1974; Eckert et al, 1976), the dog saphenous vein (Paiva and Guimaraes, 1978) and the dog mesenteric artery (Garrett and Branco, 1977).

The lack of Ca^{++} in the bathing medium did not modify the above pattern of uptake and metabolism of NA in the reserpinised artery strips (segments not examined). One qualification is that the retention of unchanged amine tended to be even less in the Ca^{++} free strips. In view of its significance to later studies on perfused segments where the efflux of metabolites is used as an index of their rates of formation, the low proportion of unchanged amine plus metabolites retained in the tissue requires emphasis. This proportion amounts to only 18% of the total ^{3}H.metabolites formed by the tissue.

The failure of Ca^{++}-lack to modify intraneuronal retention and metabolism of unchanged amine undoubtedly reflects the negligible role played by vesicular binding of NA in the reserpine pretreated artery. Ca^{++} has been shown to be not essential to the cocaine-sensitive uptake process by which NA is transported into the axoplasm of the nerve from the extracellular space. However, Ca^{++} is essential for uptake and binding of NA in the neuronal vesicles (Trendelenburg, 1980).

The absence of an effect of Ca^{++}-lack on O-methylated metabolite formation is somewhat surprising in view of the evidence that the omission of Ca^{++} decreased the O-methylation of ISO in the rat submaxillary gland by 25% (Major et al, 1978). Trendelenburg (1980) also found that the omission of Ca^{++} caused a small significant reduction (by 20%) in the extraneuronal uptake of ^{3}H.NA by the perfused rat heart which was both reserpinised and MAO and COMT inhibited.

The kinetic data shows that the amounts of each metabolite formed, and the amounts which then effluxed into the bathing medium, are linearly related to the substrate (^{3}H.NA) concentration over a range of 0.018 to 0.18μM. Furthermore, with the exception of OMDA, these amounts approximate fairly closely to the rates of formation and efflux of the individual metabolites. Presumably the linear relationship reflects the fact that the concentrations are well below the Km for neuronal uptake (rabbit aorta 2.3μM; Henseling, 1980a) and the Km for extraneuronal uptake (rabbit aorta 3.6μM; Henseling, 1980a).

Prazosin was included in a number of the experiments to reproduce the conditions in perfused artery segments (Chapter 4) where it was present to prevent the constrictor response to NA. It had little effect on the metabolite effluxes, implying that constriction and/or α-receptor blockade did not influence metabolite formation in the artery strip preparation.

The potent inhibitory effects of cocaine on metabolite effluxes are consistent with the earlier reports of Head et al (1975) and Head (1976) that DOPEG and DOMA are largely neuronal in origin in this vessel. However, unlike the results of Head et al, a significant inhibitory effect of cocaine on OMDA formation is apparent in the present study. The difference may be because Head et al measured only the tissue levels of metabolites; or alternatively it may reflect the different experimental conditions, since Head et al used artery segments, from non-reserpinised rabbits, incubated in Ca^{++} Krebs solution. The significance of the inhibitory effects on OMDA efflux and the enhancement of NMN efflux, are considered in Chapters 6 and 4, respectively.

The selective inhibitory effects of hydrocortisone on NMN efflux implies that O-methylation of NA occurs by a corticosteriod-sensitive extraneuronal uptake of the amine into a COMT-containing compartment, similar to that shown by Head et al (1980) for the O-methylation of isoprenaline. Since hydrocortisone did not effect OMDA efflux, it seems likely that OMDA formation does not involve the steroid-sensitive extraneuronal uptake of NA; this question is considered further in Chapter 4.

CHAPTER 4

DIFFUSION AND METABOLISM OF ^{3}H.NA IN PERFUSED ARTERY SEGMENTS

CHAPTER 4.

DIFFUSION AND METABOLISM OF ^{3}H.NA IN PERFUSED ARTERY SEGMENTS

INTRODUCTION

This chapter presents the major study of the thesis, describing the influence of the surface of entry (either intimal or adventitial) of noradrenaline (NA) on its metabolism in reserpine-pretreated rabbit ear arteries. As set out in the General Introduction (Chapter 1), this study was prompted in the first instance by the possibility that the relative rates of formation of the metabolites of neuronal origin might indicate the relative concentrations which either INT or EXT NA achieved in the region of the nerve terminals, and hence indicate the magnitude of the gradient of concentration of NA existing between the surface of entry and the opposite surface. Furthermore, the study has provided an opportunity to explore the regional differences in metabolism of NA within the vessel wall under conditions where the adventitia and media were intact, as opposed to the studies on the separated adventitia and media of the aorta (described in Chapter 1).

Also indicated in this chapter are the influences of neuronal and extraneuronal uptake, and of constrictor tone, on the patterns of metabolite efflux from INT and from EXT NA. Earlier studies from the author's laboratory, and also in those of Prof. U. Trendelenburg reported in part by de la Lande et al (1980), had shown that DOPEG was the major metabolite of NA applied to the adventitia of the artery. It was also known that both an inhibitor of neuronal uptake (cocaine) and an inhibitor of extraneuronal uptake (DOCA), increased the flux of unchanged EXT NA into the lumen of the vessel. However, the influence of the extraneuronal uptake inhibitor on the metabolite formed from NA

had not been precisely defined in this vessel, nor was the influence of either agent on the metabolism of intraluminal NA known. Further, it was known that in artery segments exposed to EXT NA, the flux of NA plus metabolites (not reported) was decreased when the artery constricted (Parker, 1977; de la Lande et al, 1980). Hence it was important to take the effect of constriction produced by ^{3}H.NA into account when comparing the relative metabolisms of intraluminal and extraluminal NA. This has been done in the present study by comparing the metabolism of INT and of EXT NA in perfused artery segments under three different experimental conditions which alter the magnitude of the constrictor response to the NA, namely (a) in Ca^{++}-free media to minimise constrictor activity of NA, (b) in Ca^{++}-free media plus prazosin (an α_1-receptor antagonist) to eliminate constrictor activity, and (c) in Ca^{++} media to maximise the constrictor activity of NA.

METHODS

The methods used in the present chapter are described in detail in the General Methods (Chapter 2). Briefly, reserpinised ear artery segments were perfused at 0.5 ml.min^{-1} (or in one study where the effect of increased flow rate, 2.0 ml.min^{-1}, was considered) with Ca^{++}-free Krebs (or in one study where the effect of Ca^{++} was considered) solution at 37^{o}C and bubbled with 95% O_2, 5% CO_2. Incubations of 30 minutes duration with (-)^{3}H.NA (0.18μM) followed at least a 60 minute pre-incubation period. Any drug treatments were applied 30 minutes prior to, and during, the incubation with ^{3}H.NA.

In those studies where ^{3}H.NA was applied to the EXT surface of the artery segment, this medium was replaced with fresh substrate at the 15 minute interval so that the substrate concentration was not greatly diminished by neuronal deamination over the 30 minute incubating

Table 4.1 INT ^{3}H.NA

The flux of unchanged ^{3}H.NA and efflux of ^{3}H.metabolites into either bathing solution of rabbit ear artery segments incubated with (-)^{3}H.NA (0.18 μM) applied to the intimal surface.
Values shown are means ± SEM.

			Metabolite Efflux nmol. g^{-1}.30 min^{-1}.												NA Diffusion Coefficient $x10^{-7}cm^2.sec^{-1}$
^{3}H.label	Treatment (n)	NA Flux	DOMA INT	DOMA EXT	DOMA Total	DOPEG INT	DOPEG EXT	DOPEG Total	NMN INT	NMN EXT	NMN Total	OMDA INT	OMDA EXT	OMDA Total	
2,5,6-C	Untreated (n=5)	0.12 ±0.03	0.07 ±0.02	0.03 ±0.01	0.10 ±0.03	0.20 ±0.06	0.33 ±0.10	0.52 ±0.13	0.40 ±0.03 *	0.25 ±0.02 *	0.65 ±0.04 *	0.34 ±0.17	0.13 ±0.01	0.46 ±0.20	0.84 ±0.21
	Prazosin (n=11)	0.23 ±0.03	0.06 ±0.01	0.05 ±0.01	0.12 ±0.02	0.26 ±0.04	0.46 ±0.05	0.72 ±0.08	0.62 ±0.04	0.33 ±0.02	0.95 ±0.06	0.24 ±0.04	0.23 ±0.03	0.47 ±0.05	0.90 ±0.09
	Cocaine (n=5)	0.96 ±0.10 *	0.07 ±0.02	0.01 ±0.00 *	0.08 ±0.02	0.05 ±0.01 *	0.02 ±0.00 *	0.07 ±0.01 *	0.58 ±0.08	0.35 ±0.05	0.91 ±0.13	0.17 ±0.06	0.20 ±0.02	0.37 ±0.07	4.12 ±0.04 *
	Hydrocortisone (n=7)	0.39 ±0.06	0.07 ±0.03	0.11 ±0.02	0.19 ±0.04	0.25 ±0.06	0.50 ±0.10	0.75 ±0.15	0.03 ±0.01 *	0.04 ±0.01 *	0.07 ±0.01 *	0.12 ±0.06	0.22 ±0.02	0.31 ±0.08	1.62 ±0.20 *
7-C	Untreated (n=5)	0.21 ±0.04	0.02 ±0.01	0.08 ±0.07	0.09 ±0.07	0.10 ±0.02	0.26 ±0.07	0.36 ±0.08	0.44 ±0.14	0.21 ±0.06	0.65 ±0.19	0.36 ±0.21	0.18 ±0.07	0.54 ±0.23	0.91 ±0.14
	Ca^{++} (n=5)	0.10 ±0.05	0.07 ±0.03	0.03 ±0.01	0.10 ±0.03	0.10 ±0.05	0.12 ±0.03	0.23 ±0.07	0.21 ±0.07	0.11 ±0.03	0.32 ±0.10	0.24 ±0.11	0.09 ±0.02	0.33 ±0.13	0.32 ±0.11
	PBZ (n=4)	1.29 ±0.33	0.06 ±0.03	0.01 ±0.01 §	0.06 ±0.04	0.07 ±0.02	0.07 ±0.06 §	0.13 ±0.06 §	0.03 ±0.01 §	0.01 ±0.00 §	0.04 ±0.01 §	0.11 ±0.08	0.07 ±0.02 §	0.18 ±0.08 §	6.09 ±1.76 §

* indicates significance ($p<0.05$) compared with Prazosin; unpaired t-test.
§ indicates significance ($p<0.05$) compared with Untreated (7-C labelled); unpaired t-test.

Table 4.2 EXT ^{3}H.NA

The flux of unchanged ^{3}H.NA and efflux of ^{3}H.metabolites into either bathing solution of perfused rabbit ear artery segments incubated with (-)^{3}H.NA (0.18 μM) applied to the adventitial surface.
Values shown are means ± SEM.

			Metabolite Efflux nmol. $g^{-1}.30min^{-1}$.												
			DOMA			DOPEG			NMN			OMDA			NA Diffusion
^{3}H.label	Treatment (n)	NA Flux	INT	EXT	Total	INT	EXT	Total	INT	EXT	Total	INT	EXT	Total	Coefficient $x10^{-7}cm^{2}.sec^{-1}$
2,5,6-C	Untreated (n=5)	0.45 ±0.07	0.11 ±0.01	0.60 ±0.06	0.71 ±0.06	0.72 ±0.08	2.61 ±0.22	3.33 ±0.29	0.10 ±0.02	0.26 ±0.03	0.36 ±0.04	0.15 ±0.04	0.48 ±0.12	0.63 ±0.14	1.92 ±0.34
	Prazosin (n=11)	0.47 ±0.06	0.08 ±0.01	0.52 ±0.06	0.61 ±0.07	0.62 ±0.05	2.24 ±0.18	2.86 ±0.22	0.11 ±0.01	0.27 ±0.03	0.38 ±0.04	0.24 ±0.05	0.32 ±0.04	0.55 ±0.07	2.45 ±0.36
	Cocaine (n=3)	1.17 ±0.09 *	0.01 ±0.00 *	0.08 ±0.04 *	0.08 ±0.04 *	0.02 ±0.01 *	0.09 ±0.07 *	0.11 ±0.07 *	0.38 ±0.03 *	0.61 ±0.19	0.99 ±0.26	0.23 ±0.01	0.13 ±0.05 *	0.36 ±0.05	4.56 ±0.43 *
	Hydrocortisone (n=4)	0.79 ±0.07	0.18 ±0.02	0.99 ±0.16	1.17 ±0.17	0.48 ±0.11	1.81 ±0.20	2.29 ±0.31	0.02 ±0.01 *	0.08 ±0.02 *	0.11 ±0.03 *	0.27 ±0.04	0.27 ±0.10	0.53 ±0.14	3.00 ±0.51
7-C	Untreated (n=5)	0.27 ±0.03	0.06 ±0.02	0.27 ±0.11	0.35 ±0.16	0.65 ±0.12	2.80 ±0.41	3.46 ±0.53	0.11 ±0.01	0.24 ±0.05	0.36 ±0.05	0.23 ±0.03	0.51 ±0.15	0.74 ±0.16	1.21 ±0.23
	Ca^{++} (n=6)	0.25 ±0.09	0.05 ±0.01	0.32 ±0.07	0.37 ±0.07	0.40 ±0.05	2.26 ±0.36	2.67 ±0.40	0.06 ±0.01	0.18 ±0.07	0.24 ±0.07	0.13 ±0.04	0.24 ±0.05	0.36 ±0.08	1.88 ±0.63
	PBZ (n=4)	1.49 ±0.21 §	0.00 ±0.00 §	0.02 ±0.01 §	0.02 ±0.01 §	0.01 ±0.00 §	0.03 ±0.02 §	0.04 ±0.02 §	0.01 ±0.01 §	0.02 ±0.01 §	0.03 ±0.01 §	0.07 ±0.02 §	0.06 ±0.05 §	0.13 ±0.05 §	6.04 ±0.19 §

* indicates significance ($p<0.05$) compared with Prazosin; unpaired t-test.
§ indicates significance ($p<0.05$) compared with Untreated (7-C labelled); unpaired t-test.

period. This replacement was found to be unnecessary in segments exposed to INT ^{3}H.NA where quantitatively less metabolism occurred.

The amount of unchanged ^{3}H.NA and ^{3}H.metabolites were then analysed by the cascade column chromatographic technique described in the General Methods (Chapter 2).

Routine measurements of length, diameter and weight of the vessels were determined as described in the General Methods (Chapter 2). These values allowed the calculation of metabolite formation, expressed in nmol.g^{-1}.30 min^{-1}, and of the diffusivity of substrate through the vessel wall, expressed as the apparent diffusion coefficient (in cm^{2}.sec^{-1}) using the equation of Crank (1956) for the diffusion of a substance through the wall of a hollow cylinder.

In parallel experiments, the efflux of metabolites from isolated artery strips, i.e., where the ^{3}H.NA entered via both surfaces of the vessel simultaneously, were examined. Each artery strip was derived from the same vessel used in the perfused segment studies and exposed to identical incubating conditions. The results of the artery strip studies have been presented in Chapter 3. The relationship between the metabolism in artery segments and strips are considered in the discussion of the present chapter and again in the General Discussion (Chapter 9).

RESULTS

(1) Metabolite distribution.

The amounts of metabolites which effluxed into the intraluminal (INT) and extraluminal (EXT) bathing solutions during separate incubations with INT or with EXT (-)^{3}H.NA (0.18μM) under a variety of conditions during a 30 minute period of incubation are shown in Tables 4.1 and 4.2 respectively. The total amount of each metabolite which effluxed from the vessel is shown; this represents the sum of the metabolites which

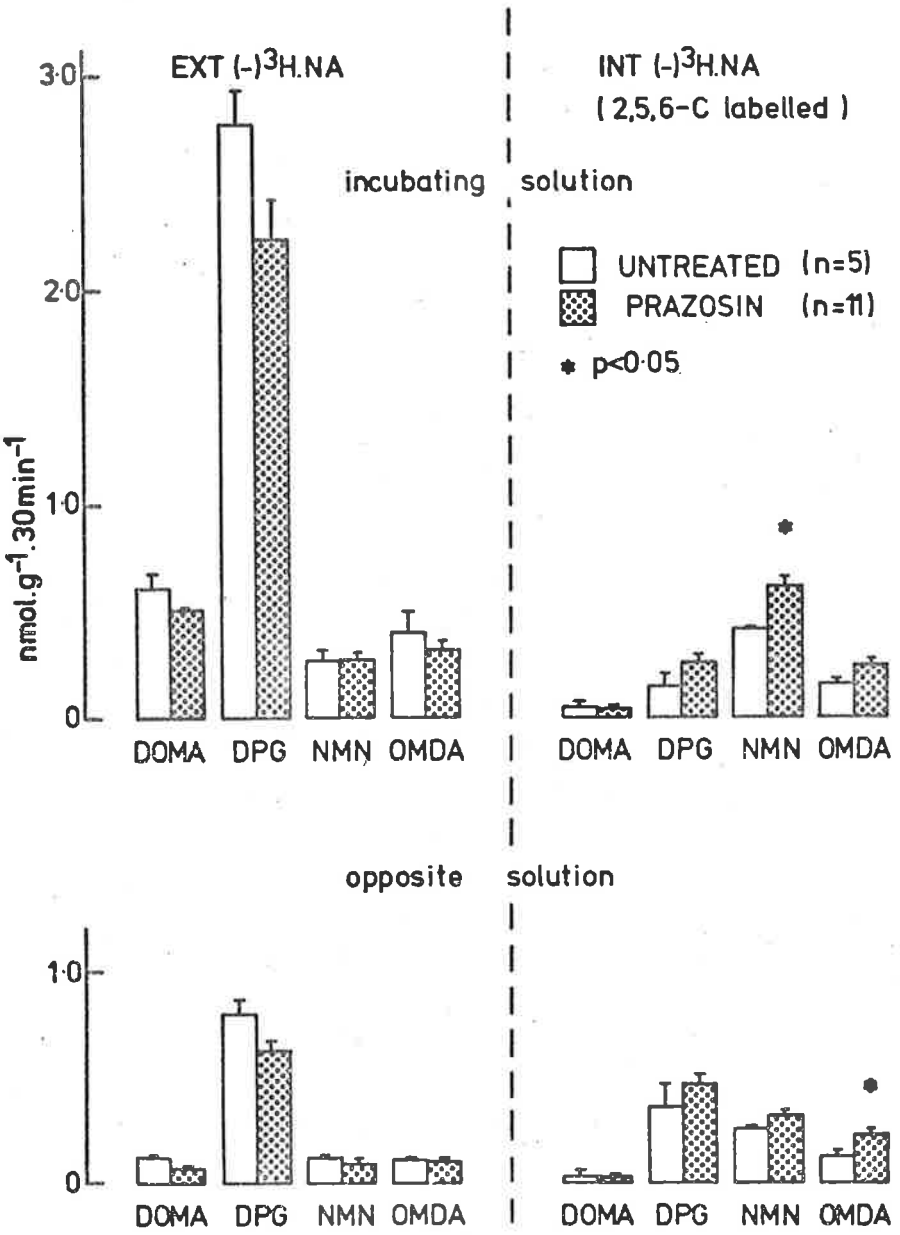

Fig. 4.1 The effect of prazosin (0.2 μM) on the efflux of ^{3}H.metabolites in nmol.g^{-1}.30min^{-1} into the Ca^{++} free medium bathing either surface of perfused ear artery segments (from reserpinised rabbits) when (-)^{3}H.NA (0.18 μM) is applied to either the adventitia (EXT) or the intima (INT).
* indicates significance ($p < 0.05$); unpaired t-test.

effluxed from the vessel and distributed into the INT and EXT bathing solutions. Some of the data in Tables 4.1 and 4.2 is also presented in Fig. 4.1 to show the pattern of metabolite effluxes in untreated (Ca^{++}-free), and in prazosin-treated (Ca^{++}-free) vessels. Although the metabolism of NA in the prazosin-treated vessels differed in minor respects from the untreated vessels (described subsequently), in both vessels the striking feature is the difference between the patterns of metabolites of INT and of EXT ^{3}H.NA. These differences will be defined quantitatively for the untreated vessels incubated with 2,5,6-C ^{3}H.NA.

In the case of EXT ^{3}H.NA the major metabolite was ^{3}H.DOPEG representing 66% of the total metabolite efflux; the other metabolites were ^{3}H.DOMA (14%), ^{3}H.OMDA (13%), and ^{3}H.NMN (7%). Unchanged ^{3}H.NA comprised 41% of the total ^{3}H in the opposite (i.e.,INT) solution. In the case of INT ^{3}H.NA, the major metabolite was ^{3}H.NMN representing 37% of the total metabolite efflux, the remaining metabolites were ^{3}H.DOPEG (30%), ^{3}H.OMDA (27%) and ^{3}H.DOMA (6%). Unchanged ^{3}H.NA comprised 16% of the total ^{3}H in the opposite (i.e., EXT) solution.

The efflux of total metabolites from segments incubated with EXT ^{3}H.NA was 3-fold greater than from segments incubated with INT ^{3}H.NA; this was due primarily to a 10.4-fold greater efflux of ^{3}H.DOPEG with EXT ^{3}H.NA. However, the total efflux of ^{3}H.NMN from vessels incubated with EXT ^{3}H.NA was only 56% of that from INT ^{3}H.NA. Surprisingly, the flux of unchanged ^{3}H.NA across the vessel wall was 2.3-fold greater than that of INT ^{3}H.NA as indicated by differences in apparent diffusion coefficients of 1.92 and 0.84 $cm^{2}.sec^{-1}$ respectively (Tables 4.1 and 4.2)

As indicated by their relative distributions between the INT and EXT solutions, the relative effluxes of the metabolites from the two surfaces were influenced in different ways by the surface of entry of

Table 4.3

Relationship between perfusion pressure and wall thickness in reserpinised rabbit ear arteries incubated with either INT or with EXT (−)^{3}H.NA (0.18 µM) in Ca^{++} and Ca^{++} free medium.
Values shown are means ± SEM.

Treatment	n	Incubating solution	Δ P(mm Hg)	Wall thickness (mm) relaxed	constricted	Δ
Ca^{++} free	9,4	INT	13 ±5	0.11±0.04	0.16±0.01	0.05
	9,4	EXT	0	0.11 0.04	0.11 0.04	0
Ca^{++}	6,4	INT	66 ±30	0.13±0.02	0.18±0.01	0.05
	6,4	EXT	10 ±6	0.13±0.02	0.15±0.02	0.02

^{3}H.NA into the vessel wall. As shown in Figs. 4.1 and 4.2 the efflux of ^{3}H.DOPEG into the EXT solution was 2 to 4-fold greater than into the INT solution irrespective of the surface of application of the amine. In contrast, the efflux of ^{3}H.NMN was approximately 2-fold greater from the surface exposed to the ^{3}H.NA than from the opposite surface. The relative effluxes of ^{3}H.OMDA were relatively independent of the surface of entry of ^{3}H.NA.

Although the above findings are based only on the total amounts of metabolite which effluxed from the tissue during a 30-minute period of incubation with ^{3}H.NA, the kinetic data from the artery strips (presented in Chapter 3) indicated that these amounts provide a reasonable estimate of both their rates of formation and rates of efflux from the vessel during this period. Hence the preceding results point to two distinct types of effect which the surface of entry has on the metabolism of ^{3}H.NA, namely, (1) it determines the rate of formation of the deaminated catecholamine metabolites but has only a minor effect on their relative rates of efflux from the two surfaces, and (2) it has a smaller effect on the rate of ^{3}H.NMN formation, but determines the direction of efflux of this metabolite from the vessel.

(2) Constriction.

In the vessels incubated in Ca^{++}-free media described above, EXT ^{3}H.NA (0.18μM) did not influence the external diameter or perfusion pressure; however, there was a persistent small constrictor response during incubations with INT ^{3}H.NA (0.18μM) as indicated by a mean increase in perfusion pressure of 13 mmHg (Table 4.3). Hence in such vessels the metabolism of EXT and of INT ^{3}H.NA were not compared under identical conditions, the metabolism of EXT ^{3}H.NA referring to

relaxed (thinner walled) vessels, and that of INT ^{3}H.NA to constricted (thicker walled) vessels. To determine the influence of the constrictor response of the vessel on the metabolism of ^{3}H.NA, two types of experiments were carried out. In one, the metabolism was compared in prazosin-treated (Ca^{++}-free) vessels, i.e., where the constrictor response was abolished, and the other where Ca^{++} was included in the bathing media to enhance the constrictor response.

The effects of these treatments on the metabolism of ^{3}H.NA are summarised in Tables 4.1 and 4.2, and Fig. 4.1. Since 2,5,6 ^{3}H.NA was used in the prazosin and 7 ^{3}H.NA in the experiments with Ca^{++}, the data on Ca^{++}-free untreated vessels are separated according to the type of label employed. However, it will be seen in Tables 4.1 and 4.2 that the type of label did not influence metabolite effluxes to significant extents, and for this reason, the two sets of data on Ca^{++}-free untreated segments were pooled to assess the significance of the treatment effects.

In Ca^{++}-free vessels incubated with EXT ^{3}H.NA, the most pronounced effect of prazosin was a tendency to decrease the total effluxes of ^{3}H.DOPEG and ^{3}H.OMDA; however, neither effect was significant. In vessels incubated with INT ^{3}H.NA, prazosin tended to increase total ^{3}H.DOPEG efflux and total ^{3}H.NMN efflux (by 50%); however, only the effect on ^{3}H.NMN efflux was significant. Prazosin was without effect on the flux of EXT ^{3}H.NA across the vessel wall, but tended to increase the flux of INT ^{3}H.NA. Although the later effect failed to reach significance ($0.1 < p < 0.5$), it is probably a genuine effect, since the diffusion coefficient of INT ^{3}H.NA was unaffected by prazosin despite a significant decrease in wall thickness.

The effects of replacing Ca^{++} in the Krebs solution are shown in Tables 4.1 and 4.2, and in Fig. 4.2. It should be noted that these experiments with Ca^{++} treated vessels were technically difficult to

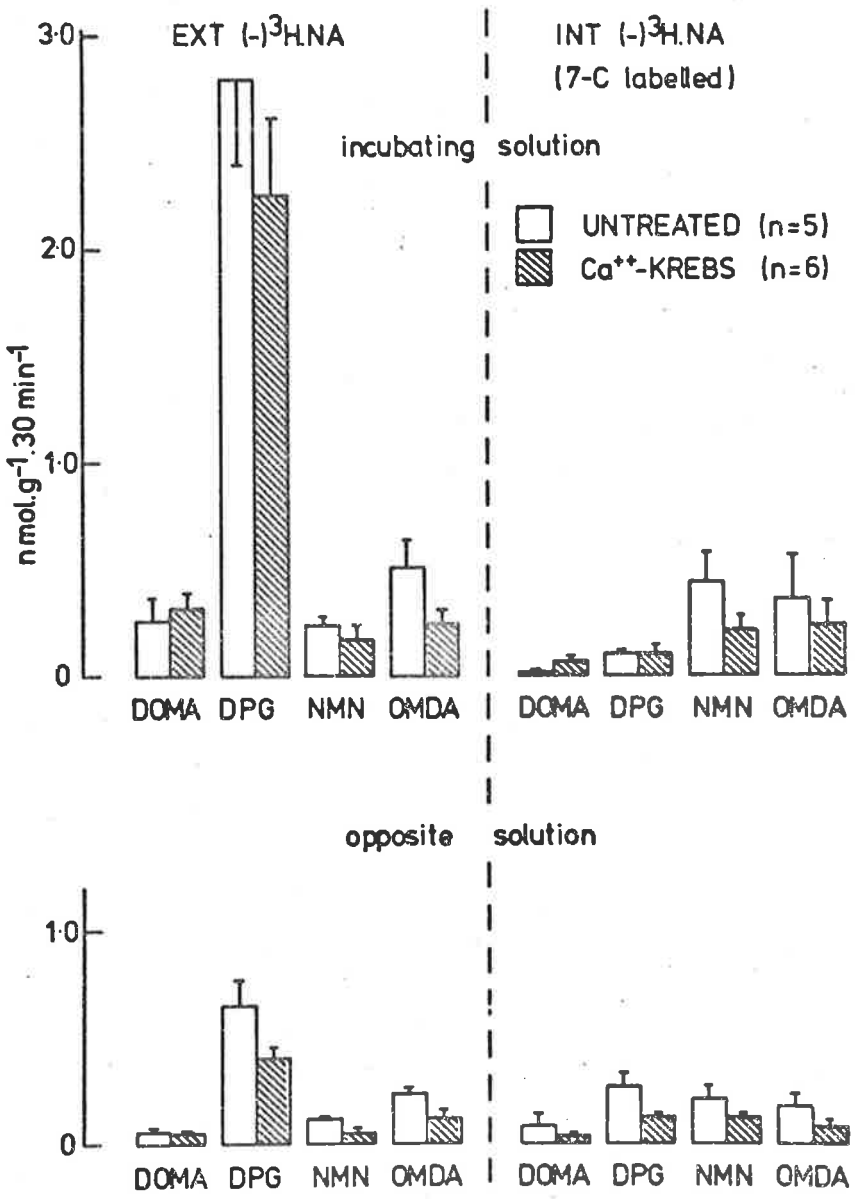

Fig. 4.2 The effect of Ca^{++} in the medium on the efflux of ^{3}H.metabolites in $nmol.g^{-1}.30min^{-1}$ into the solution bathing either surface of perfused ear artery segments (from reserpine pretreated rabbits) where $(-)^{3}H.NA$ (0.18 μM) is applied either to the adventitia (EXT) or to the intima (INT). Untreated segments had no Ca^{++} in the bathing media.

Table 4.4 — Ca^{++} treated vessels

The effect of replacing the Ca^{++} in the medium on the flux of unchanged $^{3}H.NA$ and the efflux of $^{3}H.$metabolites in perfused rabbit ear artery segments incubated with either INT or EXT (-)$^{3}H.NA$ (0.18μM). Values for Ca^{++} free samples are combined data from 7-C and 2,5,6-C labelled NA; and 7-C only for Ca^{++} samples.
Values shown are means ± SEM.
* indicates significance ($p<0.05$); unpaired t-test.

Incubating Solution	Treatment (n)	NA Flux	Metabolite Efflux nmol. g^{-1}. $30min^{-1}$. DOMA INT	DOMA EXT	DOMA Total	DOPEG INT	DOPEG EXT	DOPEG Total	NMN INT	NMN EXT	NMN Total	OMDA INT	OMDA EXT	OMDA Total	NA Diffusion Coefficient $x10^{-7}cm^{2}.sec^{-1}$
INT $^{3}H.NA$	Ca^{++} free (n=10)	0.16 ±0.03	0.04 ±0.01	0.05 ±0.03	0.10 ±0.03	0.15 ±0.03	0.29 ±0.06	0.44 ±0.08	0.42 ±0.03	0.29 ±0.06	0.65 ±0.09	0.35 ±0.13	0.15 ±0.04	0.50 ±0.14	0.78 ±0.16
	Ca^{++} (n=5)	0.10 ±0.05	0.07 ±0.03	0.03 ±0.01	0.10 ±0.03	0.10 ±0.05	0.12 ±0.03	0.23 ±0.07	0.21 ±0.07	0.11 ±0.03 *	0.32 ±0.10 *	0.24 ±0.11	0.09 ±0.02 *	0.33 ±0.13	0.32 ±0.11
EXT $^{3}H.NA$	Ca^{++} free (n=10)	0.36 ±0.05	0.09 ±0.01	0.43 ±0.08	0.54 ±0.07	0.63 ±0.05	2.71 ±0.22	3.39 ±0.29	0.11 ±0.01	0.25 ±0.03	0.36 ±0.03	0.19 ±0.03	0.49 ±0.09	0.69 ±0.10	1.56 ±0.23
	Ca^{++} (n=6)	0.25 ±0.09	0.05 ±0.01	0.32 ±0.07	0.37 ±0.07	0.40 ±0.05 *	2.42 ±0.18	2.67 ±0.40	0.06 ±0.01 *	0.18 ±0.07	0.24 ±0.07	0.13 ±0.04	0.24 ±0.05	0.36 ±0.08 *	1.88 ±0.63

carry out, and a number of arteries had to be rejected when the INT ^{3}H.NA caused an excessive rise in perfusion pressure (greater than 150 mm Hg). In two experiments this difficulty was circumvented by reducing the concentration of ^{3}H.NA so that the increase in perfusion pressure was within the desired range (50-100 mm Hg); in view of the linear relationship between metabolite efflux and substrate concentration in the studies on strips (Chapter 3), it was considered justified to adjust the experimentally derived metabolite effluxes to correspond to a concentration of ^{3}H.NA of 0.18μM.

Replacing Ca^{++} in Krebs solution tended to decrease the effluxes of all metabolites, irrespective of their surface of efflux, or the surface of entry of the ^{3}H.NA (Tables 4.1, 4.2; Fig. 4.2). The only exception was ^{3}H.DOMA efflux during incubation with INT ^{3}H.NA. However, the only changes which were significant (when compared with the pooled data on Ca^{++}-free untreated vessels) were as follows: (a) during incubation with EXT ^{3}H.NA, decreases in total efflux of ^{3}H.OMDA, and in the effluxes of ^{3}H.NMN and ^{3}H.DOPEG from the intimal surface, and (b) during incubation with INT ^{3}H.NA, the decreases in both the total effluxes of ^{3}H.NMN and its efflux from the EXT surface. Although the fluxes of EXT ^{3}H.NA and INT ^{3}H.NA were also less in Ca^{++} than in the Ca^{++}-free vessels, the difference being particularly marked in the case of INT ^{3}H.NA, these differences were not statistically significant.

Assuming that the above effects of prazosin and of Ca^{++} were related to their effects on the constrictor responses to NA, the preceding findings imply that constriction is associated primarily with a decreased efflux of ^{3}H.NMN, particularly from the INT surface, and in the case of EXT ^{3}H.NA, with a decreased efflux of ^{3}H.DOPEG from the INT surface.

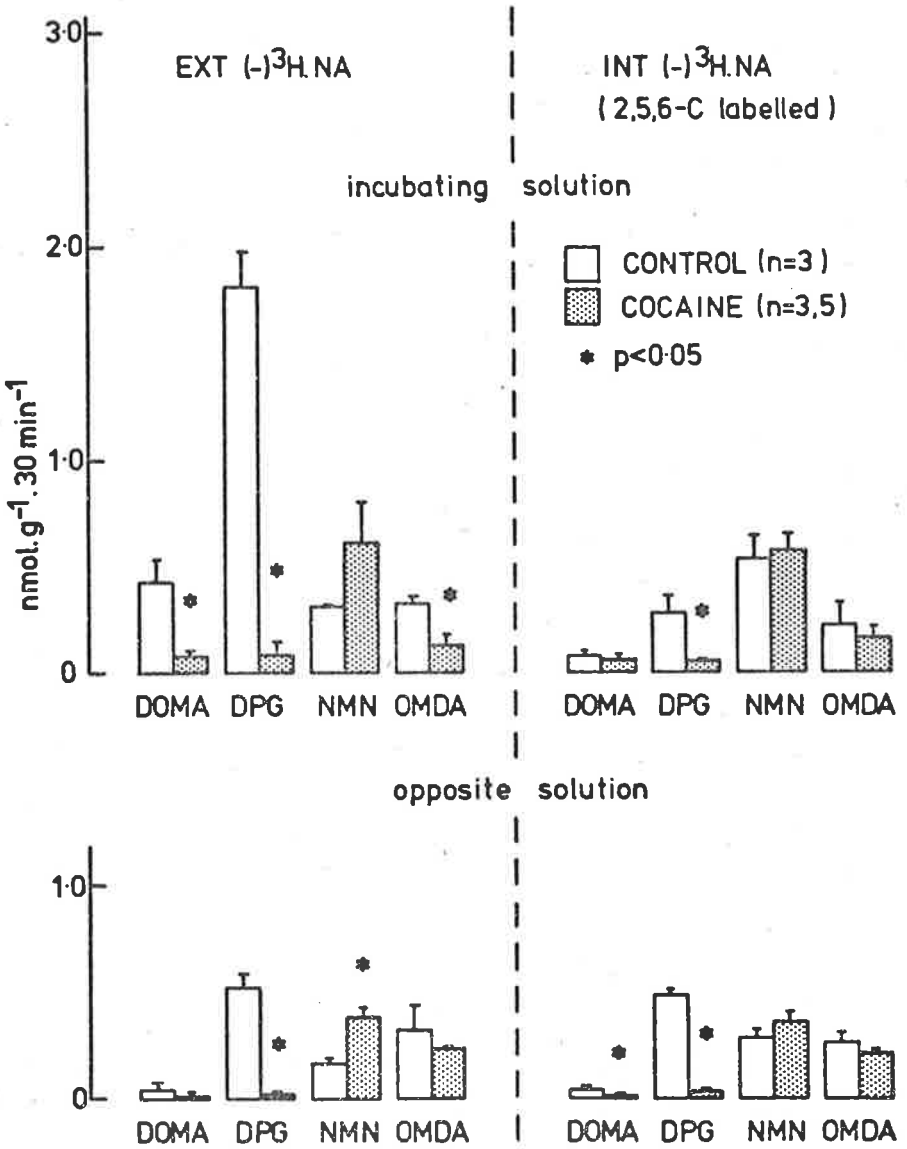

Fig. 4.3 The effect of cocaine (29 μM) on the efflux of ^{3}H.metabolites in nmol.g^{-1}.30min^{-1}into the Ca^{++} free medium bathing either surface of relaxed (prazosin, 0.2 μM) perfused ear artery segments (from reserpine pretreated rabbits) where (-)^{3}H.NA (0.18 μM) is applied either to the adventitia (EXT) or to the intima (INT).
* indicates significance ($p < 0.05$); unpaired t-test.

(3) DOPEG formation ratio.

Since studies on artery strips presented in Chapter 3 showed that ^{3}H.DOPEG formation was linearly related to substrate concentration, and as shown subsequently, that ^{3}H.DOPEG was largely neuronal in origin, then the ratio of the amount of ^{3}H.DOPEG formed from EXT ^{3}H.NA to that formed from INT ^{3}H.NA (termed the DOPEG formation ratio) should indicate the ratios of the concentrations INT and EXT ^{3}H.NA achieve in the region of the nerve terminals.

In the relaxed (prazosin-treated) arteries this ratio was low (4.4 $\pm$ 0.5), in the untreated Ca^{++}-free vessels which constricted slightly to INT ^{3}H.NA it was 3-fold greater (10.4 $\pm$ 1.7) and in the Ca^{++}-treated vessels it was 6-fold greater (23.6 $\pm$ 11.4). The great variability in the ratio in the latter vessels may have reflected the difference in the magnitude of the constrictor responses of individual vessels, since the vessel with the highest ratio of 63 also showed the greatest constrictor response (120 mmHg).

From the results in Tables 4.1 and 4.2, it will be evident that the increases in these ratios in the more constricted vessels were due primarily to the decreased rate of ^{3}H.DOPEG efflux from INT ^{3}H.NA, rather than an increased rate from EXT ^{3}H.NA.

(4) Neuronal and Extraneuronal Uptake Inhibition.

(a) Cocaine

The effects of cocaine (29μM) and of hydrocortisone (413μM) were examined to provide an insight into the influences of neuronal and extraneuronal uptake processes on the differing patterns of metabolite formations from INT and from EXT ^{3}H.NA. The effects were studied in relaxed (prazosin-treated) segments bathed in Ca^{++}-free media. The results (Tables 4.1 and 4.2 and also Fig. 4.3) indicated that in

arteries incubated with EXT ^{3}H.NA, cocaine significantly decreased the efflux of ^{3}H.DOPEG and ^{3}H.DOMA by 96% and 87% respectively, and increased the efflux of ^{3}H.NMN by 2.6-fold (significant only in the case of ^{3}H.NMN effluxing into the INT solution). A decrease in ^{3}H.OMDA efflux by 35% resulted from a significant decrease in its efflux (by 50%) into the EXT solution (considered in more detail later in the present chapter and also in Chapter 6)

Cocaine also decreased ^{3}H.DOPEG efflux by 90% when the ^{3}H.NA was applied to the INT surface; this effect was manifested by decreased rates of efflux of the metabolite into both the INT and the EXT solutions. Cocaine also significantly decreased ^{3}H.DOMA efflux, but only into the EXT solution. Cocaine was without effect on ^{3}H.NMN and ^{3}H.OMDA effluxes.

Based on estimates of the diffusion coefficients, cocaine also exerted quantitatively different effects on the fluxes of EXT and of INT ^{3}H.NA, the former being increased by a factor of 1.9 and the latter by 4.6. The net effect of these increases was that cocaine eliminated the differences between the fluxes of EXT and of INT ^{3}H.NA, and with one noteworthy exception, it also eliminated the difference between metabolite effluxes in segments incubated with EXT or with INT ^{3}H.NA. The exception was that in cocaine-treated segments ^{3}H.NMN still effluxed at a more rapid rate (1.6-fold) from the surface to which the ^{3}H.NA was applied.

From these results it was concluded that ^{3}H.DOPEG and ^{3}H.DOMA were largely neuronal in origin irrespective of the surface of entry of ^{3}H.NA and that this factor was responsible for their more rapid effluxes into the EXT solution and for their higher rates of efflux in vessels incubated with EXT ^{3}H.NA.

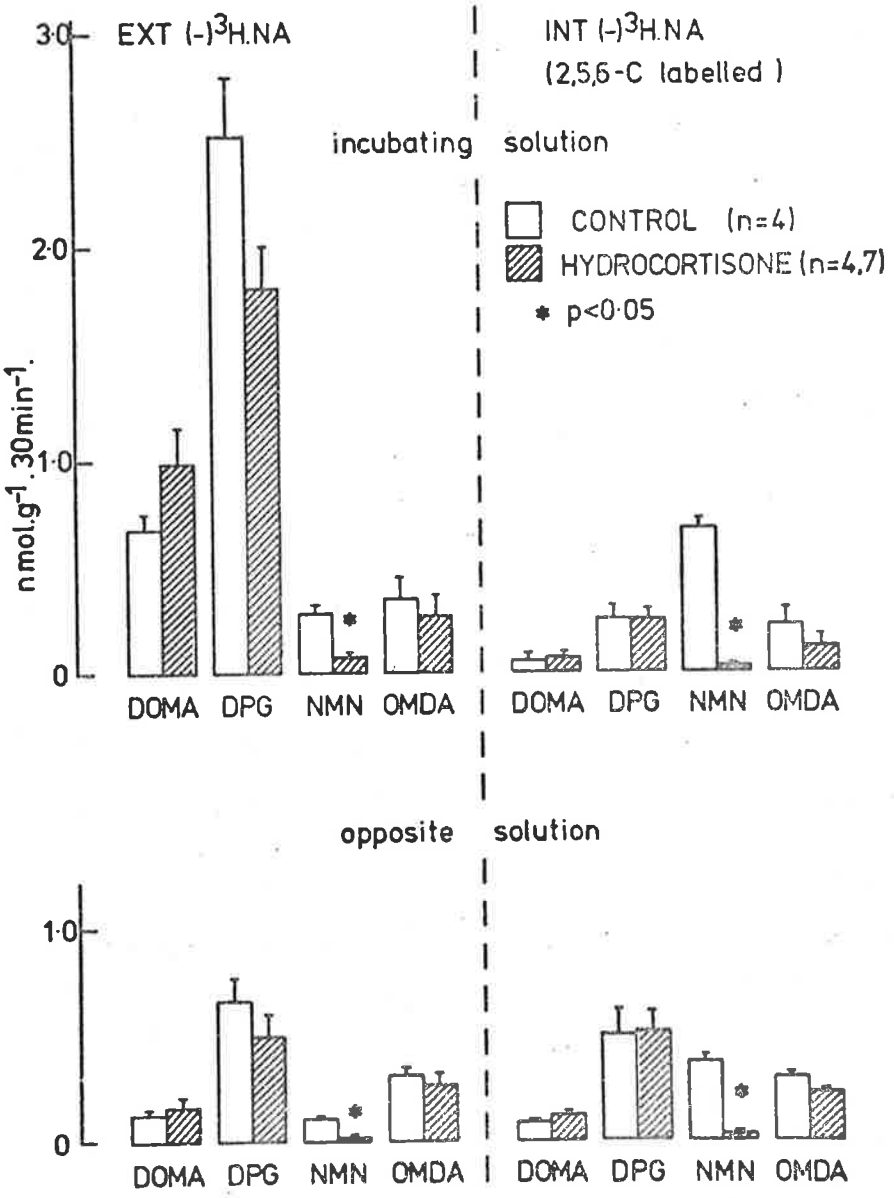

Fig. 4.4 The effect of hydrocortisone (413 µM) on the efflux of ^{3}H.metabolites in nmol.g^{-1}.30min^{-1} into the Ca^{++} free medium bathing either surface of relaxed (prazosin 0.2 µM) perfused ear artery segments (from reserpine pretreated rabbits) where (-)^{3}H.NA (0.18 µM) is applied either to the adventitia (EXT) or to the intima (INT).
* indicates significance ($p < 0.05$); unpaired t-test.

(b) Hydrocortisone

Hydrocortisone (413μM) inhibited the formation of ^{3}H.NMN with EXT or with INT ^{3}H.NA by 71% and 93% respectively; the inhibition was equally marked in the efflux of ^{3}H.NMN into both bathing solutions. As shown in Tables 4.1 and 4.2 as well as Fig. 4.4, it was associated with a small but significant (1.8-fold) increase in the NA diffusion coefficient with INT ^{3}H.NA, with no significant change in the EXT ^{3}H.NA diffusion coefficient. The lack of an effect on the efflux of the other metabolites ^{3}H.DOPEG, ^{3}H.DOMA and ^{3}H.OMDA implied that their formation was independent of a steroid-sensitive pathway.

The ^{3}H.DOPEG formation ratio of 3.1 was lowest in the hydrocortisone treated segments (compared with other segments perfused at 0.5 ml.min^{-1}, when the deaminating pathway was available). This low ratio reflected a tendency for the rate of efflux of ^{3}H.DOPEG from EXT ^{3}H.NA to decrease rather than for the rate of its efflux from INT ^{3}H.NA to increase. The failure of hydrocortisone to increase ^{3}H.DOPEG efflux from INT ^{3}H.NA was surprising since its effect on ^{3}H.NMN efflux and on ^{3}H.NA flux indicated that it had eliminated extraneuronal sites of loss of ^{3}H.NA in the artery wall. This result may possibly reflect a partial neuronal uptake inhibiting action of hydrocortisone (as also suggested in Chapter 3 in artery strips).

In summary, while confirming the extraneuronal origin of ^{3}H.NMN, the effect of hydrocortisone failed to reveal any dramatic role of extraneuronal O-methylation in influencing the patterns of the remaining metabolite formations and distributions in segments incubated with INT or with EXT ^{3}H.NA.

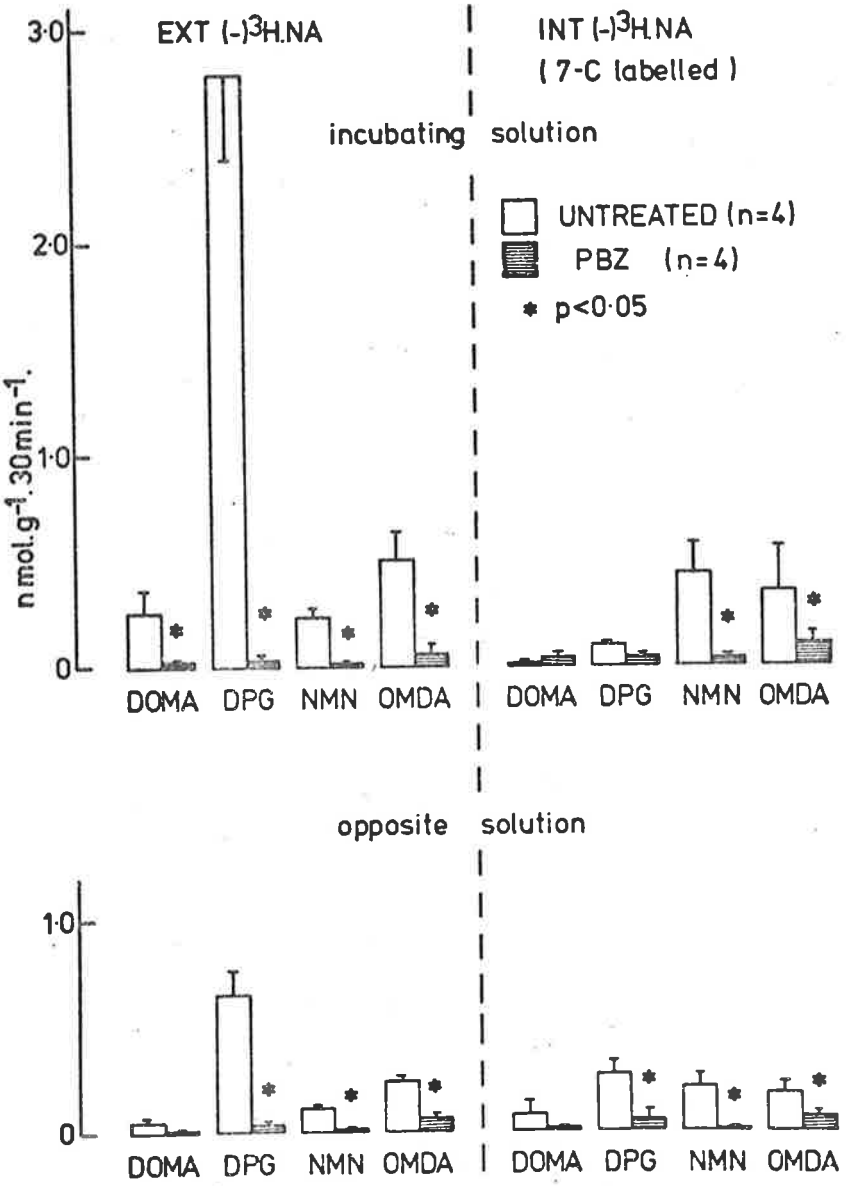

Fig. 4.6 The effect of phenoxybenzamine (PBZ, 33 μM) on the efflux of $^{3}H.$ metabolites in nmol.g^{-1}.30min^{-1} into the Ca^{++} free medium bathing either surface of ear artery segments (from reserpine pretreated rabbits) where (-)$^{3}H.NA$ (0.18 μM) is applied either to the adventitia (EXT) or to the intima (INT).
* indicates significance ($p < 0.05$); unpaired t-test.

Table 4.5

INT PLUS EXT ^{3}H.NA

The efflux of ^{3}H.metabolites from rabbit ear artery segments incubated with (-)^{3}H.NA (0.18μM) applied to both surfaces of the vessel simutaneously in Ca^{++} free medium with prazosin (0.2μM).
Values shown are means ± SEM.
* indicates significance ($p<0.05$); unpaired t-test.

		Metabolite Efflux nmol.g^{-1}.30 min^{-1}											
	Treatment	DOMA			DOPEG			NMN			OMDA		
^{3}H-label	(n)	INT	EXT	Total	INT	EXT	Total	INT	EXT	Total	INT	EXT	Total
2,5,6-C	prazosin (n=4)	0.09 ±0.03	0.45 ±0.10	0.54 ±0.12	0.61 ±0.04	2.42 ±0.16	3.05 ±0.17	0.65 ±0.03	0.65 ±0.10	1.30 ±0.08	0.44 ±0.04	0.72 ±0.02	1.16 ±0.03
	Cocaine (n=4)	0.07 ±0.00	0.10 ±0.01 *	0.17 ±0.01 *	0.07 ±0.03 *	0.12 ±0.05 *	0.19 ±0.06 *	0.92 ±0.04 *	0.85 ±0.03	1.78 ±0.06 *	0.28 ±0.03 *	0.30 ±0.05 *	0.58 ±0.05 *

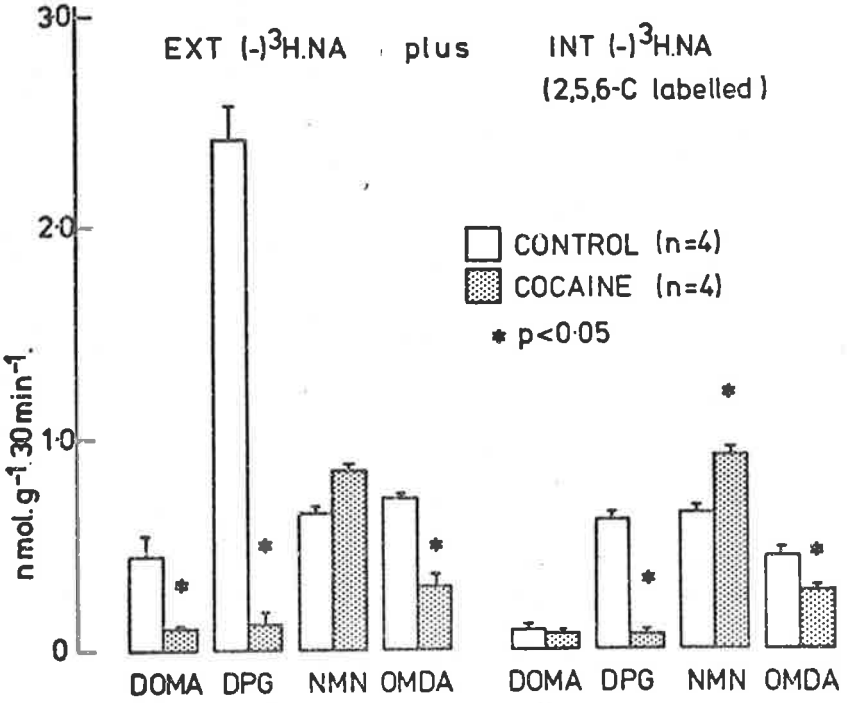

Fig. 4.7 The effect of cocaine (29 μM) on the efflux of ^{3}H.metabolites in nmol.g^{-1}.30min^{-1} into the Ca^{++} free medium bathing either surface of relaxed (prazosin 0.2 μM) ear artery segments (from reserpine pretreated rabbits) where (-)^{3}H.NA (0.18 μM) is applied both to the adventitia (EXT) and to the intima (INT).
* indicates significance ($p < 0.05$); unpaired t-test.

(c) Phenoxybenzamine

The effect of phenoxybenzamine (PBZ) treatment (which inhibits both neuronal and extraneuronal uptake pathways as well as antagonising α-receptors) on the efflux of metabolites and flux of amine was examined in vessels incubated in Ca^{++}-free media with EXT or with INT ^{3}H.NA (0.18μM). These results are also presented in Tables 4.1 and 4.2 as well as in Fig. 4.6. It can be seen that PBZ-treatment was associated with marked reductions in the effluxes of each of the metabolites regardless of the surface of entry of the amine such that the total metabolite efflux with INT ^{3}H.NA or with EXT ^{3}H.NA were reduced by 80% and 95% respectively. This was associated with marked increases in the diffusivity of NA by 6.4-fold and 2.4-fold, for INT and for EXT ^{3}H.NA respectively. In a similar manner to cocaine, PBZ eliminated the difference in flux of amine when applied to either surface of the vessel.

(5) Gradient of concentration.

In the preceding experiments ^{3}H.NA was applied to only one surface of the artery segment. Hence the concentration of the amine was not uniform within the vessel wall, but declined between the surface of entry and the opposite surface. To examine the influence of this gradient of concentration on metabolism of ^{3}H.NA, segments were studied where the amine was applied to both surfaces simultaneously. Theoretically, the concentration of NA in these segments should be uniform throughout the vessel wall. In the four vessels examined, it can be seen from the results (Table 4.5 and Fig. 4.7) that, in the case of ^{3}H.DOPEG and ^{3}H.DOMA, their respective effluxes into the INT and EXT solutions were little different from those segments incubated with EXT ^{3}H.NA alone. This result indicated that the gradient of concentration

Table 4.6

A comparison of total ^{3}H.metabolite efflux when ^{3}H.NA (0.18 μM) is applied to both surfaces simutaneously, or to either the INT or the EXT surface separately, of perfused rabbit ear artery segments.
Values are means ± SEM.

Incubating solution	Treatment (n)	Metabolite Efflux nmol.g^{-1}.30 min^{-1}. DOMA	DOPEG	NMN	OMDA
INT (a)	Prazosin (n=11)	0.12 ±0.02	0.72 ±0.08	0.95 ±0.06	0.47 ±0.05
EXT (b)	Prazosin (n=11)	0.61 ±0.07	2.86 ±0.22	0.38 ±0.04	0.55 ±0.07
(a) + (b)		0.73 ±0.08	3.58 ±0.30	1.33 ±0.09	1.02 ±0.11
INT and EXT	Prazosin (n=4)	0.54 ±0.12	3.05 ±0.17	1.30 ±0.08	1.16 ±0.03
INT (c)	Cocaine (n=5)	0.08 ±0.02	0.07 ±0.01	0.91 ±0.13	0.37 ±0.07
EXT (d)	Cocaine (n=3)	0.08 ±0.04	0.11 ±0.01	0.99 ±0.26	0.36 ±0.07
(c) + (d)		0.16 ±0.06	0.18 ±0.02	1.90 ±0.39	0.73 ±0.14
INT and EXT	Cocaine (n=4)	0.17 ±0.01	0.19 ±0.06	1.78 ±0.06	0.58 ±0.05

Table 4.7 — INT OR EXT ^{3}H.NA (2.0 ml. min^{-1})

The effect of increasing the flow rate to 2.0 ml. min^{-1} on the flux of unchanged ^{3}H.NA and efflux of ^{3}H.metabolites into either bathing solution of perfused rabbit ear artery segments incubated with (-)^{3}H.NA (0.18 µM) applied either to the intimal or to the adventitial surface of the vessel.
Values shown are means ± SEM.
* indicates significance ($p < 0.05$); unpaired t-test.

Incubating Solution	Treatment (n)	NA Flux	Metabolite Efflux nmol. g^{-1}. 30 min^{-1}. DOMA INT	DOMA EXT	DOMA Total	DOPEG INT	DOPEG EXT	DOPEG Total	MMN INT	MMN EXT	MMN Total	OMDA INT	OMDA EXT	OMDA Total	NA Diffusion Coefficient x10^{-7}cm^{2}.sec^{-1}
INT ^{3}H.NA	Untreated (n=3)	0.37 ±0.04 *	0.07 ±0.02	0.08 ±0.02	0.15 ±0.03	0.37 ±0.06	0.72 ±0.01 *	1.09 ±0.06	0.85 ±0.12 *	0.51 ±0.02 *	1.36 ±0.11 *	0.19 ±0.03	0.35 ±0.01 *	0.54 ±0.03	1.23 ±0.21
EXT ^{3}H.NA	Untreated (n=4)	0.38 ±0.03 *	0.09 ±0.00	0.46 ±0.02	0.55 ±0.02	0.68 ±0.06	2.30 ±0.15	2.98 ±0.20	0.14 ±0.01	0.33 ±0.02	0.47 ±0.03	0.32 ±0.05	0.13 ±0.04 *	0.45 ±0.09	1.08 ±0.06 *

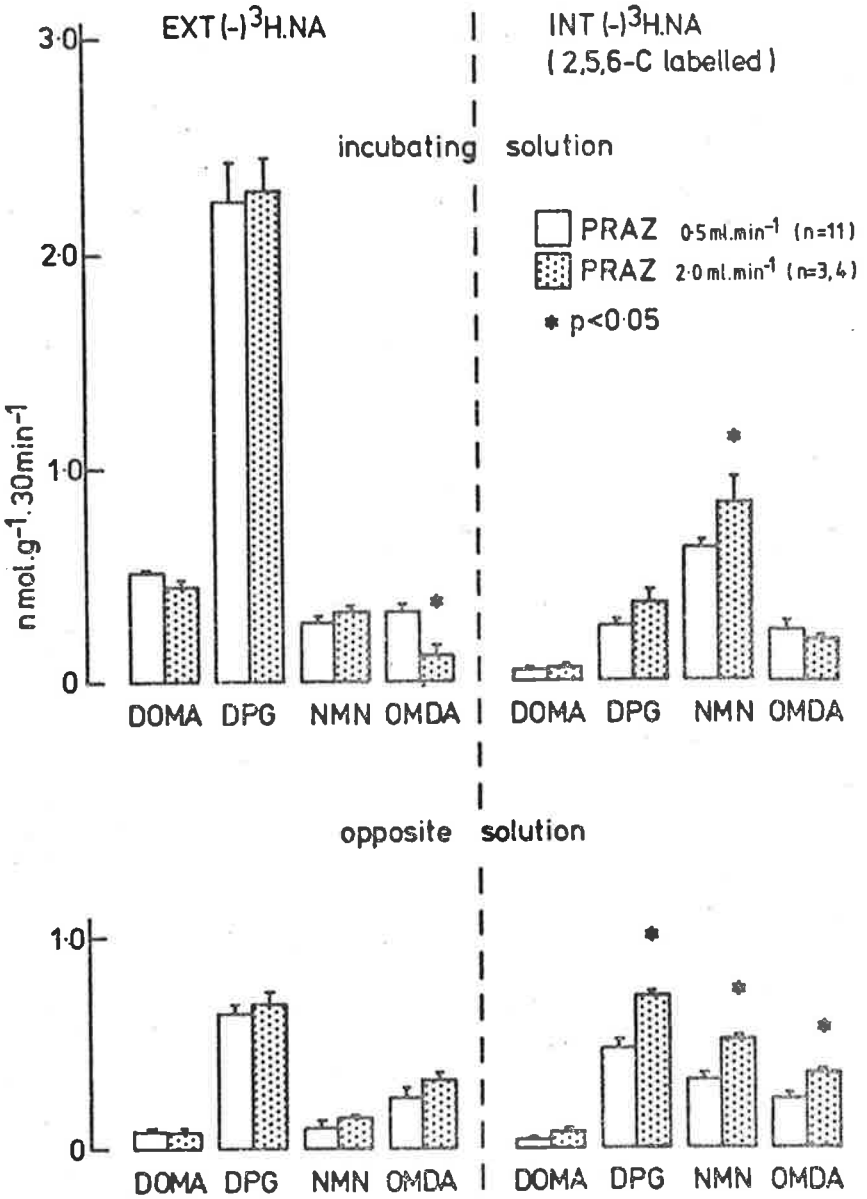

Fig. 4.8 The effect of increased flow rate (to 2.0ml.min^{-1}) on the efflux of ^{3}H.metabolites into the Ca^{++} free medium bathing either surface of relaxed (prazosin, 0.2 μM) ear artery segments (from reserpine pretreated rabbits) where (-)^{3}H.NA (0.18 μM) is applied either to the adventitia (EXT) or to the intima (INT). * indicates significance ($p < 0.05$); unpaired t-test.

was not the primary factor responsible for the greater efflux of these metabolites into the EXT solutions in vessels incubated with EXT (or INT) ^{3}H.NA. It also implied that the concentration which EXT ^{3}H.NA attains in the region of the nerves is close to the uniform concentration achieved throughout the vessel wall when the amine is applied to both surfaces; this is consistent with the rapid diffusion of NA through the adventitia (see Discussion).

To determine the influence of the gradient of concentration on extraneuronal formation of metabolites, the above experiments were repeated in the presence of cocaine. As shown in Table 4.6 the total efflux of each metabolite in the vessels incubated with INT plus EXT ^{3}H.NA in the presence of cocaine was twice the efflux from segments where ^{3}H.NA was applied separately to either surface. However, unlike its distribution in the latter segments, ^{3}H.NMN (as well as the other metabolites) was now uniformly distributed between the INT and EXT solutions. These results indicated that the concentration gradient of ^{3}H.NA within the vessel wall has a major influence on ^{3}H.NMN formation and on its relative effluxes from the two surfaces of the artery.

(6) Influence of flow rate.

The metabolism of (-)^{3}H.NA (0.18μM) was examined in four artery segments where the flow rate was 2.0 ml.min^{-1} instead of 0.5 ml.min^{-1} used in all the preceding experiments. As shown in Table 4.7 as well as Fig. 4.8, the increase in flow rate was without effect on the amounts of ^{3}H.DOPEG, ^{3}H.DOMA and ^{3}H.NMN which effluxed from the two surfaces of the vessels incubated with EXT ^{3}H.NA; the exception was that proportionally less ^{3}H.OMDA effluxed from the adventitial surface at the higher flow rate. In contrast, in segments incubated with INT ^{3}H.NA, the effluxes of all metabolites, with the exception of ^{3}H.OMDA effluxing

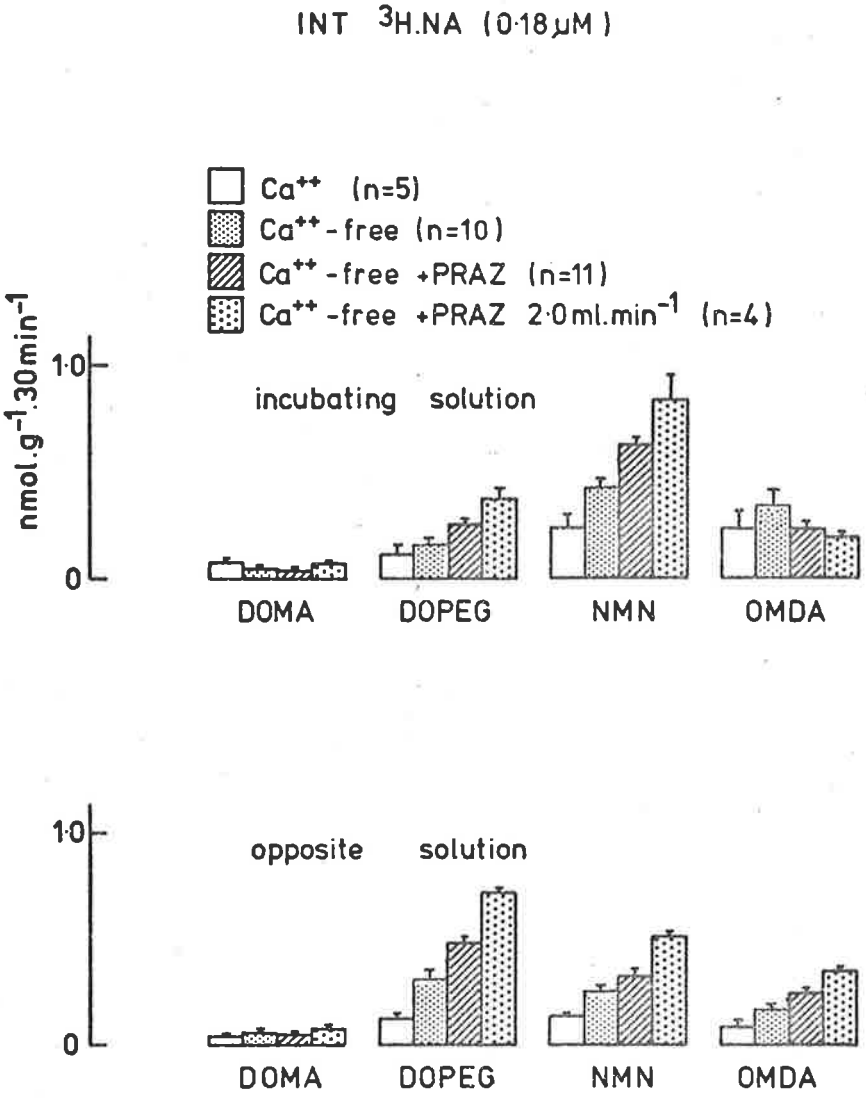

Fig. 4.9 The influence of decreasing wall thickness on the efflux of ^{3}H.metabolites in nmol.g^{-1}.30min^{-1} into the medium bathing either surface of perfused ear artery segments (from reserpine pretreated rabbits) where (-)^{3}H.NA (0.18 µM) is applied to the intima (INT). This data is also shown separately in preceding figures. The wall thickness is greatest for the Ca^{++} studies (0.18mm) and least for the Ca^{++} free prazosin vessels where the flow rate was 2.0ml.min^{-1} (0.09mm).

from the intimal surface, were increased by 40-50%. The net effect was that the DOPEG formation ratio was decreased from 4.4 to 2.7. Despite the increase in INT ^{3}H.NA metabolism, the total metabolite efflux from EXT ^{3}H.NA was still 40% greater than from INT ^{3}H.NA. However, the difference between the fluxes of INT ^{3}H.NA and EXT ^{3}H.NA was much less evident than at the lower flow rate, due to the increase in flux of unchanged INT ^{3}H.NA and a decrease in flux of EXT ^{3}H.NA.

The effects of the higher flow rate on metabolite effluxes from vessels incubated with INT ^{3}H.NA are summarised in Fig. 4.9. The effects of prazosin, and Ca^{++} are included for comparison. It will be seen that the effluxes of NMN and DOPEG in Ca^{++} medium increased progressively with the omission of Ca^{++}, the addition of prazosin, and increasing the flow rate. Interestingly, these increases approximately parallel those in the fluxes of INT ^{3}H.NA (from Tables 4.1 and 4.7).

DISCUSSION

(1) ORIGIN OF METABOLITES.

The results confirmed earlier evidence that DOPEG and DOMA are largely neuronal in origin and that NMN is largely extraneuronal in origin in the rabbit ear artery (Head et al, 1980; de la Lande et al, 1978). The results further indicated that the surface of entry of NA does not influence the origins of these metabolites; this was apparent from findings that cocaine strongly inhibited DOPEG and DOMA efflux, and hydrocortisone strongly inhibited NMN efflux, irrespective of whether the segments were incubated with INT or EXT ^{3}H.NA. The OMDA fraction appeared exceptional, in that cocaine significantly decreased its efflux into the EXT solution when incubated with EXT ^{3}H.NA, but not

from INT ^{3}H.NA. A possible explanation is presented in Section (5) of the present discussion (and also examined in greater detail in Chapter 6).

The more rapid efflux of DOPEG and DOMA from the adventitia was also independent of the surface of entry of NA, since it was apparent in segments incubated with INT ^{3}H.NA, EXT ^{3}H.NA, and INT plus EXT ^{3}H.NA. This finding is consistent with evidence that the sites of origin, i.e. the sympathetic nerves, are located at the junction of the adventitia and the media, and with evidence which suggests that the diffusivity of NA and its metabolites is considerably higher in the adventitia than the media. The evidence stems from the observation in several laboratories that during nerve stimulation, 80-90% of the released transmitter and metabolites effluxes from the adventitial surface (reviewed by de la Lande, 1975; Parker, 1977). Although the greater efflux could also be explained in terms of the proximity of the nerves to the outer surface of the artery, there is evidence that (in the rabbit aorta) the diffusion coefficient of NA in the isolated adventitia (4×10^{-6} $cm^2.sec^{-1}$) is 5.7-fold greater than that of the isolated media (7.3×10^{-7} $cm^2.sec^{-1}$) (Bevan and Török, 1970; Bevan and Su, 1973). Although the diffusion coefficients of the metabolites of NA have not been determined, it is not unreasonable to assume that they are similar to those of NA in view of their similar molecular sizes.

(2) DIFFUSION MODEL.

In contrast to its minor influence on the efflux pattern of ^{3}H.DOPEG and ^{3}H.DOMA, the surface of entry of the amine exerted a marked influence on the rates of formation of these metabolites, the rates being 3-12 fold greater (depending on the experimental conditions) when the amine entered via the adventitia. The lowest ratio (in vessels perfused at 0.5 $ml.min^{-1}$) was in the hydrocortisone-treated arteries,

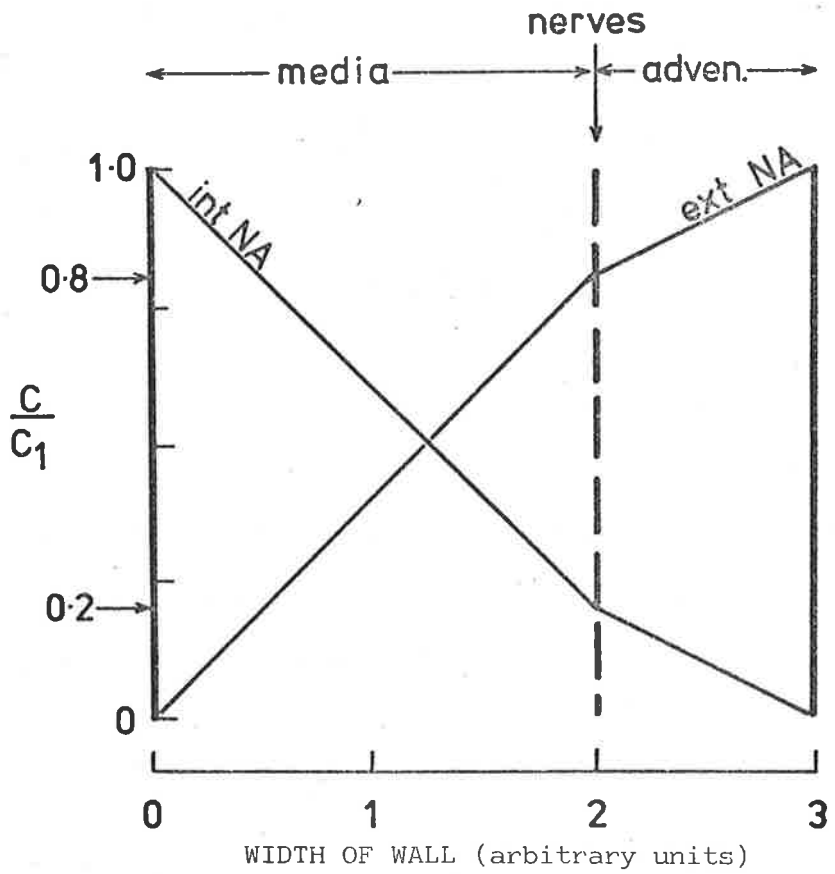

Fig. 4.10 A model of the theoretical gradients of concentration across the artery wall where NA enters either via the intimal (INT) or via the adventitial (EXT) surface. This model assumes that,

(a) the media is twice as thick as the adventitia,
(b) the diffusivity of NA in the media is twice that in the media, and
(c) the wall behaves like the depth of a plane sheet with respect to the diffusivity of NA.

Hence, this indicates that the concentration of NA at border of the media and the adventitia (the site of the sympathetic nerves) is 20%, or 80%, lower than the concentration at surface where the substrate entered depending whether the amine entered via the adventitia, or via the intima, respectively.

C= concentration of NA at any point within the wall,

C_1= concentration of NA applied to the surface.

and the greatest in the Ca^{++} constricted vessels. The difference implies that under steady-state conditions EXT ^{3}H.NA achieves a correspondingly higher concentration than INT ^{3}H.NA at the sites of ^{3}H.DOPEG and ^{3}H.DOMA formation, i.e. in the region of the nerve terminals. These differences may also be related to regional differences in diffusivity since, in theory, the ratio of the two concentrations would depend primarily on the relative distances of the nerves from the inner and outer surfaces, and on the diffusivity of NA in the intervening regions (media and adventitia). The relationship between these two factors is illustrated by the model in Fig. 4.10. This model shows that if,(a) the artery wall is treated as the depth of a plane sheet, (b) the media is twice as thick as the adventitia (as estimated by Bevan and Su, 1974) and (c) the diffusivity of NA is uniform throughout the wall, then the predicted ratio of concentrations of EXT to INT NA at the boundary between the media and the adventitia is 2.0. However, if the diffusivity in the adventitia is twice that in the media, the ratio becomes 4.0. The actual ratio of the diffusion coefficients in either the media or the adventitia of the rabbit ear artery is not known since it is not possible to separate the two regions mechanically in this vessel. However, it is probable that the value of 1.5×10^{-6} $cm^2.sec^{-1}$ obtained by Bevan and Su (1974) when NA was applied for 10 seconds to the intima refers to diffusivity in the media only. If it is assumed that the diffusivity in the adventitia of the rabbit ear artery is similar to that in the rabbit aorta (4×10^{-6} $cm^2.sec^{-1}$), the predicted ratio of the concentration of EXT to INT NA at the boundary is 5.3. In the case of the more analogous model of a cylinder (rather than a plane sheet), where inner and outer radii are those estimated in the present

study (0.29 and 0.41mm, respectively), the ratio will be slightly higher (6.7). Although this ratio is within the range of the experimentally determined ratios, quantitative extrapolation from this model is probably premature. A puzzling discrepancy between the values of the above diffusion coefficient in the inner wall obtained by Bevan and Su (1974) and a considerably lower estimate from our laboratory based on steady state flux of NA across the whole wall (0.8×10^{-6} $cm^2.sec^{-1}$) (de la Lande et al, 1980) needs to be resolved. Furthermore, one of the assumptions in the model is that neuronal uptake does not influence the steady-state concentration of NA at the boundary between media and adventitia. However, the experimentally determined ratios of concentrations were determined from measurements which depended on neuronal uptake of NA (i.e., DOPEG formation). If as seems probable, diffusion of NA is limited in the media compared with the adventitia, it is possible that the uptake of INT NA, relative to that of EXT NA, is diffusion-limited; if so the steady-state concentration in the region of the nerves will be determined, not only by the concentration gradient, but also by the flux of INT amine into that region.

Despite these limitations, the model provides a useful basis with which to interpret most features of the metabolic data. The absence of a significant difference between the rates of ^{3}H.DOPEG efflux, when ^{3}H.NA is applied to the EXT surface only and to both surfaces simultaneously, accords with the prediction that the concentration which EXT NA achieves in the region of the nerves will be little different from the concentration when the latter is uniform throughout the wall and equal to the concentration in the incubating solution.

This model also provides a useful basis for interpreting the patterns of extraneuronal metabolite formation and efflux from NA. The efflux of NMN was 1.9 and 2.5-fold greater from the surface of entry of the

INT and the EXT amine, respectively, than from the opposite surface in the relaxed (prazosin-treated) preparations. This ratio is sufficiently close to the theoretical ratio(i.e. 2.0) if it is assumed that (a) the concentration of parent amine declines uniformly across the media, (b) the sites of O-methylation are uniformly distributed across the media, but are not present in the adventitia, (c) the rates of O-methylation are directly proportional to the parent amine concentration within the vessel wall, and (d) the diffusivity of amine and metabolites in the adventitia approximate to those in free solution. The theoretical derivation of this ratio is presented in Appendix 1. The observation that the ratio of ^{3}H.NMN efflux (in the presence of cocaine) from INT plus EXT ^{3}H.NA was almost exactly twice the rate from INT or EXT ^{3}H.NA separately, also accords with the above assumptions(a) to (d). Since the present studies provide evidence that NMN formation was proportional to substrate concentration (Fig. 3.1), and there is evidence (in the rabbit aorta) that COMT activity is uniform throughout the media (Verity et al, 1972), the quantitative and qualitative agreement between the theoretical and observed ratio of NMN efflux adds considerable support to the argument that the concentration of parent amine does, in fact, decline uniformly across the media. The only qualification is the report by Lowe and Creveling (1978) that in the rat aorta and coronary blood vessels, COMT activity, as demonstrated by an immunohistochemical technique, was confined to the intima. It does not seem possible to reconcile such a distribution with any of the observed features of the catecholamine metabolism in the present study. For example, if COMT were distributed in this manner in the rabbit ear artery, then the surface of ^{3}H.NMN efflux should be independent of the surface of entry of ^{3}H.NA. It will be seen from the data presented that clearly this is not the case. The data

presented here for the rabbit ear artery is in accord with that of Branco et al (1981a), who showed, using a sensitive autoradiographic technique that the smooth muscle cells of the rabbit aorta corresponded to the steroid-sensitive extraneuronal O-methylating site(s).

(3) CONSTRICTION AND FLOW RATE.

In the constricted artery perfused at 0.5 $ml.min^{-1}$, the difference between the rates of ^{3}H.DOPEG efflux from EXT and from INT ^{3}H.NA was more pronounced than in the relaxed artery. Thus the ^{3}H.DOPEG formation ratio was 4.4 in the relaxed (prazosin-treated) perfused artery, and 10.4 in Ca^{++}-free vessels in the absence of prazosin, where the artery was slightly constricted in response to INT ^{3}H.NA. The mean ratio was 24 when the constrictor response to INT ^{3}H.NA was greatest, i.e., in the Ca^{++}-treated arteries which also constricted to EXT ^{3}H.NA. The increase in this ratio was due largely to a selective decrease in the rate of ^{3}H.DOPEG efflux from INT ^{3}H.NA. In the presence of prazosin, the rates of ^{3}H.DOPEG efflux from EXT and from INT ^{3}H.NA were increased and decreased respectively, although in neither case was the change significant. Nevertheless, the possibility cannot be excluded that prazosin exerted a mild inhibitory effect on the neuronal uptake and deamination of NA which tended to mask an increase in ^{3}H.DOPEG formation from INT ^{3}H.NA resulting from the prazosin induced relaxation of the vessel.

Since the rate of ^{3}H.DOPEG formation is linearly related to the concentration of ^{3}H.NA (Chapter 3), the above findings imply that the concentration which INT ^{3}H.NA achieves in the region of the nerve terminals decreases as the artery constricts. This phenomenom can be explained in terms ofone or more of the factors (a) to (c) below.

(a) An increase in thickness of the media, if proportionally greater than that of the adventitia, would steepen the gradient of concentration of INT ^{3}H.NA between the intima and the nerve terminal region.

(b) Constriction may decrease the diffusivity of NA within the media. This possibility seems excluded by the finding that, although prazosin increased the flux of ^{3}H.NA across the wall, this was due primarily to the decrease in wall thickness; i.e., prazosin did not alter the diffusion coefficient of the unchanged amine. Nevertheless, in an earlier study, it was shown that the diffusion coefficient of ^{14}C-sorbitol (which distributes only in the extracellular space) was less in the constricted than in the relaxed artery (de la Lande et al, 1980). Furthermore, a decrease in diffusivity of ^{3}H.NA in the constricted artery may also explain the effects of prazosin, and of Ca^{++}, on ^{3}H.NMN efflux from the vessel. In Ca^{++} free segments, where only INT ^{3}H.NA caused constriction, blockade of this constriction by prazosin was associated with an increase in ^{3}H.NMN efflux from INT ^{3}H.NA, but not from EXT ^{3}H.NA. This result suggests that constriction of the artery is associated with a decreased capacity of the artery to O-methylate NA, a suggestion which is supported by the comparisons between Ca^{++} and Ca^{++} free perfused segments. The Ca^{++} segments, although constricting to INT ^{3}H.NA, also constricted to EXT ^{3}H.NA. These effects were associated with decreased effluxes of ^{3}H.NMN in vessels incubated with either INT or EXT ^{3}H.NA. The simplest explanation of these findings is that constriction, by decreasing the diffusivity of NA within the media, reduced the concentration of NA available to the O-methylating system in the vessel wall.

(c) A third possibility is limitation of entry of INT ^{3}H.NA into the vessel wall. This may conceivably result if, for example, the lumen became convoluted, as the result of folding of the intima, to the extent that the overlying media was not uniformly exposed. This possibility is suggested by the finding that when the resting

perfusion pressure was increased by increasing the flow rate to 2.0 $ml.min^{-1}$, the rates of efflux of all metabolites during incubation with INT NA, although not during incubation with EXT NA, was increased. The increase in total metabolite efflux at the higher flow rate was approximately 40%. As a result, the difference between the rates of ^{3}H.DOPEG formation from EXT and from INT ^{3}H.NA was less pronounced, the DOPEG formation ratio being decreased from 4.4 to 2.7.

At this stage, the data does not permit the contributions of these factors (a) to (c) to the metabolic changes associated with constriction to be assessed. However, the results highlight the need for more detailed morphological studies on the changes in shape of the artery during constriction, plus further measurements of the volume of the extracellular component of the tissue at various levels of constriction.

(4) UPTAKE INHIBITION.

(a) Cocaine

The surface of entry markedly influenced the rate of metabolism of NA as indicated by an approximately three-fold greater efflux of total metabolites (i.e., the sum of the individual metabolites) during incubation with EXT ^{3}H.NA. Cocaine eliminated the influence of the surface of entry of NA by (a) its inhibitory action on ^{3}H.DOPEG and ^{3}H.DOMA efflux (already discussed) and (b) by increasing the efflux of ^{3}H.NMN from EXT ^{3}H.NA by (2.6-fold), but not from INT ^{3}H.NA. The latter observation can be explained in terms of the location of the sympathetic nerves at the medial-adventitial junction, so that inhibition of neuronal uptake permits the EXT ^{3}H.NA to achieve a higher concentration in the underlying media where the sites of O-methylation (assumed to be the smooth muscle cells) are located, but has little effect on the

concentration which INT ^{3}H.NA achieves in the media as the amine is only exposed to neuronal uptake after it diffuses through the media. This explanation places primary importance on the sequential arrangement of the neuronal deaminating and extraneuronal O-methylated pathways within the artery wall. However, the sequential arrangement cannot explain the finding that cocaine also increased ^{3}H.NMN formation (although only to a small extent) when the ^{3}H.NA entered both surfaces simultaneously since, theoretically, the concentration of the ^{3}H.NA should then be uniform throughout the artery wall. The effect of cocaine under these conditions is best explained in terms of the two pathways acting as alternative mechanisms for inactivating NA, as proposed by Hughes (1972) to account for similar observations on the rabbit vas deferns.

In the relaxed (prazosin-treated) vessel the flux of unchanged EXT ^{3}H.NA was two-fold greater than that of INT ^{3}H.NA. However, in the presence of cocaine the flux of INT ^{3}H.NA increased by a factor of 4, and that of EXT ^{3}H.NA only by a factor of 2.5; hence the difference between the fluxes is related in some way to the activity of the neuronal uptake system. The estimate of flux was based on the assumption that the content of accumulated amine in the opposite solution after the segment was incubated for 30 minutes with ^{3}H.NA, represents the total amount of unchanged amine which diffused across the artery wall in that time. Hence, it is possible that, in the untreated artery, the flux from INT ^{3}H.NA may have been underestimated due to the metabolism of some of the unchanged amine after it had diffused into the opposite solution and subsequently re-entered the adventitia. However, if this factor was solely responsible for the difference in fluxes of INT and of EXT ^{3}H.NA, it would mean that at least two-thirds of the amine which reached the opposite solution underwent metabolism, whereas the proportion of

EXT ^{3}H.NA (0.18μM) metabolised during this period was only 20%. As indicated by the studies on artery strips (Chapter 3), the proportion of ^{3}H.NA metabolised remained the same at a ten-fold lower concentration of amine. Hence the assumption involved in estimating the flux is unlikely to account for the 2.5-fold greater flux of EXT ^{3}H.NA.

A second possibility is that proportionally more INT ^{3}H.NA than EXT ^{3}H.NA was removed by neuronal uptake as the amine fluxes through the region of the nerve terminals. The magnitude of the increase in flux of unchanged amine produced by cocaine implies that, in the case of INT ^{3}H.NA, 80% was removed in this way, and that, in the case of EXT ^{3}H.NA, the proportion was 40%. The INT ^{3}H.NA which was removed appears to have been converted entirely to ^{3}H.DOPEG and ^{3}H.DOMA since in the cocaine-treated segments the increase in the content of unchanged amine in the EXT solution was approximately equal to the decrease in total flux of ^{3}H.DOPEG and ^{3}H.DOMA. It is difficult to account for the apparently more efficient removal of NA by neuronal uptake when it diffused from the internal surface except in terms of the possibility, considered already, that the removal of INT ^{3}H.NA by neuronal uptake is more diffusion-limited than the removal of EXT ^{3}H.NA.

A factor to be considered in the interpretation of the effects of cocaine is the effect already discussed of increasing flow rate on metabolite efflux in segments incubated with INT NA. The latter finding indicated that at the higher flow rate (2.0 ml.min^{-1}), the flux of INT NA across the vessel wall was increased and the flux of EXT NA decreased. Hence it is possible that the efficiency of neuronal uptake in removing INT NA may also be related in some way to the intraluminal flow rate. Analysis of the effects of neuronal uptake inhibition at various flow rates may resolve this question.

(b) Hydrocortisone

Hydrocortisone exerted a potent inhibitory effect on ^{3}H.NMN efflux, but did not otherwise influence the pattern of metabolite efflux from INT and from EXT ^{3}H.NA. In particular, the failure of hydrocortisone to increase ^{3}H.DOPEG efflux from INT ^{3}H.NA suggests that the influence of the corticosteroid-sensitive O-methylating system on the concentration which INT ^{3}H.NA achieves in the region of the nerve terminals was a minor one. Some influence cannot be excluded since the steroid tended to decrease the rate of ^{3}H.DOPEG efflux from EXT ^{3}H.NA. This latter effect may have masked the predicted effect of eliminating a site of loss of NA within the media, namely an increase in ^{3}H.DOPEG formation from INT ^{3}H.NA. The failure of hydrocortisone to increase the flux of EXT ^{3}H.NA is also puzzling, since in an earlier study (de la Lande, 1980) it was shown that DOCA (in the presence of cocaine) caused a two-fold increase in flux of NA compared with vessels treated with cocaine alone. The explanation of the difference may be in the experimental conditions since the latter study employed non-reserpinised arteries incubated with Ca^{++} Krebs solution in the presence of phentolamine (0.3μM) at a flow rate of 1.0 ml.min^{-1}.

(c) Phenoxybenzamine

Phenoxybenzamine (PBZ) illustrated the effects of combined α-receptor blockade and inhibition of neuronal and extraneuronal uptake on the metabolism and flux of NA. Comparison of the untreated (Ca^{++} free) and PBZ-treated segments indicated that the sequential processes of uptake (neuronal and extraneuronal) followed by enzymic-inactivation account for 80% and 90% of the metabolism of INT and of EXT NA respectively, and that these processes normally reduce the fluxes of unchanged INT and EXT ^{3}H.NA by 84% and 58% respectively. The diffusion coefficients of INT and of EXT ^{3}H.NA in the PBZ-treated vessels were not significantly different. Their value (both 0.6 x 10^{-6} cm^{2}.sec^{-1}) is

however, less than the value (0.8×10^{-6} $cm^2.sec^{-1}$) measured in an earlier study for EXT ^{3}H.NA (de la Lande et al, 1980). The difference may reflect a difference in the experimental conditions; the larger value was derived from arteries of non-reserpinised rabbits under conditions where the intraluminal perfusate (Ca^{++} Krebs) was continuously replaced.

(5) OMDA FORMATION

In segments incubated with Ca^{++}-free and Ca^{++} media there was considerable variability in estimates of the OMDA contents of the solution containing the substrate (^{3}H.NA). This variability was not evident when prazosin was present and for this reason the discussion of the influence of the surface of entry of NA on its conversion to O-methylated-deaminated metabolites will be restricted to prazosin-treated preparations. Although the composition of the OMDA fraction was not analysed, its high medium to tissue ratio (5.0) suggests that, under the experimental conditions employed, the major proportion of the OMDA fraction is MOPEG. When normal segments of artery are incubated in Ca^{++} Krebs solution (Head, 1976) the medium to tissue ratios of MOPEG and VMA are 10.3 and 0.8 respectively; this suggests that in the present study MOPEG predominates in the OMDA fraction. There was little difference between the relative effluxes of OMDA into the INT and EXT solutions, nor was there any effect of hydrocortisone on these effluxes. However, it has been pointed out by Fiebig and Trendelenburg (1978b), that corticosteroid-sensitivity does not preclude the possibility that DOPEG, after its efflux from the sympathetic nerves, may be O-methylated in the corticosteroid-sensitive extraneuronal compartment, since its high lipophilicity (Mack and Bönisch, 1979) would enable it to diffuse directly

across the cell membrane and thus by-pass the steroid-sensitive extra-neuronal uptake system which transports NA into the effector cell (considered in greater detail in Chapter 6). The present results suggest that this mechanism also operates in the rabbit ear artery. The evidence is that the efflux of OMDA was inhibited more strongly by cocaine in vessels incubated with EXT ^{3}H.NA (by 35%). Furthermore, the efflux of OMDA from the adventitial surface was more sensitive to cocaine than efflux from the intimal surface. Such a selective effect suggests that cocaine-sensitive OMDA formation was localised in the outer regions of the vessel wall, i.e., those regions where, due to its origins in sympathetic nerves, the concentration of DOPEG was greatest. By the same argument the relative insensitivity of OMDA efflux from the intimal surface to cocaine implies that a second pathway, which is insensitive to cocaine, predominates in the inner region of the wall. In view of the low concentration of DOPEG in the inner regions of the vessel wall (i.e., near the intima) it is suggested that this second mechanism is not dependent on DOPEG which effluxes from sympathetic nerves. The cocaine-insensitive pathway of OMDA formation, although insensitive to hydrocortisone, appears to be sensitive to PBZ. This possibility was suggested by the 62% decrease in OMDA efflux produced by PBZ treatment in segments incubated with INT ^{3}H.NA. These segments, like those treated with prazosin, did not constrict in response to ^{3}H.NA because PBZ also blocked the post-synaptic α-receptors.

In view of the above results, it is suggested that cocaine-insensitive OMDA formation occurs in an extraneuronal compartment into which NA is transported by a corticosteroid-insensitive, but PBZ-sensitive, transport process. Although these results provide no indication of the morphological

site of such a compartment, there is evidence in the rabbit aorta that OMDA is formed in non-neuronal structures in the adventitia by a process which is insensitive to both cocaine and corticosteroids (Schrold and Nedergaard, 1981); whether this process is PBZ-sensitive has not been reported.

(6) SUMMARY

In summary, the results of this chapter show that the surface of entry of NA exerts a profound effect on its metabolism; inactivation by the neuronal deaminating pathway predominating when NA enters via the adventitia, and extraneuronal O-methylating pathway when NA enters via the intima.' Intraneuronal deamination of NA occurs only after its transport into the nerve by the cocaine-sensitive uptake process, and O-methylation of NA occurs only after its transport into an extraneuronal compartment (presumably the smooth muscle cells) by a corticosteroid-sensitive uptake process. The O-methylated-deaminated metabolites appear to be formed in part by O-methylating DOPEG released from nerve terminals and in part by a purely extraneuronal mechanism which, although corticosteroid-insensitive, is sensitive to PBZ (considered in more detail in Chapter 6).

The different rates of deaminated metabolite formation from INT and from EXT NA, and the different pattern of effluxes of NMN from the two surfaces can be explained in terms of a gradient of concentration of NA between its surface of entry, and the opposite surface. The greater efflux of the deaminated metabolites from the adventitial than from the intimal surface appears to be independent of the gradient of concentration of NA but is consistent with evidence of regional differences in diffusivity of NA, and presumably its metabolites.

Based on the different rates of DOPEG formation from INT and from EXT NA, it is suggested that the decrease in concentration of INT NA between the intima and the nerve terminals is greater in constricted than in relaxed vessels. A possible explanation is that constriction may decrease the diffusivity of NA within the vessel wall; this explanation is supported by the association of constriction with a decreased efflux of NMN from the vessel wall.

CHAPTER 5

DIFFUSION AND METABOLISM OF ^{3}H.ISO IN PERFUSED ARTERY SEGMENTS

CHAPTER 5.

DIFFUSION AND METABOLISM OF ^{3}H.ISO IN PERFUSED ARTERY SEGMENTS

INTRODUCTION

In the preceding chapter the influence of the surface of entry of NA on its O-methylation to NMN in reserpinised rabbit ear arteries was examined. Since, conceivably, the formation of NMN may have been influenced in some way by the associated conversion of NA to O-methylated-deaminated metabolites, it was desirable to examine the patterns of efflux of the O-methylated derivative of isoprenaline (ISO) in vessels incubated with (±)^{3}H.ISO. The low affinity of ISO for neuronal uptake and high affinity for extraneuronal uptake was described by Iversen (1967). Furthermore, it forms only one metabolite, namely 3-methoxy-isoprenaline (MeOISO) by the activity of extraneuronal COMT but it is not deaminated by MAO (Hertting, 1964). The kinetics of O-methylation of ISO in the rabbit ear artery was defined by Head et al (1980), who showed that the O-methylation occurred in a single corticosteroid-sensitive extraneuronal compartment (K_m = 2.7μM).

In the present study, the distribution of ^{3}H.MeOISO between the solutions bathing the INT and the EXT surfaces of perfused segments of rabbit ear arteries incubated with (±)^{3}H.ISO is described.

METHODS

The experimental technique was identical to that used in Chapter 4 and described in the General Methods (Chapter 2). Briefly, ear arteries from reserpinised rabbits were perfused with either INT (±)^{3}H.ISO (0.18μM) or bathed in EXT (±)^{3}H.ISO (0.18μM) in Ca^{++} free Krebs solution. Cocaine (29μM) was present throughout these studies to exclude any involvement of neuronal inactivation.

Table 5.1

The flux of unchanged (±)^{3}H.ISO and efflux of ^{3}H.MeOISO into the solutions bathing either surface of perfused rabbit ear artery segments when (±)^{3}H.ISO (0.18 μM) is applied to either the intimal or the adventitial surface.
Values are means ± SEM.
* indicates significance ($p<0.05$); unpaired t-test.

Incubating solution	Treatment (n)	ISO Flux	MeOISO efflux nmol.g^{-1}.30min^{-1} INT	EXT	Total	ISO Diffusion Coefficient x10^{-7}cm^{2}.min^{-1}
INT ^{3}H.ISO	Untreated (n=5)	0.59 ±0.11	1.34 ±0.13	0.51 ±0.06	1.85 ±0.16	2.01 ±0.43
	Hydrocortisone (n=5)	1.17 ±0.11 *	0.29 ±0.03 *	0.13 ±0.02 *	0.42 ±0.04 *	3.97 ±0.47 *
EXT ^{3}H.ISO	Untreated (n=5)	0.47 ±0.05	0.52 ±0.04	0.99 ±0.14	1.51 ±0.18	1.85 ±0.35
	Hydrocortisone (n=5)	1.25 ±0.12 *	0.14 ±0.02 *	0.28 ±0.07 *	0.42 ±0.09 *	5.32 ±1.03 *

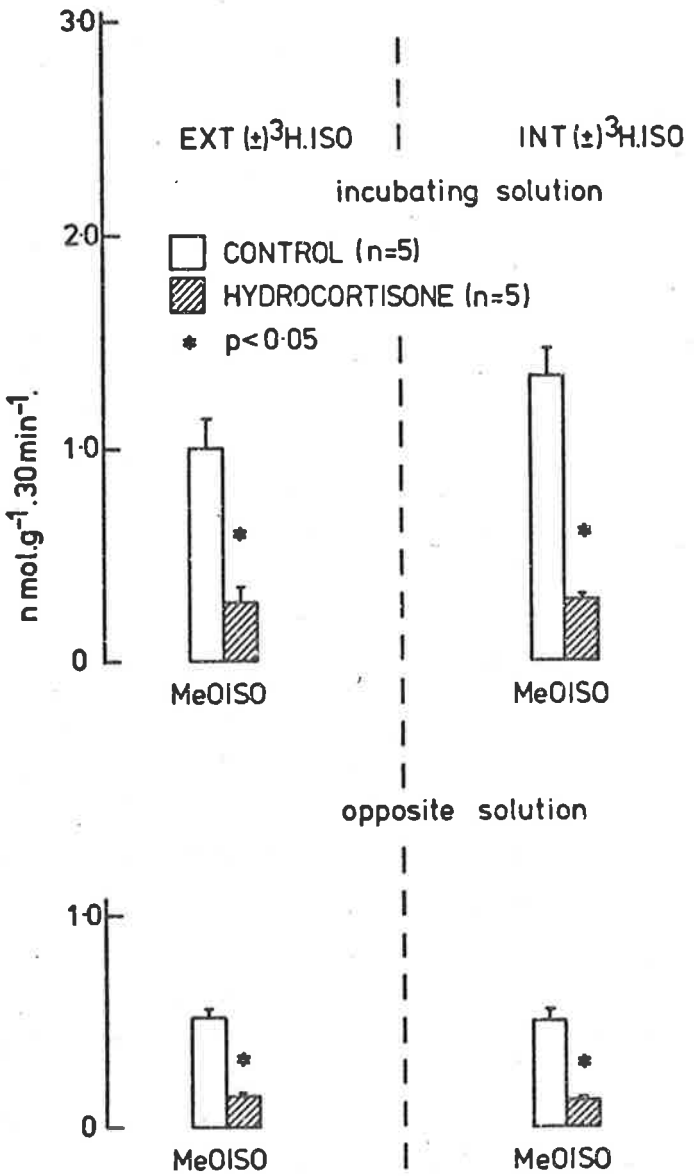

Fig. 5.1 The effect of hydrocortisone (413 μM) on the efflux of ^{3}H.metabolites in nmol.g^{-1}.30min^{-1} into the Ca^{++} free medium bathing either surface of perfused ear artery segments (from rabbits which were not reserpine pretreated) where (±)^{3}H.ISO(0.18 μM) is applied either to the adventitia (EXT) or to the intima (INT). Note that cocaine is present throughout these studies to eliminate any neuronal involvement.
* indicates significance ($p < 0.05$); unpaired t-test.

In all experiments a second incubation was carried out on each vessel after a 60 minute washout period which included a 30 minute pre-incubation with hydrocortisone (413μM). The ^{3}H.ISO was then applied to the same surface of the artery as in the first incubation in the presence of hydrocortisone. The amount of unchanged ^{3}H.ISO which fluxed through the vessel wall, and the amount of ^{3}H.MeOISO which effluxed into the two bathing solutions were then assayed by the modified cascade column chromatographic method described in the General Methods (Fig. 2.4). The recoveries and crossovers are shown in Table 2.3.

RESULTS

The relative effluxes of ^{3}H.MeOISO from the two surfaces during incubation with INT or with EXT ^{3}H.ISO (0.18μM) are shown in Table 5.1 and also in Fig. 5.1. The major feature is that a greater proportion of the metabolite (^{3}H.MeOISO) effluxed into the solution containing the substrate. These proportions are 2.6 and 1.9 in vessels incubated with INT and with EXT ^{3}H.ISO respectively. The total efflux of ^{3}H.MeOISO from vessels incubated with INT ^{3}H.ISO was greater than that from vessels incubated with EXT ^{3}H.ISO (values of 1.85 and 1.51 nmol.g^{-1}.30 min^{-1} respectively), however this difference was not statistically significant at the 5% level.

The effect of hydrocortisone (413μM) treatment was to reduce ^{3}H.MeOISO formation by 77% and 72% when ^{3}H.ISO entered via the INT and EXT surfaces respectively. Further, hydrocortisone-treatment was associated with an increase in both the flux and the diffusivity (as estimated by the apparent diffusion coefficient) of ^{3}H.ISO by 2.0 and 2.8 fold for INT and EXT ^{3}H.ISO respectively.

DISCUSSION

The total effluxes of ^{3}H.MeOISO from segments incubated with INT or with EXT ^{3}H.ISO are approximately 2.0 and 1.5 fold greater, respectively, than those of ^{3}H.NMN from vessels incubated with ^{3}H.NA under similar conditions (i.e., in the case of ^{3}H.NA incubations, where the vessel was treated with prazosin to ensure relaxation and with cocaine to inhibit neuronal uptake). The inhibitory effects of hydrocortisone on these effluxes are in accord with earlier results with non-perfused segments (Head et al, 1980) which indicated that the major proportion of ^{3}H.MeOISO was derived from a corticosteroid-sensitive extraneuronal uptake and O-methylating compartment. The present result suggests, furthermore, that the ^{3}H.MeOISO was derived from the same compartment, irrespective of the surface of entry of ^{3}H.ISO into the vessel wall.

In view of earlier evidence from artery strips that approximately 80% of the MeOISO which was formed escaped into the bathing solution during incubation with ^{3}H.ISO (Head et al, 1980), the difference between the total effluxes of ^{3}H.MeOISO and ^{3}H.NMN implies a corresponding difference between the rates of O-methylation of the two amines by the vessel.

The difference is in accord with the reported higher affinity of the steroid-sensitive extraneuronal uptake system for ISO than for NA. A contributory factor may be the conversion of NA to other O-methylated derivatives (the OMDA fraction) besides NMN. If so this conversion appears to be more important when the parent amine enters via the adventitial surface. Under these latter conditions the total efflux of ^{3}H.NMN plus ^{3}H.OMDA (1.25 nmol.g^{-1} as shown in Table 4.2) was close

to that of ^{3}H.MeOISO (1.51 nmol.g^{-1}). However, when the amine entered via the intimal surface the efflux of ^{3}H.NMN plus ^{3}H.OMDA was only two-thirds that of ^{3}H.MeOISO (i.e., 1.21 nmol.g^{-1} compared with 1.85 nmol.g^{-1} respectively).

The distribution of MeOISO efflux between the intimal and adventitial surfaces was qualitatively identical with that of NMN (the latter, both in the case of untreated and cocaine-treated segments) in that, like NMN, MeOISO effluxed preferentially from the surface of entry of the parent amine. The ratio of the effluxes from the two surfaces was somewhat higher in the case of MeOISO (2.6 and 1.9 for INT and EXT ^{3}H.ISO respectively, compared with ratios of NMN in cocaine-treated vessels of 1.7 and 1.6 for INT and EXT ^{3}H.NA respectively). Nevertheless, these ratios are still close to the theoretical ratio of 2.0 predicted on the basis of the assumptions already discussed in Chapter 4, and whose theoretical derivation is presented in Appendix 1.

In this respect, the data adds weight to the argument that the concentration of the parent amine when applied to either surface declines uniformly across the media. It is of particular interest that the ratio of effluxes of ^{3}H.MeOISO from the two surfaces tended to be greater for INT than EXT ^{3}H.ISO since such a tendency is a predictable consequence of the presence of an adventitia which is deficient in sites of O-methylation compared with the media.

Subsequent to this study, Branco et al (1981a) have reported that, per unit mass, the isolated media of the rabbit aorta possesses about twice the O-methylating activity (using ^{3}H.ISO, 2μM, as the substrate) than the isolated adventitia. Although their results suggest that the isolated adventitia does possess significant ISO O-methylating activity,

Their ratio of activity to that in the media is not very meaningful as the process of separating the media and adventitia, as these authors clearly indicate, markedly decreased (by 60%) the O-methylating capacity of the intact aorta (when compared with the O-methylating capacity of the isolated media). If the damaging effect of the separation process was largely upon the O-methylating system in the media, then the two-fold ratios of COMT activities in the separated regions would greatly overestimate the quantitative importance of adventitial O-methylation.

Two other features of their study are relevant to the present results. Firstly, the O-methylating activity in the isolated adventitia in the rabbit aorta differed from that in the isolated media by being much less sensitive to inhibition of COMT (by U0521) and inhibition of extraneuronal uptake (by cortexone). Hence it might be argued that the small residual MeOISO formation persisting in the presence of hydrocortisone in the rabbit ear artery represents O-methylation of ISO in the adventitia. However, this possibility seems unlikely as the hydrocortisone-insensitive MeOISO efflux from the adventitial surface did not differ significantly from that from the medial surface (Fig. 5.1). In the case of NMN, the hydrocortisone-resistant efflux represented only 8% and 11% of the total efflux from INT and from EXT NA respectively. These observations do not exclude, but render less likely, the possibility that the adventitia contributed to the O-methylating capacity of the ear artery to the extent which the relative data of Branco et al on the rates of O-methylation in the isolated adventitia and isolated media of the rabbit aorta might suggest.

The second feature of their data is that, using autoradiographic techniques, they showed that the smooth muscle cells of the media represented almost exclusively the sites of corticosteroid-sensitive

accumulation of ^{3}H.ISO. In this respect, their data adds further support to the assumption (Chapter 4) that the sites of O-methylation are uniformly distributed across the media.

In summary, the pattern of efflux of ^{3}H.MeOISO from the two surfaces of the rabbit ear artery resembles that of ^{3}H.NMN both quantitatively and qualitatively. It seems unlikely therefore, that this pattern (in the case of ^{3}H.NMN) was greatly influenced by the associated conversion of ^{3}H.NA to other O-methylated metabolites.

CHAPTER 6

METABOLISM OF (-)^{3}H.DOPEG IN ISOLATED ARTERY STRIPS

CHAPTER 6.

METABOLISM OF (-)^{3}H.DOPEG IN ISOLATED ARTERY STRIPS

INTRODUCTION

As explained in Chapter 4, the O-methylated-deaminated metabolites of NA can be, in theory, formed by the O-methylation of the deaminated metabolites DOPEG, and DOMA, after the latter have effluxed from the sympathetic nerves. However, direct evidence that these metabolites can in fact be O-methylated in a peripheral organ, with cell structure intact, is lacking. Evidence for such a mechanism has been sought in the present study by incubating artery strips with ^{3}H labelled DOPEG, and analysing the incubating medium and tissue for unchanged DOPEG and its O-methylated derivative. The study was restricted to DOPEG for two reasons. Firstly, since it is formed at a much higher rate than DOMA, it seemed likely that O-methylation of DOPEG would represent a more important pathway of OMDA formation than O-methylation of DOMA; and secondly, as pointed out by Mack and Bönisch (1979), DOPEG is much more lipophilic than DOMA and hence a mechanism of O-methylation involving direct diffusion of the deaminated metabolite into a COMT containing compartment is much more likely to apply to DOPEG than to DOMA.

Another reason for studying O-methylation of DOPEG was purely technical. Neither ^{3}H.DOPEG or ^{3}H.DOMA are commercially available. As reported in this Chapter, ^{3}H.DOPEG was prepared by incubating rat vas deferens with (-)^{3}H.NA, and purifying the appropriate chromatographic fraction. The high yield of ^{3}H.DOPEG, plus the low crossover of ^{3}H.NA into the ^{3}H.DOPEG fraction (0.03%) made this procedure technically feasible, but posed some difficulties in the case of ^{3}H.DOMA

where the yield is much lower, and where the crossover of ^{3}H.NA is considerably higher (2.5%).

METHODS

Preparation of ^{3}H.DOPEG.

(a) The principal of the method was that a readily available tissue (the rat vas deferens), known to possess a high rate of ^{3}H.DOPEG formation (Graefe et al, 1973), was incubated with (-)^{3}H.NA and the metabolites fractionated by the column chromatographic method described in the General Methods (Chapter 2). The ^{3}H.DOPEG was isolated in the relevant fraction (fraction 3) as described below .

(b) Four rat vas deferens (250mg) were incubated in 4ml of ascorbic Krebs solution containing (-)^{3}H.NA (1.2μM) for 60 minutes at 37^{o}C and bubbled with 95% O_2, 5% CO_2. At the end of this period, the incubating medium was acidified (0.4ml of 0.1M HCl and 0.04ml 0.6M ascorbic acid) and fractionated by the cascade column chromatographic method. The only minor modification was that the 2ml of 0.2M acetic acid effluent (containing most of the ^{3}H.DOPEG) was not further diluted with the usual 2ml of water. The ^{3}H in this ^{3}H.DOPEG fraction (termed A) was then measured to determine the amount of ^{3}H.DOPEG formed. A typical experimental yield was approximately 0.6 nmol of ^{3}H.DOPEG. This was sufficient for approximately 10 artery strip incubations when diluted with 4 parts of unlabelled DOPEG. In exploratory experiments this solution (A) was treated in one of three ways,

(i) the solution was subjected again to cascade column chromatography in the normal way; (ii) the solution was reduced to dryness by vortex evaporation at room temperature and the dried material redissolved in ascorbic Krebs solution to give a final concentration of 0.18μM. This was again subjected to the normal column chromatography; (iii) the solution was further purified by alumina chromatography only. After

adsorption onto alumina, the ^{3}H.material (i.e., ^{3}H.DOPEG) was eluted with 2ml of 0.1M HCl.

The reason that (ii) and (iii) were undertaken was to devise a method of removing the acetic acid so that artery strips could be incubated in ^{3}H.DOPEG in Krebs solution. The results of (i) indicated that when the original fraction (A) was not further purified and reanalysed by column chromatography, 76% of the total ^{3}H recovered was in the 'DOPEG' fraction (termed B) and 9% appeared in the 'DOMA' fraction (mean of 4 values). When the ^{3}H.DOPEG solution (B) was treated again as in (i), again not all the ^{3}H was recovered in the 'DOPEG' fraction, since 9% still appeared in the 'DOMA' fraction. This result suggested that there was a consistent crossover of 9% of the ^{3}H.DOPEG into the 'DOMA' fraction, rather than that the ^{3}H.DOPEG was only 76% pure. In (ii) where the ^{3}H.DOPEG solution (A) was vortex evaporated to dryness the corresponding yield of ^{3}H in the 'DOPEG' and 'DOMA' fractions were 34% and 38% respectively (mean of 5 values). This result indicated that evaporation was associated with significant degradation of the ^{3}H.DOPEG and so this procedure was abandoned. In (iii) where the ^{3}H.DOPEG solution (A) was purified on alumina a second time, 88% of the applied material was recovered in the 'DOPEG' fraction and 8% in the 'DOMA' fraction. In two experiments where this material was assayed by column chromatography (i.e., a third adsorption onto alumina), 80 and 82% of the ^{3}H appeared in the 'DOPEG' fraction and a consistent 15% in the 'DOMA' fraction. This result implied that there was either a persistent crossover into the 'DOMA' fraction or some degradation of the ^{3}H.DOPEG during the four day period between the original preparation and purification of the ^{3}H.DOPEG sample and application onto alumina on the day of the experiment. But clearly the

Table 6.1

The accumulation and O-methylation of (-)^{3}H.DOPEG (0.18 μM) in rabbit ear artery strips. Values shown are means ± SEM.
* indicates significance ($p < 0.05$); unpaired t-test.

Treatment	n	Tissue DOPEG	MOPEG Formation nmol.g^{-1}.30min^{-1} medium	tissue	total	medium/tissue ratio
Untreated	14	0.12 ±0.01	0.39 ±0.03	0.05 ±0.00	0.04 ±0.03	7.8
Cocaine	10	0.10 ±0.01	0.35 ±0.02	0.05 ±0.00	0.40 ±0.02	7.0
Cocaine + hydrocort.	10	0.10 ±0.01	0.31 ±0.01	0.05 ±0.01	0.36 ±0.01 *	6.2
U0521	6	0.13 ±0.03	0.07 ±0.02	0.02 ±0.00	0.09 ±0.02 *	3.5

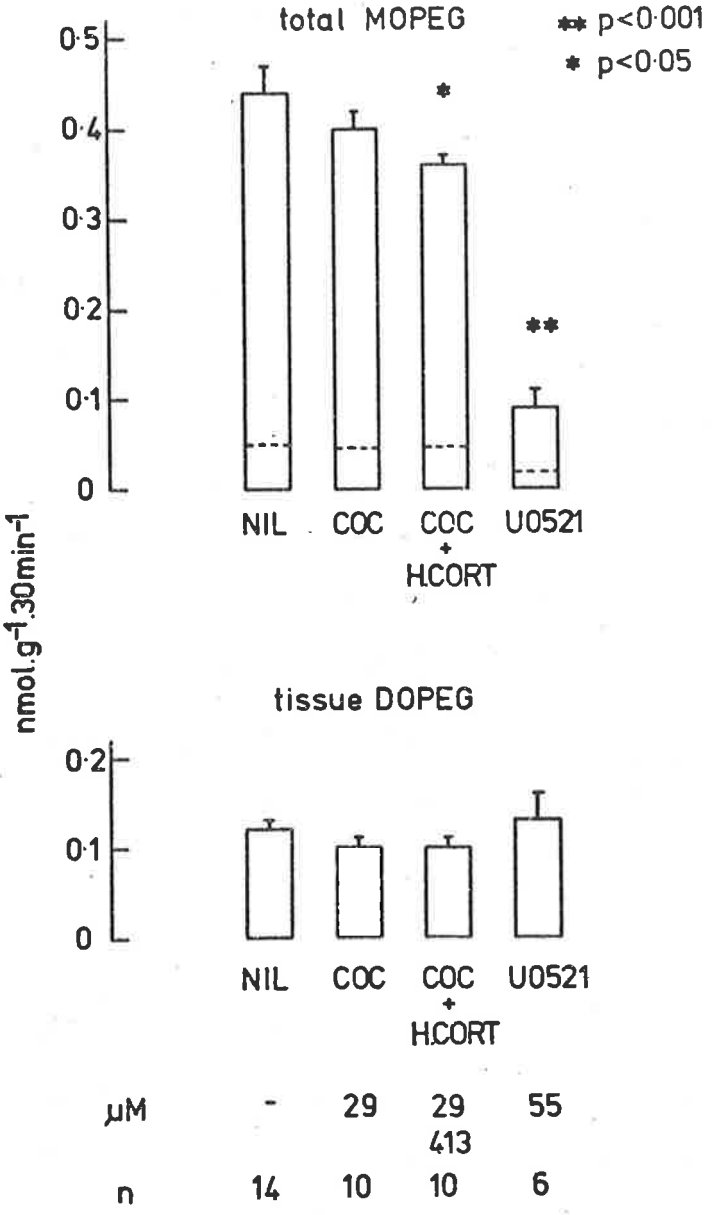

Fig. 6.1 The accumulation of ^{3}H.DOPEG (0.18 µM) and the formation of its O-methylated product (^{3}H.MOPEG) in nmol.g^{-1}.30min^{-1} in rabbit ear artery strips. The dashed line indicates the tissue content of ^{3}H.MOPEG at the end of the incubation period. Also shown are the effects of cocaine (29 µM), of cocaine plus hydrocortisone (413 µM) and of U0521 (55 µM).
* indicates significance ($p < 0.05$); unpaired t-test.
** " " ($p < 0.001$); " "

method outlined in (iii) was far superior to the evaporation method in (ii) as a means of removing the acetic acid and was therefore adopted in the present study.

Note:- In the light of subsequent experiments, the apparently high crossover of ^{3}H.DOPEG into the 'DOMA' fraction (9%) may have been related to the particular batch of alumina used at the time of these experiments. The crossover of unlabelled DOPEG into the 'DOMA' fraction with this batch of alumina was 4.5%, which was approximately twice the crossover observed with other batches of alumina used in other experiments before and after this series, and shown in Table 2.2 (Chapter 2). Further, that the material appearing in the 'DOMA' fraction was probably DOPEG was indicated by additional comparisons using the TLC method of Head et al (1976), which showed 86% of the ^{3}H appearing at the spot corresponding to DOPEG which was comparable with the 78% recovery quoted by Head et al for pure unlabelled DOPEG.

RESULTS

The results, summarised in Table 6.1 and also Fig. 6.1, show that the artery strips O-methylated ^{3}H.DOPEG at a rate of 0.44±0.03 nmol.g^{-1}. 30 min^{-1}. The major proportion (85-90%) of the O-methylated product effluxed into the incubating medium after its formation. The tissue did not accumulate unchanged ^{3}H.DOPEG to a significant extent. The content of ^{3}H.DOPEG remaining in the tissue at the end of the incubation was only 0.12±0.01 nmol.g^{-1}. 30 min^{-1}; i.e., 67% of the incubating concentration (0.18μM). This is close to this content if the DOPEG in the incubating medium were distributed mainly into the extracellular space, estimated by de la Lande et al (1980) to be 0.6ml.g^{-1}.

Table 6.2

A comparison of the rate of O-methylation of ^{3}H.DOPEG with that of ^{3}H.ISO and ^{3}H.NA under identical incubating conditions, in rabbit ear artery strips. Values shown are means ± SEM.

Substrate (μ M)	Treatment	O-methylated product	nmol.g^{-1}.30min^{-1}	n
^{3}H.DOPEG (0.18)	Nil	^{3}H.MOPEG	0.44 ±0.03	14
^{3}H.ISO (0.18)	Nil	^{3}H.MeOISO	1.66 ±0.15	4
^{3}H.NA (0.18)	Nil	^{3}H.NMN	0.51 ±0.04	4
^{3}H.NA (0.18)	Prazosin Ca^{++} free reserpine	^{3}H.NMN	0.49 ±0.04	21

The evidence that the product measured in the 'OMDA' fraction was O-methylated ^{3}H.DOPEG (i.e., ^{3}H.MOPEG) was indicated by the 80% reduction in its formation when an inhibitor of COMT (i.e., U0521, 55μM) was also present. However, this inhibition was not associated with an increase in the amount of unchanged ^{3}H.DOPEG in the tissue.

As shown in Fig. 6.1, neither cocaine (29μM) or hydrocortisone (413μM) effected O-methylation, or tissue retention, of ^{3}H.DOPEG to significant degrees. The only significant effect was a decrease in the amount of O-methylated DOPEG formed in the cocaine plus hydrocortisone treated vessels compared with untreated vessels. However, this reduction was small (only 18%).

The rate of O-methylation of ^{3}H.DOPEG is compared with that of ^{3}H.NA and ^{3}H.ISO in Table 6.2. The data on ^{3}H.NA and ^{3}H.ISO is taken both from the earlier studies in this thesis, and from other investigations (Head et al, 1980).

DISCUSSION

The potent inhibitory effect of U0521 on the formation of ^{3}H.material which was present in the 'OMDA' fraction suggests strongly that it is an O-methylated product of DOPEG formed by the activity of COMT. The medium to tissue ratio of the product (8:1) is comparable with that of MOPEG (10:1) and very much higher than that of VMA (0.8:1). The values for MOPEG and VMA are from de la Lande et al (1978). Hence there seems little doubt that the O-methylated product in the present experiments is MOPEG.

The relative insensitivity of MOPEG formation to cocaine and hydrocortisone implies that MOPEG formation from DOPEG does not involve neuronal uptake, or the corticosteroid-sensitive extraneuronal uptake system. In this respect the results add support to the proposals of Henseling et al (1978b) in the rabbit aorta and Fiebig and Trendelenburg (1978b) in

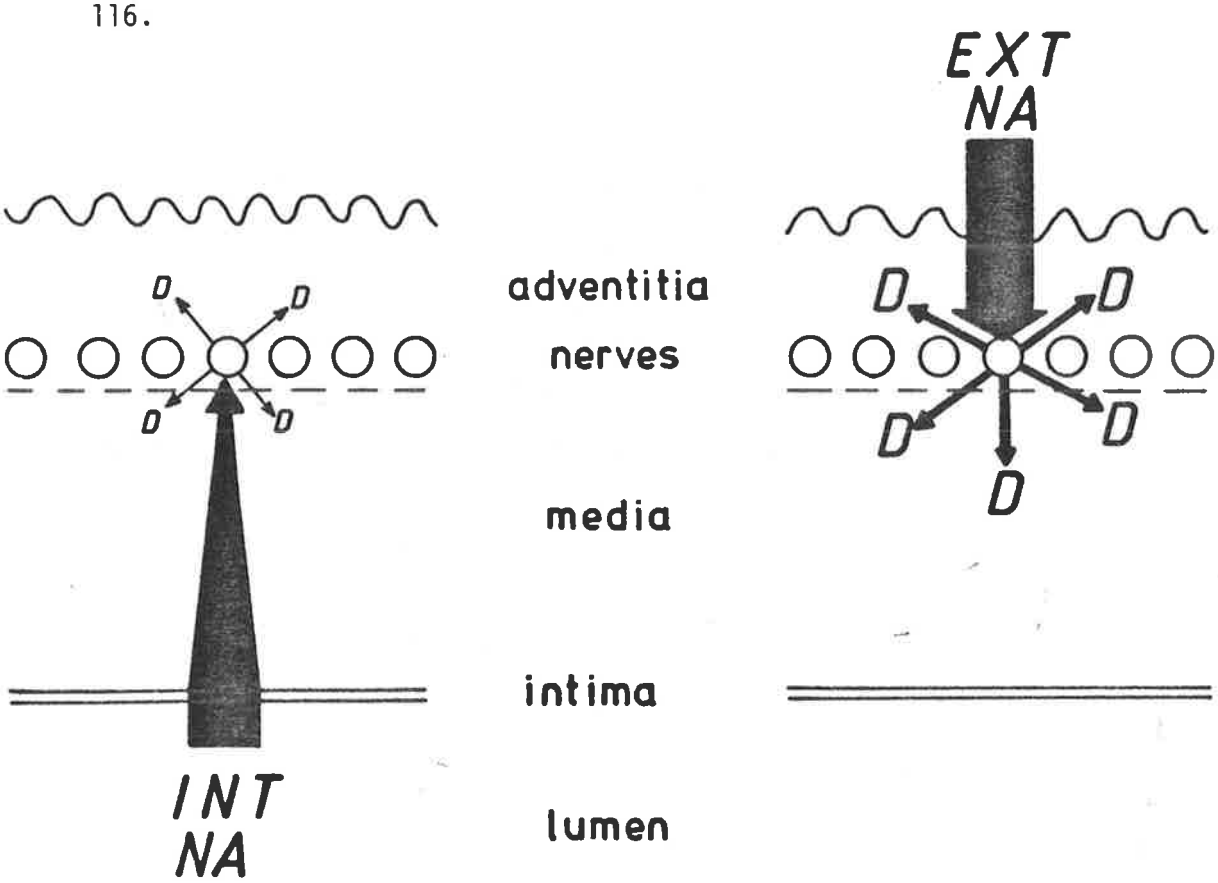

Fig. 6.2 A diagrammatic representation of the relative concentrations of NA entering the artery wall either via the intima (left hand panel) or via the adventitia (right hand panel) near the nerve terminals. This shows that the media, but not the adventitia, limits the penetration of NA to the nerves. The "D" stands for DOPEG and the arrows indicate its rate of formation; ie, the concentration of DOPEG is greater when NA enters the wall via the adventitia since the access of the substrate is not impeded by the presence of the media.

the rat heart, that DOPEG is capable of diffusing directly into an extraneuronal COMT compartment and thus bypassing the extraneuronal corticosteroid-sensitive uptake system. However, the data does not exclude the possibility that the extraneuronal compartment involved in O-methylating DOPEG may be quite distinct from that which O-methylated NA or ISO. Analysis of the effects of NA or ISO on DOPEG O-methylation would help to resolve this question.

It should be noted that a neuronal O-methylating compartment is excluded by the evidence that COMT activity is not present in the sympathetic nerves of the rabbit ear artery (Head et al, 1975; de la Lande et al, 1978; Head et al, 1976).

Irrespective of the nature of the extraneuronal compartment in which O-methylation occurred, the data does provide the first direct demonstration that, in the intact tissue, DOPEG is a substrate for COMT. It therefore helps to explain the sensitivity of OMDA formation to cocaine when NA enters via the adventitia (Chapter 4) since the formation of DOPEG by the tissue is approximately 4 fold greater when NA enters via the adventitia, than when it enters via the intima (Chapter 4, Tables 4.1 & 4.2). This is shown diagrammatically in Fig. 6.2.

The rate of O-methylation of DOPEG is comparable with that of NA under identical conditions, and also with that of NA in the prazosin-treated (Ca^{++}-free) reserpinised vessels, but is only about one quarter that of ISO.

It is interesting to speculate whether the rate of O-methylation of DOPEG is sufficient to account for the cocaine-sensitive component of OMDA formation. In preliminary experiments where ^{3}H.DOPEG (0.18μM) was applied to the adventitia of an untreated ear artery in normal Krebs solution, it was found that 1.14 $nmol.g^{-1}.30\ min^{-1}$ diffused into the

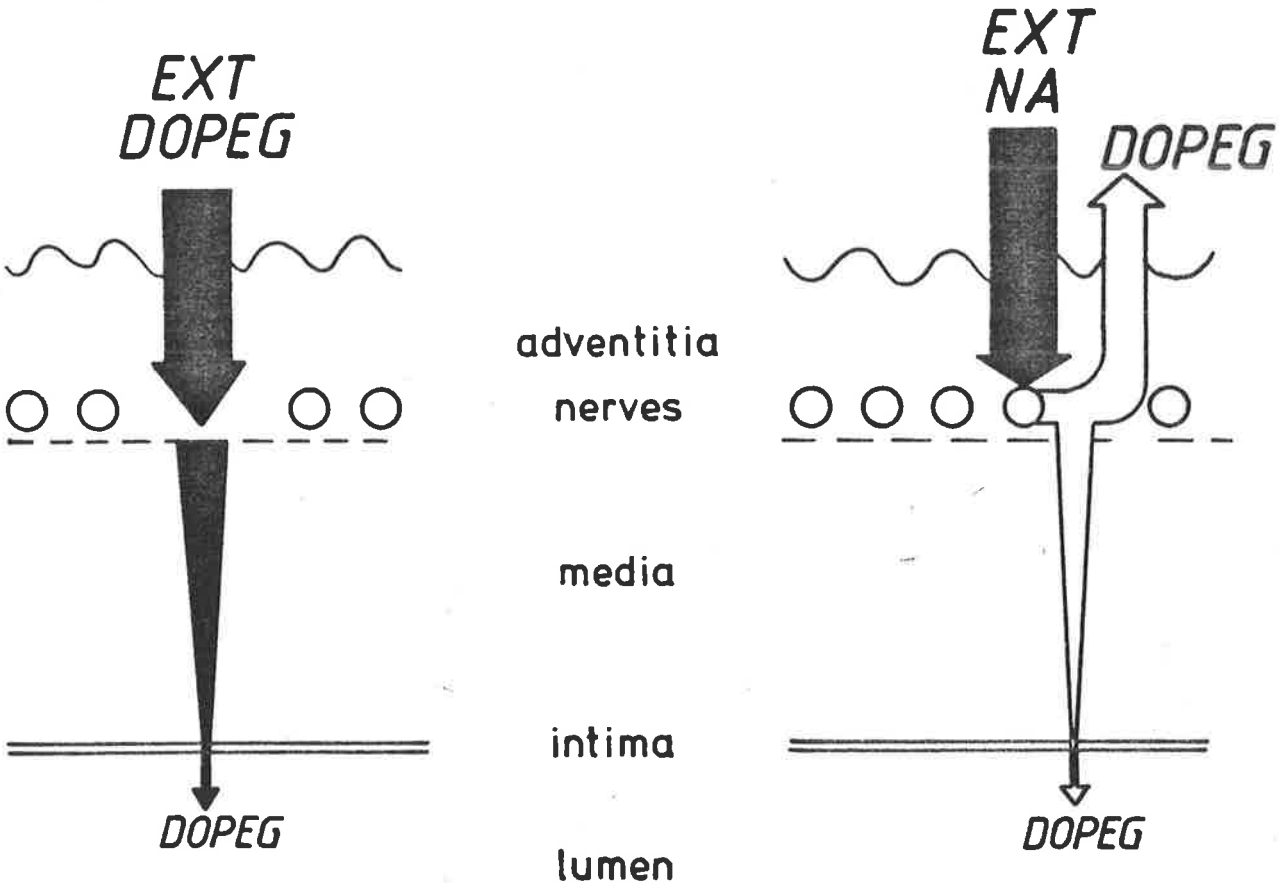

Fig. 6.3 Shows how an estimate was made of the concentration of DOPEG in the region of the nerve terminals, where NA enters via the adventitial surface of the artery. The left-hand panel shows ^{3}H.DOPEG applied to the adventitial surface, diffusing freely through the adventitia, but not the media. The amount of this DOPEG which reached the lumen in 30 minutes was used as an indication of the relative concentration of ^{3}H.DOPEG, formed from NA entering via the adventitia, in the region of the nerves (as shown in the right-hand panel).

Table 6.3 Cocaine-sensitive OMDA

The effect of cocaine (29 μM) on the efflux of O-methylated-deaminated metabolites in four different preparations, incubated with (-)^{3}H.NA (0.18μM). Values shown are means ± SEM.
* indicates significance ($p < 0.05$); unpaired t-test.

Preparation	Incubating solution	OMDA Efflux nmol.g^{-1}.30min^{-1} Untreated	Cocaine	difference	n
Perfused segment	EXT	0.55 ±0.07	0.36 ±0.05	0.19	11, 3
	INT	0.47 ±0.05	0.37 ±0.07	0.10	11, 5
	INT + EXT	1.16 ±0.03	0.58 ±0.05 *	0.58	4, 4
Artery strip	INT + EXT	0.71 ±0.06	0.33 ±0.09 *	0.38	21, 7

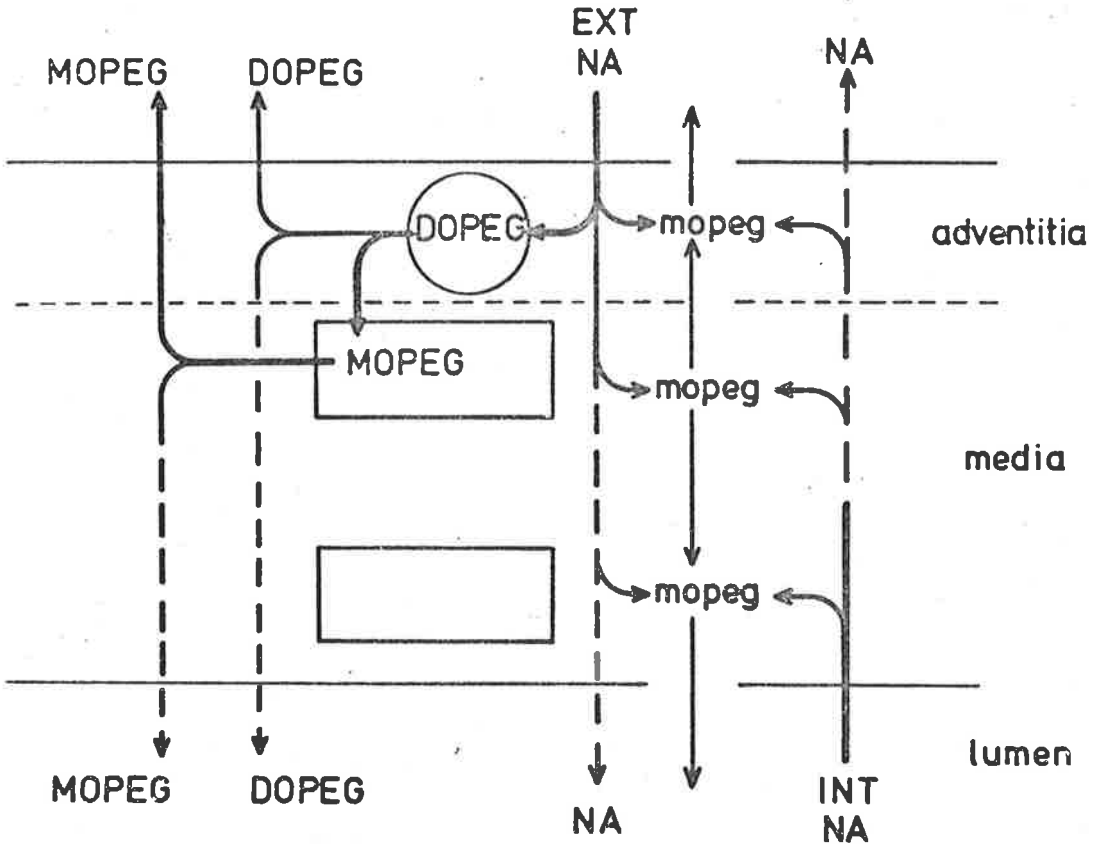

Fig. 6.4 This shows two possible mechanisms for the formation of MOPEG in the artery wall. The first (suggested by the present study) is DOPEG effluxing from the nerves entering nearby effector cells where it is O-methylated to MOPEG, which effluxes primarily via the adventitia. This mechanism predominates in the outer regions of the wall when the concentration of DOPEG is high (such as occurs when NA is applied to the adventitia), is sensitive to cocaine (which inhibits DOPEG formation) but insensitive to hydrocortisone (presumably because the highly lipid soluable DOPEG can diffuse directly into the effector cell). The second mechanism (with little experimental evidence to indicate its origin) operates throughout the wall, is insensitive to cocaine or hydrocortisone but sensitive to PBZ, and predominates in the inner region of the wall regardless of the surface of application of NA.

opposite (i.e., INT) solution. If it is assumed that this flux is directly proportional to the concentration in the EXT solution, it can be estimated from the standard diffusion equation that the flux of DOPEG into the INT solution (0.5 $nmol.g^{-1}.30\ min^{-1}$) during incubation with EXT $^{3}H.NA$ (0.18μM) diffused from a site (i.e., a narrow zone in the region of the nerves) where the mean concentration of DOPEG was $\frac{0.5}{1.14} \times 0.18$, i.e. 0.10μM (Fig. 6.3). Assuming approximate linearity between MOPEG formation and DOPEG concentration, the rate of MOPEG formation from DOPEG (0.10μM) would be $\frac{0.10}{0.18} \times 0.44$, i.e. $0.24\ nmol.g^{-1}\ 30\ min^{-1}$. This is close to the cocaine-sensitive component of OMDA formation from EXT $^{3}H.NA$ of $0.19\ nmol.g^{-1}.30\ min^{-1}$ (Table 6.3). This agreement adds further support to the argument that O-methylation of DOPEG is an important metabolic pathway in the rabbit ear artery.

As indicated in Chapter 4, there is a significant component of OMDA formation which is insensitive to cocaine. This component appears to represent the major pathway of formation when NA enters via the intima, but is still evident when NA enters via the adventitia. The insensitivity to cocaine excludes the possibility that it involves O-methylation of DOPEG after the latter is released from sympathetic nerves. Other than its sensitivity to PBZ, the mechanism remains unknown. Fig. 6.4 diagrammatically incorporates the various pathways of OMDA formation (illustrated by MOPEG only) in relation to the morphology of the vessel, as suggested by the results in this chapter and in Chapter 4.

CHAPTER 7

DIFFUSION OF NA ACROSS THE ARTERY WALL, STUDIED BY THE TECHNIQUE OF OIL IMMERSION

CHAPTER 7.

DIFFUSION OF NA ACROSS THE ARTERY WALL, STUDIED BY THE TECHNIQUE OF OIL IMMERSION

INTRODUCTION

The study in this chapter was intended to assess pharmacologically, whether extraneuronal inactivation of NA, entering via the intimal surface, can influence the steepness of the gradient of concentration of the amine across the wall of the rabbit ear artery.

The technique of oil immersion has been applied to the perfused artery in the expectation that, when the external bathing medium was oil, a uniform gradient of NA would exist across the artery wall (i.e., no concentration gradient) when the constrictor response to the INT NA (in aqueous medium) reached steady-state. This technique of immersing only one surface in oil must be distinguished from that of total immersion of tissues in oil. The latter technique was first used by Kalsner and Nickerson (1969) to study the kinetics of inactivation of biogenic amines in strips of rabbit aorta. The rate of recovery of the preparation in oil, from the previous vasoconstrictor response applied in aqueous media, was used as a measure of the rate of removal of the amine from the receptor biophase by uptake, binding and metabolism.

The present technique of exposing only one surface to oil permits the amine to be applied to one surface in aqueous media while the opposite surface was bathed in oil and hence is analogous to the technique of applying a silicone grease coating to one surface of rabbit aortic strips as used by Pascual and Bevan (1979). The reason for restricting the efflux of NA into the EXT solution was to ensure a

more uniform concentration of the amine in the wall and to establish whether this was associated with a greater constrictor response. It was reasoned that the increased response would be due primarily to a greater recruitment of smooth muscle cells in the outer region of the media, reflecting the increase in concentration in this region. If the inactivation of INT NA normally limited penetration into this region, then inhibition of inactivation should augment the increase in response to INT NA produced by the oil. In the present study, the role of corticosteroid-sensitive extraneuronal uptake and O-methylation was studied in this fashion. Observations have also been made on the possible influence of neuronal uptake on the concentration of NA within the media.

METHODS

(1) Perfusion system.

Untreated rabbit ear artery segments were prepared as described by de la Lande and Rand (1965). The principal of the method is that artery segments were cannulated at both ends, set up in organ baths under 1g tension and bathed in normal Krebs solution bubbled with 5% CO_2 in O_2 at 37°C. The intraluminal (INT) surface was perfused with a constant flow peristaltic pump at 2.0 $ml.min^{-1}$. Hence, the solution bathing the extraluminal (EXT) surface did not mix with the INT perfusate. Constrictor responses to NA applied to either surface were measured as an increase in perfusion pressure by a Statham pressure transducer lineated between the pump and the tissue, and recorded on Rikidenki double channel pen recorder.

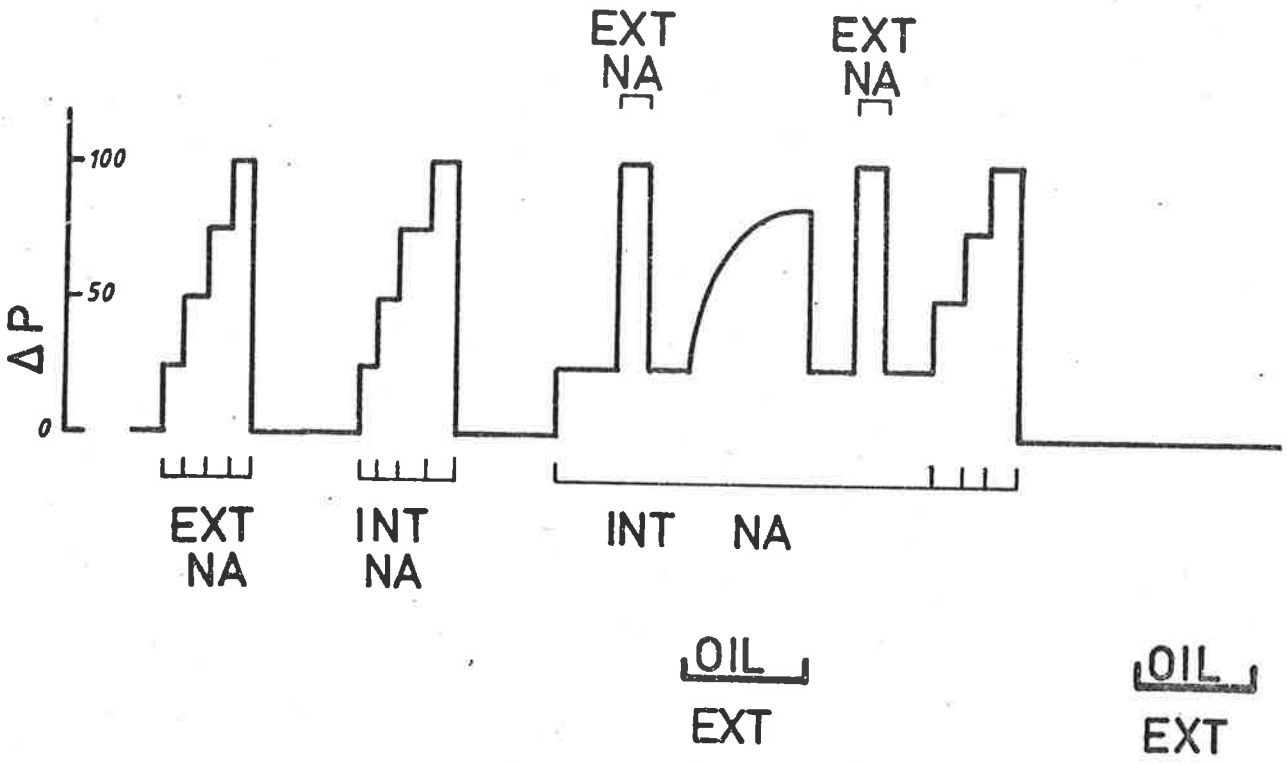

Fig. 7.1 A diagrammatic representation of the experimental protocol, showing cumulative dose-response curves to extraluminal (EXT) and to intraluminal (INT) NA. This was followed by a further dose of INT NA which would give a small response (less than 50 mmHg) which was maintained during responses to the same concentration of EXT NA applied before and after the EXT medium was replaced with oil. Finally the small INT NA response was increased until a response of equal or greater magnitude than that to EXT NA was obtained. When all NA was washed out of the system, the EXT medium was again replaced with oil.
ΔP = increase in perfusion pressure (mm Hg).

(2) Experimental.

Cumulative dose-response curves were first obtained to both INT and to EXT NA. The subsequent procedures are shown diagrammatically in Fig. 7.1. When a steady-state response to a concentration of INT NA (chosen from the initial dose-response curve so as to give a perfusion pressure of 20-40 mmHg) was obtained (Table 7.1), the NA-free EXT solution was replaced with the same solution (containing NA) as was perfusing the INT surface.

Table 7.1.

Concentration of INT NA required to increase perfusion pressure (20-40 mmHg) (means ± SEM)

Treatment	INT NA Concentration (μM)	n
UNTREATED	0.28 ± 0.11	5
COCAINE	0.09 ± 0.01	9
COC + DOCA	0.05 ± 0.01	13

Under these conditions the concentration of NA at either surface of the artery was identical. The EXT NA was washed out after the new steady-state response was obtained. The EXT aqueous solution was then replaced with paraffin oil (bubbled with 95% O_2 - 5% CO_2 and at 37°C). The oil was allowed to remain until another steady-state response was obtained. The oil was then replaced with fresh Krebs solution. When the preparation had recovered, another response was obtained to the EXT NA, in the same concentration as used previously. The EXT NA was then replaced with fresh NA-free Krebs solution. The INT NA concentration was then progressively increased until a response approximately equal to that seen during the application of EXT NA was

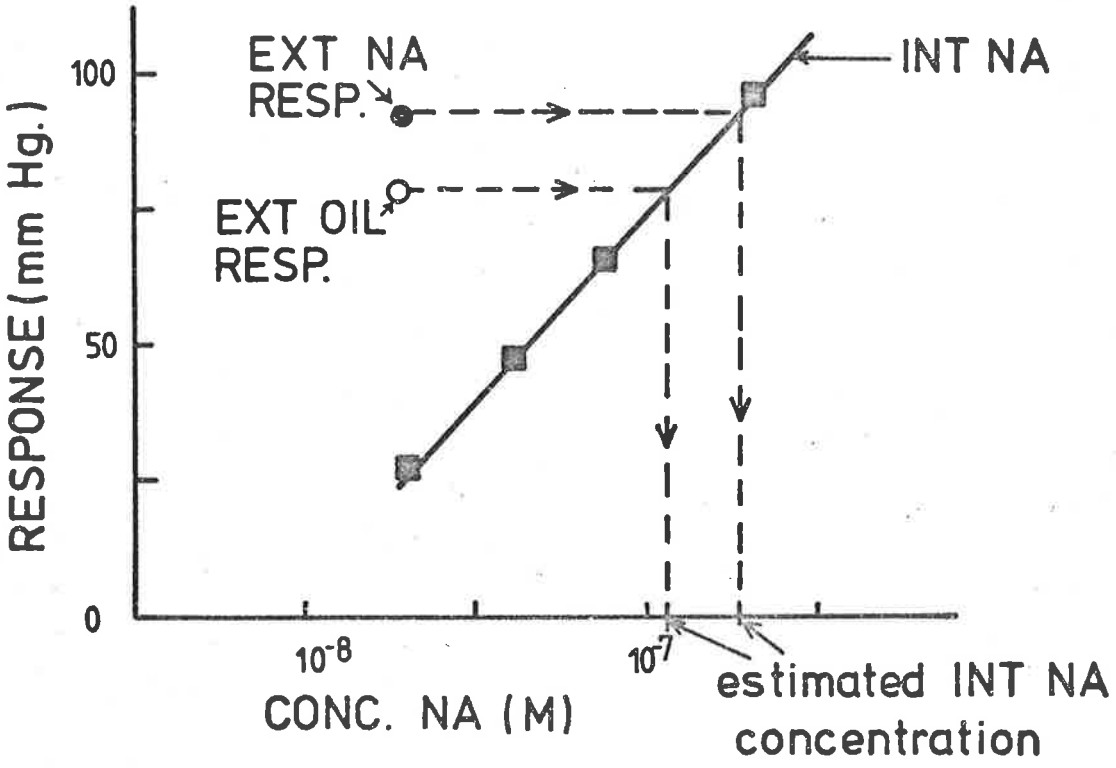

Fig. 7.2 This shows the dose-response curve to intraluminal (INT) NA (solid line). The dashed lines indicate how the concentration of INT NA required to produce the same response was estimated either where the same concentration of NA was added to the adventitial surface, or where the EXT aqueous medium was replaced with paraffin oil, both during steady-state responses to INT NA.

obtained. This protocol was repeated in the presence of cocaine (29μM) and cocaine plus DOCA (13μM). In most experiments the effect of EXT oil alone (i.e., without NA) was examined to test the possibility that paraffin oil may have intrinsic pharmacological action.

The increases in sensitivity produced by the EXT NA, and the oil, were expressed in terms of the increases in concentration of INT NA producing the same responses as EXT NA, and oil. (Fig. 7.2). This assumes that the dose-response curve to EXT NA, and to EXT oil, parallel those to INT NA.

RESULTS

In five untreated arteries, the addition of EXT NA during the steady-state response to INT NA produced a further constriction which was sustained during the period of application (2-3 minutes). This response was equivalent to an increase in sensitivity to INT NA of 1.8 fold (Table 7.2). Replacing the EXT aqueous medium with oil produced a much smaller increment in the response than did EXT NA; the increased response was not sustained over a 3-5 minute period in 4 of the 5 vessels examined. Based on the peak of the transient response only, the oil increased the sensitivity to INT NA by a factor of approximately 1.4. The theoretical significance of the latter estimate is dubious since it is not based on comparisons of steady state responses; nevertheless it does show that even at its maximum, the response to oil was small compared with that to EXT NA.

Table 7.2.

Potentiation of INT NA response by EXT treatment with either the same concentration of NA or paraffin oil. (Geometric means ± SEM)*

Treatment	EXT NA	EXT OIL	n
UNTREATED	1.84 ± 0.26	1.44 ± 0.11	5
COCAINE	3.44 ± 0.31	1.46 ± 0.16	9
COC + DOCA	2.38 ± 0.26	1.68 ± 0.17	13

* values were estimated as shown in Fig. 7.2.

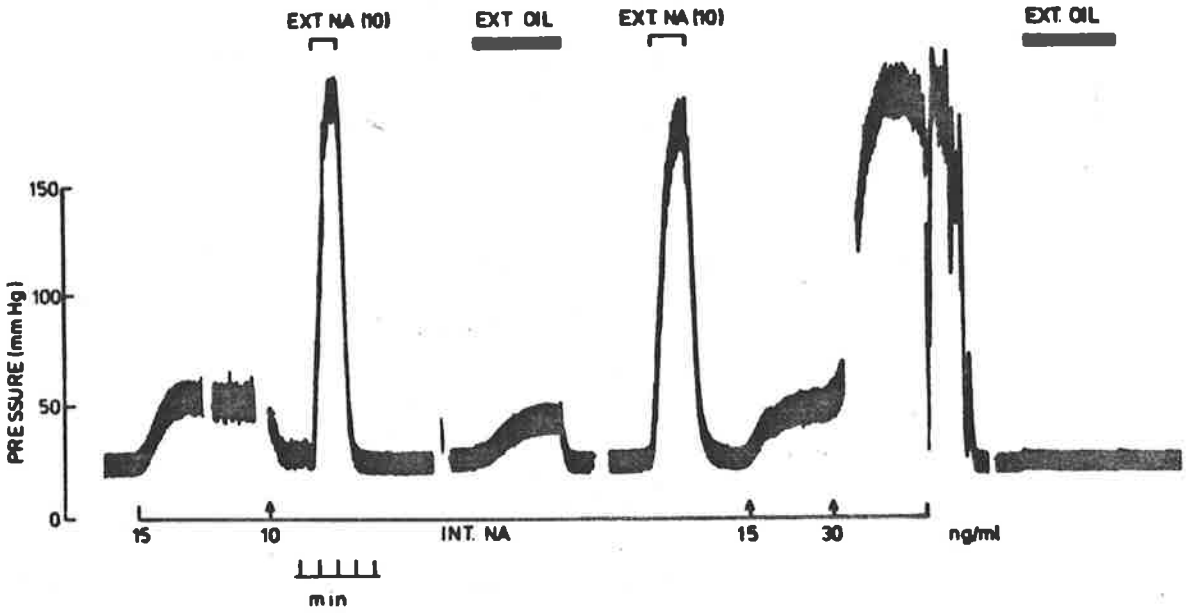

Fig. 7.3 This shows a typical trace with the effects of EXT oil and of EXT NA on the responses of the perfused rabbit ear artery to INT NA. Arrows indicate the periods of application of INT NA.

In 9 cocaine (29μM) treated arteries the effects of EXT NA, and of EXT oil, were qualitatively similar to their effects in untreated vessels. Thus, in contrast to the sustained response to EXT NA in each of the preparations, only one of the vessels showed a sustained response to oil. In the cocaine-treated vessels, the equivalent increase in sensitivity to EXT NA was 3.4-fold; i.e., approximately double that seen in the untreated preparation. As in the case of the untreated vessel, the effect of EXT oil, based on the initial peak response only, was equivalent to an increase of 1.5 fold to INT NA. Hence, cocaine treatment augmented the effect of EXT NA, but did not lead to a more pronounced response to the oil.

In contrast, in the presence of DOCA and cocaine (Fig. 7.3) the response to oil was well sustained in 13 of the 16 segments examined. Kinetically, the response to EXT NA and to EXT oil during the steady-state response to INT NA differed markedly. The response to EXT NA achieved steady-state rapidly (within 30 seconds), whereas the response to oil was slower in onset, taking 3-4 minutes to plateau. Quantitatively, the increase in sensitivity produced by EXT oil was on average only 70% of that produced by EXT NA.

DISCUSSION

The observation that, during the response to INT NA, the cocaine-treated rabbit ear artery constricted further when NA was applied to the EXT surface confirms the earlier finding of Kalsner (1972). The simplest explanation is that proposed by Kalsner, namely, that the concentration which INT NA achieves in the outer region of the smooth muscle is less than that in the inner region, i.e., close to the surface of application of NA. The fact that the effect of EXT NA was greater

in the presence than in the absence of cocaine suggests that neuronal uptake may have been one of the factors responsible for the lower concentration of INT NA in the outer region of the media. However, a more likely explanation is that cocaine, by inhibiting neuronal uptake, permitted EXT NA to achieve a higher concentration in the underlying (i.e., outer) region of the media. In the presence of cocaine and DOCA, the effect of EXT NA was not further augmented, but tended to be less than when cocaine alone was present. Although the concentration of DOCA used (13μM) was less than that previously shown to cause profound inhibition of extraneuronal uptake (Johnson and de la Lande, 1978), it was nevertheless the concentration which produced near maximum potentiation of the responses to catecholamines in the study of Johnson and de la Lande (1978). Enhanced sensitivity to INT NA (approximately 2-fold) was also apparent in the present study when the concentration of INT NA which produced equivalent constriction in cocaine alone, and cocaine plus DOCA preparations are compared (Table 7.1). Hence the failure of DOCA to further augment the effect of EXT NA when the latter was added during the response to INT NA implies that extraneuronal uptake in the media was probably not the major factor responsible for the decreasing concentration of INT NA in the outer media. However, this conclusion does not take into account the effects of oil.

Oil was used to prevent NA from escaping from the adventitial surface. Under conditions where NA is not inactivated in the vessel wall, and NA cannot escape from the adventitia, INT NA should attain the same concentration throughout the artery wall.

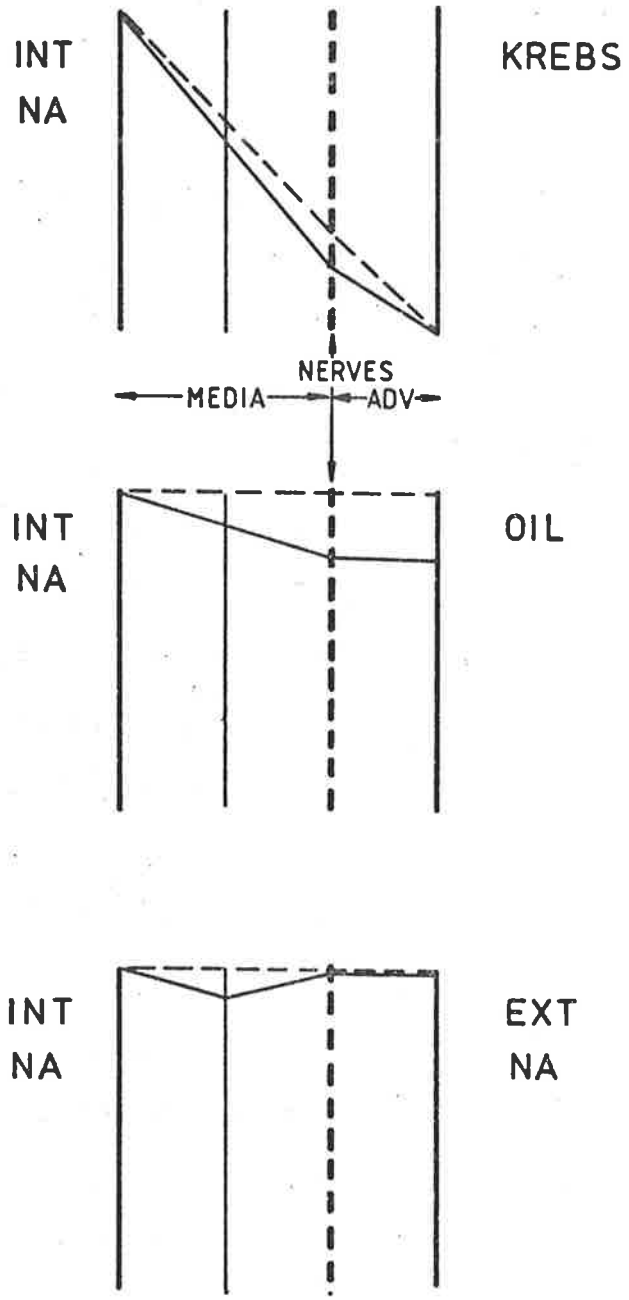

Fig. 7.4 A diagrammatic representation showing the effect of extraneuronal uptake in the media on the gradient of concentration of INT NA when extraneuronal uptake is active (solid line) and when it is blocked by DOCA (13 μM) (dashed line). The top panel shows normal Krebs solution bathing the adventitia, the middle panel shows the EXT aqueous medium replaced with paraffin oil and the lower panel where the same concentration of NA is applied to the adventitia.

The relevant finding here is that an augmented steady-state response to INT NA was produced by oil only when DOCA was present. This is precisely what would be expected if extraneuronal uptake was a significant factor which prevented INT NA from achieving a uniform concentration throughout the wall.

Hence, the conclusions drawn from the effects of EXT NA, and of oil, on sensitivity to INT NA appear to be at variance with each other; the former effects implying that extraneuronal uptake does not contribute to the decline in concentration of INT NA as it diffuses across the wall, while the effects of oil imply that extraneuronal uptake does have a major influence. A partial explanation is illustrated diagrammatically in Fig. 7.4. It shows that in theory, the influence of extraneuronal uptake on the concentration of NA within the media (treated as a plane sheet) will be twice as great when NA enters from one surface under conditions where it cannot escape from the opposite surface, than when NA enters from both surfaces simultaneously. This factor may contribute to the different effects of DOCA on the sensitivity increases produced by EXT NA and oil.

Other evidence which suggests that the effects of oil reflect a role of extraneuronal uptake stems from the kinetics of the response. In the DOCA treated preparation, the response to oil was characterised by a slow onset ($t_{\frac{1}{2}}$ = 2-3 min.) and a rapid offset (Fig. 7.3). The slower onset accords with the time required for the INT NA to diffuse throughout the outer regions of the muscle mass; the fast offset time is explained by the fact that once the oil is replaced by Krebs, the NA in the outer region can now diffuse freely from the nearby adventitial surface. Presumably the more rapid onset of the response to EXT NA is

because the amine has only to transverse the adventitia, in which it is freely diffusible, to reach the outer smooth muscle layers of the media.

If the above arguments are accepted, then the relatively smaller effect of oil, than of EXT NA, on the sensitivity to INT NA can be explained in one of two ways; either that extraneuronal uptake was not completely blocked by DOCA, or that there are other NA inactivating processes in the media which are not sensitive to DOCA. The latter possibility is supported by the evidence in Chapter 4, where it was shown that the formation of O-methylated-deaminated metabolites of NA were insensitive to another corticosteroid (hydrocortisone) and that this lack of sensitivity was particularly evident when NA entered via the intimal surface.

There remain a number of puzzling features of the data for which there is not an obvious explanation. The main one is (as previously indicated) the tendency for the effects of EXT NA to be less in the presence of cocaine plus DOCA than in the presence of cocaine alone.

CHAPTER 8

UPTAKE AND METABOLISM OF CATECHOLAMINES IN THE NORMOTENSIVE AND DOCA-SALT HYPERTENSIVE RAT TAIL ARTERY AND LEFT ATRIUM

CHAPTER 8

UPTAKE AND METABOLISM OF CATECHOLAMINES IN THE NORMOTENSIVE AND DOCA-SALT HYPERTENSIVE RAT TAIL ARTERY AND LEFT ATRIUM

INTRODUCTION

Early investigations in the author's laboratory were originally aimed at examining the inactivation of NA both in normotensive and hypertensive rabbit ear arteries. However, considerable difficulty was experienced in the developing of a reproducible hypertensive model in rabbits without excessive wastage of animals (Johnson, 1975). For this reason the rat was chosen instead since hypertension can be readily induced by a variety of techniques. The model chosen was that of DOCA-salt treatment in the nephrectomised rat since it offered an opportunity to assess, not only the relationship between inactivation of NA and hypertension, but also the effects of chronic treatment with an inhibitor of extraneuronal uptake. Pharmacological and biochemical evidence that DOCA inhibited extraneuronal uptake in the rabbit ear artery was presented by Head et al (1975). The possibility that the inhibitory action of DOCA on extraneuronal uptake might contribute to the hypertensive response in DOCA-salt treated rats does not appear to have been previously examined. The only relevant evidence which could be found in the extensive literature on this model was in the study of de Champlain in 1967. His data indicated that the isolated hearts of DOCA-salt hypertensive rats, when perfused with $(\pm)^{3}$H.NA, did not retain as much ^{3}H.NMN (a product of extraneuronal COMT activity) as did the hearts of the normotensive controls.

The main vessel selected for the present study was the rat tail artery. This was shown by Hodge and Robinson (1972) to possess a dense sympathetic innervation which was confined to the border of the media and the adventitia (as in the rabbit ear artery). Since the rat tail artery is a smaller, thinner walled vessel than the rabbit ear artery, it is technically more difficult to cannulate and perfuse. However, Venning and de la Lande (1981) demonstrated that it was possible to examine this vessel pharmacologically in a similar way to the rabbit ear artery described by de la Lande and Rand (1965). The former workers showed that the rat tail artery closely resembles the rabbit ear artery with respect to its sensitivity to intraluminally and extraluminally applied catecholamines and the relative influences of neuronal uptake on these sensitivities. Furthermore, these workers showed that the vessels from the hypertensive rats were 3-5 fold more sensitive to NA than vessels from normotensive controls. Their results, together with those of Wyse (1976), using the technique of oil immersion, had indicated that the neuronal uptake had exerted a much greater influence on the pharmacological response to NA than did extraneuronal uptake. However, the metabolic pathways of NA inactivation had not been examined by the more direct procedure of measuring the accumulation of unchanged amine and the formation of the metabolites when the tissue was incubated with ^{3}H.NA.

The experimental investigation in this chapter has, as its primary aim, the analysis of the metabolism of catecholamines in the rat tail artery. Wherever practical, comparative studies were carried out on tail arteries from DOCA-salt hypertensive rats at a time of treatment when hypertension was well developed, i.e., after three weeks of treatment (unless specified otherwise in the text).

As mentioned in the General Introduction (Chapter 1) it was hoped to extend this study to an analysis of the metabolism in perfused segments of tail arteries at various stages of the development of hypertension. However, since many of the studies in the perfused rabbit ear artery had to be repeated, due to the supply of "falsely labelled" (-)^{3}H.NA, indicated in the report of Starke et al (1980), time did not allow this phase of the study to be undertaken. Hence the present study refers only to the metabolism of NA, and of ISO, in non-perfused segments of the rat tail artery.

In view of the evidence of de Champlain et al (1967), described above, comparisons were also made with heart tissue (the isolated left atrium was chosen) of hypertensive and normotensive rats.

METHODS

(1) Incubation studies.

Incubation studies were carried out as described in the General Methods. The principal of the method was as follows.

Rats were killed by a blow on the head and bleeding. Tissues were rapidly removed and placed in vials containing normal Krebs solution and bubbled with 95% O_2 and 5% CO_2 at 37^{o}C. Test drugs were added 30 minutes prior to incubation with (-)^{3}H.NA or (±)^{3}H.ISO for a further 30 minutes. At the end of the incubation tissues were washed for 5 seconds in 2ml of ^{3}H-free Krebs and extracted overnight in 0.4M $HClO_4$ (containing 3mM EDTA and 10mM Na_2SO_3) at 4^{o}C. The incubation medium was acidified with 0.2ml of 0.1M HCl and 0.02ml of 0.6M ascorbic acid immediately after the tissue had been removed at the end of the incubation period, and stored at 0^{o}C before being assayed. The ^{3}H content of 0.1ml aliquots of each of the acidified incubating medium and tissue acid extract was determined before fractionating the unchanged ^{3}H.NA and ^{3}H metabolites by the cascade column

chromatographic method as described in the General Methods (Chapter 2). In studies using ^{3}H.ISO, the incubation procedure was identical; however, the column separation was abbreviated, as described in the General Methods, since only one metabolite separation was required (^{3}H.MeOISO appearing in fraction 2). In some early experiments ^{3}H.ISO and ^{3}H.MeOISO were separated by the TLC method of Head et al (1976), this also allowed comparisons between the two chromatographic methods.

(2) DOCA-salt model.

Porton derived male rats (70-100g) were unilaterally nephrectomised on the left side under pentobarbital anaesthesia (50mg.Kg^{-1}) and, after a one week recovery period, injected subcutaneously twice weekly with DOCA (20mg.Kg^{-1}) and allowed normal saline to drink ad libitum. Litter mates were sham operated, injected in the same manner with the vehicle (benzyl alcohol: peanut oil, 1:20) and allowed tap water to drink. The group receiving DOCA-alone were injected with the same regimen as above, without having a kidney removed or saline to drink. Indirect blood pressure estimations were taken using a tail cuff coupled with a sphygmomanometer, and a doppler flow probe coupled with a DC amplifier and headphone set. This procedure was similar to that described by Reichle (1971). Measurements were taken once or twice weekly prior to and during the treatment period. Rats were familiarised with the restraint cages prior to the commencement of indirect blood pressure determinations.

(3) Endogenous Catecholamine assay.

The endogenous NA, adrenaline (A) and dopamine (DA) contents of perchoric acid (0.15M) extracts of tissues were determined by the perchloric radioenzymatic assay of Da Prada and Zürcher (1976), as modified by Crabb et al (1980). The method is described in detail in the General Methods (Chapter 2). The procedure followed was that tissues were rapidly removed

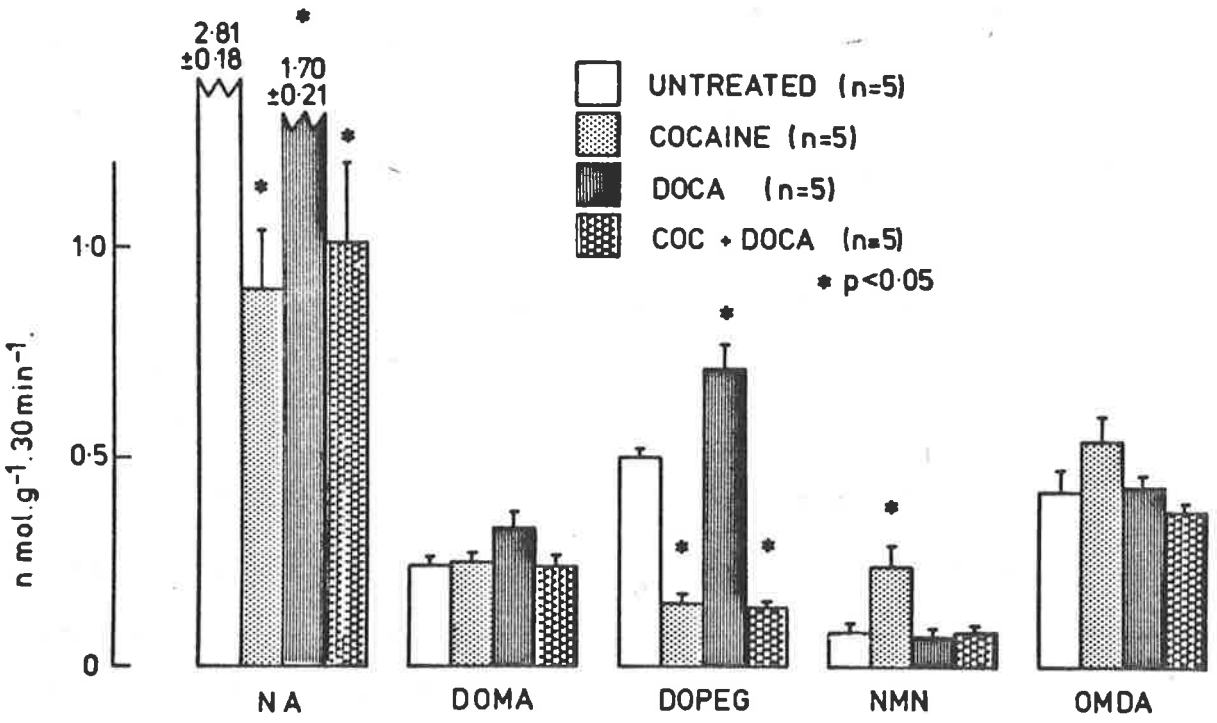

Fig. 8.1 This shows the uptake and metabolism of 7-C labelled (-)^{3}H.NA (0.18 μM) in the rat tail artery in nmol.g^{-1}.30min^{-1}. Also shown are the effects of cocaine (29 μ M), DOCA (27 μ M), and cocaine plus DOCA on ^{3}H.NA uptake and metabolite formation.

* indicates significance ($p<0.05$); unpaired t-test.

from the rats and held at 4°C. Tissues were blotted and weighed before the catecholamines were extracted in 0.15M $HClO_4$ at 4°C. The principal of this assay involves the O-methylation of the catecholamines by COMT with ^{3}H.S-adenosylmethionine providing the ^{3}H-labelled methyl group in a favourable incubating environment. The remainder of the assay involves a series of solvent and aqueous extractions, and a purification step, before the three ^{3}H.O-methylated products are separated by thin layer chromatography (TLC), and the tritium content of the regions corresponding to unlabelled carriers determined by liquid scintillation spectrometry. The levels of the catecholamines of the unknown samples are then determined by comparison with their respective standard curves constructed by assaying, in parallel, samples with known amounts of the three catecholamines. The assay was verified by the recovery, in parallel samples, of known amounts of commercially aquired ^{3}H.metabolites (^{3}H.normetanephrine, ^{3}H.metanephrine and ^{3}H.methoxytyramine). Results were then expressed as arithmetic means (± the standard error of the mean) in nmol.g^{-1} wet weight. The sensitivity of the assay was greater than required to detect the endogenous catecholamine contents of tissues; in fact the acid extracts were normally diluted (1:5 or 1:10) to coincide their levels with the most linear part of the standard curves (i.e. less than 12pmol.ml^{-1}).

RESULTS

(1) Normotensive tissues.

(a) Noradrenaline. As shown in Fig. 8.1, the metabolism of (-)^{3}H.NA (0.18μM) in the rat tail artery was characterised by a 15-fold accumulation of unchanged ^{3}H.NA and the formation of ^{3}H.DOPEG as the major metabolite (40%), followed by ^{3}H.OMDA (34%), ^{3}H.DOMA (19%) and ^{3}H.NMN (6%). Of the ^{3}H.NA removed from the tissue, approximately two thirds accumulated unchanged and one third metabolised. The ratio of individual metabolites

Table 8.1

A comparison of two chromatographic techniques for separating catecholamine metabolites

(a) Tail artery incubated with (-)^{3}H.NA (0.36 μM) (incubating medium only)

Method	n	DOMA	DOPEG	NMN	VMA	MOPEG
COLUMN	3	0.33 ±0.08	1.52 ±0.25	0.18 ±0.09	0.87 ±0.06	
TLC	3	-	1.50 ±0.15	0.13 ±0.08	0.64 ±0.31	0.09 ±0.00

(b) Tail artery incubated with (±)^{3}H.ISO (0.81 μM).

Method	n	Tissue ^{3}H.ISO	Medium ^{3}H.MeOISO	Tissue ^{3}H.MeOISO
COLUMN	10	1.86 ±0.17	4.49 ±0.35	0.59 ±0.02
TLC	10	1.83 ±0.15	3.90 ±1.22	0.72 ±0.08

Values shown are means ± SEM in nmol.g^{-1}.30 min^{-1}.

appearing in the incubating medium to those retained by the tissue was lowest in the case of ^{3}H.DOPEG (17%) and highest in the case of ^{3}H.NMN (60%). A similar pattern of ^{3}H.NA uptake and ^{3}H.metabolite formation was apparent when the incubating medium was assayed by both column and thin layer chromatographic methods (Table 8.1). This comparison also indicated that ^{3}H.VMA was the major component of the ^{3}H.OMDA fraction.

Inhibition of neuronal uptake by cocaine (29μM) (Fig. 8.1) was associated with, (i) a decrease in both the accumulation of ^{3}H.NA and the formation of ^{3}H.DOPEG (each by approximately 70%), (ii) no significant effect on the ^{3}H.OMDA or ^{3}H.DOMA formation, and (iii) a three-fold increase in ^{3}H.NMN formation. In the absence of cocaine,inhibition of extraneuronal uptake by DOCA (27μM), (i) significantly decreased the accumulation of ^{3}H.NA by 40%, (ii) did not affect ^{3}H.NMN formation, and (iii) significantly increased the formation of ^{3}H.DOPEG by 40%. In the presence of cocaine, DOCA significantly decreased the accelerated rate of ^{3}H.NMN formation and ^{3}H.OMDA formation when compared with arteries treated with cocaine alone, to a level similar to that seen in the untreated vessels. Similarly, in the presence of DOCA, cocaine significantly decreased the accelerated rate of ^{3}H.DOPEG formation seen in the preparation treated with DOCA alone, to a level similar to that in the preparation treated with cocaine alone. The formation of ^{3}H.DOMA was not significantly influenced by either cocaine and/or DOCA, however, treatment with DOCA alone tended to increase ^{3}H.DOMA formation. The formation of ^{3}H.OMDA was only significantly decreased by DOCA in the cocaine treated preparation.

These results point to a largely neuronal origin of the processes involved in ^{3}H.NA accumulation and ^{3}H.DOPEG formation in the untreated rat tail artery.

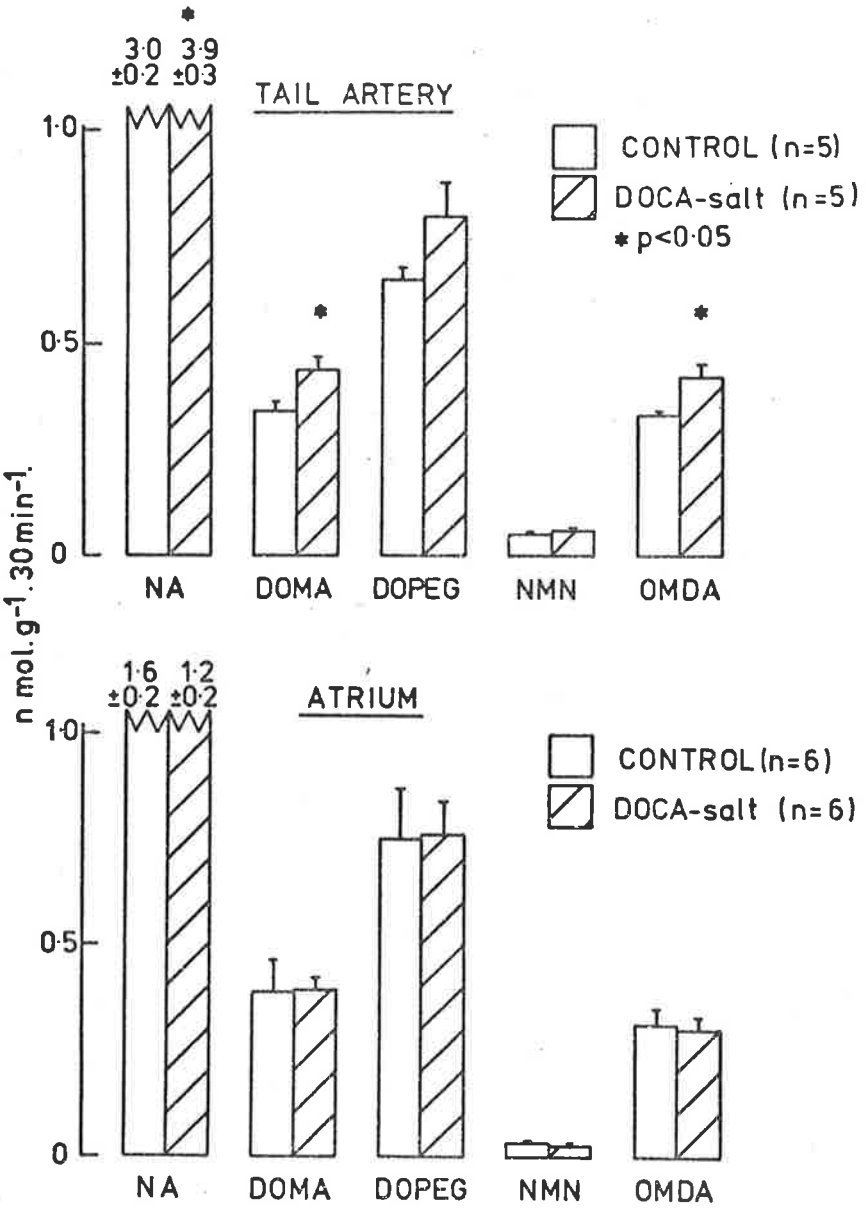

Fig. 8.2 The uptake and metabolism of 2,5,6-C labelled (-)^{3}H.NA (0.18 μM) in nmol.g^{-1}.30min^{-1} in normotensive and DOCA-salt hypertensive rat tail arteries and left atria. This shows similar patterns of metabolite formations in the two tissues, despite the lower uptake of unchanged ^{3}H.NA by the atria.

* indicates significance ($p < 0.05$); unpaired t-test.

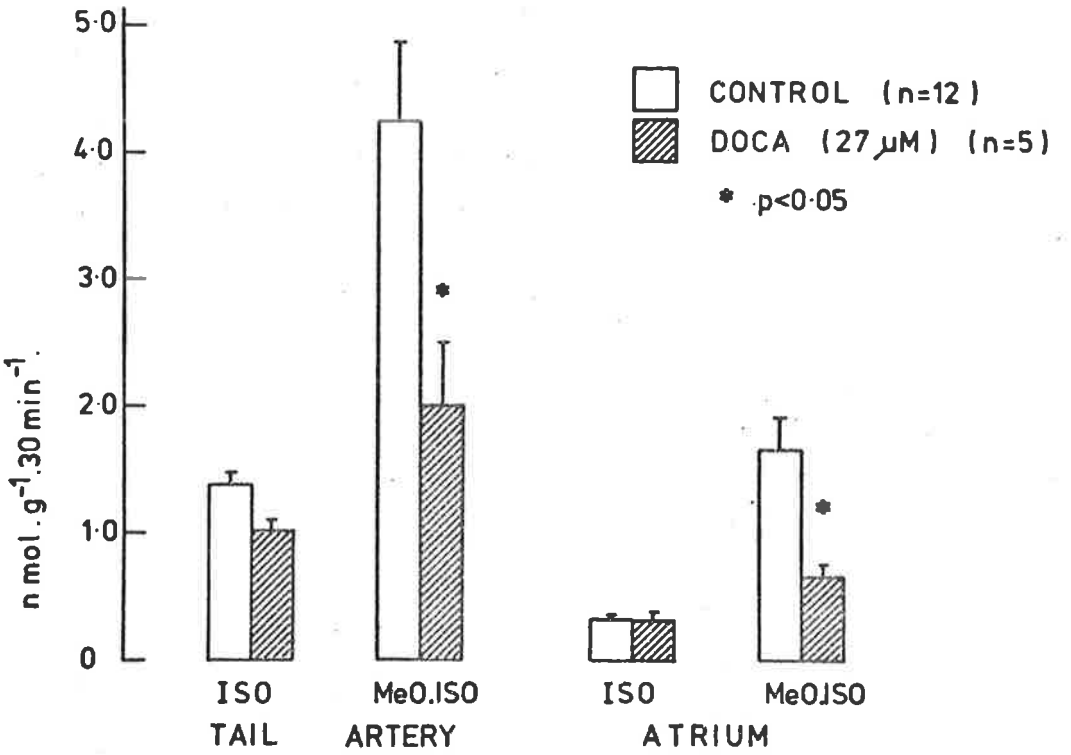

Fig. 8.3 The effect of DOCA (27 μM) on the accumulation and metabolism of (±)^{3}H.ISO (0.81 μM) in rat tail artery and left atrium.

* indicates significance ($p < 0.05$); unpaired t-test.

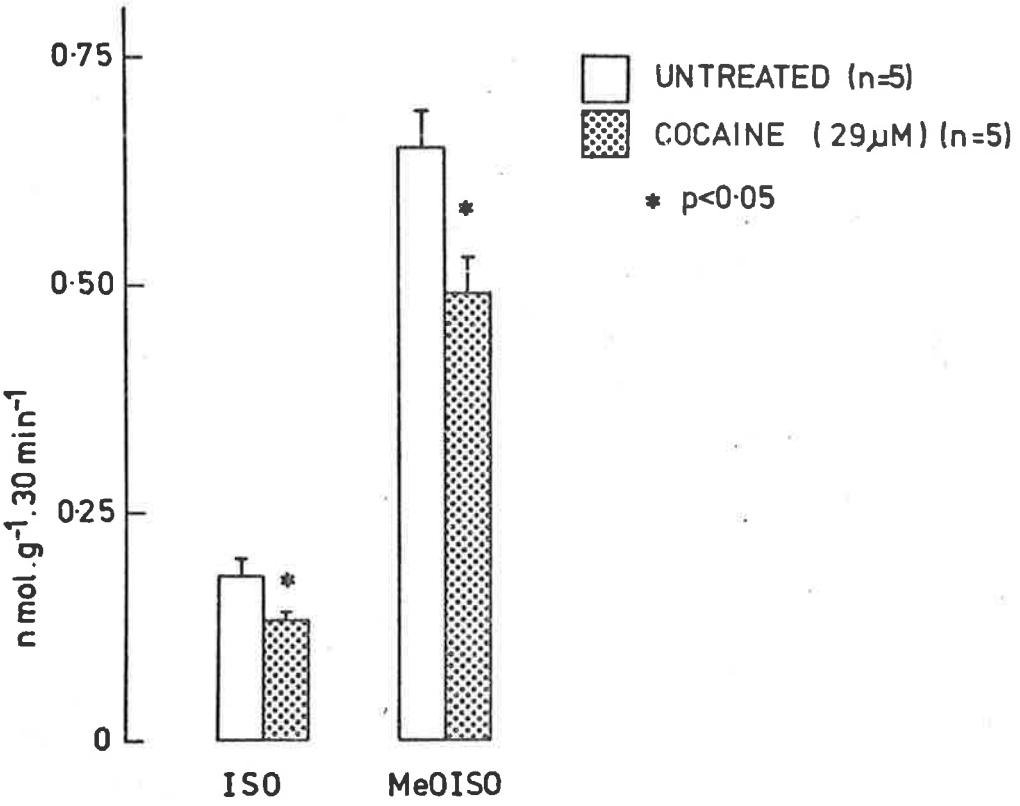

Fig. 8.4 The effect of cocaine (29 μM) on the accumulation and metabolism of (±)^{3}H.ISO (0.18 μM) in nmol.g^{-1}. 30min^{-1} in the isolated rat tail artery.

* indicates significance ($p < 0.05$); unpaired t-test.

Table 8.2

The effect of DOCA (27 μM) on the accumulation and metabolism of (±)^{3}H.ISO in tissues from normotensive and DOCA-salt hypertensive rats.
Values shown are means ± SEM.
* indicates significance ($p<0.05$) compared with controls; unpaired t-test.

Tissue	Treatment	n	BP (mm Hg)	ISO (μM)	Tissue ^{3}H.ISO		Total ^{3}H.MeOISO nmol. g^{-1}.30min^{-1}.	
					Nil	DOCA	Nil	DOCA
TAIL ARTERY	Control	6	101 ± 3	0.81	0.73 ± 0.15	1.01 ± 0.11	3.60 ± 0.05	2.02 ± 0.53
	DOCA-salt (3-week)	6	149 ± 6 *	"	0.92 ± 0.11	0.72 ± 0.13	5.35 ± 0.95	0.65 ± 0.11 *
ATRIUM	Control	7	101 ± 3	0.81	0.21 ± 0.02	0.32 ± 0.05	1.91 ± 0.51	0.66 ± 0.09
	DOCA-salt (3-week)	7	149 ± 6 *	"	0.19 ± 0.02	0.19 ± 0.04	2.52 ± 0.39	1.01 ± 0.25 *

In the isolated left atrium, the accumulation of unchanged ^{3}H.NA was about half that seen in the tail artery (as indicated in normotensive control tissues in Fig. 8.2), but the pattern of ^{3}H.metabolites was similar both qualitatively and quantitatively to that seen in the tail artery at the same substrate concentration. The source of ^{3}H.metabolites was not examined in the atrium in view of the extensive evidence in the rat heart that the deaminated catechol metabolites (DOPEG and DOMA) are neuronal in origin and the O-methylated metabolite (NMN) is extraneuronal in origin (Fiebig and Trendelenburg 1978b).

(b) Isoprenaline.

In contrast to (-)^{3}H.NA, there was only a small accumulation of (±)^{3}H.ISO by the rat tail artery; the major metabolite was ^{3}H.MeOISO (Fig. 8.3 and Table 8.2). A minor proportion of ^{3}H appeared in the 'OMDA' fraction when assayed by the column chromatographic method, but the "metabolite(s)" responsible was not identified as it was not apparent in tissues analysed by the thin layer chromatographic method. As shown in Table 8.1, the results obtained by the two methods were quantitatively similar and both indicated ^{3}H.MeOISO as the major metabolite. Of the ^{3}H.ISO removed by the tissue, approximately 80% was metabolised. The amount of ^{3}H.ISO metabolised was approximately half that of ^{3}H.NA at the same substrate concentration. The largely extraneuronal origin of ^{3}H.MeOISO was indicated by a 60% reduction in its rate of formation in the presence of DOCA (27μM) (Fig. 8.3 and Table 8.2). However, DOCA was without a significant effect on either the slight accumulation of ^{3}H.ISO, or on the formation of the "metabolite(s)" appearing in the 'OMDA' fraction. Cocaine (29μM) caused a small significant decrease (by 25%) in both ^{3}H.ISO accumulation and ^{3}H.MeOISO formation in the tail artery (Fig. 8.4), suggesting a minor proportion of ^{3}H.ISO accumulated and was O-methylated in neuronal structures.

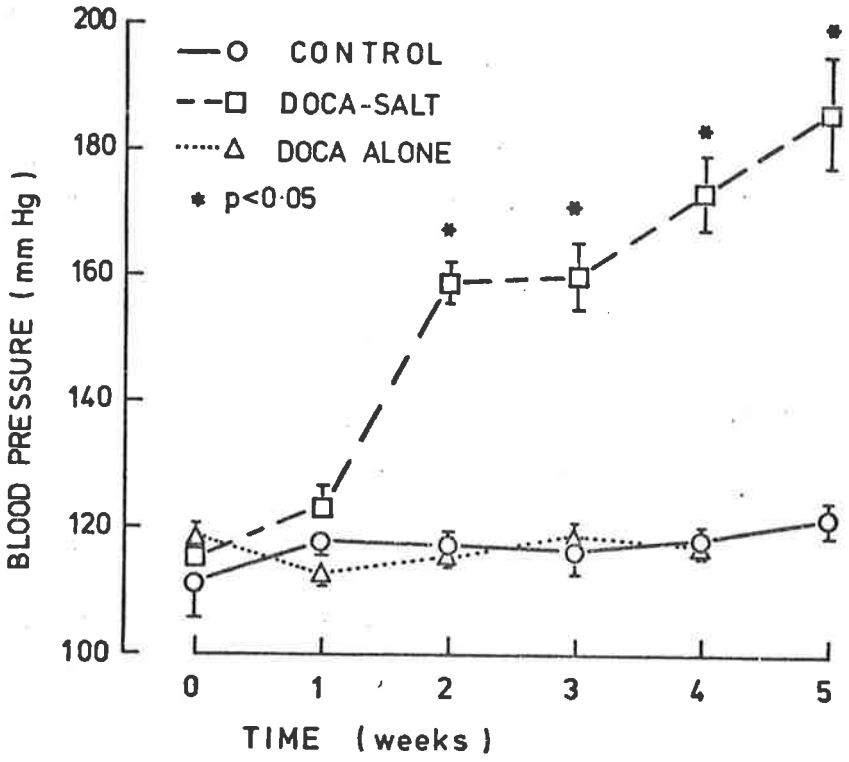

Fig. 8.7 The effect chronic DOCA-salt treatment on the blood pressure (measured indirectly), compared with sham-operated rats, injected with the vehicle alone, and rats injected with the same dose of DOCA alone. The number of observations at each point varies. For the DOCA-salt rats n=18, 49, 54, 75, 9 and 9 for each point; for the control rats n=11, 50, 58, 81, 14 and 9; and for the DOCA alone rats n=6 at each point.

* indicates significance ($p < 0.05$); unpaired t-test.

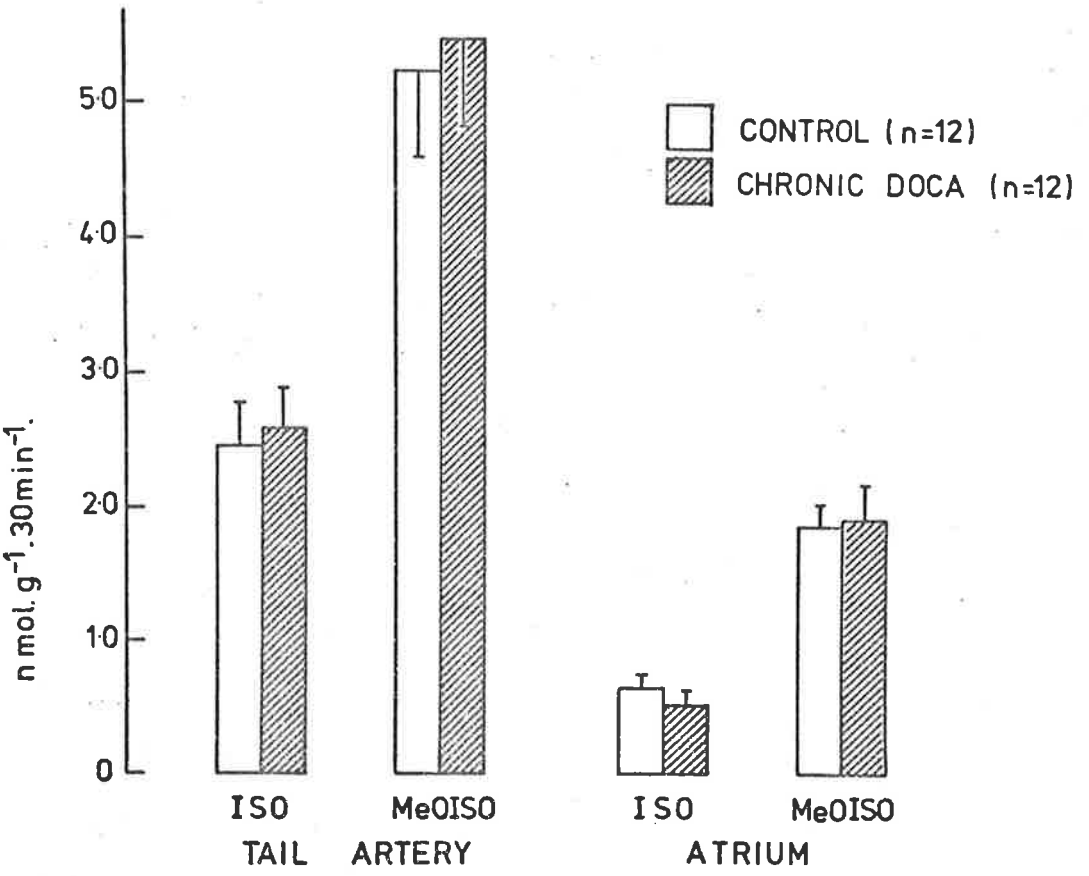

Fig. 8.6 The effect of chronic DOCA (20mg.Kg^{-1} injected subcutaneously twice weekly) on the accumulation of unchanged (±)^{3}H.ISO and the formation of ^{3}H.MeOISO in nmol.g^{-1}.30min^{-1} in the isolated rat tail artery and left atrium incubated with (±)^{3}H.ISO (0.81 μM).

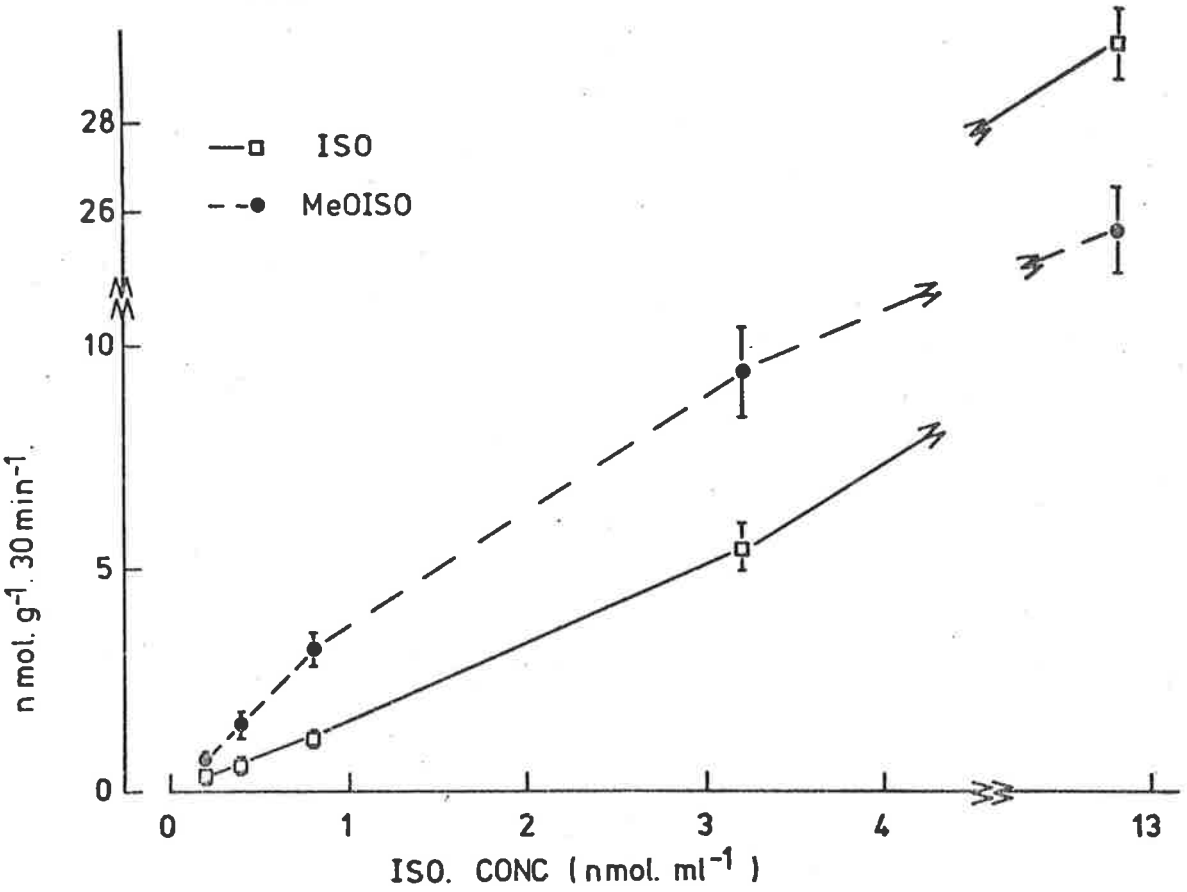

Fig. 8.5 The accumulation of unchanged (±)^{3}H.ISO and the formation of ^{3}H.MeOISO in nmol.g^{-1}.30min^{-1} in the isolated rat tail artery with substrate concentrations ranging from 0.18 to 12.8 µM (n=6). This shows that saturation of these pathways had not occurred at the substrate concentrations routinely used in the present study; ie, 0.18 and 0.81 µM.

Table 8.3 The accumulation and metabolism of (-)^{3}H.NA in normotensive and DOCA-salt hypertensive rat tail arteries. Metabolite values in brackets refer to efflux into the incubating medium only. Values are means ± SEM. * indicates significance ($p < 0.05$)

Treatment	n	BP (mm Hg)	NA μM (^{3}H.label)	Tissue NA	Metabolite Formation nmol.g^{-1}. 30min^{-1} DOMA	DOPEG	NMN	OMDA
Control	9	116 ± 1	0.12 (7-C)	1.82 ± 0.06	0.18 ± 0.02	0.59 ± 0.02	0.08 ± 0.01	0.34 ± 0.03
DOCA-salt (3-week)	7	179 ± 5 *		1.82 ± 0.05	0.21 ± 0.02	0.56 ± 0.03	0.06 ± 0.01	0.41 ± 0.03
Control	5	135 ± 4	0.18 (2,5,6-C)	3.02 ± 0.21	0.34 ± 0.02	0.65 ± 0.04	0.05 ± 0.00	0.33 ± 0.01
DOCA-salt (3-week)	5	193 ± 7 *		3.88 ± 0.29 *	0.44 ± 0.03 *	0.80 ± 0.08	0.60 ± 0.01	0.42 ± 0.03 *
Control	9	142 ± 3	0.59 (2,5,6-C)	-	(1.08 ± 0.26)	(2.40 ± 0.17)	(0.05 ± 0.01)	(0.69 ± 0.10)
DOCA-salt (3-week)	9	206 ± 6 *		-	(1.06 ± 0.18)	(2.15 ± 0.17)	(0.04 ± 0.01)	0.73 ± 0.07)
Control	12	120 ± 3	0.59 (7,8-C)	-	(0.52 ± 0.05)	(1.69 ± 0.14)	(0.13 ± 0.04)	(1.67 ± 0.18)
DOCA-salt (3-week)	12	173 ± 5 *		-	(0.61 ± 0.05)	(2.06 ± 0.14)	(0.16 ± 0.03)	(2.19 ± 0.17)
Control	9	120 ± 2	0.18 (7,8-C)	3.15 ± 0.22	0.16 ± 0.01	0.61 ± 0.04	0.12 ± 0.01	0.73 ± 0.07
DOCA-salt (6-week)	7	200 ± 9 *		2.92 ± 0.25	0.14 ± 0.01	0.46 ± 0.05 *	0.10 ± 0.02	0.99 ± 0.05 *
Untreated	5	-	0.18	2.81 ± 1.81	0.24 ± 0.02	0.50 ± 0.02	0.08 ± 0.02	0.42 ± 0.05

In a separate experiment, the kinetics of ^{3}H.ISO accumulation and ^{3}H.MeOISO formation were examined over a concentration range of 0.18 to 12.8μM . As shown in Fig. 8.5, neither the rate of ^{3}H.ISO accumulation nor ^{3}H.MeOISO formation were approaching saturation at the concentrations of ^{3}H.ISO more routinely used in the present study (0.18 and 0.81μM).

The amounts of unchanged ^{3}H.ISO retained by the tissue and the formation of ^{3}H.MeOISO were considerably lower in the isolated left atrium than in the tail artery (Table 8.2 and Fig. 8.3). The content of ^{3}H.ISO per gram of tissue was only 40-70% of the ^{3}H.ISO content per ml of the bathing medium which, in view of the 5-second wash, suggests that it was largely confined to the extracellular compartment. The extraneuronal origin of the ^{3}H.MeOISO was indicated by the inhibitory effect of DOCA, which was comparable with that of the tail artery (Table 8.2 and Fig. 8.3).

Chronic treatment with DOCA alone for five weeks had no effect on either the accumulation of ^{3}H.ISO (0.81μM) or the formation of ^{3}H.MeOISO in the tail artery or atrium (Table 8.5 and Fig. 8.6). This DOCA treatment was not associated with changes in blood pressure (Fig. 8.7).

(2) Hypertensive tissues.

The rise in blood pressure associated with the DOCA-salt treatment is shown in Fig. 8.7. After three weeks of treatment their mean systolic blood pressure was 160mmHg, compared with 117 mmHg in control rats (means of approximately 75 animals), although the magnitude of this increase did fluctuate with different treatment series (as seen in Table 8.3).

In initial experiments the accumulation and metabolism of (-)^{3}H(7-C) NA (0.12μM) was compared in tail arteries from a group of three week DOCA-salt hypertensive rats and their normotensive controls (Table 8.3).

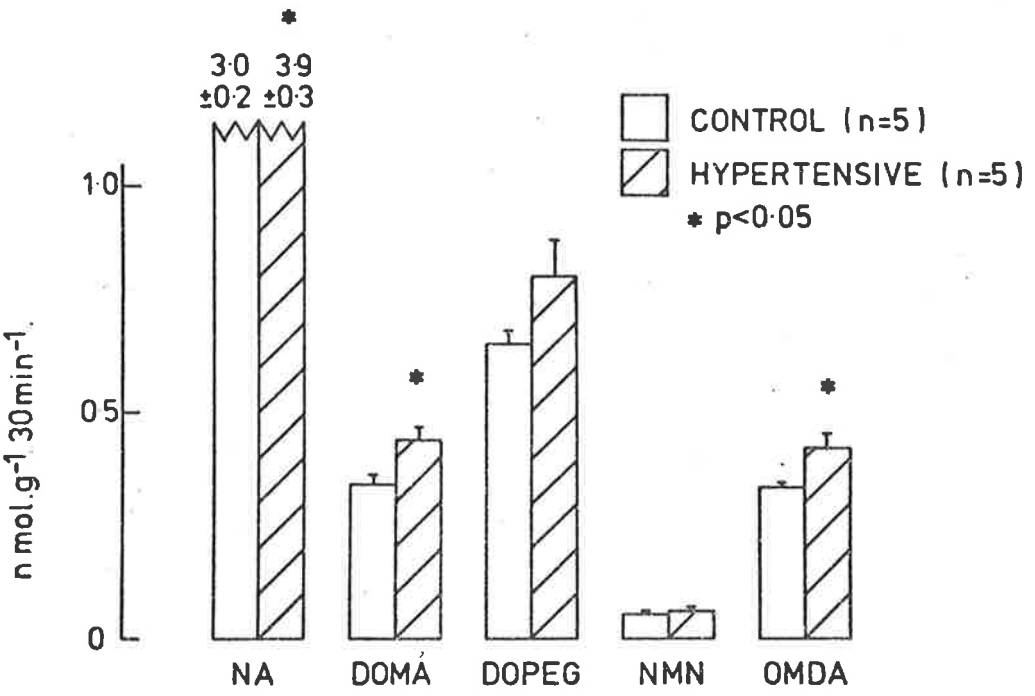

Fig. 8.8 The uptake of unchanged ^{3}H.NA and the formation of the ^{3}H. metabolites in nmol.g^{-1}.30min^{-1} in isolated tail arteries from control and DOCA-salt rats treated for 3 weeks, incubated with 2,5,6-C labelled ^{3}H.NA (0.18 µM).

* indicates significance ($p < 0.05$); unpaired t-test.

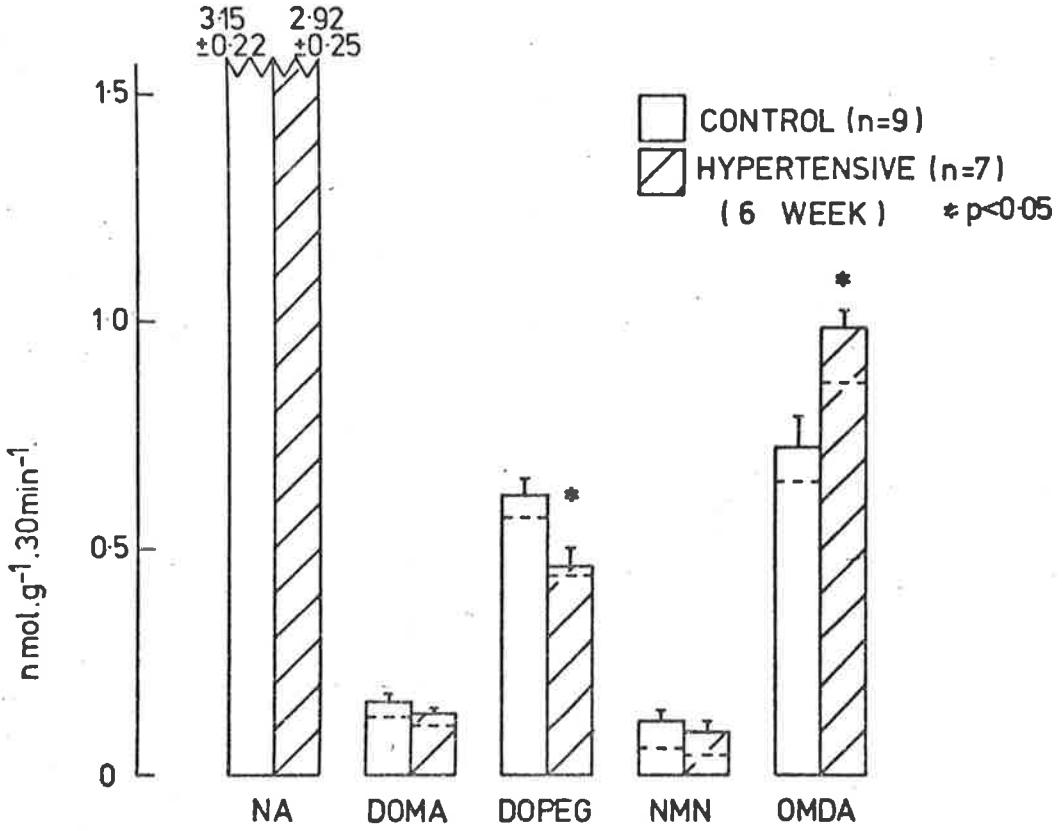

Fig. 8.9 The uptake of unchanged ^{3}H.NA and the formation of ^{3}H.metabolites in nmol.g^{-1}.30min^{-1} in isolated tail arteries from control and DOCA-salt rats treated for 6 weeks, incubated with 2,5,6-C labelled (-)^{3}H.NA (0.18 µM). The dashed line indicates the amount which effluxed into the bathing medium, compared with that retained by the tissue.

* indicates significance ($p < 0.05$); unpaired t-test.

The comparison was repeated in a separate experiment at a higher substrate concentration (0.59μM), but it was subsequently learned that the (-)^{3}H.NA used (from a different commercial source) was partially labelled on the 8-C atom (Starke et al, 1980; confirmed in personal communication with NEN). For this reason, the above comparisons were repeated in two further experiments using (-)^{3}H.NA labelled on the 2,5 and 6-C atoms on the aromatic ring to avoid doubts about the location of the ^{3}H on the 7-C or 8-C atom. These latter comparisons, involving a total of 23 normotensive and 21 hypertensive rats failed to reveal any consistent differences in either the accumulation of unchanged amine or the formation of ^{3}H.metabolites. A possible exception is, at the low substrate concentration, the mean values of ^{3}H.DOMA and ^{3}H.OMDA formation were increased (by approximately 30%) in the hypertensive vessels, although these increases were only significant in the case of the comparison using the 2,5,6-C labelled ^{3}H.NA. In the latter comparison (shown in Fig. 8.8, and Table 8.3), the accumulation of unchanged ^{3}H.NA was also significantly increased by 30%. There were no significant differences in the rates of ^{3}H.DOPEG or ^{3}H.NMN formation in either comparison.

Only one comparison was carried out on six-week DOCA-salt treated rats (Table 8.3 and also Fig. 8.9). The only significant changes were a 38% increase in ^{3}H.OMDA and a 34% decrease in ^{3}H.DOPEG formation. However, these changes were small, and since 7,8-C labelled ^{3}H.NA was used in these experiments, it is possible that the higher rates of ^{3}H.OMDA formation (compared with the 2,5,6-C ^{3}H.NA) reflected varying amounts of tritiated water in this fraction (as explained previously).

The accumulation and metabolism of 2,5,6-C labelled (-)^{3}H.NA (0.18μM) in isolated left atria were compared in one group of three-week hypertensive rats and their normotensive controls. These results (Fig. 8.2 and Table 8.4)

Table 8.4

The accumulation and metabolism of (-)^{3}H.NA in normotensive and DOCA-salt hypertensive rat left atria.
Values shown are means ± SEM.
* indicates significance ($p<0.05$); unpaired t-test.

Treatment	n	BP (mm Hg)	NA µM (^{3}H.label)	Tissue NA	Metabolite Formation nmol.g^{-1}. 30min^{-1}. DOMA	DOPEG	NMN	OMDA
Control	6	135 ± 4	0.18 (2,5,6-C)	1.61 ± 0.24	0.39 ± 0.07	0.75 ± 0.12	0.03 ± 0.01	0.31 ± 0.05
DOCA-salt	6	193 ± 7 *		1.19 ± 0.16	0.39 ± 0.03	0.76 ± 0.09	0.03 ± 0.00	0.30 ± 0.03

Table 8.5

The accumulation and metabolism of (±)^{3}H.ISO in tissues from normotensive and DOCA-salt hypertensive rats. Values shown are means ± SEM.
* indicates significance ($p<0.05$); unpaired t-test.

Tissue	Treatment	n	BP (mm Hg)	ISO (μM)	Tissue ISO	Total ^{3}H.MeOISO nmol.g.$^{-1}$.30min^{-1}
TAIL ARTERY	Control	18	119 ± 2	0.81	1.36 ± 0.09	4.25 ± 0.06
	DOCA-salt (3-week)	20	191 ± 9 *	"	1.61 ± 0.19	3.70 ± 0.44
	Control	12	118 ± 1	0.81	2.47 ± 0.30	5.25 ± 0.64
	DOCA-alone (5-week)	12	118 ± 2	"	2.59 ± 0.28	5.48 ± 0.65
	Untreated	5	-	0.18	0.18 0.02	0.65 0.04
ATRIUM	Control	18	119 ± 2	0.81	0.32 ± 0.02	1.66 ± 0.27
	DOCA-salt (3-week)	20	191 ± 9 *	"	0.33 ± 0.03	1.48 ± 0.20
	Control	12	118 ± 1	0.81	0.63 ± 0.10	1.86 ± 0.18
	DOCA-alone (5-week)	12	118 ± 2	"	0.51 ± 0.10	1.90 ± 0.27

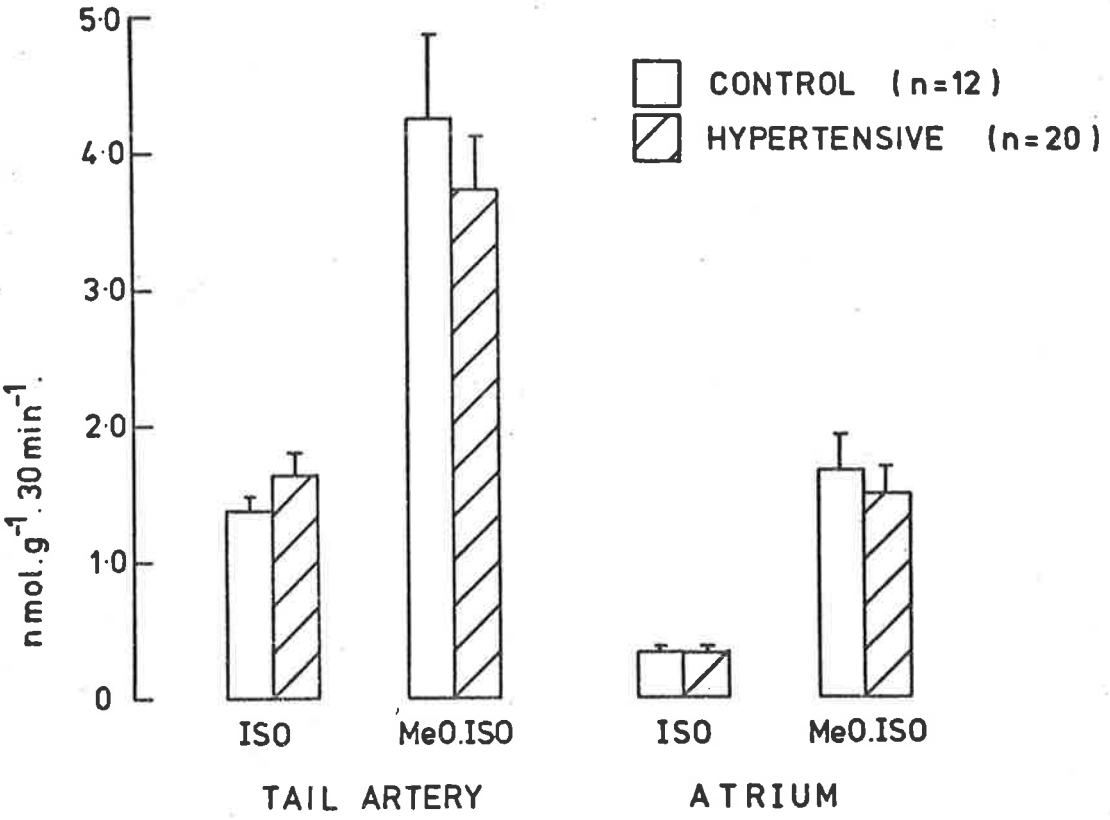

Fig. 8.10 The accumulation of unchanged ^{3}H.ISO and the formation of the ^{3}H.metabolite, ^{3}H.MeOISO, in nmol.g^{-1}.30min^{-1} in isolated tail arteries and left atria from control and DOCA-salt rats treated for 3 weeks, incubated with (±)^{3}H.ISO (0.81 μM).

Table 8.6

The endogenous catecholamine contents of tissues from normotensive and DOCA-salt hypertensive rats. Values shown are means ± SEM in nmol.g^{-1}wet wt. * indicates significance ($p<0.05$); unpaired t-test.

Treatment	n	NA	A	DA	Weight(mg)
(a) Tail artery:-					
Control	6	33.2 ±4.0	0.30 ±0.05	0.48 ±0.10	13.0 ±0.8
DOCA-salt (3 week) A	6	38.2 ±4.4	0.39 ±0.13	0.30 ±0.05	11.7 ±0.1
Control	11	22.9 ±2.0	0.24 ±0.05	0.38 ±0.08	7.7 ±0.6
DOCA-salt (3 week) B	10	22.8 ±1.8	0.25 ±0.05	0.36 ±0.05	6.9 ±0.4
Control	6	29.3 ±1.0	0.20 ±0.03	0.25 ±0.05	15.3 ±1.0
DOCA-salt (6 week)	6	40.1 ±3.4*	0.44 ±0.05*	0.44 ±0.05	19.0 ±1.3*
(b) Heart:-					
Control	6	3.3 ±0.2	0.06 ±0.01	0.07 ±0.01	880 ±50
DOCA-salt (3 week) A	6	1.9 ±0.2*	0.04 ±0.01*	0.03 ±0.00*	1070 ±30*
Control	11	2.7 ±0.2	0.05 ±0.01	0.06 ±0.01	790 ±30
DOCA-salt (3 week) B	10	2.0 ±0.2*	0.03 ±0.00*	0.05 ±0.01	832 ±141
Control	6	1.4 ±0.2	0.04 ±0.00	0.02 ±0.00	910 ±20
DOCA-salt (6 week)	6	1.2 ±0.2	0.03 ±0.00	0.02 ±0.00	1150 ±60 *
(c) Left atrium:-					
Control	4	11.7 ±1.3	0.24 ±0.02	0.24 ±0.04	18.0 ±2.3
DOCA-salt (3 week) B	3	5.5 ±0.8*	0.12 ±0.03*	0.15 ±0.03	21.4 ±4.1

did not reveal any significant changes in the accumulation of unchanged amine or the rates of ^{3}H.metabolite formation in the hypertensive rat atria.

The accumulation and metabolism of ^{3}H.ISO (0.81μM) in the tail artery and left atrium (Table 8.5 and Fig. 8.10) revealed no significant differences between the tissues from normotensive and DOCA-salt hypertensive rats.

(3) Endogenous Catecholamine contents.

The contents of NA, adrenaline and dopamine in tail arteries, hearts and atria are summarised in Table 8.6. In normotensive rats, the tail arteries had high levels of NA but negligible contents of adrenaline (A) and dopamine (DA). The comparison between two separate groups (A and B), representing proximal segments in A and distal segments in B, when assayed, suggested that the NA content diminished distally.

There were no significant differences between the endogenous catecholamine contents of three-week normotensive and hypertensive vessels, nor was there any evidence of hypertrophy in the latter tail arteries. After six weeks of treatment the hypertensive vessels showed evidence of hypertrophy and had significantly higher NA and adrenaline contents.

In normotensive rats, the contents of NA, adrenaline and dopamine in the whole heart were approximately one tenth those of the tail artery. The contents of the left atria were approximately four-fold greater than those of the whole heart. The hearts of the hypertensive rats were significantly hypertrophied at three and at six-weeks; the hypertrophy at three weeks was associated with significant decreases in the NA, adrenaline and dopamine contents. The comparison between a small number of atria suggested that changes in the atrial contents reflected those in the whole heart.

DISCUSSION

(1) Origin of metabolites in normotensive tissues.

(a) Tail artery. The pattern of accumulation and metabolism of NA in the tail artery is consistent with evidence, based on histochemical observations (Hodge and Robinson, 1972) and pharmacological evidence (Wyse, 1973), confirmed by the endogenous NA contents (this study), that the rat tail artery has a dense sympathetic innervation. Thus the effects of cocaine indicated that the removal of NA by this tissue was largely via uptake into sympathetic nerves and its subsequent deamination represents an important pathway of metabolism. The extraneuronal inactivation appears to be quantitatively important since the net removal and metabolism of NA by the tissue in the presence of cocaine was about half that in the absence of cocaine. However, the contribution of the corticosteroid-sensitive extraneuronal pathway in inactivating NA appears to be quantitatively less significant since, although DOCA significantly reduced NA accumulation by 40%, it was without effect on NMN formation when neuronal uptake was unimpaired. Extraneuronal inactivation appeared to be of greater importance when neuronal uptake was blocked. Hence it may be concluded that, in the rat tail artery, the extraneuronal uptake system is quantitatively less important than the neuronal system in the inactivation of NA. The effects of cocaine, and of DOCA, indicated that ^{3}H.DOPEG was largely neuronal in origin, and at least 67% of the ^{3}H.NMN was formed extraneuronally in a corticosteroid-sensitive O-methylating compartment. Although in the cocaine-treated vessel, ^{3}H.OMDA formation was significantly decreased by DOCA, this decrease was small (30%). Hence the origin(s) of the major proportion of the OMDA fraction, and also the small amount of DOMA formed, cannot be identified with either neuronal or corticosteroid-sensitive extraneuronal compartments. Insensitivity of OMDA formation to corticosteroids has been reported in other tissues.

As already discussed in Chapter 4, it has been proposed that DOPEG which effluxes from the nerves may diffuse directly into effector cells and undergo O-methylation in the rat heart (Fiebig and Trendelenburg,1978b). A similar conclusion was arrived at by Henseling et al (1978b in rabbit aortic strips. Schrold and Nedergaard (1981) showed that OMDA could be formed in the isolated adventitia of the rabbit aorta, possibly by fibroblasts which have been reported in the adventitia of this tissue by Levin (1974) and contain both the enzymes COMT and MAO (Jacobowitz, 1972). Conceivably access of NA into these cells is not affected by steroids.

In view of the seemingly minor role played by the extraneuronal O-methylating system in the inactivation of NA, the capacity of the tail artery to O-methylate ISO is surprisingly high. The rate of formation of MeOISO was 56% of that seen in the rabbit ear artery which is regarded as having a well developed extraneuronal uptake system (Head et al, 1980). Furthermore, the tail artery O-methylated ISO at a rate which was approximately one-half the total rate of metabolism of NA and 2.7 fold greater than the rate of O-methylation of NA in the cocaine treated vessels where, presumably, only the extraneuronal O-methylating pathway was available. These observations suggest that the steroid-sensitive extraneuronal inactivating system of the rat tail artery is not poorly developed, but rather that it has a low affinity for NA compared with that for ISO. However, the possibility must also be considered that not all of the ISO was metabolised in a steroid-sensitive O-methylating compartment. The small but significant inhibitory effect of cocaine suggested that 25% of the MeOISO may have been derived from a neuronal compartment. On the other hand, it is also probable that the 60% reduction of MeOISO formation by DOCA is an underestimate of the steroid-sensitive extraneuronal contribution, since,from studies in the rat heart (Iversen and Salt, 1970)

and the rabbit ear artery (Johnson and de la Lande, 1978), it seems likely that the steroid is a competitive inhibitor of extraneuronal O-methylation.

(b) Atrium. Although the origins of the metabolites of NA in the atria were not investigated, the fact that the ratio of the relative accumulations of unchanged amine in the atria and tail artery (0.57) was similar to the ratio of their endogenous contents (0.35) suggests that the major proportion of the amine accumulated in the atria was in sympathetic nerves. Nevertheless, with the possible exception of ^{3}H.NMN, the rates of metabolite formation in the atria were strikingly similar to those in the tail artery. This finding suggests that, in the atria, proportionally more of the amine which is removed by neuronal uptake is deaminated than is the case in the tail artery. The formation of ^{3}H.NMN was less than in the tail artery and represented only 2% of the total metabolite formation.

The accumulation of ISO and its rate of O-methylation was also markedly less in the atrium than in the tail artery. This result was surprising in view of the capacity of the whole rat heart to accumulate and metabolise ISO (Bönisch and Trendelenburg,1974b). The values in the atria also differ from those in ventricular slices reported by Bönisch et al (1974) in that, in the atria, the accumulation of ISO was markedly less while the rate of MeOISO formation was approximately four-fold greater. It is conceivable that the lower values in the atria and ventricular slices reflect more limited diffusion of the substrate into the latter tissues (thickness approximately 0.5mm) compared with the tail artery (wall thickness approximately 0.07mm). As in the case of the tail artery, DOCA decreased but did not abolish MeOISO formation (by 60%). In view of evidence in whole heart (Bönisch et al, 1974), it is probable that this finding again reflects the competitive nature of the action of DOCA.

(2) DOCA-salt treatment.

The most reproducible of the changes in metabolism of NA in the hypertensive tail artery was the increase in OMDA formation at low substrate concentrations. This increase, although only 30%, was significant in one of the two groups of three-week hypertensive vessels. The interpretation of this effect is complicated by a number of factors, (a) the 'OMDA' fraction is the least 'pure' of the metabolite fractions obtained by the cascade column chromatographic method, and (b) in the case of 7,8-C labelled amine (as used in the six-week hypertensive vessels) the 'OMDA' fraction would have also included tritiated water derived from the action of MAO on the 8-C ^{3}H molecules. As already discussed, the origin of the OMDA fraction in this tissue is unknown in view of the relative failure of both cocaine and DOCA to significantly modify its formation. However, the present results do suggest that the apparent increase in OMDA formation is not due to an increase in the steroid-sensitive O-methylating pathway of the vessel, since the increases in OMDA formation were not associated with increases in the formation of NMN, or in the formation of MeOISO from ISO. Similarly, it seems unlikely that increases in OMDA were associated with increased neuronal deaminating activity, since the major product of neuronal deamination (DOPEG) was enhanced in only one of the three-week comparisons, and then not significantly so. Further, the increase in OMDA formation in the six-week hypertensive vessels were associated with a significant decrease in DOPEG formation. Hence, the present study can offer little support for the association of NA inactivation in the tail artery and the hypertension induced by DOCA-salt treatment. Hence these metabolic data provide no firm evidence that the increase in sensitivity of the hypertensive tail artery to NA (reported by Venning and de la Lande, 1981) resulted from an alteration in the neuronal

inactivation pathway. In this respect the results support the conclusions of these workers derived from the failure of cocaine to influence this increased sensitivity.

The failure to observe differences in accumulation and neuronal deamination of NA in atria of hypertensive rats is more puzzling in view of the hypertrophy and reduced endogenous NA content associated with the hypertension. A possible explanation is that the hypertrophy is accompanied by parallel increases in the activity of the neuronal deaminating and extraneuronal O-methylating enzymes.

In general, the absence of changes in the rates of O-methylation of NA, or ISO, in both the tail artery and the atria argues strongly against the possibility that the activity of the extraneuronal O-methylating pathway is modified during DOCA-salt treatment. These results differ from those of de Champlain (1967) who observed a significant reduction in the retention of NMN in isolated whole hearts of DOCA-salt hypertensive rats perfused with $(\pm)^{3}$H.NA. However, as indicated above, differences between isolated cardiac tissues and the whole perfused heart may reflect more adequate perfusion and diffusivity of substrate and metabolites in the latter preparation.

Finally, it should be noted that the data suggests that chronic treatment with corticosteroid is without effect on the activity of the steroid-sensitive extraneuronal uptake system. This conclusion is based on the failure of chronic DOCA treatment to influence the O-methylation of ISO, both when the treatment is associated with an increase in blood pressure (after DOCA-salt) and when it is without effect on blood pressure (after DOCA alone). The effect of chronic DOCA treatment on NA metabolism was not examined.

CHAPTER 9

GENERAL DISCUSSION

CHAPTER 9

GENERAL DISCUSSION

(1) Resumé of biochemical data.

(a) Rabbit ear artery.

The studies in Chapters 3 and 4 established that the surface of entry of NA into the artery wall exerted a major influence on its metabolic fate. Entry via the adventitial surface was associated with a predominantly neuronal deamination inactivating pathway, while entry via the intima was associated with predominantly extraneuronal O-methylation. The difference was explained in terms of the interaction between a number of factors of which the main ones were:

(i) the location of the sympathetic nerves at the medial-adventitial border, together with evidence that the nerves are the principle site of formation of the deaminated metabolites;

(ii) the uniform distribution of the majority of the O-methylation sites throughout the media;

(iii) the probability of a high diffusivity of NA and its metabolites in the adventitia compared with the media; and

(iv) the presence of a declining concentration of NA from its surface of entry into the vessel wall and the opposite surface.

The DOPEG formation ratio (i.e., the ratio of the total amounts of DOPEG effluxing from the vessel when incubated with EXT NA, and with INT NA) was used as an index of the concentrations achieved by EXT and by INT NA in the region of the nerve terminals. Hence, this ratio served as an approximate measure of the magnitude of the decline in concentration of INT NA between the intima and the nerve terminals.

Qualitatively, the difference in metabolism of EXT and INT NA appeared to be independent of wall thickness and perfusion pressure (i.e., the changes accompanying constriction), the presence or absence of Ca^{++}, the presence or absence of α_1 receptor blockade, or the rate of intraluminal flow. However, quantitatively the patterns of NA metabolism, particularly that of INT NA, was altered by these factors. Thus the DOPEG formation ratio was greatest (24) in the Ca^{++} media, 10 in Ca^{++} free media, 4.4 in Ca^{++} free media with prazosin, and lowest (2.7) when the flow rate was 2.0 $ml.min^{-1}$. The effect of constriction on the efflux of NMN from both surfaces differed from that of DOPEG in that efflux of NMN tended to decrease with increasing constriction during incubation with EXT NA, as well as during incubation with INT NA.

These results suggested that the constrictor tone of the vessel was capable of modifying, in a substantial way, the metabolism of INT NA and the O-methylation of EXT NA, although having little influence on the formation of deaminated metabolites from EXT NA. Factors which may have been responsible for these differences were considered in the Discussion of Chapter 4. These included: (a) to explain the effects of constriction; changes in the thickness of the media compared with the adventitia, folding of the lumen and changes in the diffusivity of NA, and (b) to explain the effects of flow rate; the higher flow rate leading to an increase in basal perfusion pressure, an expanded lumen and a decrease in wall thickness. For the most part, these possible explanations were speculative since data which would enable their critical assessment was unavailable. However, there is evidence from other studies in this laboratory relating to the possible roles of changes in perfusion pressure and of diffusivity during constriction. De la lande et al (1980)

Table 9.1 The efflux of metabolites from rabbit ear arteries incubated with (-)^{3}H.NA (0.18 μM) (present study), compared with the formation of metabolites in the rabbit aorta incubated with (-)^{3}H.NA (0.30 μM) (Levin, 1974).

Tissue	Preparation	Metabolism nmol.g^{-1}.30min^{-1}. DOMA	DOPEG	NMN	OMDA
ear artery	INT ^{3}H.NA	0.12 ±0.02 (5%)	0.72 ±0.08 (32%)	0.95 ±0.06 (42%)	0.47 ±0.05 (21%)
	EXT ^{3}H.NA	0.61 ±0.07 (14%)	2.86 ±0.22 (65%)	0.38 ±0.04 (9%)	0.55 ±0.07 (13%)
	INT & EXT ^{3}H.NA	0.54 ±0.12 (9%)	3.05 ±0.17 (50%)	1.30 ±0.08 (21%)	1.16 ±0.03 (19%)
aorta	isolated media	0.03 ±0.01 (2%)	0.13 ±0.11 (8%)	1.17 ±0.09 (74%)	0.26 ±0.03 (16%)
	isolated adventitia	0.11 ±0.02 (7%)	1.23 ±0.11 (76%)	0.13 ±0.03 (8%)	0.14 ±0.02 (9%)
	intact aorta	0.04 ±0.00 (3%)	0.58 ±0.05 (43%)	0.54 ±0.05 (40%)	0.19 ±0.02 (14%)

found that in arteries perfused at a constant rate, the diffusion coefficient of EXT ^{14}C.sorbitol decreased significantly during constriction, i.e., when both wall thickness and perfusion pressure increased. However, the decrease in diffusivity did not occur in vessels perfused at a constant pressure, i.e., when only the wall thickness decreased. Their results imply that an increase in intraluminal perfusion pressure alone can decrease the diffusivity of a substance diffusing from the adventitia to the intima. Such an effect could be explained in terms of an increased bulk flow of solution across the wall from the intima to the adventitia which would oppose the diffusion of NA resulting from its concentration gradient. The problem with this explanation is that, if correct, the flux of INT NA across the wall should be greater in the constricted than in the relaxed vessel. The present data indicates that the opposite, in fact, occurs. Further experiments designed to measure the diffusion coefficient of INT ^{14}C.sorbitol at various levels of constriction, and perfusion pressure, may help to resolve these apparent paradoxes.

The different metabolisms of INT and of EXT NA complement the earlier findings of Levin (1974) on the relative metabolisms in the isolated adventitia and isolated media of the rabbit aorta. The relative proportions of metabolites are compared in Table 9.1. It should be noted that the values shown for rabbit ear artery segments represents the total efflux into the bathing media of vessels incubated with (-)^{3}H.NA (0.18μM), whereas values from rabbit aorta represents total metabolite formation (i.e., efflux into the bathing medium plus the tissue extract) in vessels incubated with (-)^{3}H.NA (0.30μM). However, in the case of DOPEG and NMN, the difference is small since these have high medium to tissue ratios (shown in Fig. 3.3, Chapter 3). Further

(as discussed later) the rate of metabolism of NA in the perfused segment of ear artery appears to be greater than that in the isolated artery strip. Nevertheless, the data indicates that in both tissues the primary site of deamination of NA (i.e., DOPEG and DOMA formation) is associated with the adventitia and specifically with the sympathetic nerves , and O-methylation (i.e., NMN formation) primarily associated with the media. In the case of the aorta, further confirmation of the sites of these pathways is reported in a recent study on the effects of surface of entry on NA metabolism by Henseling (1980b). His abstract does not report the method used to restrict the entry of NA to one surface only.

The origin of the OMDA fraction (i.e., MOPEG and VMA) appear to be more complex than those of the remaining metabolites. In the present study evidence was presented that a significant proportion of MOPEG is formed by the O-methylation of DOPEG. Evidence of such a mechanism was not possible in Levin's study on the isolated adventitia and media of the aorta since this mechanism relies on DOPEG formed in the adventitia, being O-methylated probably in the media. The quantitative contribution of the DOPEG O-methylation mechanism of MOPEG formation may, however, be less important in the aorta since its media is approximately 3-fold thicker than the media of the ear artery. This is because the present evidence indicates that DOPEG O-methylation is more important in smooth muscle cells close to the sympathetic nerves than in more distant cells. A second O-methylation pathway of OMDA formation was resistant to hydrocortisone and appeared to account entirely for the formation of this fraction when NA entered via the intima. Since this pathway predominated in the regions more distant from the nerve terminals, it may well play a more important

role in the thicker walled aorta. However, it might also be argued that, as a result of reserpine pretreatment of the rabbit, the quantitative contribution of the O-methylation of DOPEG to MOPEG formation in the rabbit ear artery was overestimated in present study. This is because reserpine pretreatment greatly increased DOPEG formation (shown in Chapter 3, Fig. 3.1).

The possible origin of VMA was not investigated. Its formation is unlikely to involve the same cocaine-sensitive corticosteroid-insensitive mechanism responsible for the O-methylation of DOPEG, since this mechanism relies on the high lipophilicity of DOPEG as the factor which enables the glycol to diffuse into the extraneuronal O-methylating compartment (Mack and Bönisch, 1979). These workers also showed that the lipid solubility of DOMA is poor compared with that of DOPEG; hence the same mechanism is unlikely to account for VMA formation. An extension of the present study to include separation of the OMDA fraction into VMA and MOPEG will be required before the site(s) of formation of these metabolites can be further analysed.

No attempt has been made to identify sulphate conjugates in the present study. Sulphate conjugates are well known in brain and liver homogenates (Meek and Neff, 1973). Further, during in vivo studies, most NA and metabolites are found in urine as conjugates (Goodall and Alton, 1968). It seems likely, therefore, that such conjugation occurs by a secondary mechanism at a site distant from the blood vessel wall, e.g., the liver (Meek and Neff, 1973). The possibility that the OMDA fraction could include sulphate conjugates seems unlikely, since Head et al (1980) could identify only O-methylated metabolites when rabbit ear arteries were incubated with ^{3}H.ISO and the incubate analysed by TLC.

A similar conclusion was drawn by Levin (1974) in studies with the rabbit aorta. He was unable to detect any metabolites of NA (other than the 5 considered in the present study) using paper chromatography, with the possible exception of a small peak containing 0.15% of the total radioactivity applied to the plate (Rf = 0.9) on chromatograms obtained from isolated adventitia, but not from isolated media. This peak did not coincide with any of the unlabelled metabolites (not conjugated).

In summary, the present metabolic studies have further defined the pathways of NA metabolism and origin of metabolites in the rabbit ear artery. An inter-relation between metabolism and constriction has been revealed. For its further understanding, there is a requirement for further experiments to expand this relationship to include more precise correlation of morphological and metabolic changes.

(b) Metabolism in artery strips and segments.

Comparison of the results on isolated artery strips, and other perfused segments incubated with INT plus EXT ^{3}H.NA, suggested that there was significantly less metabolite formation in the artery strip. Although the strips were taken from the immediately distal region of the artery from which the segment was derived, it cannot be argued that regional differences in metabolism are responsible, since, in certain experiments where proximal strips were used (Chapter 3, Table 3.1), the metabolism of NA in these strips did not differ significantly from that of more distal strips measured in other experiments (Chapter 3, Table 3.4).

The lower rate of metabolism of NA in strips is difficult to explain, since the effect of cutting an artery segment to form a strip was found to have little effect on metabolite efflux in the non-perfused preparations

(Chapter 3, Table 3.1). The possibility that the artery strip was constricted is excluded, since many of the studies using strips were carried out in Ca^{++}-free media with prazosin present. The possibility that entry of substrate into the strip may have been limited by inadequate stirring seems unlikely since the continuous bubbling (with 95% O_2, 5% CO_2) ensured that the strip was constantly agitated in the incubating medium. Furthermore, these incubation conditions correspond exactly with those to which the adventitia was subjected in the perfused segment.

There remains the possibility that the pulsations of the wall of the perfused segment, imposed by the effect of the roller pump on the intraluminal flow, may have caused a type of 'stirring' of the solutes in the extracellular compartment, thus enabling the substrate to penetrate more freely to the inactivation sites and promote more rapid efflux of metabolites from these sites. This question could be resolved by further experiments in which a non-pulsating pump was employed. These results do, however, raise an important aspect of technique which has been largely ignored in most studies of *in vitro* metabolism in blood vessels. It is suggested that such studies should be carried out under conditions more closely related to the physiological situation, i.e., segments should be used and these should be perfused intraluminally if quantitative extrapolations of the *in vitro* data to the *in vivo* situation are to be meaningful.

(c) Rat tail artery.

Although somewhat tangential to the main theme of this study, the study on the metabolism of NA, and ISO, in the rat tail artery showed that, again, DOPEG was the major metabolite and was neuronal in origin, whereas as NMN and MeOISO were extraneuronal in origin, and steroid sensitive. The main difference between this preparation and the rabbit

ear artery appears to be in the resistance of OMDA formation to cocaine. This may conceivably be due to the fact that the tail arteries were not derived from reserpine pretreated rats, so that the absolute rate of DOPEG formation was considerably less than in the reserpinised rabbit ear arteries, i.e., the contribution of the DOPEG O-methylation mechanism would be less. However, another factor to be taken into account is that the major component of the OMDA fraction, present in incubates of this vessel, was VMA and not MOPEG, unlike the rabbit ear artery.

The failure to detect consistent changes in the metabolism of NA, or ISO, in tail arteries of the DOCA-salt hypertensive rats does not necessarily imply that such changes do not occur. In view of the observation that, in the rabbit ear artery, the metabolism of NA in perfused segments exceeded that in artery strips, it is quite possible that the use of non-perfused segments in the rat studies meant that the conditions were not optimal for detecting such changes. Extensions of this study to include perfused segments would appear to be desirable.

(2) Pharmacological Implications.

(a) Perfused segments

As outlined in the General Introduction (Chapter 1) the influence of the surface of entry on the steady-state vasoconstrictor response to NA, comprising a 10-20 times greater sensitivity to INT NA than to EXT NA, has been related primarily to the location of the sympathetic nerve terminals at the medial-adventitial border. The lesser effect of EXT NA was explained in terms of its removal by the nerves as it diffused from the adventitia to the media, leading to a lower concentration at the receptors in the media.

The results of the present studies provide biochemical evidence in support of the modified model (shown in Fig. 1.2). Thus the neuronal inactivation of NA, as indicated by the formation of DOPEG and DOMA, was quantitatively much greater when NA entered via the adventitia. This finding provides a further indication that the original model proposed by de la Lande et al (1967) (shown in Fig. 1.1), where the concentration of INT NA was assumed to be uniform throughout the wall, was an over-simplification. It supports the concept that the minor role of neuronal uptake in the response to INT NA is due, in part at least, to the relative failure of INT NA to penetrate to the region of the nerve terminals.

The effect of cocaine on the inactivation of NA is, qualitatively at least, in agreement with the pharmacological data. The pharmacological experiments showed that, in the presence of cocaine, the sensitivity to EXT NA was greatly enhanced and approached that to INT NA (de la Lande and Waterson, 1967). Similarly, the present results showed that cocaine, by its potent inhibitory effect on DOPEG and DOMA efflux, eliminated the differences between the metabolisms of INT and of EXT NA. There are, however, some quantitative discrepancies. The magnitude of the decrease in concentration of EXT NA resulting from neuronal uptake, as deduced from pharmacological studies, was 10-20 fold (de la Lande et al, 1967). However, in the present study, the total increase in flux of EXT NA plus the increase efflux of NMN into the INT perfusate, produced by cocaine, was only 2.5 fold. This difference may be partly due to the different experimental conditions used in the present study, where the INT NA was recirculated continuously through the lumen for the 30 minute period

of incubation. In the pharmacological studies, the intraluminal perfusate was not recirculated. De la Lande and Graefe (included in de la Lande et al., 1980) observed that in three of four arteries perfused at the same rate as in the present study (0.5 $ml.min^{-1}$), but in which the intraluminal perfusate was not recirculated, cocaine produced increases in flux of EXT NA of the order of six fold. These workers used a Ca^{++}-free medium, but may not have prevented constriction completely, as an α-receptor blocking agent was not present. However, in view of the present evidence of Chapter 4, that when the artery constricts, i.e., increases its wall thickness, the flux of amine across the wall is decreased, the presence of an α-receptor antagonist in the present experiments cannot explain the relatively small effect of cocaine on the flux of EXT NA. This is because cocaine normally enhances the constrictor response to NA; in the experiments of de la Lande and Graefe, such an effect of cocaine would tend to reduce the flux of EXT NA across the vessel wall, below that occurring when the artery is relaxed.

A third factor to be taken into account is that, in the present experiments, the vessels were derived from reserpinised rabbits. More amine may be removed by neuronal uptake in normal arteries where the amine, after its uptake, is bound in neuronal vesicles. However, in some as yet unpublished pharmacological experiments, de la Lande and Jonsson (1981) found that, in six reserpinised vessels, cocaine potentiated the sensitivity to EXT NA by approximately 25 fold; i.e., to about the same extent as in normal arteries perfused at the same rate. These experiments were carried out at a flow rate of 2.0 $ml.min^{-1}$ in Ca^{++} medium.

Finally, it should be noted that the discrepancies between the effects of neuronal uptake on removal of EXT NA, as deduced from pharmacological and biochemical experiments, will occur if part of cocaine's effect on sensitivity to NA is due to an extraneuronal action. The metabolic experiments provided no evidence of such an action in that NMN formation from INT NA was not affected by cocaine. From its pharmacological actions, it can be argued that if an extraneuronal action of cocaine is present, it is of minor importance. This is because the increase in sensitivity to INT NA produced by cocaine was only 1.5 fold (de la Lande, 1975). However, even the argument that this small increase is primarily extraneuronal is weakened by evidence, indicated previously in Chapter 1, that cocaine does not potentiate the response of the rabbit ear artery to another α_1-receptor agonist, namely methoxamine.

(b) Role of flow rate

The influence of flow rate was examined only at a late stage of the study. It was not examined earlier in view of evidence that the diffusion coefficient of ^{14}C. sorbitol when applied to the adventitia was unaffected by increasing the flow rate from 0.25 ml.min^{-1} to 1.0 ml.min^{-1} (de la Lande et al, 1980). Furthermore, the metabolic data (Chapter 4) indicated that, in the absence of neuronal uptake, there was little difference between the metabolisms of INT and EXT NA; i.e., between the metabolism of NA when added to a well-stirred solution bathing the adventitia, and its metabolism when perfusing the lumen. However, the results of some recent pharmacological experiments (de la Lande and Jonsson, unpublished) do suggest that flow rate did influence the vasoconstrictor response to INT NA. It was found that, in reserpine

pre-treated vessels, the vasoconstrictor activity to INT NA, although not that to EXT NA, was less when the vessels were perfused at 0.5 ml.min^{-1} than at 2.0 ml.min^{-1}. This phenomenon, still under investigation, prompted the extension of the present study to include vessels perfused at 2.0 ml.min^{-1}. As shown in Chapter 4, the metabolism of INT NA, although not that of EXT NA, was increased by approximately 40% at the higher flow rate. Together with the pharmacological results, this finding implies that at the higher flow rate, INT NA penetrates more readily into the vessel wall. However, even if this is the case, the biochemical data derived from the vessels perfused at 0.5 ml.min^{-1} appears of pharmacological significance. This is because the reserpinised Ca^{++}-free vessels, perfused at 0.5 ml.min^{-1}, still displayed a marked difference between their sensitivity to INT and EXT NA (Table 4.3, Chapter 4).

(c) The magnitude of the gradient of concentration.

As indicated in the General Introduction (Chapter 1), the pharmacological and histochemical evidence suggested that, when NA was applied to one surface only, there was at least a 10-fold decline in concentration of NA across the vessel wall. The index of this ratio in the present study, i.e., the DOPEG formation ratio, in vessels perfused at 0.5 ml.min^{-1}, ranged from 4.4 in the relaxed vessel to 24 in constricted vessels. The higher value is probably more relevant to the pharmacological and histochemical studies since the latter studies were carried out on constricted vessels. This was inherent in the nature of the pharmacological studies, where estimates of sensitivity are based on ratios of INT and EXT NA which are equipotent in producing constriction. In the case of histochemical studies of de la Lande et al (1970), the vessels (reserpinised) were exposed to high concentrations of NA (3μM);

when perfused intraluminally at this concentration, the vessels were strongly constricted. Hence there is reasonable agreement between the estimates of the gradient of concentration of INT NA derived from the present studies, and those from earlier pharmacological studies. This agreement, that the ratio is high in constricted vessels, highlights the probability first emphasised by Kalsner (1972a), that the response to NA (and to other agonists) is mediated primarily by those smooth muscle cells closest to the surface of entry.

Possible explanations of the apparently smaller gradient, i.e., smaller decline in concentration of INT NA, when the vessel is relaxed, were considered in the discussion of Chapter 4 and will not be further considered here. As suggested in Chapter 4, further experiments which define the changes in the morphology of the vessel during constrictor responses, and also define the extracellular space before and at different stages of constriction appear to be the most fruitful lines of further study of the relationship between the gradient of concentration and the level of constriction.

(d) Role of extraneuronal uptake

Previously Johnson and de la Lande (1978) showed that an inhibitor of extraneuronal uptake, DOCA, caused only small, and barely significant, potentiations of the responses to INT and to EXT NA (1.1 and 1.2 fold, respectively). Potentiation by DOCA was somewhat more pronounced in the presence of cocaine, being 1.4 fold in the case of INT NA and 2.0 fold for EXT NA. Hence there appears to be a quantitative discrepancy with the pharmacological result of Johnson and de la Lande and the metabolic data of the present study, where in the case of INT NA, NMN accounted for

43% of the total metabolite efflux. That NMN was formed in a steroid sensitive extraneuronal compartment was evident by its virtual elimination by hydrocortisone. When the biochemical data is related to the pharmacological data, the results imply that, although uptake and O-methylation of NA into this compartment is the major pathway of inactivation of INT NA, this process has only a minor effect on the concentration of NA at its receptors in the smooth muscle. The difference may reflect the reported low affinity of NA for the extraneuronal uptake process (Iversen, 1967). In support, isoprenaline was taken up and O-methylated at approximately three times the rate of NA (shown in Table 6.2). The difference between the rates of O-methylation of NA and ISO accords well with the findings of de la Lande and Johnson that DOCA potentiated the dilator (β_2-receptor mediated) response to ISO on the vessel by a factor of 2.5.

As discussed in Chapter 4, the O-methylation pathway involved in the formation of the OMDA fraction was insensitive to hydrocortisone. Hence, the effect of this inactivating pathway on the concentration of NA at its receptors will not be tested by the action of DOCA on the pharmacological response to NA. Nevertheless, an indirect guide to the pharmacological importance of the OMDA pathway would be provided by the relative pharmacological data on the effects of COMT inhibition and steroid-sensitive extraneuronal uptake inhibition. Inspection of the results of Johnson (1975) shows that U0521 potentiated the response to INT and EXT NA by factors of 1.6 and 2.2 respectively (compared with 1.1 and 1.2 for DOCA under the same conditions). It would appear from these results, that the steroid-insensitive O-methylation system plays a role at least as great as that of the steroid-sensitive system in controlling the level of NA at its receptors. Unfortunately the further pharmacological

evaluation of the importance of the OMDA pathway is limited by the absence of a specific inhibitor. The present study indicated that phenoxybenzamine is an inhibitor, but its α-receptor blocking action and its lack of specificity precludes its use in such a pharmacological evaluation.

By a different approach to that of Kalsner(1972a), the studies in Chapter 7 confirmed that the concentration of NA at its receptors was significantly increased when, in the absence of neuronal and steroid-sensitive extraneuronal uptake, INT NA distribution in the wall became more uniform because it was unable to escape from the adventitial surface when the EXT bathing medium was replaced with oil. The greater effect of EXT NA, than oil, on the response to INT NA, is compatible with the presence of an inactivating mechanism which is neither cocaine or steroid sensitive. This could conceivably correspond to the OMDA formation which was insensitive to these agents.

APPENDIX 1

THE DIFFUSION OF A SUBSTANCE THROUGH A SLAB, WITH INTERNAL GENERATION OF A METABOLITE

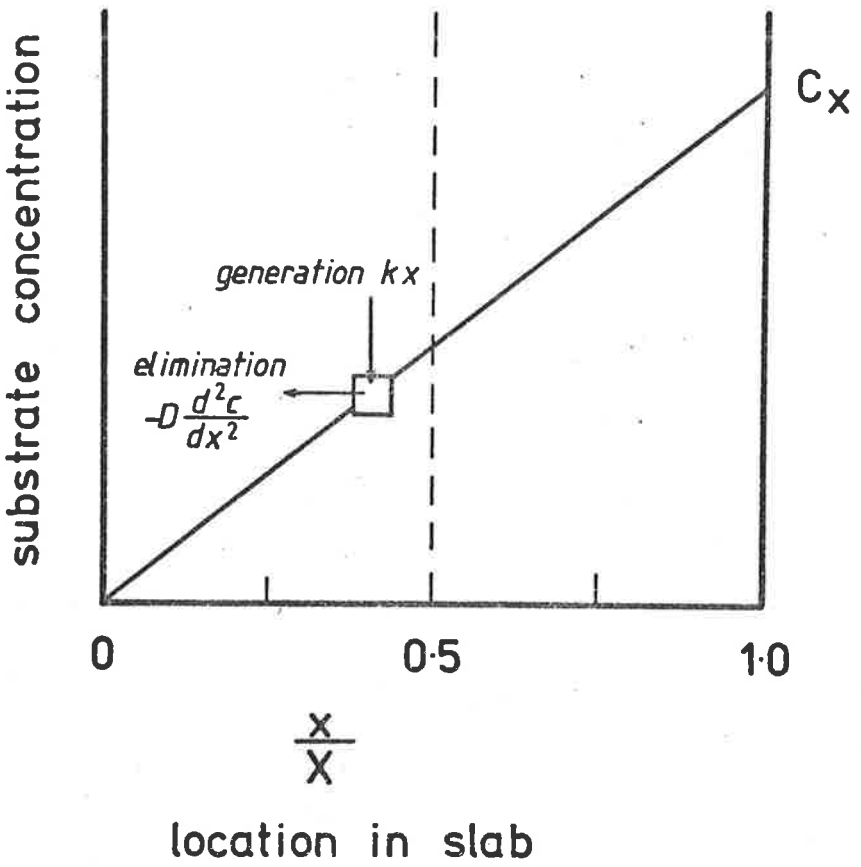

Fig. A .1 The distribution of a substance in steady diffusion through a slab of thickness X. The concentration at location x represents a balance between the rate of generation from the substrate and the rate of elimination by diffusion.

APPENDIX 1

The diffusion of a substance through a slab, with internal generation of a metabolite.

A substrate with a concentration c is applied to the face x = X of a slab of material while the face x = 0 is maintained at a concentration of zero. Under conditions of simple, steady diffusion through the slab, the profile of concentration is linear. The concentration of the substance at any position within the slab is proportional to the distance x from the face at zero concentration is shown in Fig. A.1 and is simply

$$c = \frac{x}{X} \quad \ldots\ldots\ldots\ldots (1)$$

If a metabolite is formed continuously by reaction of the substrate within the slab, it must pass from the slab by diffusion and must develop a profile of concentration to do so. The shape of the profile is determined by the condition that the rate of generation of metabolite at a particular locality and its rate of diffusion from that locality must reach equilibrium.

The rate of generation of the metabolite is presumed to be proportional to the local concentration of the substance which is, in turn, proportional to position, x, in the slab. The rate of diffusion is dictated by Fick's Law of Diffusion. The necessary equilibrium is

$$kx = -D\frac{d^2c}{dx^2} \quad \ldots\ldots\ldots (2)$$

where k is the rate constant for generation, D is the diffusivity of the metabolite and c is its local concentration.

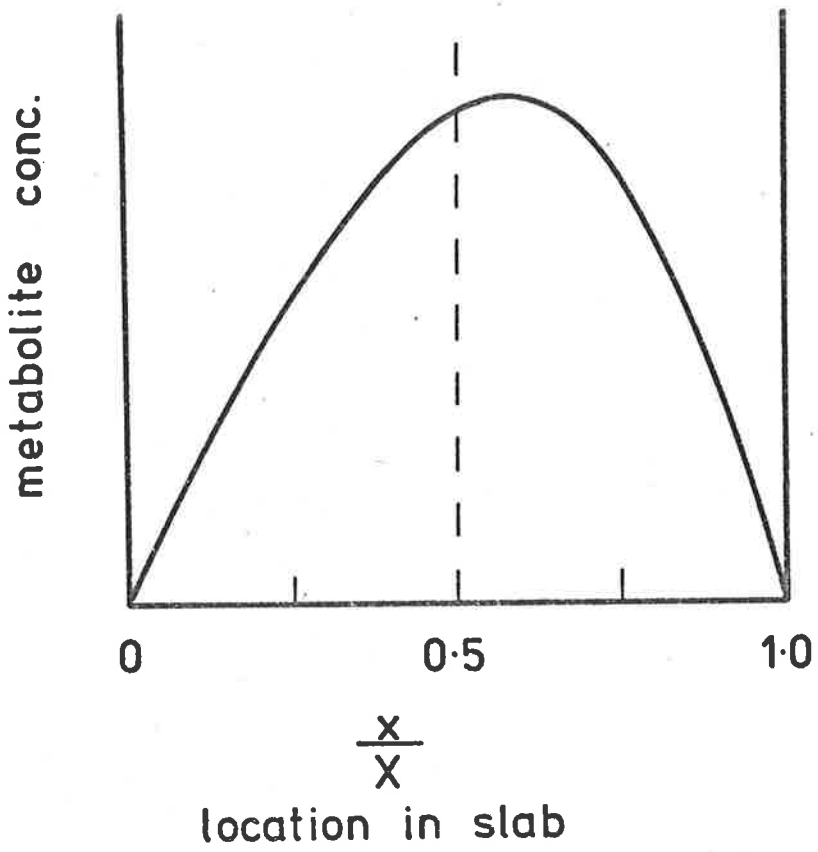

Fig. A .2 The distribution of metabolite in the slab. The maximum value is at location $^{x}/_{X}$ = 0.577. The concentration gradient and flux from face x=X is twice that from face x=0.

Integrating equation (2) from x = 0 to x = X gives

$$\frac{kx^2}{2} = -D\left(\frac{dc}{dx} - \left(\frac{dc}{dx}\right)_0\right) \quad \ldots\ldots\ldots \quad (3)$$

in which $\left(\frac{dc}{dx}\right)_0$ is the concentration gradient at the face x = 0. Integrating again yields

$$c = x\left(\frac{dc}{dx}\right)_0 - \frac{k}{D}\,\frac{x^3}{6} \quad \ldots\ldots\ldots \quad (4)$$

for the distribution of metabolite through the slab.

Invoking the conditions that the concentration of metabolite is zero at each of the faces, x = 0 and x = X, gives values for the gradient at each face of

$$\left(\frac{dc}{dx}\right)_0 = \frac{k}{D}\,\frac{X^2}{6} \quad \ldots\ldots\ldots \quad (5)$$

and

$$\left(\frac{dc}{dx}\right)_X = -\frac{k}{D}\,\frac{X^2}{3} \quad \ldots\ldots\ldots \quad (6)$$

and the formula

$$c = \frac{k}{D}\,\frac{X^3}{6}\left(\frac{x}{X} - \left(\frac{x}{X}\right)^3\right) \quad \ldots\ldots\ldots \quad (7)$$

for the distribution of concentration.

The profile of concentration is illustrated in Fig. A.2 and shows a maximum value at $\frac{x}{X} = 0.577$. The fluxes of metabolite from the two faces of the slab may be found by applying the formula

$$Q = -D\,\frac{dc}{dx} \quad \ldots\ldots\ldots\ldots\ldots \quad (8)$$

to the respective values of the concentration gradient in (4) and (5) above. The fluxes are $\frac{-kX^2}{6}$ at the face x = 0, and $\frac{kX^2}{3}$ at the face x = X.

The flux of metabolite from the face at which the substrate enters is exactly twice as great as the flux from the other face.

To apply this result to diffusion through the media of an artery, some account of the presence of the adventitia at one of the surfaces must be taken. The effect of such adventitia must be to impede diffusion from that particular surface. The balance of flows of metabolite would be shifted in favour of the opposite surface.

APPENDIX 2

DRUGS AND CHEMICALS

APPENDIX 2

Drugs and Chemicals

(1) Krebs bicarbonate solution. The following analytic grade chemicals were used to prepare this physiological bathing medium.

NaCl	120mM
KCl	4mM
$NaHCO_3$	25mM
K_2HPO_4	1mM
Glucose	6mM
EDTA	0.01mM
$CaCl_2$ *	2mM
$MgCl_2$	0.5mM
ascorbic acid **	9mM

* In many experiments $CaCl_2$ was omitted from the bathing medium.

** Ascorbic acid was normally added to warmed, gassed Krebs solution 2-3 minutes before the preparation of the ^3H.catecholamine incubating solution.

(2) Cocaine HCl (McFarlane Smith Ltd.) was dissolved in normal saline (154mM) giving a stock concentration of 2.9mM such that 0.01ml per ml of bathing medium yielded a final concentration of 29μM.

(3) Hydrocortisone sodium succinate ("Solu-Cortef", Upjohn Co., Kalamazoo) was dissolved in aqueous solution containing phosphate buffers (sodium phosphate, 3.8mM, and anhydrous sodium diphosphate, 37mM) and benzyl alcohol (46mM) giving a stock concentration of hydrocortisone of 103mM; 0.004ml of this solution per ml of bathing medium yielded a concentration of 413μM hydrocortisone.

(4) Phenoxybenzamine HCl (Smith, Kline and French Laboratories) was dissolved in normal saline (154mM) giving a stock concentration of 3.3mM; 0.01ml of this solution per ml of bathing medium yielded a concentration of 33μM.

(5) U0521 (3,4-dihydroxy-2-methyl propiophenone) (The Upjohn Co., Kalamazoo) was dissolved in normal saline (154mM) containing ascorbic acid (0.6mM), giving a stock solution of 5.5mM; 0.01ml of this solution per ml of bathing medium yielded a concentration of 55μM.

(6) Deoxycorticosterone acetate (4-pregnen-21-ol-3,20-dione, Koch-Light Laboratories Ltd., Coinbrook Bucks, England) was dissolved in ethanol (analytical grade) giving a stock solution of 5.4mM; 0.005ml of this solution per ml of Krebs solution yielded a concentration of 27μM.

(7) Reserpine (Serpasil, Ciba) was dissolved with phosphoric acid, ascorbic acid, 'Versene Fe3', in propylene glycol and sterile water for injection. Each ampoule (1ml) contained 4.1mM reserpine.

(8) Alumina (Merck-AG) was prepared by the method of Crout (1961). The principal of this method involves gently boiling 200-300g in 1.0 litre of 2M HCl for 30 minutes. When cool the acid was decanted and the alumina washed 12 to 15 times with distilled water, allowing 5 minutes between washes. The pH was adjusted to 4-5 before drying the alumina in an evaporation dish for 2-3 hours in an oven at 100^{o}C.

(9) DOWEX-50W (Sigma Chemical Company) hydrogen form (4% cross linked and dry mesh 200-400) was purified as described by Graefe et al (1973). The principal of this method involves repeated washing of 200-300g of the resin with 2M NaOH (containing 1% EDTA) at 50°C until the supernatant is clear; the resin is then washed 3-4 times with (i) distilled water, and (ii) with 6M HCl:ethanol (1:1), and then equilibrated to pH = 2 with 0.01M HCl.

(10) (-)noradrenaline bitartrate (l-arterenol bitartrate, Sigma Chemical Company) was dissolved in normal saline (154mM) containing ascorbic acid (0.6mM), giving a stock solution of 0.6M (as the base). Dilutions were made as required.

(11) (±)Isoprenaline HCl (dl-isoproterenol HCl, Sigma Chemical Company) was prepared as in (10).

(12) Paraffin oil (F.H. Faulding and Co. Ltd., Adelaide) was warmed to 37°C and bubbled with 95% O_2, 5% CO_2 prior to use.

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