METABOLISM OF CATECHOLAMINES IN THE RABBIT EAR ARTERY

A THESIS SUBMITTED FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

by

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SOUTH AUSTRALIA

November 1976

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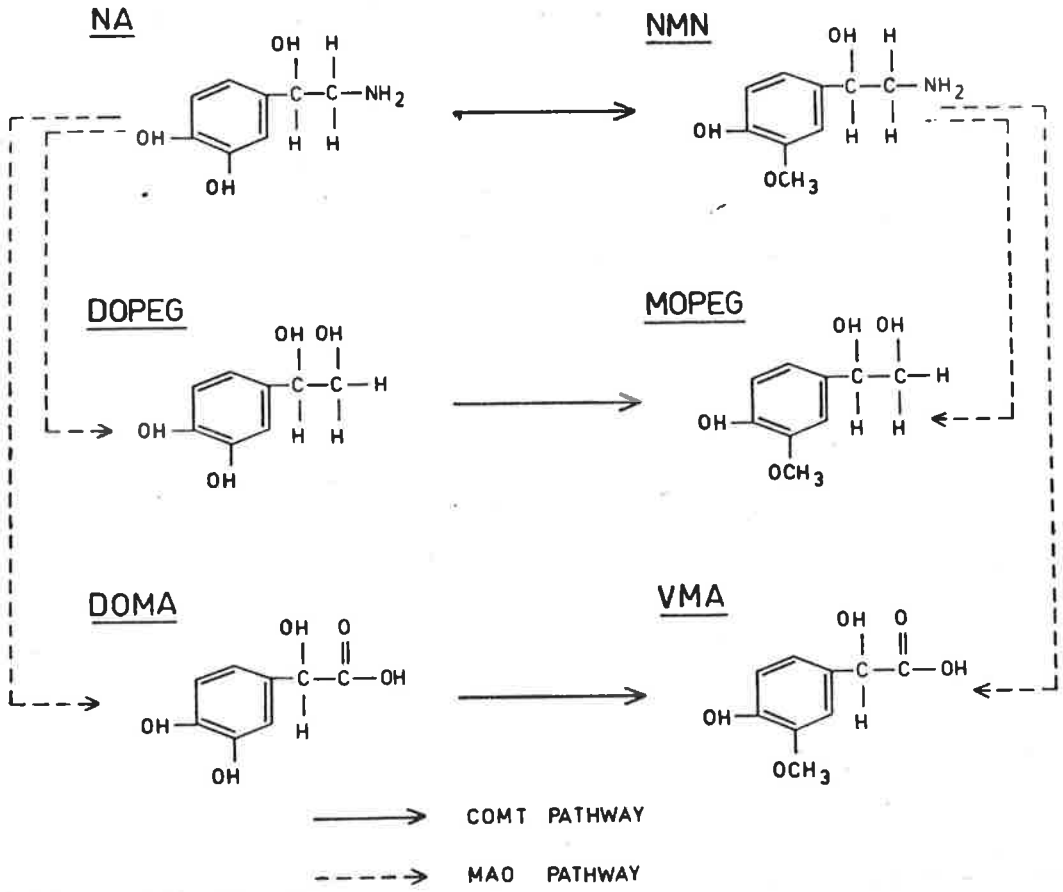

Structures of the metabolites of noradrenaline referred to in this thesis.

SUMMARY

(1) This thesis describes a series of biochemical investigations on the rabbit ear artery. Their purpose was to determine the neuronal and extraneuronal distributions of the enzymes monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) and to assess their importance in the metabolism of catecholamines (CA's).

(2) MAO activity as determined by deamination of tyramine in homogenates of arteries was approximately 90% extraneuronal in distribution. COMT activity appeared to be entirely extraneuronal.

(3) Studies on the uptake of tritiated noradrenaline (^{3}HNA) showed that the metabolites formed were: 3,4-dihydroxyphenyl glycol (DOPEG), 3,4-dihydroxy mandelic acid (DOMA), 4-hydroxy-3-methoxy phenyl glycol (MOPEG), 4-hydroxy-3-methoxy mandelic acid (VMA) and normetanephrine (NMN).

(4) The proportion of metabolites that accumulated in the solution bathing the arteries greatly exceeded the proportion in the tissue. DOPEG was the major metabolite formed.

(5) Based on the change in distribution of metabolites in the tissue caused by chronic denervation or cocaine it was concluded that DOPEG was the main metabolite formed by neuronal MAO and that the formation of NMN was entirely extraneuronal. This conclusion was consistent with studies on the efflux of the metabolites from arteries in the presence of PBZ; these studies showed that inhibition of MAO did not significantly divert metabolism of NA from the MAO pathway to the COMT pathway.

(6) The function of the extraneuronal COMT was further studied using tritium labelled isoprenaline (^{3}HISO) as substrate instead of ^{3}HNA. Isoprenaline was used in view of other evidence that this amine is not taken up by sympathetic nerves. The results showed that ^{3}HISO is accumulated by this artery and metabolised by COMT to form 3-methoxy isoprenaline (MeOISO). Inhibition of COMT with the inhibitor 3,4-dihydroxy-2-methyl propiophenone (U0521) caused an increase in accumulation of ^{3}HISO that was not sensitive to cocaine but sensitive to the extraneuronal uptake inhibitor deoxycorticosterone acetate (DOCA). It was concluded that extraneuronal accumulation of ^{3}HISO was limited by the activity of COMT.

(7) The preceding study revealed a small but significant decrease in the artery content of unchanged ^{3}HISO following denervation, suggesting that, in the artery at least, there is some neuronal uptake of isoprenaline. In case neuronal factors had influenced the inhibitory effect of DOCA on the O-methylation of isoprenaline, the effect of DOCA on uptake and O-methylation of isoprenaline in chronically-denervated arteries was examined. The results were identical to those obtained using innervated arteries, thus establishing beyond doubt that DOCA's inhibition of O-methylation is entirely extraneuronal in nature. This action was explained in terms of impairment by DOCA of access of ^{3}HISO to COMT.

(8) The kinetics of efflux of ^{3}HISO from arteries was examined to determine whether it was consistent with a multicompartmental system for the uptake and O-methylation of this amine. The results were consistent with an efflux of ^{3}HISO from two compartments of which the more rapid was similar to that from which ^{3}HMeOISO effluxed.

(9) In further experiments on COMT inhibited arteries it was shown that DOCA served to decrease the rate of efflux of ^{3}HISO from the compartment from which ^{3}HISO effluxes rapidly. It was concluded that the site of action of DOCA in the rabbit ear artery is a single compartment from which both ^{3}HISO and ^{3}HMeOISO efflux.

(10) Some preliminary studies on the metabolism of ^{3}HNMN by MAO are described which suggest that part of this metabolism is attributable to neuronal MAO. The metabolism of ^{3}HNMN by extra-neuronal MAO occurs in a compartment which is sensitive to DOCA.

(11) The results of this thesis are considered in; (a) relation to earlier pharmacological studies on the functional significance of neuronal and extraneuronal MAO and of extraneuronal COMT, and (b) in relation to studies on the distribution of NA and its metabolites in other sympathetically innervated tissues.

(12) Included in this thesis are studies relating to methodological aspects of experiments involving catecholamines and their metabolites. These comprised:

(a) development of a semi-automated colourimetric assay for the estimation of NA and its metabolites designed as an adjunct for experiments involving chromatographic separation of these compounds.

(b) development of a thin layer chromatographic procedure for the separation of catecholamines and their metabolites based on the principal of sodium borate chelation of catechols.

(c) a study relating to the use of the semi-automated tri-hydroxyindole assay for the measurement of catecholamines in biological samples.

(d) a study of the purity and stability of high specific activity tritiated catecholamines.

(e) a description of an electrolytic oxidative O-demethylation process for the formation of catecholamines from 3-O-methyl catecholamines.

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.

Richard John Head

November 1976

PUBLICATIONS

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

Proc. Aust. Phys. Pharm. Soc. 5: No. 1 80-81 (1974).

Clin. exp. Pharm. and Physiol. Suppl. 2: 39-42 (1975).

Proc. Aust. Phys. Pharm. Soc. 6: No. 2 143-144 (1975).

Aust. J. exp. Biol. med. Sci. 54: 35-42 (1976).

Proc. Aust. Phys. Pharm. Soc. 7: 2 III (1976).

Clin. exp. Pharm. and Physiol. In press.

J. chromatog. Sci. In press.

ACKNOWLEDGEMENTS

My supervisor Professor I.S. de la Lande, for his invaluable advice and criticisms throughout the course of this study. Professor R.E. Stitzel with whom I collaborated for some of the experiments described in Chapter 5.

Of the studies described in the main body of the thesis I wish to acknowledge the skilful assistance of Miss Jennifer Kennedy and expert advice of Mr. R.J. Irvine in the application of the thin layer chromatographic procedure to the separation of radioactively labelled catecholamines. Miss Julie Fischer who assisted in completing the studies described in Chapter 9.

Dr. D.B. Frewin and Mr. G.A. Crabb with whom I collaborated in experiments described in Appendix I, Dr. D.A.S. Parker and Mr. G.A. Crabb with whom I collaborated for experiments described in Appendix II and Appendix III.

Mrs. Barbara Harrison for skilful typing, Mr. G.A. Crabb and Mrs. A. Raymond for expert assistance in the preparation of figures.

The Life Insurance Medical Research Fund of Australia and New Zealand for financial support during the years 1972 to 1976.

I wish to acknowledge again the assistance of Mrs. Barbara Harrison, Miss Jennifer Kennedy, Miss Julie Wilkins, Mr. R.J. Irvine and Mr. G.A. Crabb for assistance in many diverse ways during the course of this study.

CHAPTER 1

INTRODUCTION

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During the past decade attention has been focussed on the role played by cellular mechanisms for the inactivation of catecholamines (CA's) in the control of vascular sensitivity of arteries. There is at present strong evidence to suggest that at least four mechanisms are involved in the control of cellular and extracellular concentrations of noradrenaline (NA) in sympathetically innervated tissues. These are (i) uptake into sympathetic nerve terminals (neuronal uptake) (ii) uptake into effector cells (extraneuronal uptake) (iii) metabolism by monoamine oxidase (MAO) (iv) metabolism by catechol-O-methyl transferase (COMT). The experiments to be described in this thesis were undertaken to investigate the nature of the inactivation mechanisms for catecholamines in the rabbit ear artery.

It is the purpose of this Introduction to summarise some properties of the relevant inactivation mechanisms. Subsequently attention will be directed toward those pharmacological properties of the rabbit ear artery upon which both the nature and direction of the experiments to be described in this thesis were based.

(i) Neuronal Uptake

It is now widely accepted that in sympathetically innervated tissues NA is removed from the extracellular fluid and actively transported into the axoplasm of sympathetic nerves. The influx of NA across the neuronal membrane is referred to as *neuronal uptake*.

The discovery of the ability of postganglionic sympathetic neurones to accumulate exogenously applied catecholamines stemmed from pioneering studies from the laboratory of Axelrod, which showed that tissue uptake served to remove intravenously administered catecholamines from the circulation (Axelrod *et al.* 1959; Whitby *et al.* 1961). These authors studied the distribution of ^{3}HNA in different tissues of the cat after infusion with tritiated NA (^{3}HNA) and found uptake of ^{3}HNA was greatest for tissues characterised by a rich sympathetic innervation (Whitby *et al.* 1961). The refinement of the techniques of autoradiography and electron microscopy, together with the advent of a specific fluorescence histochemical procedure for the cellular localisation of catecholamines, enabled investigators to establish the precise sites of accumulation of exogenous NA in tissues. Perhaps the most convincing evidence that catecholamine uptake can occur in sympathetic nerves came from the studies of Gillespie and Kirpekar (1965) who used techniques similar to those described above to demonstrate that part of the ^{3}HNA infused into the cat spleen accumulated in the nerve fibres which contain the endogenous catecholamine.

The elucidation of the properties of this uptake process has been the subject of numerous investigations in the past decade. Iversen (1963, 1965) established that neuronal uptake (referred to as $Uptake_1$ by that author) in the isolated perfused rat heart was a saturatable process and obeyed Michaelis-Menten kinetics. This process was characterised by a high affinity for the l isomer of NA (Km 0.27×10^{-6} M; Iversen 1967), a lower affinity for the d isomer (Km 1.39×10^{-6} M) and a marked dependence on sodium ion concentration (Iversen and Kravitz 1966). Burgen and Iversen (1965) examined the effects of various sympathomimetic amines on the uptake of NA in the isolated perfused rat heart and estimated the concentrations of these compounds required to produce a 50% inhibition of uptake of NA. In this way they were able to reveal the structure-action relationships that characterised this uptake process. They observed that N-substitution of NA decreased the affinity of the amine for the uptake site, thus NA had a higher affinity for neuronal uptake than did adrenaline (A) or isoprenaline (ISO). O-methylation of phenolic hydroxyl groups also served to decrease the affinity of the amine for the uptake site. Other compounds structurally unrelated to sympathomimetic amines were also shown to inhibit this uptake process and these included cocaine and the adrenergic blocking agent phenoxybenzamine (PBZ). There is now considerable evidence supporting the view that much of the NA in sympathetic nerves is located within specific storage vesicles. Von Euler and Hillarp (1956) showed that portion of the NA present in bovine splenic nerves was associated with particles that could be isolated by differential centrifugation.

Since then numerous studies have provided evidence for the distribution of exogenously applied radioactively labelled NA into intraneuronal storage sites. Potter and Axelrod (1963) showed that the subcellular distribution of ^{3}HNA in the rat vas deferens closely paralleled that of endogenous NA. A significant amount of both labelled and non-labelled NA was associated with microsomal particles which contained storage vesicles. The importance of these vesicles in the accumulation of NA by sympathetic nerves has been demonstrated by the use of reserpine, a drug which interferes with the incorporation of NA into storage vesicles. Kopin *et al.* (1962) showed that isolated hearts from rats treated with reserpine rapidly lose ^{3}HNA from the tissues after infusion of the amine ceases. Iversen *et al.* (1965) confirmed these earlier observations and concluded that although the amount of NA taken up from the perfusing medium was the same as for untreated hearts the amine failed to accumulate in the tissue. Moreover, Kopin (1962) showed in reserpine treated hearts that NA is released not as the unchanged amine but as a deaminatedmetabolite suggesting that storage vesicles play an important role in controlling the level of substrate available to neuronal MAO.

Evidence that neuronal uptake (under certain conditions) is an important factor in reducing the concentration of NA at adrenergic receptors has been reviewed recently by Trendelenburg (1966, 1972). This evidence includes the findings that the morphological degeneration of sympathetic nerve terminals in the cat nictitating membrane (van Orden 1967) and the decrease in ability of degenerating nerve

terminals to retain exogenous NA (Smith *et al.* 1966) are temporarily related to denervation supersensitivity of this tissue to NA. The influence of vesicular structures within sympathetic nerves (under certain conditions) in influencing the concentration of NA at adrenergic receptors has also been investigated in the cat nictitating membrane (Trendelenburg 1971) and the rabbit ear artery (de la Lande and Jellet 1972).

(ii) Extraneuronal Uptake

There is now strong evidence supporting the view that NA is taken up extraneuronally into the effector cells of sympathetically innervated tissues. The process of transfer of NA from extracellular fluid onto or into a cell other than neurons is referred to as *extraneuronal uptake*.

Evidence for the extraneuronal binding of NA is derived from the studies of Fischer *et al.* (1965) who noted that a fraction of the uptake of NA into the salivary gland persisted after denervation and treatment with reserpine. This observation supported an earlier suggestion by Anden *et al.* (1963) that some extraneuronal binding of NA occurred in this tissue. In studies concerned with the uptake of NA in the isolated perfused heart, Iversen (1965) noted that when hearts were perfused with media containing NA or A an abrupt and rapid uptake of amine occurred at about 5 μmol l^{-1}. This phenomenon occurred at concentrations which were greater than those shown previously by Iversen to saturate neuronal uptake ($Uptake_1$). This new process was also characterised by its ability to lead to tissue contents greater than those attributable to simple diffusion of amine throughout the tissue. Furthermore the initial rates of

uptake could be described by Michaelis-Menten kinetics. Iversen referred to this new process as $Uptake_2$ to distinguish it from neuronal uptake ($Uptake_1$). The properties of this process were investigated and shown by Iversen to differ markedly from those seen for neuronal uptake. $Uptake_2$ exhibited a low affinity for dlNA (approximately 250 µmol) but a high capacity for binding of amine. This process lacked stereochemical selectivity and both A and ISO accumulated in the rat heart at rates greater than those seen for NA at comparable concentrations. In contrast to $Uptake_1$ this process was insensitive to cocaine and most of the amine (NA or A) accumulated by $Uptake_2$ was readily removed by perfusing the tissue with an amine-free medium. Inhibitors of this process included the O-methylated derivatives of CAs (viz. normetanephrine (NMN) and metanephrine (MN)) and PBZ. A summary of some of these properties together with a comparison of the properties of neuronal uptake appears in Table 1.1.

Extraneuronal binding of NA by collagen and elastin and accumulation of NA in the cytoplasm of smooth muscle cells following incubation of the rabbit ear artery with NA in high concentrations was described by Avakian and Gillespie (1968). Gillespie (1968) summarised the properties of smooth muscle uptake and compared them with those of $Uptake_1$ and $Uptake_2$ of Iversen. Close similarities were apparent between smooth muscle uptake and $Uptake_2$ (Table 1.1, from Gillespie 1968). Subsequently Gillespie and Muir (1970) found considerable species variation in the ability of smooth muscle to accumulate NA. This accumulation was most prominent in the mouse and rabbit and poorly developed in the guinea pig.

There is now considerable evidence suggesting that the fate of NA in effector cells is related to the metabolism of this amine by the enzymes MAO and COMT. Lightman and Iversen (1969) incubated the rat heart with low concentrations of substrate (NA < 14.8 μmol l^{-1}; A < 4.5 μmol l^{-1}) and showed that, when these metabolizing enzymes were inhibited, the contents of metabolites were stoichiometrically replaced by an accumulation of unchanged amine. On this basis, they revised the theory of a threshold phenomenon of extraneuronal uptake to accommodate the possibility that extraneuronal uptake occurred at all amine concentrations in the rat heart. Consistent with this finding was the histochemical observation by Burnstock *et al.* (1971) that inhibition of COMT and MAO in the rabbit ear artery lowered the threshold for uptake of NA from 59 μmol l^{-1} to 0.59 μmol l^{-1}.

The physiological importance of extraneuronal uptake remained obscure due primarily to the diversity of actions of compounds known to inhibit $Uptake_2$. However, recently it has been established that certain steroids reduce both the accumulation of NA and the formation of metabolites in the rat heart (Iversen and Salt 1970). These authors concluded that these compounds served to inhibit $Uptake_2$. This new class of inhibitor has been used recently by several groups of investigators to establish the physiological significance of extraneuronal uptake. In essence these studies emphasized the significance of O-methylation as the major extraneuronal pathway and pointed to an intimate relationship between COMT activity and extraneuronal uptake. A more detailed account of this relationship appears in the discussion to Chapter 8 and in Chapter 10.

TABLE 1.1*

Comparison of some properties of uptake$_1$, uptake$_2$ and smooth muscle uptake of noradrenaline

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

* Based on that of Gillespie (1968)

PBZ = phenoxybenzamine

NMN = normetanephrine

(iii) MAO

Monoamine oxidase includes a group of enzymes which oxidatively deaminate aliphatic and aromatic amines to their corresponding aldehydes. The properties of MAO have been extensively reviewed (Blashko 1966; Gorkin 1966; Blashko 1972 and Tipton 1972). A summary of some of these properties that are relevant to its role in the inactivation of intracellular catecholamines in sympathetically innervated tissues is shown in Table 1.2. Many derivatives of phenylethylamines are substrates for MAO including NA and its 3-O-methyl metabolite, normetanephrine (NMN, Tipton 1972). MAO exhibits stereochemical specificity and has a greater affinity for laevo than for the dextro isomer of NA (Pratesi and Blashko 1959). Among the catecholamines which are not deaminated by MAO are the α-methyl derivitive of noradrenaline (α-methyl noradrenaline, Blashko *et al.* 1937) and the N-isopropyl derivative of noradrenaline (Isoprenaline, Hertting 1964).

A large number of compounds which inhibit MAO have been described and many of the biochemical aspects of these inhibitors are discussed in a recent review by Ho (1972). Two of these inhibitors, nialamide and pargyline have been used frequently in biochemical and pharmacological studies concerned with the functional role of MAO.

There is extensive evidence supporting the existence of multiple forms of MAO although it is not entirely clear whether these forms represent MAO from different populations of cells or different forms of the enzyme within one cell population. Various

studies have suggested that these multiple forms exhibit different physico-chemical properties which include differences in electrophoretic mobilities (Youdim and Sandler 1967, Kim and D'orio 1968) substrate specificities (Youdim *et al.* 1969) and thermal stabilities (Youdim and Sourkes 1965). There is accumulating evidence that suggests for some tissues that the MAO associated with sympathetic nerves is different than that associated with extraneuronal structures. This difference has been demonstrated in the rat pineal gland (Goridis and Neff 1971, Goridis and Neff 1972), the rat mesenteric artery (Goridis and Neff 1973) and the rat vas deferens (Jarrott 1971). Much of the evidence is based on the fact that the MAO associated with sympathetic nerves (referred to as type A MAO by Goridis and Neff) is completely inhibited at low concentrations of the MAO inhibitor clorgyline, is relatively heat stable and deaminates NA and 5-hydroxytryptamine (5HT). In contrast the extraneuronal enzyme (called type B MAO) is heat labile and does not appreciably deaminate NA or 5 HT. The MAO substrates (Tyramine and Tryptamine) commonly used in biochemical and histochemical procedures for estimation of MAO activity are metabolised by both Type A and Type B MAO (Squires 1972).

The availability of histochemical procedures for the direct cellular localisation of MAO has helped in determining the cellular distribution of this enzyme in sympathetically innervated tissues. Extraneuronal MAO has been demonstrated histochemically in a variety of tissues which include the rat salivary gland (Almgren *et al.* 1966 rat heart (Horita and Lowe 1972) and rabbit ear artery (de la Lande *et al.* 1970). Histochemical evidence for neuronal MAO includes

evidence for the movement of MAO down the sciatic nerve (Dahlström *et al.* 1969).

Less direct evidence for the presence of neuronal and extra-neuronal MAO in sympathetically innervated tissues has come from biochemical studies in which a decrease in the MAO activity of homogenates was observed in some tissues following chronic denervation. Tissues which exhibited this decrease included the rat pineal gland (Snyder *et al.* 1965), and the cat nictitating membrane (Jarrott and Langer 1970). Significantly however in some tissues the use of histochemical and biochemical procedures have not revealed a decrease in the MAO activity of denervated tissues. These have included histochemical studies on the rabbit ear artery (de la Lande *et al.* 1970)and biochemical investigations of the rabbit ear artery (Armin 1953) the rat kidney, heart and small intestine (Jarrott 1971). Reasons for the failure to detect decreases in MAO activity in denervated tissues have been offered by Jarrott (1971). This explanation relates to the minimal amounts of MAO in sympathetic nerves compared to the activities in other cells of the tissue, the degree of sympathetic innervation of the tissues studied and the affinities of the substrates used in these experiments to demonstrate neuronal and extraneuronal MAO. Evidence for the presence of neuronal and extraneuronal MAO in blood vessels includes studies in which the MAO activity in homogenates of isolated adventitial and medial structures of the rabbit aorta have been determined (Verity *et al.* 1972). In that study MAO activity was detected in the adventitia (mainly neuronal) and media (mainly extraneuronal), the predominant activity being associated with the media. MAO activity has been demonstrated in homogenates of the rabbit ear artery

(Burnstock *et al.* 1972, Armin 1953) and shown histochemically to be present in the smooth muscle cells of the rabbit ear artery (de la Lande *et al.* 1970). Similar evidence for a neuronal origin of this activity was lacking at the time at which the studies described in this thesis were commenced.

The specific role of MAO in the metabolism of NA has been elucidated by biochemical studies using intact isolated sympathetically innervated tissues. Although it has been shown that MAO catalyses the formation of aldehydes from amines in tissue homogenates it is now well established that in many intact tissues these aldehydes are of transient existence and either oxidized to their corresponding acid derivatives or reduced to the corresponding alcohol. It is believed that this oxidation is catalysed by an aldehyde dehydrogenase (Erwin and Deitrich 1966) and the reduction of the aldehyde catalysed by an aldehyde reductase (Tabakoff and Erwin 1970). In keeping with this concept it has been shown that in many sympathetically innervated tissues including the rat heart, rat vas deferens and rabbit aorta that the deaminated metabolites of NA may include 3-methoxy-4-hydroxy phenylglycol (MOPEG), dihydroxyphenylglycol (DOPEG), 3-methoxy-4-hydroxy vanillyl mandelic acid (VMA), dihydroxy mandelic acid (DOMA).

A comparison of the pattern of these metabolites between innervated and denervated or cocaine-treated tissues has enabled the relative contributions of neuronal and extraneuronal MAO to the metabolism of NA to be determined. This will be discussed in greater depth in the discussion section of Chapter 6.

Table 1.2

Some properties of MAO

Reaction catalysed	deamination of amines including noradrenaline, normetanephrine, tyramine and tryptamine to form corresponding aldehydes
Cofactor requirement	O_2
Cellular localisation	Mitochondria
Inhibitors include	nialamide and pargyline

(iv) COMT

Catechol-O-methyl transferase is a cytoplasmic enzyme that effects the 3-O-methylation of catecholamines and utilizes the methyl group donor S-adenosyl methionine (S-AMe) for this transmethylation reaction. Since its discovery by Axelrod (1957) there have been extensive investigations into the properties and physiological function of this amine. These investigations have been reviewed recently by Guldberg and Marsden (1975). The functions and regulatory importance of the cofactor S-AMe have also been reviewed (Lombardini and Talalay 1967).Some of the properties of this enzyme that are relevant to this thesis are summarized in Table 1.3. The enzyme is not stereochemically specific and exhibits similar affinities for the d and l isomers of NA (Axelrod and Tomchick 1958). There is evidence for multiple forms of this enzyme although the biological function and significance of this multiplicity is uncertain (Guldberg and Marsden 1975). A variety of inhibitors of COMT have been described and include derivatives of pyrogallol, catechol and compounds that are isoteric with catechols. Inhibitors used in biochemical and pharmacological studies are 3,4 dihydroxy-2-methyl propiophenone (UO521), pyrogallol and tropolone. Some commonly employed pharmacological agents have been tested for an action on this enzyme. Thus ascorbic acid was shown to be a weak inhibitor of COMT (Blaschke and Hertting 1971). PBZ and cocaine were shown not to be inhibitors of COMT (Eisenfeld *et al.* 1967 and Holtz *et al.* 1960). It is of interest that the principal metabolites of oestrogens, the 2-hydroxylated oestrogens have been shown to be inhibitors of rat liver COMT (Knuppen *et al.* 1969). This is because the parent

steroids are included amongst steroids which are inhibitors of extraneuronal accumulation of NA. These parent steroids are apparently without effect on COMT activity in tissue homogenates (Hapke and Green 1970).

The relative neuronal and extraneuronal disposition of COMT is not completely defined. The main reason seems to be the lack of a histochemical procedure for the cellular localisation of this enzyme. The predominantly medial origins of COMT activity in the rabbit aorta (Verity *et al.* 1972) and the persistance of all or part of the COMT activity of many tissues following denervation has been taken as evidence for a predominantly extraneuronal origin of this enzyme.

In some studies a decrease in COMT activity has been demonstrated after chronic denervation. In an early study Crout and Cooper (1962) showed a decrease in COMT activity of the sympathetically denervated cat heart. Subsequently many other groups failed to demonstrate a decrease in COMT activity in a variety of denervated peripheral tissues. One of the reasons may have been that the conditions for measurement of COMT were not optimal. This follows from the findings of Jarrott (1971) who showed that an optimal concentration of S-AMe was required to demonstrate a decrease in COMT activity in the denervated rat vas deferens. Jarrott demonstrated a decline in COMT activities after denervation of the rabbit and rat vas deferens and cat nictitating membrane. A significant decline was not seen for other tissues investigated which included the vas deferens of the guinea pig. The functional significance of extraneuronal COMT

relates to the metabolism of cytoplasmic NA in the effector cells. The functional significance of neuronal COMT is not clear. In the cat nictitating membrane the presence of neuronal and extraneuronal COMT as demonstrated by Jarrott and Langer (1971) in homogenate studies is in good agreement with the findings that suggest that in the intact tissue ^{3}HNA is O-methylated at both intra- and extra-neuronal sites (Langer 1970). However in the rat vas deferens which also exhibits neuronal COMT activity in homogenates (Jarrott and Iversen 1971) their is no evidence of neuronal O-methylation of ^{3}HNA in the intact tissue (Langer 1970). A possible relationship between neuronal O-methylation and the biosynthesis of NA has been suggested by Rubio and Langer (1973). This was based on the observation that DOPEG, a metabolite formed by neuronal MAO inhibits tyrosine hydroxylase activity. Neuronal O-methylation of this glycol may indirectly regulate the activity of tyrosine hydroxylase and in this way the rate of synthesis of NA. Prior to the commencement of the studies described in this thesis it was shown by Burnstock *et al.* (1972) that the rabbit ear artery possessed COMT activity similar to that observed for other tissues of the rabbit. There was no information regarding the neuronal or extraneuronal distribution of the COMT activities of this artery. More recently Levin (1974) has characterized the nature of the metabolites of NA formed in intact and isolated adventitial and medial segments of the rabbit aorta. The results of these experiments will be discussed in subsequent chapters.

Table 1.3

Some properties of COMT

Reaction catalysed	O-methylation of catechols including noradrenaline, dihydroxyphenyl glycol and dihydroxymandelic acid
Cofactor requirement	S-adenosyl methionine
Cellular localisation	Cytoplasmic
Inhibitors include	Catechol derivatives and tropolones

(v) Relationship to pharmacology of the rabbit ear artery

Early pharmacological studies on the rabbit ear artery (de la Lande *et al.* 1967) indicated that neuronal uptake was important in determining the sensitivity to NA when it was applied extra-luminally (to the adventitia) but not when it was applied intra-luminally (to the intima). Their evidence was that cocaine treatment or denervation markedly potentiated extra-luminal NA but not intra-luminal NA. Since the nerves are located in the adventitia, the relatively small influence of neuronal uptake on intra-luminal NA was interpreted as being due to NA having its effect on α receptors before reaching the site of neuronal uptake (Figure 1.1).

However it was also possible that extraneuronal uptake processes and metabolism in the media smooth muscle cells may have limited the access of NA to neuronal uptake sites when NA was applied intra-luminally (Figure 1.2). An influence of extraneuronal uptake and metabolism on NA's access to the sympathetic nerves was suggested by the histochemical data of de la Lande *et al.* (1974). Application of intraluminal NA (3 μmol l^{-1}) did not restore fluorescence in MAO inhibited arteries which had been depleted of NA with reserpine. However some fluorescence was restored when MN (an inhibitor of extraneuronal uptake) and UO521 (an inhibitor of COMT) were present.

Pharmacological studies in cocaine treated ear arteries by Johnson (Ph.D. thesis 1975) indicated a two fold potentiation of NA responses when COMT was inhibited with UO521.

Although histochemical evidence (de la Lande *et al.* 1970) suggests that MAO is mainly extraneuronal in the rabbit ear artery the pharmacological evidence of de la Lande and Jellet (1972)

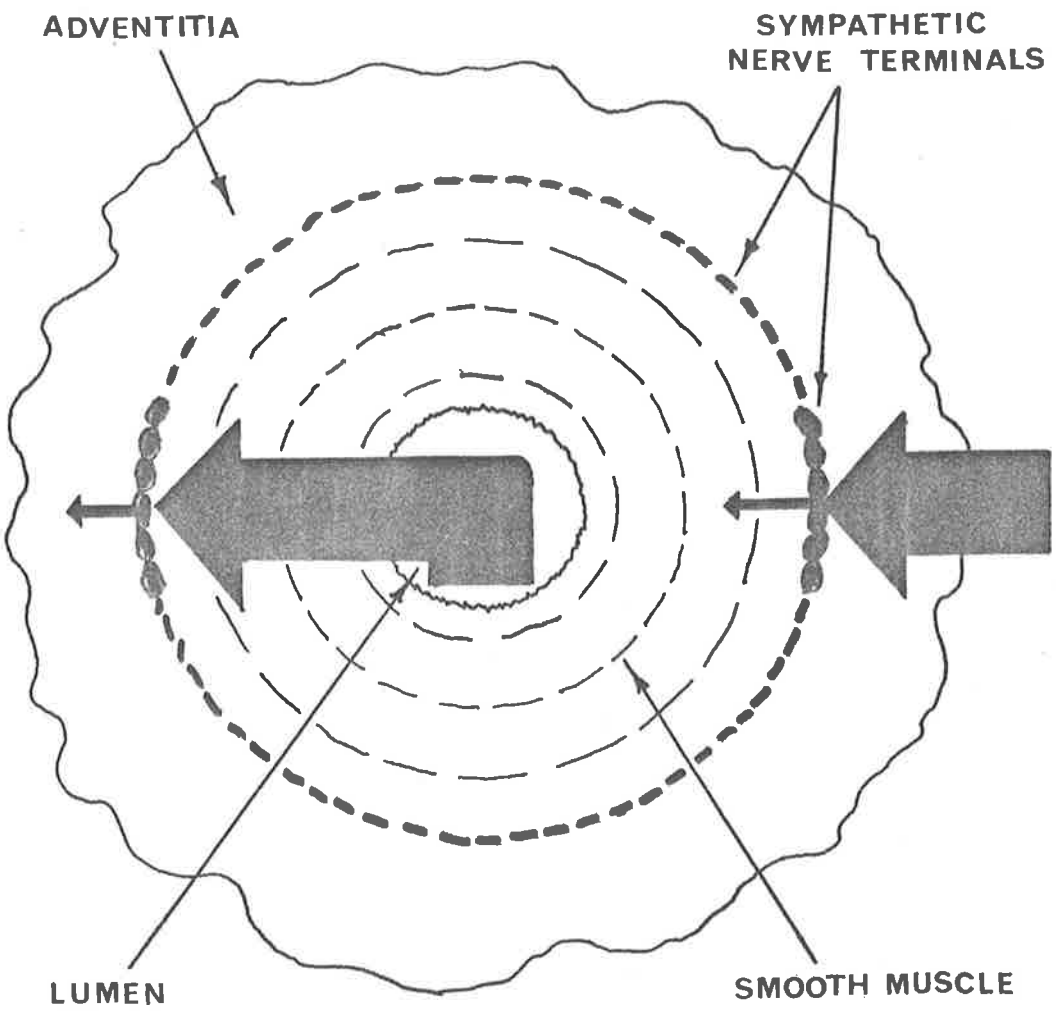


Fig. 1.1 Diagrammatic representation of the influence of uptake by the sympathetic nerve terminals on the concentration of NA in the smooth muscle of the artery. The direction of the arrows indicates the direction of diffusion of NA. Thicknesses of arrows represent concentrations of NA. The model implies free penetration of both extraluminal NA (through the adventitia) and intraluminal NA (through the media) to the sympathetic nerve terminals.

suggests that it is the neuronal MAO which is important in determining the sensitivity to NA. Potentiation of NA by MAO inhibition occurred only with extra-luminal NA and was lost after cocaine treatment or denervation. Two questions arising from these pharmacological studies were:

(i) What is the disposition of COMT and MAO between neuronal and extraneuronal sites in the rabbit ear artery?

(ii) What is the relationship between these enzyme activities and the neuronal and extraneuronal accumulation of NA?

Answers to these questions have been sought in the studies described in this thesis.

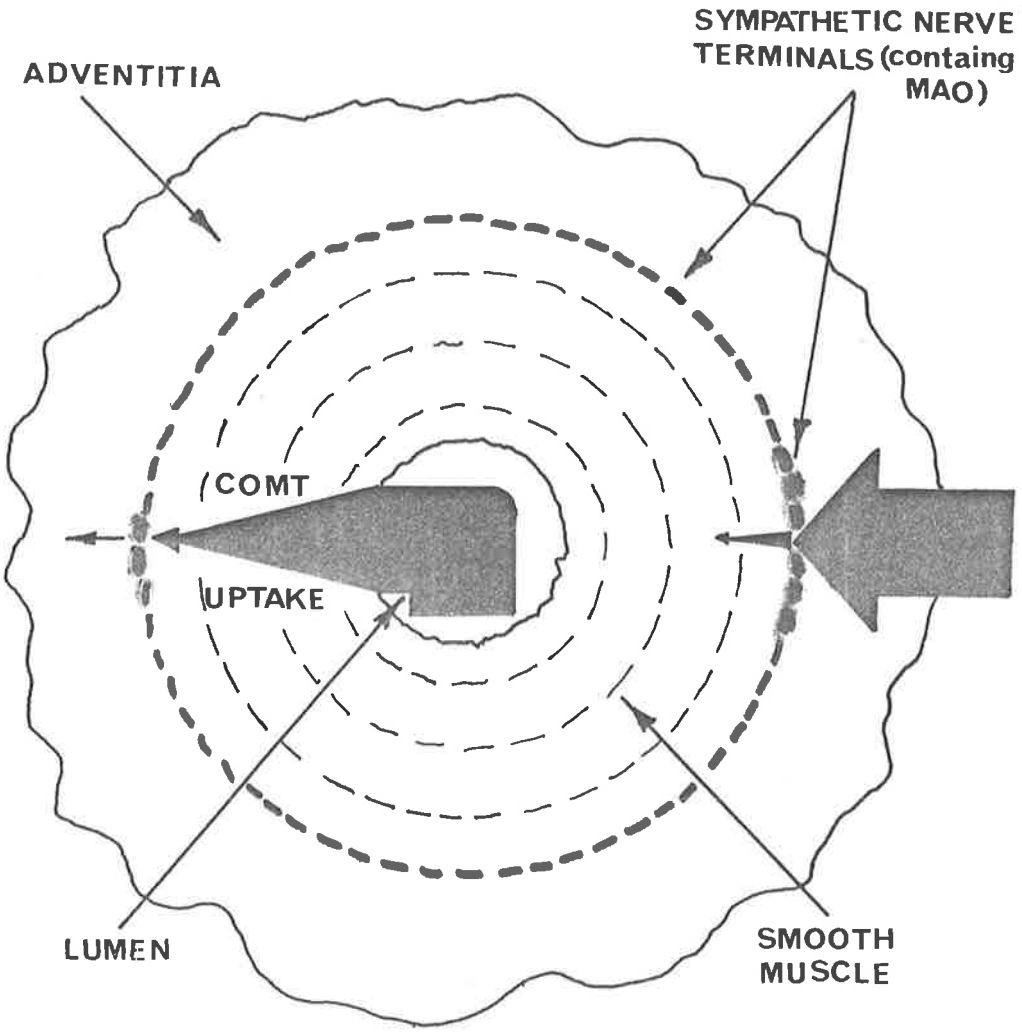


Fig. 1.2 Diagrammatic representation of the influence of neuronal uptake, as indicated in Fig. 1.2. The model differs from that shown in Fig. 1.1 in that it incorporates the concept of restricted passage of NA through the media, partly as a consequence of extraneuronal uptake, and metabolism by COMT.

CHAPTER 2

GENERAL METHODS

CHAPTER 2

GENERAL METHODS

The procedures described in this chapter include:-

(i) isolation of the rabbit ear artery

(ii) techniques for incubation of isolated segments of artery

(iii) homogenisation of arteries and enzyme assays

(iv) technique of surgical denervation

(v) liquid scintillation spectrometry

(vi) tests of significance

(i) *ISOLATION OF THE RABBIT EAR ARTERY*

Male and female semi-lop-eared rabbits, weighing 1.5-2.5 kg were used throughout the course of this study. They were bred at the Central Animal House of the University of Adelaide.

Rabbits were stunned, bled and an incision made in the skin at the base of the ear. The entire segment of artery (illustrated in Fig. 2.1) was exposed by blunt dissection and cleaned of adhering tissue. Tissues were kept moist with warm Krebs solution (the composition of Krebs solution will be described later) and the ear

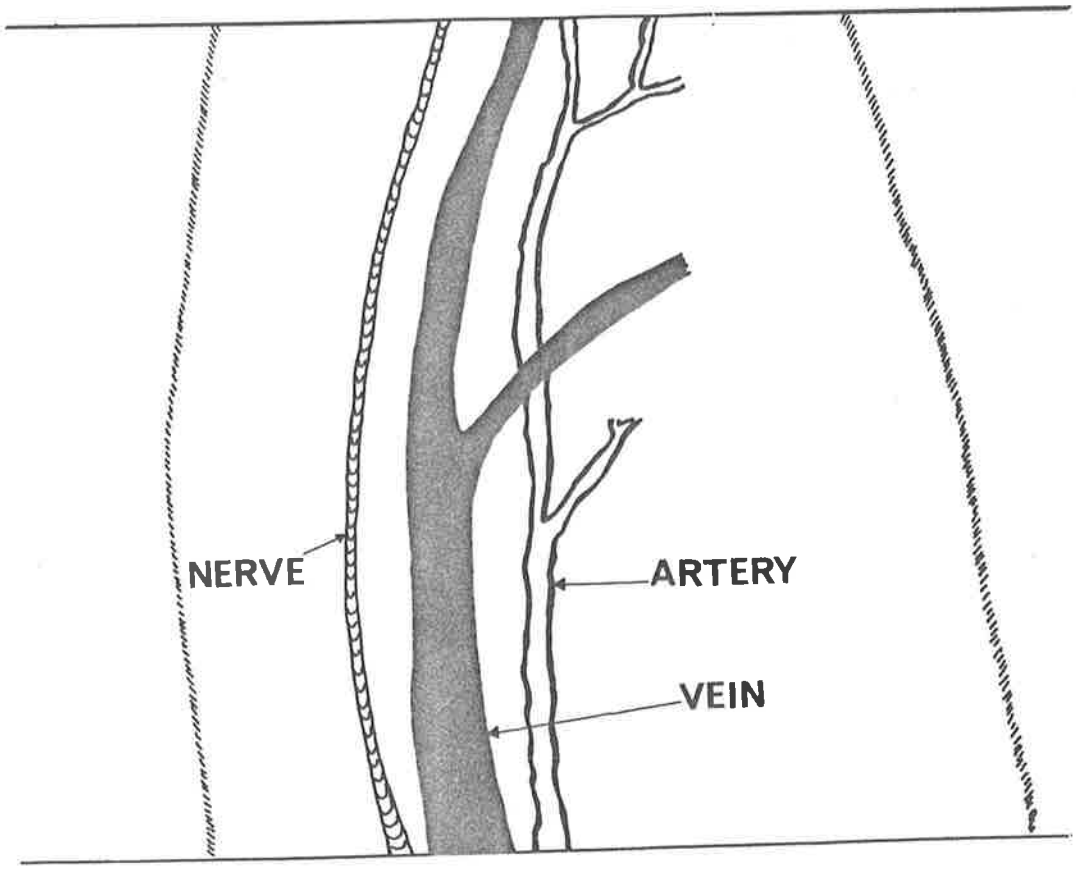


Fig. 2.1 Diagrammatic representation of the convex surface of the left ear of a semi-lopeared rabbit, indicating the relative positions of the central artery, central vein and auricular nerve.

artery segment transferred to beakers containing gassed (95% O_2 and 5% CO_2) Krebs solution. The artery was then removed from this solution, blotted lightly on filter paper moistened with Krebs solution and transferred to a Parafilm disc on the weighing pan of a balance. After weighing, the artery was transferred to gassed (95% O_2 and 5% CO_2) Krebs solution at 36^{o}C.

For some experiments (those designed to measure the efflux of tritium (^{3}H) from arteries in wash solutions, described in Chapter 8) single cannulated arteries were used. Segments of artery were removed from the ear and weighed using the same conditions as described above. Immediately after weighing a single polythene cannulae was inserted into the proximal end of the artery. The polythene cannulae was then attached to a glass micro-pipette which in turn was connected to a perfusion apparatus (see Fig. 2.3). The glass micro-pipette served to permit ease of handling of the artery during its transfer into wash solutions (i.e. eliminated the use of forceps), and at the same time permitted continual intra-luminal washing of the artery. It should be noted that under these conditions the perfusate flowed into the external bathing solution.

(ii) *PROCEDURES USED FOR INCUBATION OF ISOLATED SEGMENTS OF ARTERY*

Arteries were incubated in glass vials containing gassed (95% O_2 and 5% CO_2) Krebs solution of the following composition:- NaCl (120 mmol l^{-1}), $NaHCO_3$ (25.0 mmol l^{-1}), Glucose (5.5 mmol l^{-1}), KCl (4.7 mmol l^{-1}), $CaCl_2$ (2.5 mmol l^{-1}), $MgCl_2$ (1.1 mmol l^{-1}), KH_2PO_4 (1.0 mmol l^{-1}), ethylene diamine tetra-acetic acid (EDTA 10.8 µmol l^{-1}) and ascorbic acid (290 µmol l^{-1}). Ascorbic acid was added after equilibration of Krebs solution with 95% O_2 and 5% CO_2. Solutions

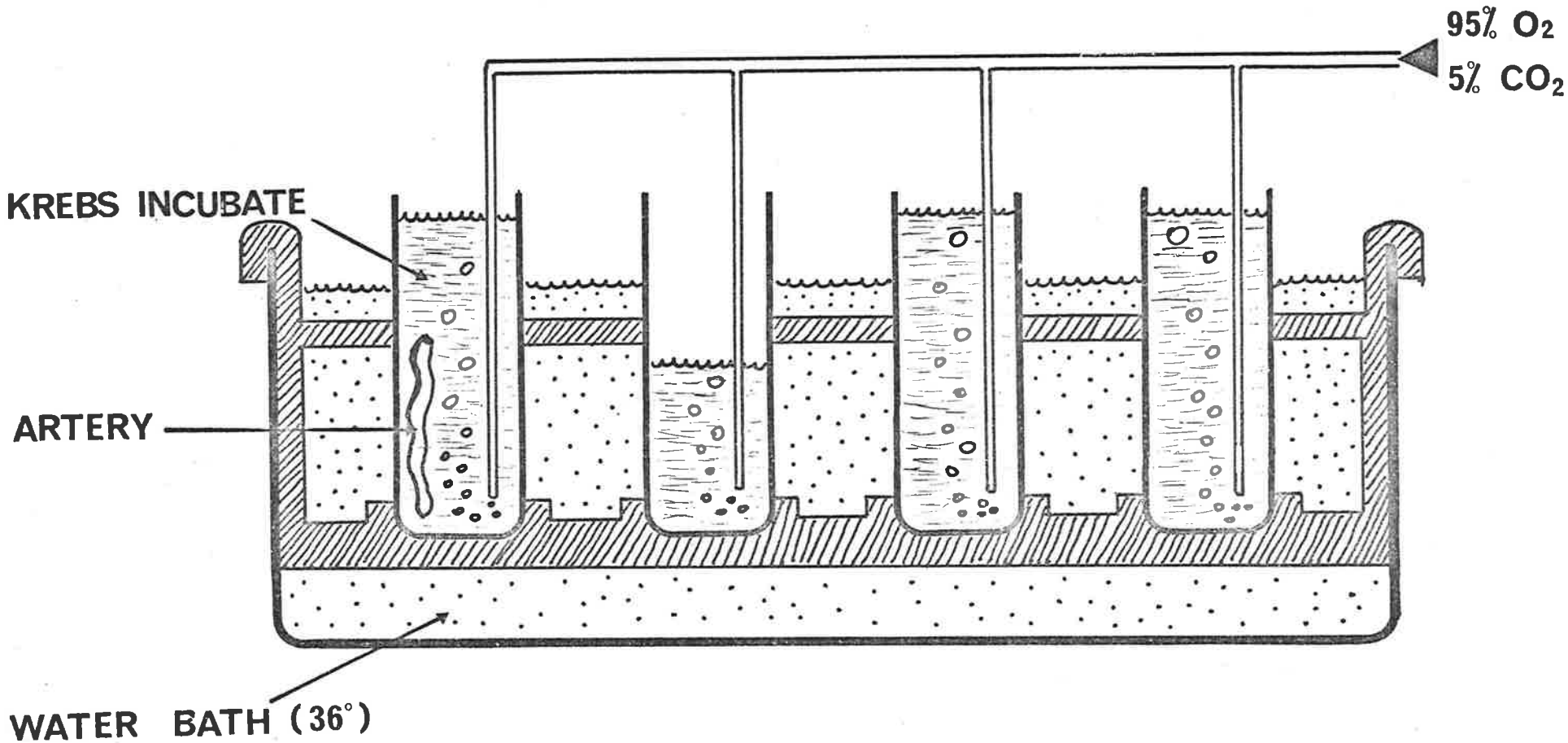


Fig. 2.2 Diagrammatic representation of the apparatus used for incubation of the rabbit ear artery.

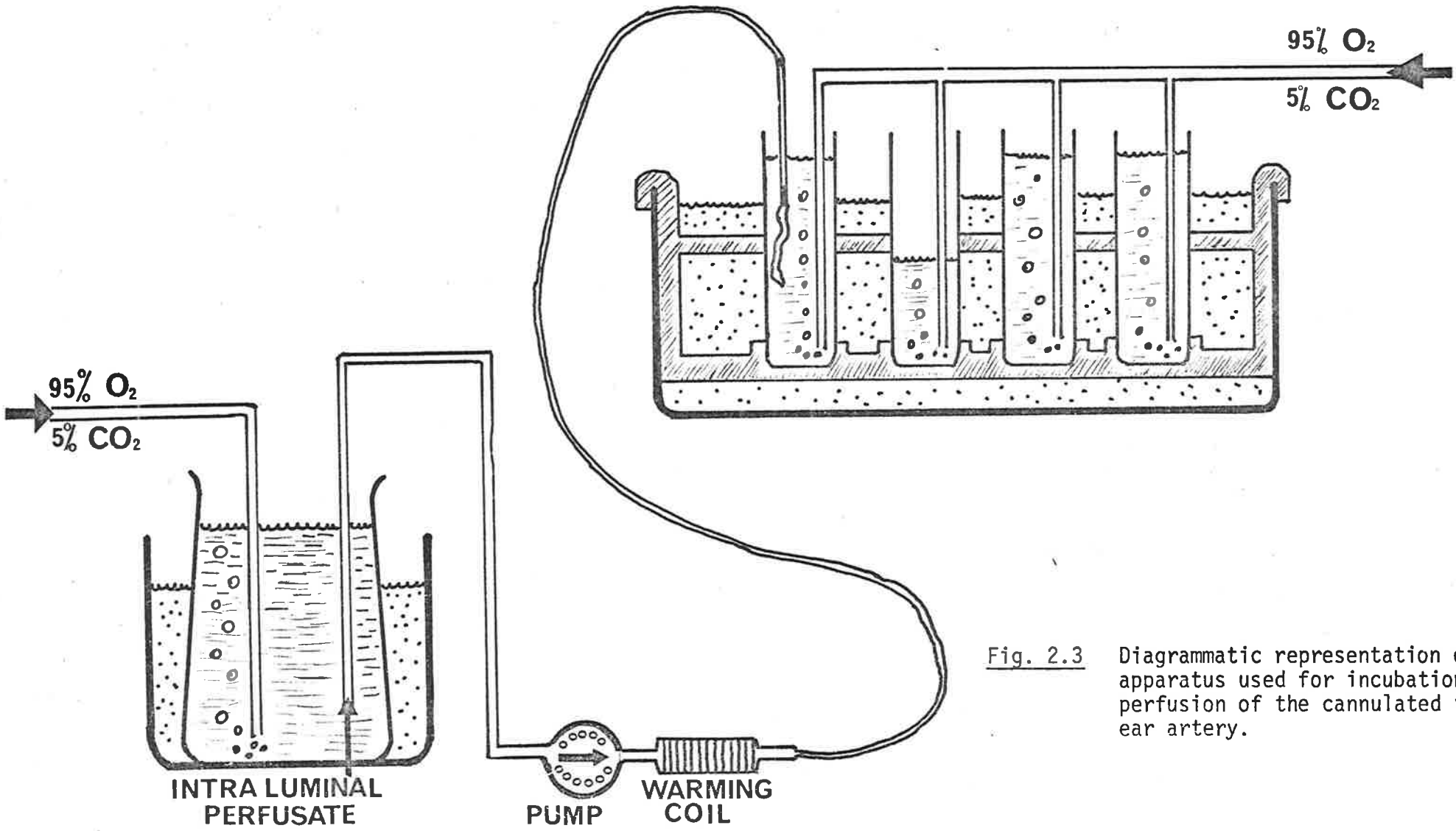


Fig. 2.3 Diagrammatic representation of the apparatus used for incubation and perfusion of the cannulated rabbit ear artery.

were added to glass vials of 2.5 ml capacity and each vial fitted with a poly-propylene tube of small internal diameter through which the gas mixture (95% O_2 and 5% CO_2) was passed continuously into the solution (see Fig. 2.2). The glass vials were placed in a test tube rack fitted to a water bath and the temperature thermostatically maintained at 36°C. The vials were arranged in the test tube rack in four parallel rows, the first of which contained 2.0 mls of Krebs solution (for the initial equilibration phase of incubation), the second of which contained 1.0 ml of Krebs solution plus the radioactive compound under investigation, and the third and fourth rows contained 2.0 mls of Krebs solution for the washing of arteries. All artery transfers were made using fine tipped forceps. The conditions of incubation used for efflux experiments, described in Chapter 8, were similar to those above except that the number of wash vials was increased to permit a 30 minute or longer period of artery wash.

In the experiments to be described in this thesis drugs were added to incubation solutions in volumes not exceeding 0.02 ml and the preparations of those most commonly used were as follows: 3',4'-dihydroxy-2-methyl-propiophenone (U0521, 2.7 mmol l^{-1}) in 0.9% (w/v) saline containing ascorbic acid (0.57 mmol l^{-1}), nialamide (17.5 mmol l^{-1}) in 0.9% (w/v) saline with the aid of gentle heat, DOCA (5.5 mmol l^{-1}) and Phenoxybenzamine (PBZ 3.3 mmol l^{-1}) in ethanol and Cocaine in 0.9% (w/v) saline.

(iii) *HOMOGENISATION AND ENZYME ASSAYS*

Ear arteries were cleared of adhering tissue and excised in the same way as described in (i) above. Immediately after weighing,

arteries (between 15 and 25 mgm) were placed in ice-chilled tubes containing sodium phosphate buffer (5 mmol l^{-1}, pH 7.0). This solution and the artery were then transferred to a motorised teflon-glass homogeniser and the artery homogenised at $4^{o}C$. Portions of this homogenate were then assayed for the activities of MAO and COMT using the methods of Jarrott (1971a, 1971b) and for protein content by the method of Lawry *et al.* (1951). The methodological procedures used for the determinations were as follows:

(a) MAO assay.

The reaction was carried out in oxygen (O_2) filled glass centrifuge tubes and the reaction mixture comprised; 0.025 ml of artery homogenate, 0.05 ml of sodium phosphate buffer (200 mmol l^{-1}, pH 7.8) containing ^{3}H tyramine (2 mmol l^{-1}, 20 μCi $(\mu mol)^{-1}$) and 0.02 ml of water. Solutions were incubated at $36^{o}C$ with continuous shaking. Blanks were prepared by replacing the homogenate with 0.025 ml of water. After 30 minutes of incubation tubes were removed and placed in an ice bath and the reaction stopped by the addition of 0.02 ml of HCl (1.5 mol l^{-1}). Ethyl acetate (0.5 ml) was added to the mixture and the tubes shaken for 5 minutes. After centrifugation, 0.4 ml of the organic layer was transferred to a centrifuge tube containing 0.1 ml of HCl (300 mmol l^{-1}) and shaken for 5 minutes. The tubes were centrifuged and 0.25 ml of the organic layer removed and added to scintillation vials containing 15 mls of Toluene based scintillant solution. The ^{3}H contents were measured by liquid scintillation spectrometry (to be described later in this chapter) and the enzyme activity expressed in nmols of product formed per hour per mg of protein. Preliminary experiments

established that under these conditions of assay the reaction proceeded linearly with time and protein (i.e. homogenate) concentration.

(b) COMT assay.

The reaction was carried out in glass centrifuge tubes and the reaction mixture was comprised of 0.05 ml of artery homogenate, 0.05 ml of sodium phosphate buffer (200 mmol l^{-1}) containing 3',4' dihydroxybenzoic acid (2 mmol l^{-1}), ^{3}HS-adenosylmethionine (100 µmol l^{-1}, 20 µCi $(\mu mol)^{-1}$) and $MgCl_2$ (50 mmol l^{-1}). The reaction was stopped after 30 minutes of incubation at 36°C by the addition of 0.02 ml of HCl (1.5 mol l^{-1}). The subsequent solvent extraction and estimation of the ^{3}H labelled O-methylated acid were exactly the same as those described for the estimation of MAO activity. The COMT activity was expressed as nmoles of O-methylated product formed per hour per mgm of protein. Preliminary experiments established that under these conditions O-methylation proceeded linearly with time and protein (i.e. homogenate) concentration.

(c) Protein estimation.

The protein contents of homogenates were measured, after dilution, by the method of Lawry *et al.* (1951). Aliquots of homogenates were diluted (usually 1:40) in sodium phosphate buffer solution (5 mmol l^{-1}, pH 7.0) and added to glass test tubes in a volume not exceeding 1.0 ml. Standard solutions of bovine serum albumin (BSA) in the concentration range 10 to 100 µg-ml^{-1} were prepared in sodium phosphate buffer solution (5 mmol l^{-1}, pH 7.0). A reagent mixture prepared by mixing 8.0 mls of a solution of $CuSO_4$ (32 mmol l^{-1})

containing sodium potassium tartrate (41 mmol l^{-1}) with 200 mls of a solution of Na_2CO_3 (217 mmol l^{-1}) in NaOH (100 mmol l^{-1}) was freshly prepared before each experiment. 5.0 mls of this solution was added, with mixing to diluted homogenates and BSA standard protein solutions. This mixture was left to stand for 10 minutes. Folin reagent (0.5 ml of commercial solution diluted 1:1 with distilled water) was then added to all tubes and the optical density (measured at 750 nm) determined 30 minutes later using a Bausch and Lomb Spectronic 20 photometer. A plot of protein concentration vs optical density was constructed for each experiment and from this curve the protein contents of the diluted homogenates determined.

(iv) *SYMPATHETIC DENERVATION*

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

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

For experiments described in Chapter 5 arteries were removed from rabbits 1-8 days after denervation, those described in Chapter 6 were removed 7 to 14 days after denervation. In addition, for experiments described in Chapter 5 a group of rabbits were subjected to sham operations which involved exposure but not excision of ganglia 48 hours prior to removal of arteries. For experiments described in Chapter 7 one group of rabbits was denervated in the normal way and another group were denervated by removal of both the superior cervical ganglia 14 days before the removal of ear arteries.

The effectiveness of sympathectomy was assessed by fluorescence microscopy examination of denervated and contralateral innervated control arteries as described by Waterson (1968). Sections of arteries approximately 5 mm in length were frozen in a mixture of acetone and dry ice and transferred to freeze dryer (Thermovac model FD3) at $-50^{o}C$ and at a pressure of 2.5-6 Nm^{-2} for 18 hours. The sections were then placed in a one litre jar containing 5 g of paraformaldehyde powder which had been stored over sulphuric acid at a relative humidity of 70% for one week. The jar was sealed and placed in an oven at $80^{o}C$ for 60 minutes. The formaldehyde-treated specimens were then vacuum infiltrated with paraffin wax at $60^{o}C$, blocked in paraffin wax, and cut in transverse

sections of 7 μm thickness, using a microtome (Leitz model 1212). After mounting in a mixture of Entellan (Merck) and xylol, the sections were examined using a Leitz microscope with a dark field condenser. Fluourescence was produced with an HBO 200 mercury vapour lamp using a 1.5 mm Schott BG12 excitation filter and 530 nm barrier filter. Photographs were taken using a Leitz orthomat camera and Kodak photoflure film.

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

(v) *LIQUID SCINTILLATION SPECTROMETRY*

The radioactivity present in sclutions containing carbon-14 labelled (^{14}C) or tritium labelled (^{3}H) compounds was measured by liquid scintillation spectrometry using a Packard Model 3310 Liquid Scintillation Spectrometer. For the measurement of radioactivity in aqueous solutions, samples (usually between 0.1 and 2.0 mls) were added to counting vials containing 15 mls of Toluene based phosphor. The latter consisted of 8.25 gm 2,5-diphenyoxyazole (PPO), 0.25 gm of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), and 500 ml Triton X100 added to one litre of toluene.

The procedure of internal standardisation was used to correct for quenching and for efficiency of counting. This involved determination of the count rate (r_1) for the test sample of unknown activity (a) followed by a second determination of count rate (r_2) after the addition

of ^{3}H toluene (for samples containing ^{3}H compounds) or ^{14}C toluene (for samples containing ^{14}C compounds) of known activity (a_k). The counting efficiency (E) was derived from $E = \frac{r_2 - r_1}{a_k}$ and the activity (a) of the test sample from $a = \frac{r_1}{E}$. The percentage efficiency of counting was 18-25% for ^{3}H and 60-65% for ^{14}C. The limit of detectability (a_D) of ^{3}H in solutions was derived from the efficiency (E) and the background count rate (r_b) (i.e. $a_D = \frac{2}{\sqrt{E^2 r_b}}$). For a count period of 1.0 minutes and a 95% probability estimation this was approximately 100 DPM for most solutions.

(vi) *TESTS OF SIGNIFICANCE*

When comparing the effects of different drugs and their effects under different experimental conditions, students' t-tests were performed on arithmetic means. The appropriate t-test used, according to whether the samples contained matched pairs or unpaired data is indicated in each case. The levels of significance (in the text as p) are shown where appropriate in the legends to figures or the footnotes to tables. For convenience the differences between observations are described in the text as either "significant" (i.e. $p < 0.05$) or "not significant" (i.e. $p > 0.05$). Where this occurs the t-test used, number of observations and the levels of significance for this data appears in the relevant tables or figures.

CHAPTER 3

SPECIFIC METHODS; PURIFICATION PROCEDURES

CHAPTER 3

SPECIFIC METHODS; PURIFICATION PROCEDURES

The techniques described in this Chapter were used to purify manufacturer's solutions of ^{3}H catecholamines and ^{3}H normetanephrine before their use in studies concerned with uptake and metabolism. Preliminary chromatographic experiments had indicated that all of these solutions of ^{3}H amines contained ^{3}H impurities. A more detailed investigation into the nature of these impurities and the stability of $^{3}HCAS$ during storage appears in the appendix (Appendix 2).

(i) Purification of solutions of ^{3}HCA's

Solutions of dl^{3}HNA, l^{3}HNA and dl^{3}HISO were purified by a batch alumina procedure based on that previously described for the purification of urinary catecholamines (de la Lande *et al.* 1967). The sample of ^{3}HCA (usually 0.5 ml or 0.5 mCi) was added to a solution (4.0 mls) of HCl (10 mmol l^{-1}). The sample was then added to a disposable polypropylene tube (30 ml capacity) containing activated alumina (700 mg), EDTA (100 mg) and ascorbic acid (100 mg). The mixture was agitated with a stream of nitrogen (N_2) bubbles and its pH adjusted to and maintained at pH 8.4 by addition of Na_2CO_3

(500 mmol l^{-1}). After 4.0 minutes the bubbling was stopped to allow the alumina to settle. The supernatant was removed and 20 mls of distilled water added to the tube, bubbled briefly with N_2 and the alumina again allowed to settle. The wash solution was discarded and the wash procedure repeated. The ^{3}HCA was eluted from the alumina by the addition of 5.0 mls of acetic acid (300 mmol l^{-1}). The mixture was bubbled with N_2 for 5 minutes, the tube centrifuged (5,000 g for 10 minutes) and 4.5 mls of the acetic eluate removed. A further 3.0 mls of acetic acid was added to the alumina, shaken briefly and centrifuged. The supernatant was removed and added to the first acetic eluate. This pooled solution was then centrifuged (9,000 g for 30 minutes, to remove alumina fines) the supernatant removed and stored in polypropylene tubes at 4^{o}C.

The contents of CA (either NA or ISO) in the acetic eluates were measured using the automated trihydroxyindole (THI) assay designed for use with alumina eluates and described in detail in the appendix (Appendix 1). The ^{3}H contents of eluates were measured by liquid scintillation spectrometry. The specific activities (expressed as Curies per mmol) of purified solutions of ^{3}HCA's were calculated from the NA and ^{3}H contents.

Comments: <u>dl^{3}HNA</u>. It will be shown later in this thesis that:

(1) manufacturers' stock solutions of dl^{3}HNA contain at least two impurities which can be isolated by ion exchange paper chromatography. The batch alumina purification procedure described above decreases the contents of these impurities in solutions of dl^{3}HNA.

(2) solutions of dl^{3}HNA deteriorate with time and the optimal conditions of storage include storage at 4^{o}C in polypropylene tubes.

dl^{3}HISO. Preliminary experiments showed a need for purification of solutions of ^{3}HISO before use in the experiments to be described in Chapter 7. When samples of non purified ^{3}HISO were chromatographed on sodium borate impregnated thin layer plates (using the procedure described in the following chapter for the separation of ISO and the methoxyisoprenaline (MeOISO)) a significant amount of the total ^{3}H applied to the thin layer plate co-chromatographed with non-labelled MeOISO. In contrast when samples of alumina purified ^{3}HISO were chromatographed much smaller amounts of ^{3}H were detected in the MeOISO region of the plate. Elution profiles illustrating this are shown in Fig. 3.1.

(ii) Purification of solutions of ^{3}HNMN

Thin layer chromatographic analysis of manufacturers' solutions of ^{3}HNMN showed the presence of ^{3}H material that co-chromatographed with the catechols NA and DOMA. Solutions of dl^{3}HNMN were purified by combined alumina and ion exchange chromatography.

The sample of ^{3}HNMN (usually 0.25 ml or 0.25 Ci) was chromatographed on alumina using the batch procedure described above for ^{3}HCA's. The effluent fraction from the alumina (i.e. the fraction containing ^{3}HNMN) was adjusted to pH 6.5 by the addition of HCl (500 mmol l^{-1}). This solution was then passed through a Dowex 50 (Na^+) column using the experimental procedures to be described in

the following chapter for the isolation of ^{3}HNMN from tissue extracts and incubation solutions. ^{3}HNMN was eluted from the ion exchange column with HCl (1 mol l^{-1}) in a volume of 15.0 mls. This eluate was rapidly frozen (with a dry ice acetone freezing mixture) and freeze dried. The dried sample was redissolved in 2.0 mls of HCl (10 mmol l^{-1}) containing EDTA (20 μmol l^{-1}) and stored in polypropylene tubes at 4^{o}C.

Samples of purified ^{3}HNMN subjected to thin layer chromatographic analysis (using the procedure to be described in the following chapter for the separation of NA and its metabolites), showed smaller amounts of ^{3}H present in the region corresponding to NA and DOMA than was the case for the non purified sample. Elution profiles illustrating this are shown in Fig. 3.1.

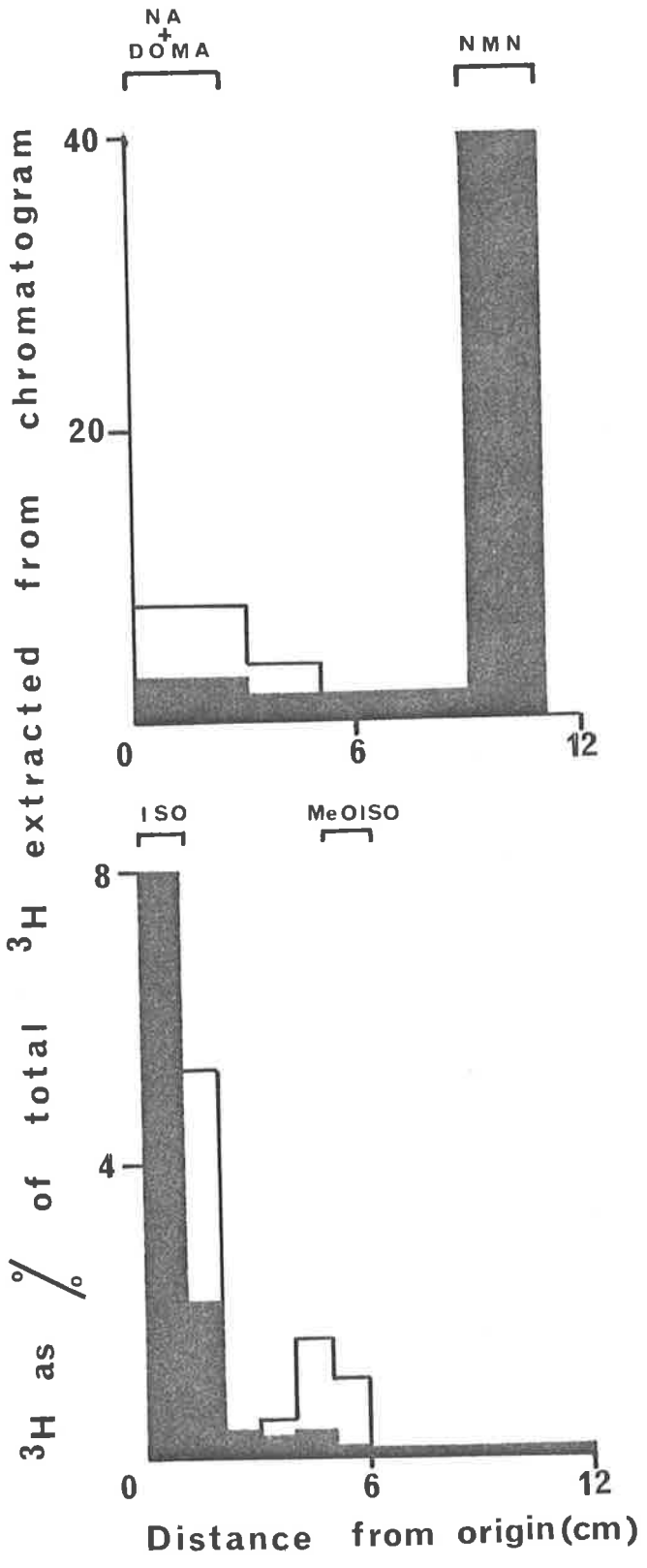


Fig. 3.1 The distribution of ^{3}H on thin layer chromatograms for samples of purified (filled histograms) and non purified (open histograms) ^{3}HNMN (upper panel) and ^{3}HISO (lower panel). The conditions of purification and chromatography are described in the text.

CHAPTER 4

SPECIFIC METHODS; RADIOCHEMICAL PROCEDURES

CHAPTER 4

SPECIFIC METHODS; RADIOCHEMICAL PROCEDURES

The procedures described in this chapter are those which were used in the studies of the accumulation and metabolism of ^{3}H amines to be described later in this thesis.

This methodology comprises:

(i) Preparation of ^{3}H catecholamines

(ii) Extraction of ^{3}H from arteries

(iii) Techniques for the assay of ^{3}H amines and their metabolites.

(i) *PREPARATION OF ^{3}H AMINES FOR INCUBATION*

After purification of the ^{3}H catecholamines (described in the previous chapter) they were present in acetic acid solutions. Lyophilisation of these solutions removed this volatile acid and permitted reconstitution of ^{3}H catecholamine directly in Krebs solution at the desired concentration.

Procedure: The appropriate quantity of ^{3}H catecholamine ($^{3}HISO$ or ^{3}HNA) was added to lyophilisation vials and rapidly frozen with an acetone dry ice mixture. Lyophilisation was usually complete within one hour. The amine was reconstituted in gassed (95% O_2, 5% CO_2) Krebs solution containing ascorbic acid (290 $\mu mol\ l^{-1}$)

Table 4.1

The efficiency of extraction of ^{3}H from arteries

Compound	% of total extracted by HCl
^{3}HNA	97.8 ± 0.2 (n = 12)
$^{3}HISO$	96.1 ± 0.9 (n = 15)

Footnotes

Values refer to the amount of ^{3}H extracted by HCl as a percentage of the total ^{3}H present in arteries (i.e. ^{3}H in HCl wash + ^{3}H in NCS extract).

EDTA (10.8 μmol l^{-1}) and bubbled immediately with 95% O_2 and 5% CO_2.

Comments:

The efficiency of this process was demonstrated by a recovery of greater than 95% for both ^3H and amine content when analysed by liquid scintillation counting and THI assay. Prolonged lyophilisation (greater than two hours) led to losses of amine content for some samples of ^{3}HISO.

(ii) *EXTRACTION OF ^3H FROM ARTERIES*

The ^3H catecholamines and their ^3H metabolites which were present in arteries at the end of incubation were extracted overnight with either of the following solutions:

(1) 1.0 ml of HCl (100 mmol l^{-1}) containing EDTA (22 μmol l^{-1})

(2) 2.5 ml of acetic acid (1.0 mol l^{-1}) containing EDTA (22 μmol l^{-1})

Comments:

HCl was used for the extraction of tritium from arteries prior to column chromatography. Acetic extracts were used for thin layer chromatography. Extraction into acid media eliminated the protein precipitation step inherent in procedures which involved the homogenisation of the tissue. The efficiency of this extraction technique was estimated by determining the amount of ^3H left in the tissue after acid extraction. This was determined after solubilisation of the tissue with NCS solubilising agent. Efficiencies of extraction of ^{3}HNA and ^{3}HISO are shown in Table 4.1.

(iii) *TECHNIQUES FOR THE ASSAY OF ^{3}H AMINES AND THEIR METABOLITES*

(a) The systems for assay ^{3}H catecholamines and their metabolites (to be described later in this section) required a method for estimating the efficiency of recovery of the ^{3}H compounds from these separative procedures.

The widely adopted native fluorescence techniques for the assay of phenolic compounds were not used because blank values and reproducibility were subject to large variations when this procedure was used in conjunction with thin layer chromatography. Instead the catecholamines and their metabolites were measured using an automated colourimetric assay based on measurement of the change of absorbance produced by diazonium derivatives of these compounds. For this purpose the para nitroanaline method of visualisation of catechols and 3-O-methylated catechols on chromatography media was adapted for use on the Technicon Autoanalyser.

Samples of CA's or metabolites in HCl (300 mmol l^{-1}) were introduced into the manifold of the Autoanalyser and made alkaline by the addition of K_2CO_3 and diazonium derivative formed after the addition of freshly mixed para nitroanaline and sodium nitrite. The coloured derivatives were passed through the flow cuvette of the colourimeter and the change in Optical Density ($O.D._{505}$) recorded. The manifold design and reagent concentrations are shown in Figure 4.1. A plot of the $O.D._{505}$ vs concentration of NA, ISO and their metabolites is shown in Figure 4.2.

Comments:

Ascorbic acid or sodium metabisulphite could be used as anti-oxidants during preparation procedures. Because sodium metabi-

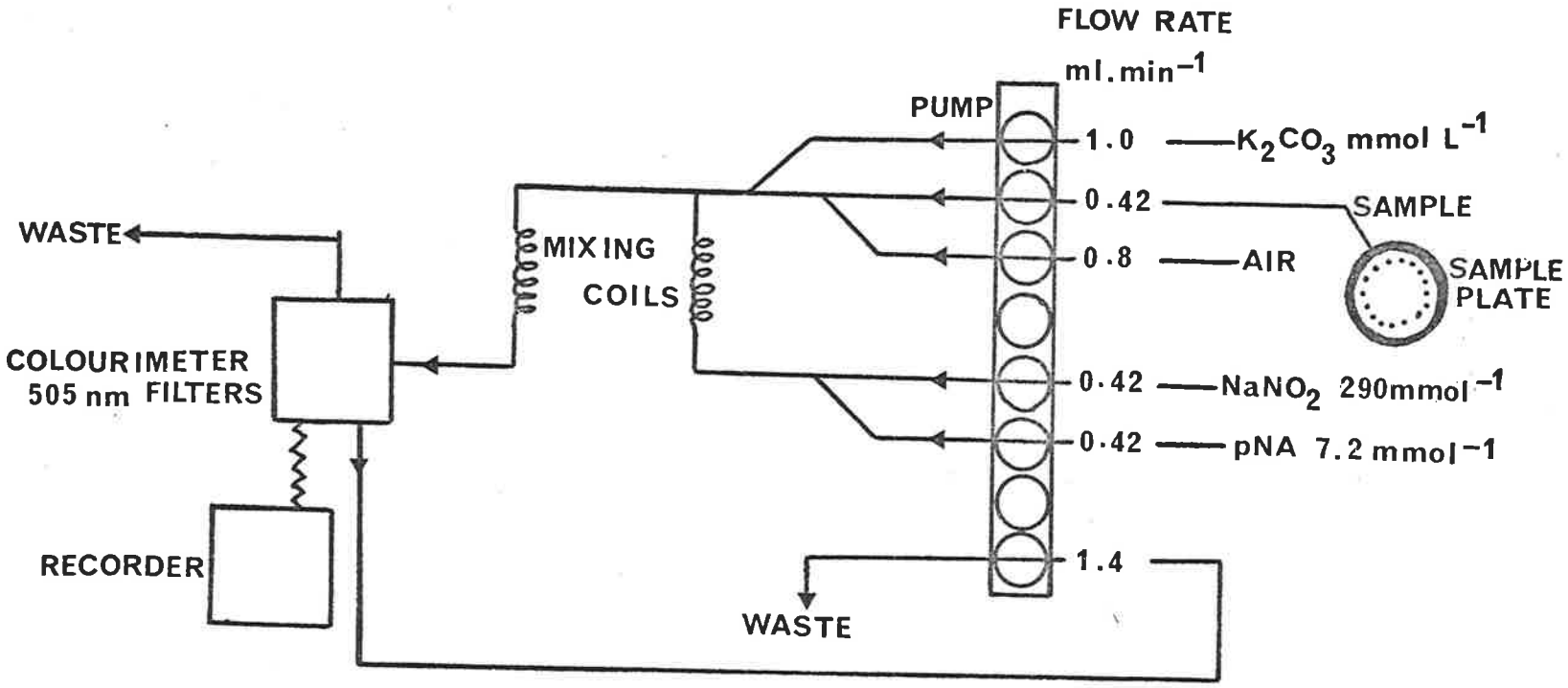


Fig. 4.1 A schematic representation of the automated colourimetric assay procedure for the measurement of catecholamines and their metabolites.

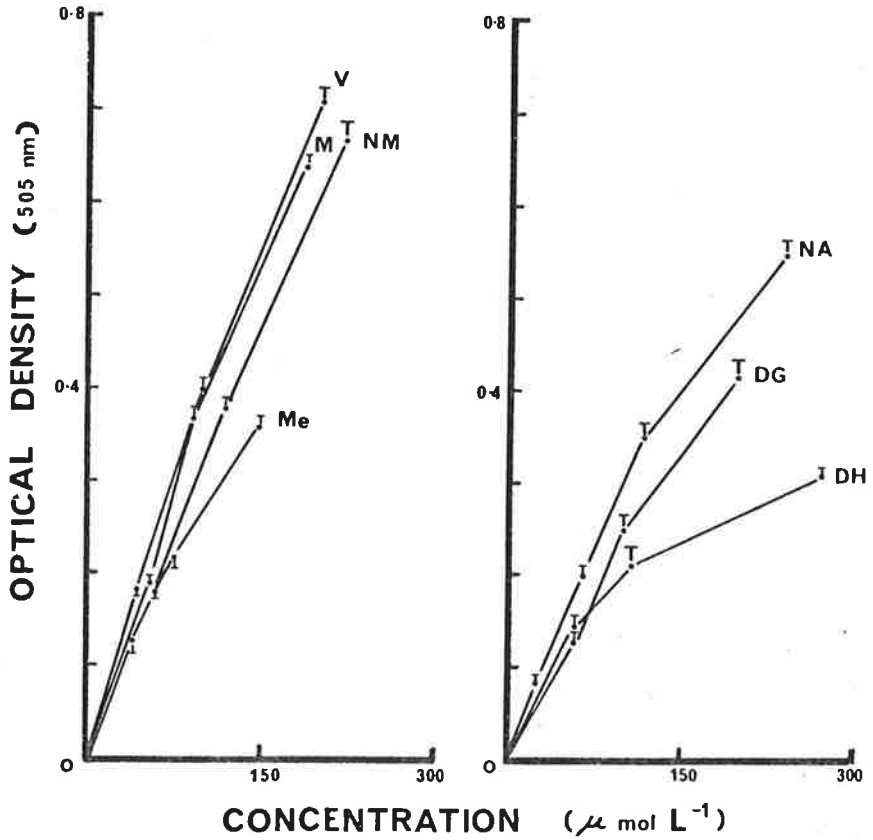


Fig. 4.2 Illustrates calibration curves for standard solutions of CA's and their metabolites obtained using the automated assay outlined in Fig. 4.1. Ordinate: optical density measured at 505 nM. Abscissae: concentration (μmol l^{-1}) of catecholamine or metabolite. Values represent the mean of 12 or more determinations for each compound.

Abbreviations:

VMA	(V)
NMN	(NM)
MN	(M)
MeOISO	(Me)
DOPEG	(DG)
DOMA	(DH)

sulphite gave the least interference in this assay it was used in preference to ascorbic acid in the following separative procedures.

(b) Method I

In some experiments the identity of ^{3}H compounds in the incubates and tissue extracts was determined by ion exchange paper chromatography.

Aliquots of the incubates and tissue extracts were applied to strips of Whatman (P81) paper and run in parallel with non labelled amines and metabolites. Test solutions were spotted onto cellulose phosphate paper strips (1.5 cm wide), together with a 20 microlitre aliquot of a solution containing 10 mmol l^{-1} of each of the following compounds in HCl (10 mmol l^{-1}): Noradrenaline, normetanephrine, 3,4-dihydroxymandelic acid and 4 hydroxy-3-methoxy-mandelic acid. The papers were air-dried and then developed for a distance of 47.0 cm with ammonium acetate (200 mmol l^{-1}, pH 6.5): isopropranolol (2:1). The strips were dried thoroughly, cut into 1.0 cm sections and each section eluted for at least 12 hours with 1.0 ml of HCl (300 mmol l^{-1}). The paper segments and the eluates were added to 15.0 mls of toluene based scintillator, and the tritium content measured. In addition separate strips containing only the cold marker spots were treated identically except after removal from the chromatography tank, dried and sprayed with diazotized paranitro-aniline reagent. The Rf values of these control marker spots were recorded for each batch of papers treated.

Comments:

The above method for separating NA and its metabolites embodies the cellulose phosphate paper chromatographic system described by Roberts (1962). The efficiency of separation was confirmed by identification of the native fluorescence of carrier marker spots under U.V. and the Rf values achieved after spraying control strips with diazonium reagent.

Co-chromatography of a major peak of tritiated material with a non labelled compound was taken as evidence for the existence of this compound in the original test solution.

(c) Methods II and III

Methods II and III were used for the separation of the following classes of compounds; bases (NA and NMN), acids (DOMA and VMA), glycols (DOPEG and MOPEG), catechols (NA, DOMA and DOPEG) and phenols (NMN, VMA and MOPEG). The principles involved in the separation were: catechols but not phenols bind to Al_2O_3 at pH 8.4, amines but not acids and glycols, bind to Dowex 50 (Na^+) at pH 6.4. NA and DOPEG elute from Al_2O_3 with a weak organic acid (viz. acetic acid), and DOMA elutes with a mineral acid (viz. hydrochloric acid). The elution of NA precedes that of NMN from Dowex 50 columns washed with HCl.

The two combinations of Dowex and Al_2O_3 used were; Al_2O_3 followed by Dowex, referred to as Method II and Dowex followed by Al_2O_3 referred to as Method III. These combinations and the identity of metabolites present in the different fractions are illustrated in Fig. 4.3 and Fig. 4.4 For both Method II and III

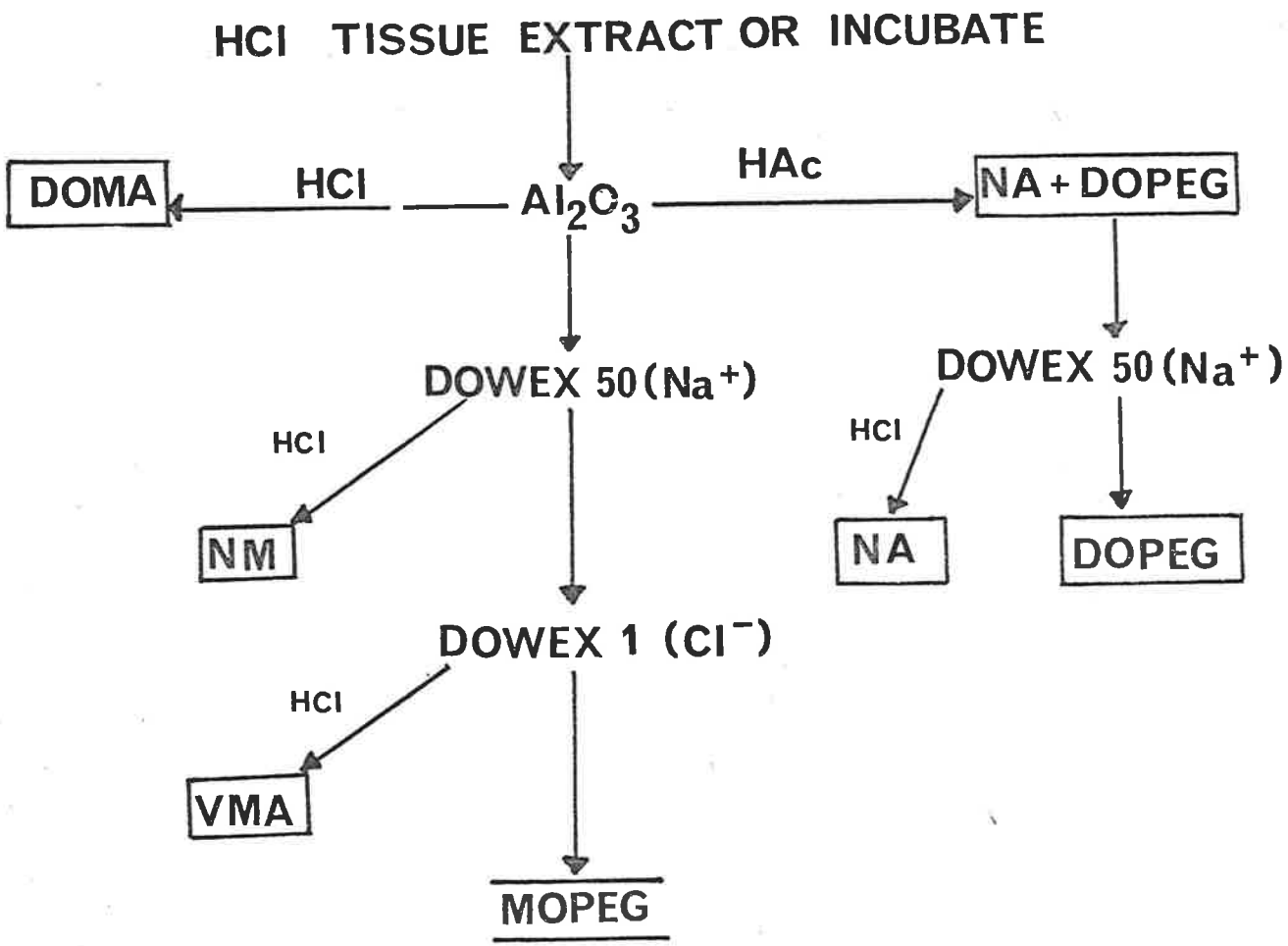


Fig. 4.3 Shows the combination of Al_2O_3 and Dowex-50 when used for Method II. The identity of metabolites in fractions is shown.

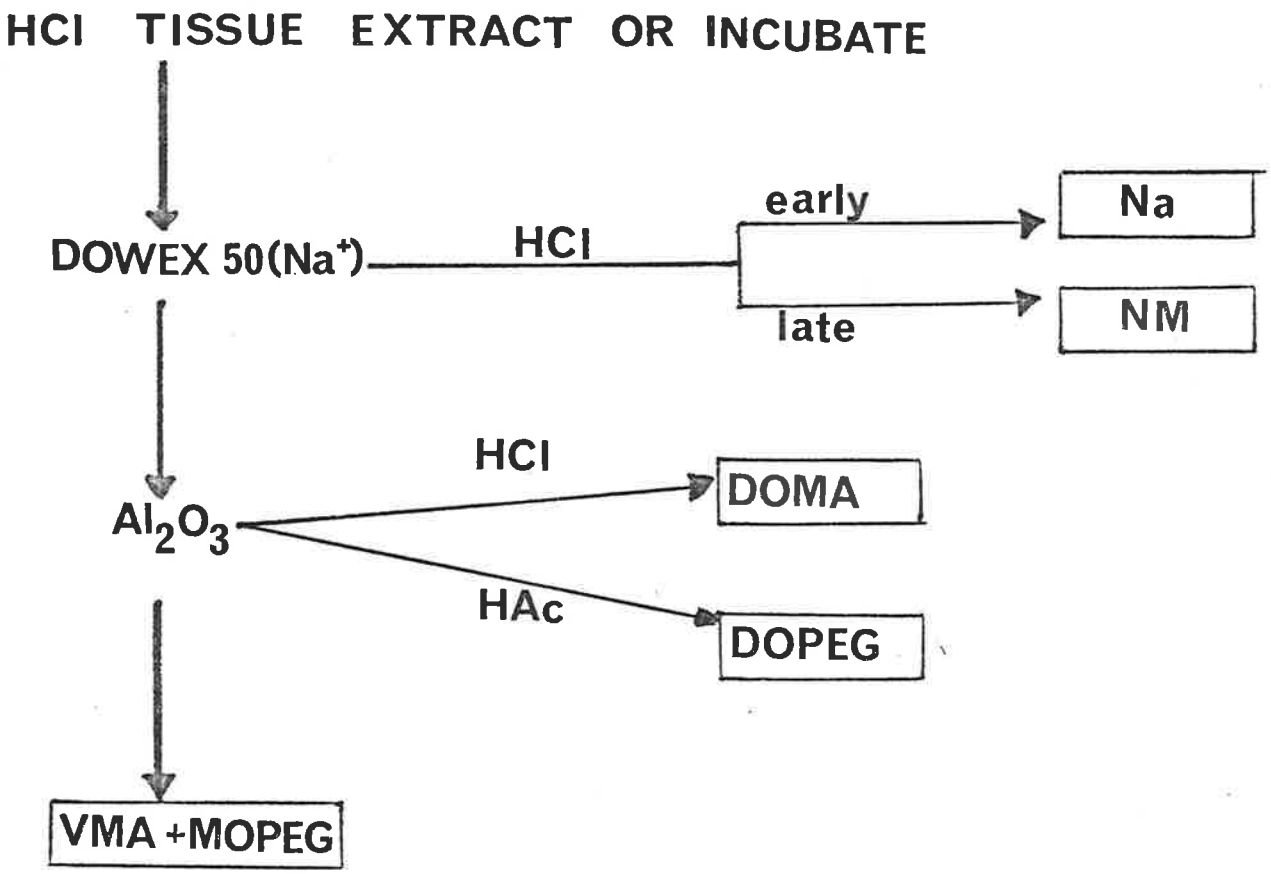


Fig. 4.4 Shows the combination of Al_2O_3 and Dowex-50 when used for Method III. The identity of metabolites in fractions is shown.

1 μmol of NA and of each of its metabolites were added to the solutions for analysis (i.e. Krebs incubation solutions and acid extracts of tissues) to permit estimation of recovery of these compounds after fractionation. Blank solutions were treated in parallel and these solutions comprised; samples of incubation media containing ^{3}HNA not exposed to tissue (i.e. incubate blank) and samples of acid extraction media containing ^{3}HNA, the ^{3}H contents of which were similar to the total ^{3}H contents of tissue extracts. Chromatographic analysis of blank samples permitted an estimate of the contamination of each metabolite fraction by ^{3}H not attributable to the metabolite.

Batch Al_2O_3 chromatography:

This method is based on that described in the previous chapter for the purification of ^{3}HCAs and involved addition of test solutions (either Krebs incubates, tissue acid extracts, or effluent samples from Dowex columns) to tubes containing Al_2O_3 (500 mg), EDTA (100 mg) and sodium metabisulphite (10 mg). Samples were adjusted to, and maintained at pH 8.4 for 4 minutes with Na_2CO_3 (2.0 mol l^{-1}). The effluent fraction was removed and its pH adjusted to pH 6.4 with HCl (5.0 mol l^{-1}) and stored at 4^{o}C prior to further analysis. The alumina was washed in two volumes (20 ml) of distilled water, one volume (5 ml) of acetic acid (300 mmol l^{-1}), two volumes (20 ml) of acetic acid (300 mmol l^{-1}) and finally 6 ml HCl (500 mmol l^{-1}). It was established that with this procedure non labelled NA and DOPEG eluted with the first (i.e. 5 ml) acetic wash and DOMA with the HCl wash.

Dowex 50 chromatography:

Columns (60 mm x 40 mm) were filled with Dowex 50 (Na^+) and washed for one hour with distilled water. For this procedure all samples and wash solutions were pumped (Technicon peristaltic pump) through the columns at a flow rate of 0.5 ml $(min)^{-1}$. Sodium metabisulphite (10 mg) was added to each test solution (1 to 2.0 ml of either Krebs solutions, tissue acid extracts, alumina effluent fractions or alumina acetic eluates). These solutions were adjusted to pH 6.4 and applied to columns. The columns were washed with 15 ml of distilled water, the effluent fractions containing acid and glycol metabolites collected, acidified (pH 4.0) with HCl (5.0 mol l^{-1}) and stored prior to further analysis. NA was eluted from the columns with HCl (0.5 mol l^{-1}) in 5 ml fractions. NMN was then eluted with HCl (1.5 mol l^{-1}) in 5 ml fractions.

For Method II VMA was separated from MOPEG by passing the solution over a column of Dowex I (Cl^-) of the same dimensions as used for Dowex 50. VMA was eluted from the column in 15 ml of a solution containing HCl (100 mmol l^{-1}) and methanol (6 mol l^{-1}). Chromatographic profiles illustrating the separation of NA and its metabolites using these methods are shown in Figures 4.5, 4.6.

Comments:

The procedures described above embodied methods reported by Laverty and Taylor (1968) and more recently by Graefe *et al.* (1973). The essential difference between the procedure described and those reported by the above authors was the use of a peristaltic pump

Fig. 4.5 The left hand panel shows the distributions of NA and its metabolites into fractions obtained with the batch Al_2O_3 procedure. Conditions of chromatography and procedures for estimation of contents of compounds in fractions are outlined in the text. Fraction 1 corresponds to the Al_2O_3 effluent, 2 and 3 to the water wash, 4 to the acetic eluate, 5 and 6 to the acetic wash and 7 to the HCl eluate.

The right hand panel illustrates the use of the batch Al_2O_3 procedure for determining the nature of ^3H labelled compounds in resting efflux of ^3H solutions from untreated arteries (open histograms) and arteries treated with cocaine during incubation (filled histograms). Conditions of incubation and drug treatments are described in Chapter 6.

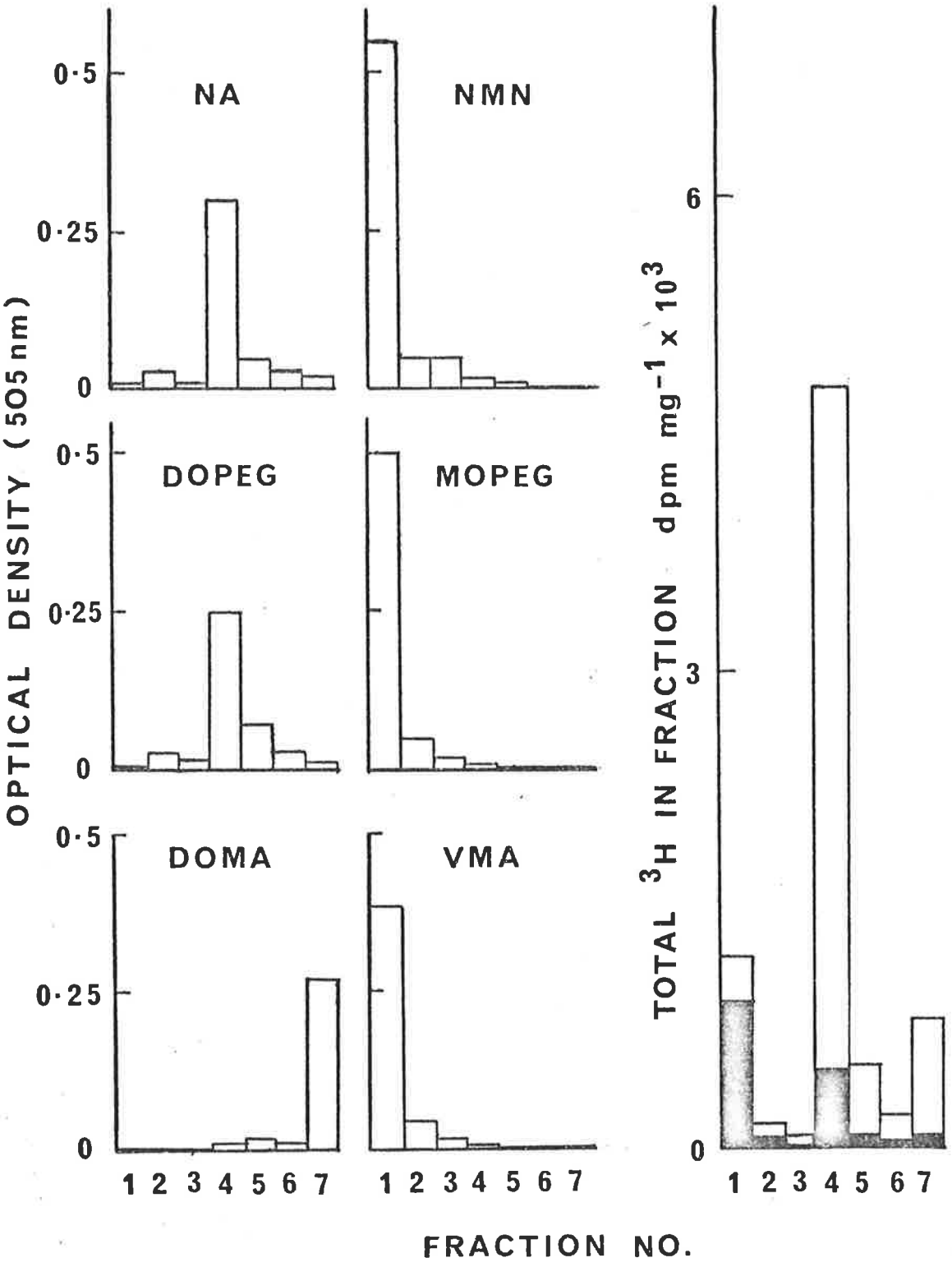
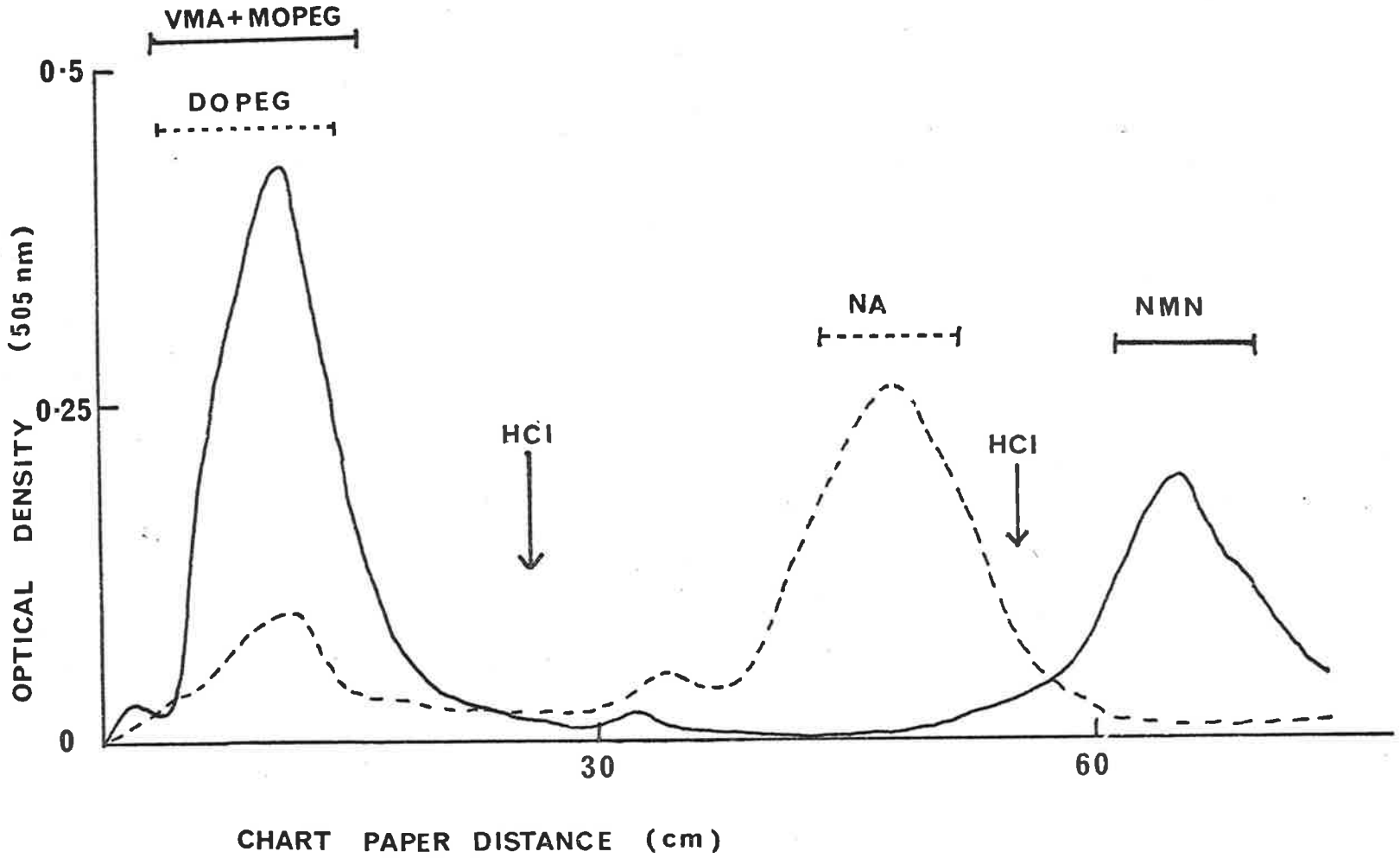

NA
NMN
DOPEG
MOPEG
DOMA
VMA
0·5
0·25
0
OPTICAL DENSITY (505 nm)
TOTAL ^3H IN FRACTION dpm $mg^{-1} \times 10^3$
6
3
0
1 2 3 4 5 6 7
FRACTION NO.

Fig. 4.6(a) Shows the elution profile obtained when a solution containing NMN, MOPEG and VMA (all 1.0 μmol l^{-1}) was chromatographed on Dowex-50 (shown as solid line). Also shown is an elution profile for a mixture of DOPEG (0.3 μmol l^{-1}) and NA (1.0 μmol l^{-1}) (shown as dotted line) obtained from a second column run in parallel. For these experiments solutions from both columns were fed into a two channel automated colourimetric assay and the changes in OD_{505} continuously recorded. Conditions of chromatography described in the text.


VMA+MOPEG
DOPEG
0·5
0·25
0
OPTICAL DENSITY (505 nm)
HCl
NA
HCl
NMN
30
60
CHART PAPER DISTANCE (cm)

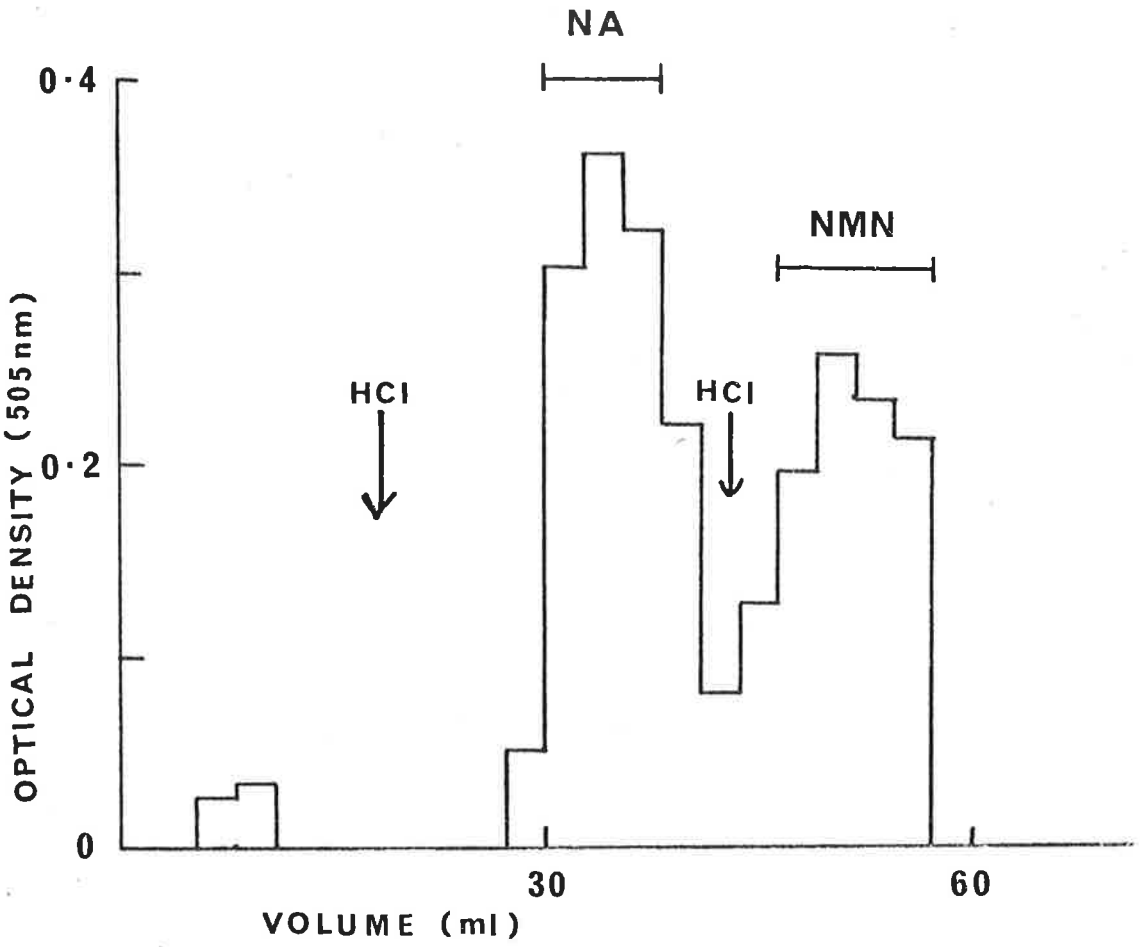


Fig. 4.6(b) Shows the elution profiles obtained when a mixture of NA and NMN was chromatographed on Dowex-50 using the conditions described in the text. Ordinate optical density of fractions (3.0 ml) measured at 505 nm.

Abscissae: Volume (ml) of solution collected from column after addition of test sample

with the ion exchange columns. This modification ensured a constant rate of flow for all columns.

Dowex 50 columns were re-used and regenerated before use by passing NaOH (2 mol l^{-1}) through the column at 0.5 ml $(min)^{-1}$ for one hour, followed by distilled water containing EDTA (26 mmol l^{-1}) for one hour, HCl (2 mol l^{-1}) for one hour, distilled water (plus EDTA) for one hour and finally phosphate buffer (200 mmol l^{-1}, pH 6.4) for one hour.

Columns were re-used for periods of up to two months and regularly checked for their ability to separate non labelled NA and its metabolites.

(d) Method IV - Thin layer chromatography

Preparation of samples prior to chromatography:

Incubating media were adjusted to pH 6.5 with HCl (1 mol l^{-1}) at the end of incubation. Prior to fractionation, non labelled CA's and their metabolites were added to solutions to permit estimation of recoveries (final contents on thin layer plates: 150 nmol of NA and each metabolite, 200 nmol of ISO and MeOISO).

Tissue extracts were concentrated by lyophilisation prior to chromatography. Tissue extracts containing ^{3}HISO were reconstituted in 0.2 ml of a mixture of HCl (100 nmol l^{-1}) and ethanol (10 mol l^{-1}). Tissue extracts containing ^{3}HNA and ^{3}HNMN were reconstituted in a mixture of acetic acid (100 nmol l^{-1}) and ethanol (10 mol l^{-1}). Non labelled metabolites were added prior to lyophilisation for recovery purposes (1 µmol ISO and MeOISO, 1 µmol NA and metabolites).

Separation of ^{3}HISO and ^{3}HMeOISO:

The principle of the method is that ISO is firmly bound to the origin of a silica gel plate which is impregnated with sodium borate; in contrast, MeOISO migrates with the organic solvent. In the absence of boric acid both amines remain at the origin. Hence by restricting the borate impregnation to the lower portion of the plate, the MeOISO migrates to the borate 'front', where it becomes highly localised (Fig. 4.7). In solutions containing ^{3}HISO the assay involves the addition of sufficient non labelled MeOISO to permit an estimate of the recovery of MeOISO from this region of the plate.

In the assay procedure the test solution (containing ^{3}HMeOISO and ^{3}HISO) is applied to the plate after addition of a known amount of carrier MeOISO. At the conclusion of the run, the discrete MeOISO region (visualized under UV light) is scraped, eluted in weak HCl and the eluate counted for tritium and assayed for the non labelled MeOISO content. The last value permits the estimate of tritium to be corrected for loss of MeOISO during chromatography and subsequent extraction. The estimation of ^{3}HISO content in tissues is obtained by subtracting the ^{3}HMeOISO content from the total uptake value. (This estimation assumes that there is only one metabolite of ^{3}HISO present in tissue extracts, evidence for this is presented in Chapter 7).

Silica gel plates were subjected to ascending chromatography in freshly prepared sodium borate (200 mmol l^{-1}) solution and the solvent front allowed to migrate for a distance of 5 to 7 cms. The plates were then removed from the tank, oven-dried (100^{o}C for

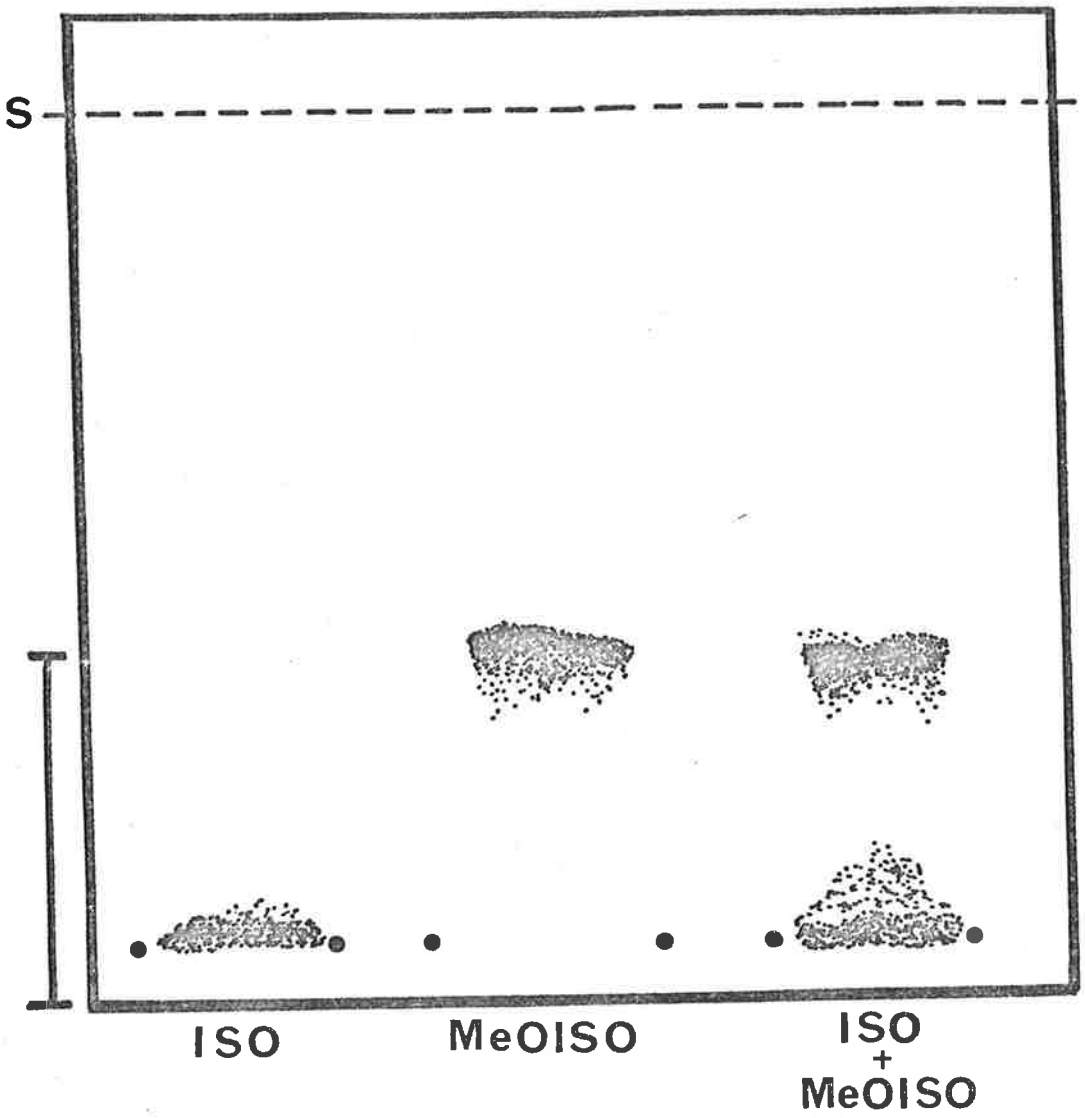


Fig. 4.7 A representation of a borate impregnated thin layer plate showing the separation of ISO from MeOISO and the migration of the 3-methoxy catecholamines to the region of the 'borate front'. The area of borate impregnation of the plates is indicated by a solid vertical bar. S indicates the position of the organic solvent front. Plates were sprayed with $K_3Fe(CN)_6/FeCl_3$.

1 hr), and stored at room temperature. The Krebs incubate (0.02 ml) and the concentrated tissue extract (0.1 ml) were spotted at the origins of the borate TLC plate and dried in a stream of cold air. The plates were then subjected to ascending chromatography, with a mixture of toluene : EtOH (1:1) for 1 or 2 hours and then air dried. The carrier compounds on the plate were visualised under UV light and the appropriate regions scraped into vials containing 2 mls of HCl (300 mmol l^{-1}). The tubes were left overnight or agitated for 6 hours, centrifuged briefly and 1.0 ml of the supernatant counted for tritium. The remainder of the solution was used for photometric assay of the total MeOISO content.

Thin layer plate blanks were prepared by applications of the same volume of Krebs incubate or tissue concentrate without carrier MeOISO and the region corresponding to MeOISO was reassayed and eluted in HCl.

Separation of ^{3}HNA, ^{3}HNMN on ^{3}H metabolites:

The contents of ^{3}H amines and their ^{3}H metabolites in test solutions were measured after their separation by thin layer chromatography. The principle of borate chelation described above also applied to this method. Silica gel plates were impregnated with sodium tetraborate as described above, the only difference being that the concentration of borate was increased to 500 mmol l^{-1} and the pH reduced to 8.5. Reducing the pH of the borate permitted some migration of one of the catechols, DOPEG and hence its separation from NA and DOMA which remained at the origin. After application

of test solutions plates were subjected to ascending chromatography with a mixture of (n-Butanol : ethanol : Tris buffer (250 mmol l^{-1}, pH 8.5 - 2:1:1). The carrier compounds on the plate were visualized and the regions scraped and eluted as described above. The contents of ^{3}H and non labelled amines and metabolites were estimated by scintillation counting and colourimetric assay. An illustration of the separation of NA and its metabolites on thin layer plates was shown in Figure 4.8.

Solvent extraction procedures

For a limited number of experiments (described in Chapter 7) ^{3}HMeOISO contents of incubation solutions and tissue acid extracts were measured after separation from ^{3}HISO by solvent extraction. This extraction procedure is based on the principle that ^{3}HMeOISO and not ^{3}HISO, can be extracted from aqueous solutions that are saturated with sodium borate.

To 0.5 ml of Krebs solution (containing ^{3}HISO and ^{3}HMeOISO) 1.0 ml of sodium borate (sodium borate, 500 mmol l^{-1} containing NaOH, 20 mmol l^{-1}) was added, shaken for 15 minutes and centrifuged (900 g for 5 minutes). 10.0 ml of the organic phase was removed and added to a counting vial containing 5 ml of concentrated phosphor. The ^{3}H content of the organic phase was estimated by liquid scintillation counting. The partitioning of ^{3}HISO from Krebs solution into the organic phase was determined by extracting incubation solutions containing ^{3}HISO, not exposed to tissues. The recovery of MeOISO was measured by estimating the amount of ^{14}CMeOISO extracted from Krebs solutions or acid extraction media containing ^{14}CMeOISO (3,000 dpm).

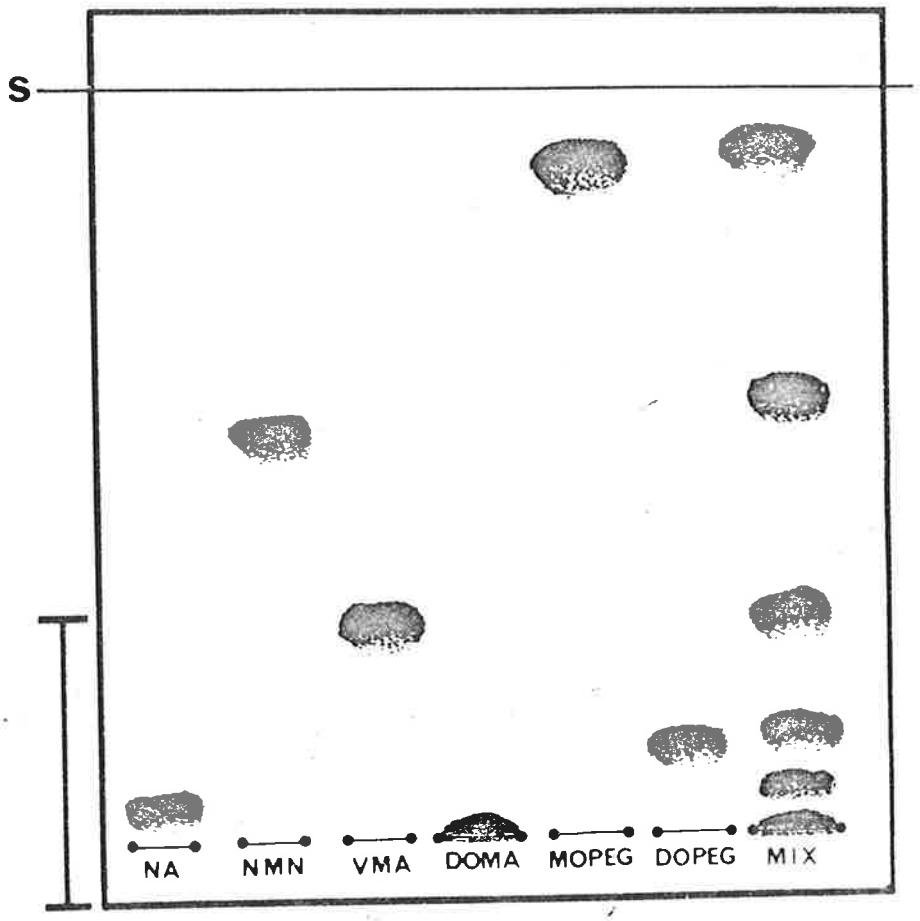


Fig. 4.8 An illustration of a borate impregnated thin layer plate showing the separation of NA from its metabolites. The area of borate impregnation of the plate is indicated by the solid vertical bar. S indicates the position of the solvent front. Plates sprayed with $K_3Fe(CN)_6/FeCl_3$

1.0 ml of an acid extract of arteries was adjusted to pH 5.0 by the addition of Na_2CO_3 (2.0 mol l^{-1}) and then treated in exactly the same way as described above.

^{14}C labelled MeOISO was prepared enzymatically by incubation of non-labelled ISO with ^{14}C SAME in the presence of COMT. The ^{14}C MeOISO was extracted from the incubation medium and purified by ion exchange paper chromatography. The purified COMT preparation used in this synthesis was prepared from rat liver COMT by the method of Axelrod and Tomchick (1959).

The incubation mixture consisted of: 0.5 mls of purified COMT, 0.1 ml of sodium phosphate buffer pH 7.8 (500 mmol l^{-1}), 0.1 ml of $MgCl_2$ (216 mmol l^{-1}), 0.1 ml of ISO (12 mmol l^{-1}), and 0.2 ml of ^{14}C SAME (16.6 mmol l^{-1}, 60 mCi $(mmol)^{-1}$) After incubation (90 mins) at 36°C the reaction was stopped with the addition of 1.5 mls of sodium tetra borate (500 mmol l^{-1}, pH 10.0) and the ^{14}C MeOISO extracted into 25 mls of a mixture of ISO amyl alcohol and Toluene (1:2). After extraction the organic phase was transferred to a tube containing 2.0 mls of HCl (100 mmol l^{-1}) and shaken for 30 minutes on a mechanical shaker. The organic phase was discarded after centrifugation and the aqueous phase containing the ^{14}C MeOISO freeze dried. The freeze dried material was redissolved in a minimal volume (approximately 0.4 ml) of HCl (100 mmol l^{-1}) and chromatographed on sheets of Whatman cellulose phosphate (P81) paper using the conditions described for Method I. The region of the paper containing the ^{14}C MeOISO was cut into 0.5 cm sections and eluted overnight in 2.0 mls of HCl (50 mmol l^{-1}). This acid eluate was removed, diluted to 30,000 dpm ml^{-1} and the solution of ^{14}C MeOISO stored at 4°C.

The recoveries of ^{14}CMeOISO from Krebs solutions and acid extraction media together with the cross contamination of ^{3}HISO in the organic solvent are summarised in Table 4.2. Due to the poor recoveries of MeOISO from Krebs solutions this procedure was modified by replacing the toluene with the solvent mixture isoamyl alcohol: toluene (1:2) and shaking the organic phase (containing MeOISO) with 3 ml of HCl (300 mmol l^{-1}). These modifications improved the recovery of MeOISO and permitted the use of the colourimetric assay procedure for determination of extracted non labelled MeOISO. This modified procedure was used in experiments described in Chapter 8 for the extraction of ^{3}HMeOISO and ^{3}HISO contents of wash solutions.

General comments

It was noted that hydrochloric acid extractions of arteries caused a consistent increase (1.66 ± 0.05 fold, n = 30) in the weight of the tissue. Arteries previously incubated with ^{3}HNA were extracted with acetic acid and not hydrochloric acid in view of the finding that NA and DOPEG in HCl solutions decomposed during the lyophilisation procedure. When hydrochloric acid solutions containing DOPEG (1 µmol) and NA (1 µmol) were lyophilised and the reconstituted acid extracts chromatographed on thin layer plates at least 4 bands of material other than NA or DOPEG were detected under U.V. light. This did not occur when acetic acid was used nor did it occur for solutions of ISO in hydrochloric acid. Fractions from Methods II and III containing minimal amounts of ^{3}H were concentrated by lyophilisation. These samples were added to lyophilisation vials and freeze dried. The residue was dissolved in 5.0 mls of a solution of HCl (100 nmol l^{-1}) and ethanol (10 mol l^{-1}), and transferred to a liquid scintillation vial containing 15 mls of phosphor.

Method II could not be applied to the estimation of ^{3}H metabolites present in incubation solutions. This was due to a relatively large and variable contamination of fractions by ^{3}H not attributable to the ^{3}H metabolites. It was noted that for acid extraction media containing only ^{3}HNA there was a large and variable contamination of the fractions corresponding to NMN and MOPEG. This was thought due to oxidation of ^{3}HNA during the preceding alumina chromatographic stage and the co-chromatography of these oxidation products with NMN and MOPEG. Similarly the fraction VMA + MOPEG as estimated by Method III was less precise than the other fractions for the same reasons. A comparison of the contents of metabolites (including VMA + MOPEG) in test solutions determined by Method III and Method IV is shown in Table 6.3 in Chapter 6 and the results bear out this conclusion. In contrast to Methods II and III the thin layer procedure (Method IV) was characterised by a small and constant ^{3}H contamination of the fractions NMN, MOPEG and VMA.

To this extent both methods complement each other and have both been used in subsequent studies to be described in Chapter 6. The ability of all three methods to account for the total ^{3}H present in artery extracts is also discussed in Chapter 6.

Table 4.2

Properties of thin layer chromatographic and solvent extraction procedures

Method	Test solution	Metabolite	Cross contamination (%)	Recovery (%)
IV	Incubate	DOPEG	1.27 ± 0.25 (17)	77.8 ± 2.1 (71)
		VMA	0.63 ± 0.09 (20)	39.5 ± 1.9 (71)
		NMN	0.23 ± 0.05 (15)	82.6 ± 1.6 (73)
		MOPEG	0.11 ± 0.11 (15)	75.7 ± 1.5 (73)
	Tissue Extract	DOPEG	2.31 ± 0.79 (11)	67.0 ± 1.8 (75)
		VMA	1.16 ± 0.48 (11)	42.8 ± 1.6 (76)
		NMN	0.51 ± 0.19 (10)	84.6 ± 1.8 (76)
		MOPEG	0.17 ± 0.05 (11)	80.6 ± 1.8 (76)
IV	Incubate	MeOISO	0.023 ± 0.005 (14)	67.9 ± 3.8 (40)
	Tissue Extract	MeOISO	0.034 ± 0.006 (8)	87.1 ± 2.5 (48)
Solvent Extraction	Incubate	^{14}CMeOISO	1.46 ± 0.39 (12)	48.9 ± 4.5 (10)
	Tissue Extract	^{14}CMeOISO	2.7 ± 0.48 (7)	65.1 ± 10.0 (10)

Footnotes

(1) value in brackets refers to the number of determinations

(2) tissue recovery values for method IV refer to efficiency of recovery through lyophilisation and thin layer procedure

(3) cross contamination refers to the amount of ^{3}H present in the fraction not attributable to the radioactively labelled metabolite as a ratio of the amount of ^{3}H present in the test solution expressed as a percentage.

CHAPTER 5

THE EFFECT OF CHRONIC DENERVATION ON THE ACTIVITIES OF MONOAMINE OXIDASE AND CATECHOL-O-METHYL TRANSFERASE AND ON THE CONTENTS OF NORADRENALINE AND ADENOSINE TRIPHOSPHATE IN THE RABBIT EAR ARTERY

CHAPTER 5

THE EFFECT OF CHRONIC DENERVATION ON THE ACTIVITIES OF MONOAMINE OXIDASE AND CATECHOL-O-METHYL TRANSFERASE AND ON THE CONTENTS OF NORADRENALINE AND ADENOSINE TRIPHOSPHATE IN THE RABBIT EAR ARTERY

INTRODUCTION

Although the study of Jarrott (1971) has indicated the presence of neuronal monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) in a variety of sympathetically innervated tissues, evidence of the distribution of these enzymes in the rabbit ear artery is lacking. The early studies of Armin *et al.* (1953) on the activity of MAO in the rabbit ear indicates that there was no change in the activity following chronic denervation. However, their technique involved manometric estimation of the increase in oxygen consumption during tyramine oxidation, and this may have been too insensitive to detect small changes in activity. In the present study, we have compared the activities of the two enzymes in innervated arteries, and in arteries at various times after chronic denervation. The assay procedures employed were those described by Jarrott (1971). In addition, the noradrenaline (NA) and adenosine triphosphate (ATP) contents have been measured. ATP was included in view of evidence that it is present in NA storage vesicles in sympathetic nerves (Schümann,

1958), and that its concentration declined in the rat vas deferens following chronic sympathetic denervation (Westfall and Stitzel, 1973).

METHODS

The central artery of one ear in each of 25 lop-eared rabbits was denervated by removal of the homolateral superior cervical ganglion as described previously in Chapter 2 (Methods). The rabbits were divided into four groups, in which the central arteries were excised 24, 48, 96 and 192 h after denervation. A fifth group of five rabbits were subjected to sham operations in which the ganglion on one side was exposed but not excised 48 h prior to removing the artery. For each rabbit the control was the contralateral innervated artery, i.e. the artery from the opposite ear.

All rabbits were stunned and bled. The central arteries in both ears were carefully cleaned of adhering connective tissue and excised at the same time. The arteries were then washed briefly in Krebs Ringer solution gassed with 95% O_2, 5% CO_2 and treated as follows:

A proximal segment of artery approximately 5 mm long was rapidly frozen in a dry-ice-acetone mixture and prepared for fluorescence histochemistry as described in the General Methods chapter. The remainder of the artery was cut into two segments of approximately equal length and each segment rapidly blotted and weighed. The segment for analysis of ATP and total catecholamines was placed in 1.0 ml of perchloric acid (400 mmol L^{-1}) at $4^{o}C$. The second segment was used for enzyme assay; it was added to 0.4 ml of sodium phosphate buffer (5 mmol L^{-1}) at pH 7.0. The time between killing the animal and placing

the segments in the extraction media was approximately 15 min. Both segments were then homogenised at $4^{o}C$ with a motorised teflon-glass homogeniser (0.8 ml capacity). The perchloric homogenate was then centrifuged (10,000 g, 30 min, $4^{o}C$) and the supernatant taken for estimation of ATP and NA content.

ATP Assay: ATP was assayed by the modification of the luminescence (firefly) assay by Stanley and Williams (1969). Luminescence was measured on a Packard Tricarb Scintillation Spectrometer with the circuitry set out of coincidence. Each counting vial contained 1.0 ml of arsenate buffer (100 mmol L^{-1}), 1.0 ml of sodium phosphate buffer (10 mmol L^{-1}) and 1.0 ml of water, in addition to 0.02 ml of sample. To the ATP standards (10^{-12} - 10^{-9} M) and enzyme blanks 0.02 ml of perchloric acid (40 mmol L^{-1}) was added to each vial in order to correct for acid quenching. The net counts accumulated for the first channel were calculated and the ATP content estimated from the standard calibration curve which was constructed for each experiment. All assays were carried out in triplicate, and the values obtained were expressed as moles of ATP per gm wet weight tissue or as the ATP content of the denervated artery expressed as a percentage of the innervated control.

NA Assay: The total catecholamine (A plus NA) contents of arteries were estimated by the fluorometric trihydroxyindole (THI) method. This followed isolation of the amines from the perchloric acid extract by means of alumina adsorption chromatography. The present method has been described in detail in the Appendix to this thesis and is essentially the same as that described earlier by de la Lande, Glover, and Head (1967), with changes in the concentration of reagents to enable adaptation of the assay to the Technicon autoanalyser. Thus the reaction

mixture comprised potassium ferricyanide (300 μmol L^{-1}, 1.6 ml min^{-1}), followed by a pre-mixed solution of sodium hydroxide (2.5 mol L^{-1}, 0.8 ml min^{-1}), and ascorbic acid (17 mmol L^{-1}, 0.32 ml min^{-1}), mixing occurring at a stage which permitted maximum lutine formation by the time the mixture reached the cuvette. The automated assay resulted in better reproducibility than that previously obtained by the manual assay and the addition of β-thioproprionic acid as a lutine-stabilising agent was found to be unnecessary. The fluorescence of the THI derivatives of NA plus A was measured at an emission wavelength of greater than 485 nm after activation at 410 nm (using a Wratten 8 filter and a 410 nM interference filter, respectively). Although under these conditions both A and NA contribute to the fluorescence, the values obtained approximate closely to the NA content of the arteries, since the proportion of A to NA in this tissue is negligible (de la Lande and Head, 1967).

MAO Assay: The MAO activity in the phosphate homogenates of arteries was estimated in an identical fashion to that method employed by Jarrott (1971). This method has been described in detail in the General Methods chapter and involves conversion of ^{3}H tyramine (2 mmol L^{-1}) to its ^{3}H deaminated products. The latter are then separated by solvent extraction and estimated in terms of their tritium content. In the present study, all samples were run in duplicate and the blank values estimated by replacing the tissue homogenate with distilled water.

COMT Assay: The COMT activity in the phosphate homogenates of arteries was assayed by the method of Jarrott (1971), which has been

described in the General Methods chapter. The method involves incubation of the homogenate with dihydroxybenzoic acid (2 mmol L^{-1}) and S-adenosyl-1-(^{3}H) methyl methionine (100 μmol L^{-1}) ($^{3}HSAME$), followed by solvent extraction and estimation in terms of tritium content of the ^{3}H-O-methylated derivative of the acid. The only change from Jarrott's procedure was an increase in the sample volume to 0.05 ml occasioned by the small quantities, and low COMT activity, of the artery. It should be noted that the homogenates used in the present study were not dialysed or fractionated prior to assay, since there was no information available as to the particular or soluble nature of the enzyme in the ear artery.

Protein: The protein contents of the phosphate homogenates were estimated after dilution by the method of Lowry *et al.* (1951) using standard solutions of bovine serum albumin. The details of this assay have also been described in the General Methods chapter.

Liquid Scintillation Spectrometry: The tritium contents of samples were measured by liquid scintillation spectrometry using a toluene based phosphor (8.25 gm 2,5-diphenyloxazole (PPO), 0.25 gm of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), and 500 ml Triton X 100 added to one litre of toluene). Samples were added to 15 ml of scintillation solution and corrections for efficiency made using ^{3}H-toluene as an internal standard.

Reproducibility: The coefficient of variation ($\frac{SD}{mean}$ %) for each of the above assays were estimated on a pooled tissue extract. They were as follows: COMT 12% (n = 12); NA 3% (n = 6); ATP 12% (n = 15).

The coefficient was not determined for MAO, since there was data available from a separate study in these laboratories on the variations between the activities in six duplicate samples of rat brain homogenates. These activities differed by less than 10%, while the coefficient of variation for the whole 12 samples was 9.9%. Accordingly, it was not considered necessary to estimate the coefficient on a single pooled sample.

RESULTS

1. NA: The monoamine fluorescence at the media adventitia border had largely disappeared in all arteries at 48 h after denervation. However, fluorescence was still present 24 h after denervation, and chemical assay of four of the 24 h denervated arteries confirmed that the decrease in content at this time was small (Table 5.1). The histochemical evidence that monoamines had largely disappeared after 48 h was also confirmed by direct assay (Table 5.1). The mean content of NA in 20 innervated arteries was 19.5 ± 1.3 mole x 10^{-9} gm^{-1}.

2. ATP: The mean ATP content (± SE) of all innervated arteries was 2.05 ± 0.02 mole x 10^{-6} gm^{-1} (n = 39). When compared with their contralateral control arteries, the denervated arteries showed a decrease in ATP content which was significant at 48, 96, and 192 h. The maximum decrease was 32% at 96 h (Table 5.1). At 192 h the results indicated a significant recovery at this stage of the ATP contents of denervated arteries to the levels prevailing both at 24 h, and in the untreated arteries. However, the ATP contents at 192 h remained significantly less than those of the contralateral control arteries, since the latter arteries also showed a significant increase

TABLE 5.1 Effect of denervation on the contents of NA and ATP in the rabbit ear artery

Constituent	Tissue Innervation	Hours after Denervation				
		24	48	48 (sham)	96	192
NA (moles$\times 10^{-9}$ gm^{-1})	Innervated	21±2 (4)	10±4 (2)	24±3 (4)	-	13±1 (2)
	Denervated	19±3 (4)	<1.0 (2)	22±1 (4)	-	<1.0 (2)
	% Denerv / Innerv	92±16	<10	99±15	-	<7.7
	Significance	N.S.	S	N.S.		S
ATP (moles$\times 10^{-6}$ gm^{-1})	Innervated	2.7±0.4 (8)	1.3±0.1 (5)	1.9±0.3 (5)	1.3±0.1 (5)	2.2±0.3 (7)
	Denervated	2.4±0.2 (8)	1.1±0.1 (5)	2.4±0.3 (5)	0.9±0.1 (5)	1.8±0.3 (7)
	% Denerv / Innerv	89±6	88±4	127±10	68±5	80±6
	Significance	N.S.	S	N.S.	S	S

Each value is the mean ± SEM for the number of arteries shown in brackets.

Significance refers to $p < 0.05$ (S) or $p > 0.05$ (N.S.) when estimated by the paired t-test for the comparisons, innervated artery vs denervated artery.

The NA and ATP contents from arteries from four untreated rabbits were 19.0±1 (moles$\times 10^{-9}$gm^{-1}, n=4) and 1.9±0.3 (moles$\times 10^{-6}$gm^{-1}, n=4) respectively.

between 96 and 192 h. The content of ATP in the contralateral control arteries at 48 h was also less than that of the control arteries at 24, and at 192 h. The cause of the difference between the various control values was not apparent. It was not due to the operating procedure, since the content of ATP in the contralateral control artery at 48 h was still significantly below the content in arteries from sham operated controls at 48 h.

3. COMT: The COMT activity proved to be low and subject to considerable variation between arteries. The mean activity ± SE in a total of 43 innervated arteries was 4.3 ± 0.3 nMole of substrate O-methylated/mg protein/h.

When the data was evaluated using the paired t-test, the COMT activities in the chronic denervated arteries were not significantly different from the activities in the contralateral control arteries irrespective of the time after denervation (Table 5.2). Pooling the data from different days (48 + 96 + 192 h, or 96 + 192 h) in order to increase the number of paired comparisons also failed to reveal a singificant effect of chronic denervation on COMT activity. Surprisingly, the COMT activities of some of the control arteries differed in that the activities at 48 h were significantly less than those at 24 h. As in the case of ATP, where a similar trend was evident, the reasons for these differences between the control activities were not apparent. The operation procedure seems excluded as a possible cause, since the activity of COMT in the 48 h contralateral control arteries remained significantly less than the activity in the corresponding arteries from sham operated rabbits.

TABLE 5.2 Effect of denervation on the activities of COMT and MAO in the rabbit ear artery

Enzyme	Tissue Innervation	Hours after denervation				
		24	48	48(sham)	96	192
COMT (nMoles of O methylated product/60mins)/mgm protein	Innervated	6.5±1.3 (5)	2.5±0.4 (6)	4.9±0.9 (5)	4.0±0.5 (4)	3.0±0.5 (6)
	Denervated	6.6±1.3 (5)	2.6±5 (6)	5.9±0.9 (5)	4.1±0.8 (4)	2.6±0.4 (6)
	% Denerv / Innerv	105±11	103±17	85±11	108±28	87±14
	Significance	N.S.	N.S.	-	N.S.	N.S.
MAO (nMoles Tyr. Deaminated/ 60 mins)/mgm protein	Innervated	228±16 (4)	182±12 (5)	-	240±28 (5)	194±22 (5)
	Denervated	230±26 (4)	170±10 (5)	-	220±28 (5)	172±14 (5)
	% Denerv / Innerv	103±15	94±3	-	91±4	91±4
	Significance	N.S.	N.S.*	-	N.S.*	N.S.*

Each value is the mean ± SEM for the number of arteries shown in brackets.

Significance refers to $p < 0.05$ (S), $p = 0.05$-0.1 (N.S.*) and $p > 0.05$ (N.S.) when estimated by the paired t-test for the comparison of innervated vs denervated arteries. The COMT activities in arteries from 4 untreated rabbits were 4.1±0.5 nMoles O-methylated/60 mins/mgm protein.

4. MAO: The activity of MAO in the innervated arteries was much greater, and the variation between arteries less, than the corresponding COMT activity. The mean MAO activity (± SE) in 16 innervated arteries was 210 ± 10 nMole of tyramine metabolised/mg protein/h. The activities of the various control arteries did not differ significantly at different times after denervation, unlike the COMT activities. However, the MAO activities of the denervated arteries at 48, 96, and 192 h after denervation were consistently less than the activities of their contralateral control arteries (Table 5.2). Although the differences approached, but did not attain, the $p = 0.05$ level of significance, comparisons between the pooled activities of all denervated arteries at and after 48 h, and their corresponding contralateral controls using the paired t-test, revealed a highly significant difference ($p < 0.01$, $n = 15$). The difference was also highly significant when the 96 and 192 h values alone were combined ($p < 0.01$, $n = 10$). The percentage changes in MAO activity following denervation, together with those of COMT activity and the changes in NA and ATP contents, are summarised in Fig. 5.1.

DISCUSSION

1. NA and ATP:

The results of fluorescent histochemical analysis, and of direct chemical assay indicated partial loss of NA at 24 h, and virtually complete loss at 48 h. The time intervals are comparable with those reported for the cat nictitating membrane (Van Orden *et al.* 1967), but greater than that for mouse and rat irides, the latter being 14 and 16 h, respectively (Sachs and Jonsson, 1973). Presumably the

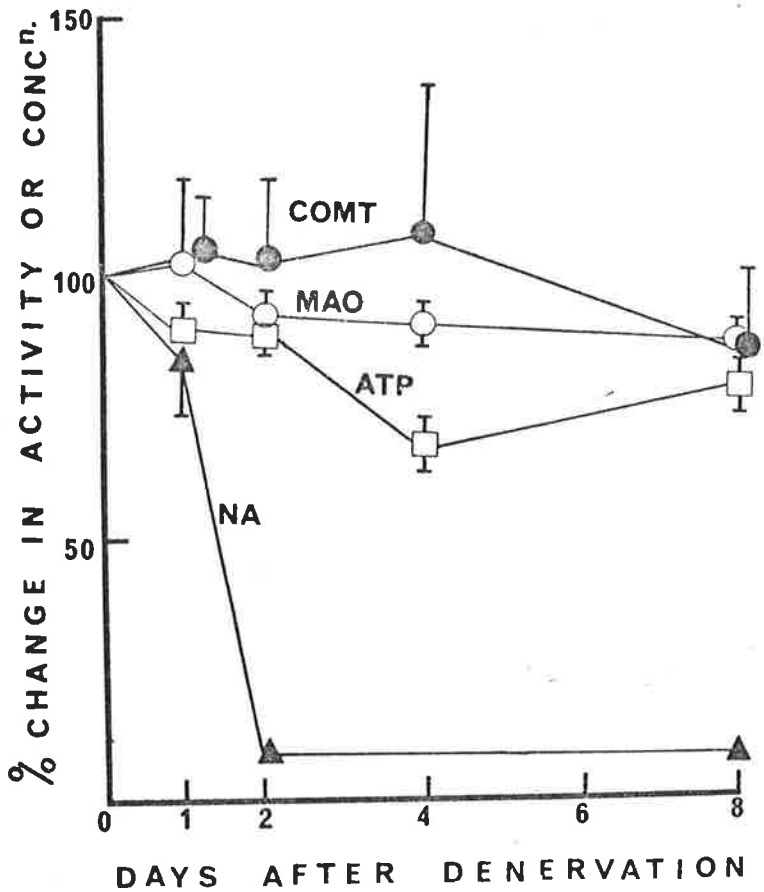


Fig. 5.1 Percentage changes in NA, ATP, COMT and MAO contents and activities of the rabbit ear artery following chronic surgical denervation. In these experiments the activities or contents of the denervated ear artery were expressed as a percentage of the value determined for the corresponding innervated artery (contralateral control artery). The percentage changes shown for ATP, COMT and MAO are derived from data based on four or more observations.

different times reflected differences in lengths of postganglionic neurones, as suggested by Sachs and Jonsson (1973).

The NA contents of the innervated arteries (19.5 ± 1.3 x 10^{-9} mole gm^{-1}) are greater than those we reported in an earlier study (5.24 x 10^{-9} mole gm^{-1}, de la Lande and Head, 1967), and by Neill *et al.* (1974), but are of the same order as those reported by Bevan *et al.* (1972) (17.88 x 10^{-9} mole gm^{-1}). The lower values in the early study of de la Lande and Head (1967) probably reflect the presence of connective tissue with the artery, since, as pointed out in that study, the arteries were not rigorously cleaned of adhering tissue. However, there remains a discrepancy between the present values and the lower values reported by Neill *et al.* (1974), namely (4.1 - 4.7 x 10^{-9} mole gm^{-1}).

The ATP contents of innervated ear arteries (2.05 x 10^{-6} mole gm^{-1}) are of the same order as those reported for the rat vas deferens (2.4 x 10^{-6} mole gm^{-1}, Westfall and Stitzel, 1973), but greater than those for the rabbit aorta (0.3 x 10^{-6} mole gm^{-1}, Needleman and Blehm, 1970).

Following denervation, the ATP content decreased. The maximum decrease was 32% at 4 days. This is less than the decrease in the ATP content of the rat vas deferens, which ranged between 50 to 80% according to the technique of denervation. The time course is also slower than that of the decrease in the vas deferens, which is maximal at 1 day (Westfall *et al.*, 1975).

Westfall and Stitzel (1973) also noted that the ATP contents tended to increase after 2 days and that this was associated with an increase in

ATP synthesis. However, in the artery a trend towards recovery of the ATP levels was not apparent until 196 h. It is conceivable that a slower time course of depletion in the ear artery may have masked the effect of an increase in synthesis of ATP.

The proportion (32%) of ATP which disappeared after denervation may have represented either ATP which was present in the nerves or ATP which was present in the smooth muscle and whose level was controlled in some way by sympathetic innervation. Our data does not distinguish between these possibilities. However, it can be calculated that the above proportion corresponds to an amount of ATP which is vastly in excess of the 1:4 ratio of ATP to NA in sympathetic nerve vesicles (Schumann, 1958). Thus the ratio of the contents of ATP to NA which disappear after denervation, namely (400 nmole g^{-1}) to NA (19 nmole g^{-1}), respectively, is approximately 20:1, i.e. about 80 fold greater than the ratio in the vesicles. Similar calculations by Westfall *et al.* (1975) led to the conclusion that the sympathetic nerve vesicles were not the source of the ATP lost in the rat vas deferens following sympathetic denervation, and clearly the same conclusion can be drawn for the rabbit ear artery. However, the possibility that the source, although not vesicular, is still neuronal, cannot be completely excluded at this stage.

2. COMT and MAO:

The activity of COMT in the arteries from untreated rabbits was low when compared with the activity of MAO, but was greater than the COMT activity of the same preparation reported by Burnstock *et al.* (1972) (4.3 c.f. 1.7 ± 1.2 nmoles/h/mg protein). The difference may have been due to the fact that the homogenates of the artery were dialysed in Burnstock's study but not in ours.

The failure of chronic denervation to influence the COMT activity indicates that a major proportion, if not all, of this activity is extraneuronal in origin. Since it is possible that the variation in COMT activity between arteries may have masked a small contribution of neuronal COMT to the total COMT activity, it should be noted that in subsequent experiments (Chapter 6) chronic denervation was shown to increase, rather than decrease, the rate of accumulation of ^{3}H normetanephrine in intact rabbit ear artery segments incubated *in vitro* with ^{3}H NA. The latter finding reinforces the evidence from this study that COMT is largely extraneuronal.

In contrast to the activity of COMT, the activity of MAO was high, showed little inter-artery variation, and showed a small but significant decrease following chronic sympathetic denervation. The small magnitude of the decrease (approximately 10%) implied that the major part of the enzyme activity is extraneuronal in distribution. This finding confirms an earlier suggestion (de la Lande *et al.*, 1970) that failure to detect neuronal MAO histochemically was a reflection of the failure of the technique to detect small changes against a background of predominantly extraneuronal MAO activity. It should be noted that evidence will be presented subsequently (Chapter 6) showing that dihydroxyphenylglycol is the major product of deamination of NA by MAO in the ear artery, and that the concentration of this metabolite in the tissue declines markedly after chronic denervation. Accordingly, the assumption that neuronal and not extra neuronal MAO is of major physiological importance in this artery has received strong support. The small nature of the decrease in activity following denervation seen in the present study may simply reflect the possibility that the neuronal MAO and extraneuronal MAO have similar affinities for tyramine, although not for NA. This

possibility is supported by recent biochemical evidence that, in rat mesenteric artery, the neuronal MAO has a higher affinity for NA and for 5-hydroxytryptamine than has the extraneuronal enzyme (Goridis and Neff, 1973). Hence analysis of enzyme activities in the intact artery employing the naturally-occurring transmitter NA as substrate would appear to offer the best prospects for further elucidating information on the functional roles of COMT and MAO in the action of NA.

In considering the significance of the small decrease in MAO activity, and absence of change in COMT activity, following denervation obtained in the present study, it should be noted that the rabbit ear artery is densely innervated compared with many sympathetically-innervated tissues. Based on NA contents reported here, its innervation is comparable with that of the rat mesenteric artery, where NA content was measured by Berkowitz *et al.* (1972). Previously, Jarrott (1971) had drawn attention to the possibility that the quantitative differences between the decreases in enzyme activities accompanying denervation in different tissues may have simply reflected different densities of innervation. However, this may be an over simplification if the data of Goridis and Neff (1973) on the rat mesenteric artery is taken into account. These workers reported a 63% decrease in MAO activity at day 9 after completing 7 days of treatment with 6-OH dopamine to effect chemical denervation. Hence it would appear that the proportion of neuronal MAO to extra-neuronal MAO differs markedly in two muscular arteries possessing about the same degree of denervation. There is one qualification to this conclusion, namely, in another study Berkowitz *et al.* (1972) were unable to detect a decrease in MAO activity in the

same rat artery subjected to similar chemical denervation. However, a decrease in NA content obtained by the latter workers was only about 50%, so that it is conceivable that the chemical denervation was not very effective in their study. Unfortunately, Goridis and Neff do not report the effect of the treatment on the NA content of the vessel.

Another possible qualification is that the time period allowed in our study may have been too brief for the decrease in enzyme activity to be maximal. However, it is of interest that Armin *et al.* (1953), in their pioneering study on the rabbit vessel, concluded that "amino oxidase persists virtually undiminished in the denervated central artery" of the rabbit ear. Their conclusion appears supported by their data for at least the period of 6 weeks after denervation. After this period, their results are too variable for interpretation; thus three groups, each of 5-6 rabbits, at 6-7 weeks after denervation showed an increase of 8%, a decrease of 26%, and a decrease of 63%, respectively. In view of the relatively crude assay method employed by these workers, there is undoubtedly an argument for reinvestigating the effects of denervation on the enzyme activities at intervals of time beyond 8 days, and using the more sensitive methods of measuring activity developed since their study.

CHAPTER 6

THE UPTAKE AND METABOLISM OF ^{3}HNA

CHAPTER 6

THE UPTAKE AND METABOLISM OF ^{3}HNA

INTRODUCTION

Following the results of the previous chapter, it was decided to use NA as substrate in the analyses of the neuronal and extraneuronal distributions and functions of COMT and of MAO. Intact segments, rather than homogenates, of arteries were used so that some of the relationships between the activities of the enzymes, and the activities of the neuronal and extraneuronal uptake systems could be studied. The uptake systems are of major importance in controlling the access of the substrate (NA) to the enzymes; their properties, and those of the enzymes, have been reviewed in the Introduction.

In the past decade there have been many investigations into the accumulation and metabolism of NA in a variety of vascular and non-vascular tissues, including the rat heart (Eisenfeld *et al.* 1967), vas deferens (Langer, 1970), cat nictitating membrane (Langer, 1970; Langer *et al.* 1972) and the rabbit aorta (Levin, 1973). In these studies segments of these tissues were incubated with high specific activity ^{3}HNA and the contents of ^{3}HNA and its ^{3}H metabolites were measured in the ^{3}H material diffusing from the tissue or present in the tissue. The major metabolites which were identified were NMN, VMA, MOPEG, DOPEG, and DOMA.

In the present study, the metabolites present in the artery segments which had been incubated with dl^{3}HNA were first identified, and their relative proportions were measured in untreated arteries, and in cocaine and chronically denervated arteries. In this way, the extent of their neuronal origin was determined. The identity and proportions of the metabolites which effluxed into the incubation medium, and into the wash fluids after bath washout of the ^{3}HNA, were also examined. Some of the efflux studies were carried out under conditions where both neuronal and extraneuronal re-uptake of the diffusing ^{3}HNA was prevented. This chapter also includes data on the effects of COMT and of MAO inhibition on the metabolite distributions.

It should be pointed out that the study is incomplete in certain respects. This was because unexpected difficulties were encountered both with the purity of the labelled NA and with the efficiency of the chromatographic separation procedures used. The problems of methodology have been already outlined in Chapter 4 and will be referred to incidentally in the Results section. The problem of the purity of the labelled NA is presented in an appendix (Appendix 2).

METHODS

Artery segments, weighing between 10 and 35 mg, were incubated for 60 minutes at 37^{o}C in Krebs solution gassed with 95% O_2 - 5% CO_2, after which they were further incubated for 30 minutes with Krebs solution containing dl^{3}HNA (1.2 μmol l^{-1}). The arteries were then washed with NA-free Krebs solution for one minute, after which they

were placed in 1.0 ml of HCl (100 mmol l^{-1}) containing EDTA (22 μmol l^{-1} at 4°C for 24 hours in order to extract the ^{3}H compounds. The acid extracts, and also the incubation medium, were analysed for ^{3}HNA and the individual metabolites by one of the four methods described in Chapter 4. For ease of presentation, the methods will be referred to in this chapter as follows:

Paper chromatography	Method I
Adsorption on alumina followed by column chromatography on Dowex-50	Method II
Column chromatography on Dowex-50 followed by adsorption on alumina	Method III
Thin layer chromatography on sodium borate-impregnated silica gel plates	Method IV

Other procedures:

Nerve stimulation: One group of experiments were carried out in collaboration with another Ph.D. student, D.A.S. Parker, in these laboratories. Mr. Parker was investigating the effect of drugs on the release of ^{3}H from the artery in response to nerve stimulation. The present author examined the composition of some of the effluxes from these arteries. The experimental setup which he used was as follows:

The artery segment was cannulated at its proximal and distal ends and perfused intraluminally at a rate of 0.30 ml min^{-1} with Krebs solution. The adventitial surface was bathed in 2 ml of Krebs solution. The arteires were incubated with extraluminal $dl^{3}HNA$ (0.6 μmol l^{-1}) for one hour, after which they were washed for a further hour with ^{3}HNA-free Krebs solution both intraluminally and extraluminally. Extraluminal bathing solutions were then collected for two successive 8-minute

intervals and the composition of the ^{3}H material in these solutions were analysed by Method III. During the first 4 minutes of the second 8-minute period, the artery was stimulated at a rate of 5 Hz for 4 minutes by means of platinum electrodes, one inserted in the luminal inflow to the artery, and the other in the extraluminal bathing medium. Care was taken to ensure that the electrode in the extraluminal bathing medium was the anode so that cathodic oxidation of the released ^{3}HNA did not occur (Appendix 3).

Chronic denervation: Arteries were denervated 7 to 14 days prior to removal by the procedure outlined in the general methods chapter (Chapter 2).

Drug treatment: Drugs which were always added to the incubation medium 15 minutes prior to adding the ^{3}HNA comprised cocaine, U0521, and deoxycorticosterone acetate (DOCA). Nialamide was always added at least 40 minutes prior to the NA. PBZ was also used in some experiments and its use is referred to in the Results.

Variations: Variations of the above conditions are described in the relevant section of the Results.

RESULTS

1. Total ^{3}H

The relative tissue contents of ^{3}H during 30 minutes of incubation with dl^{3}HNA under identical conditions are summarised in Table 6.1. This Table summarises the pooled data from the experiments carried out with dl^{3}HNA described in the following pages. The results show that

the tritium was accumulated approximately six fold above the levels to be expected if the ^{3}H were simply distributed through the tissue water. The results also indicate that chronic sympathetic denervation or incubation with cocaine decreased the tissue levels of ^{3}H by approximately 66%, while PBZ caused a decrease of 90%.

Inhibition of MAO by nialamide tended to increase, where inhibition of COMT by U0521 tended to decrease the accumulation of ^{3}H; neither tendency was significant.

These results suggested that the major proportion - approximately 80%, of the ^{3}H was sympathetic neuronal in origin, so that the extra-neuronal proportion constituted approximately 20% of the accumulated ^{3}H.

TABLE 6.1

Accumulation of ^{3}H (expressed as NA nmole g^{-1})
in arteries incubated with dl ^{3}HNA

Treatment	Nil	Cocaine	Denerv^{n}	Cocaine + Denerv^{n}	PBZ	PBZ + Denerv^{n}	Nial.	U0521
Conc. ^{3}H	8.23 ±0.59	2.85 ±0.41	2.30 ±0.38	1.34 ±0.36	0.77 ±0.11	0.62 ±0.10	8.60 ±1.05	6.46 ±0.43
N	27	8	13	8	9	8	5	5

Footnote:

1. The data refers to the ^{3}H accumulation in the artery after incubating for 30 min with ^{3}HNA 1.2 $\mu mole^{-1}$ l^{-1} and washing for one minute. All changes produced by these treatments were significant ($p < 0.05$) using an unpaired t-test, with the exception of those produced by Nialamide and U0521.

2. Metabolites in the tissue

The main metabolite fractions in arteries incubated with dl^{3}HNA were identified using paper chromatography (Method I). In these experiments 6 artery segments were incubated with dl^{3}HNA (3.0 μmol l^{-1}, 2.6 curies $mmol^{-1}$) for 15 minutes, washed for 30 seconds with amine-free Krebs solution, and then washed for a further 15 minutes prior to assay of the tissue, and of the wash fluid. A typical chromatogram is shown in Fig. 6.1, and the results are summarised in Fig. 6.2. They indicated that 59% of the label extracted from the chromatogram of the tissue extract was ^{3}HNA, 15% was in the fraction (DOMA + DOPEG), 4.6% was NMN, and 4.5% in the fraction (VMA + MOPEG). In the wash fluids, the percentages of NA, (DOMA + DOPEG), NMN and (VMA + MOPEG) were 30, 22, 11 and 12% respectively.

In subsequent experiments, the three further separation procedures described in the Methods were used in an attempt to achieve better resolution and quantitation of each of the individual metabolites. The three procedures were : (a) alumina adsorption followed by Dowex-50 column chromatography (Method II); (b) Dowex-50 column chromatography followed by alumina adsorption (Method III); and (c) thin layer chromatography on sodium borate-impregnated silica gel plates.

The artery segments were treated identically, namely, the segment was incubated with dl^{3}HNA (1.2 μmol l^{-1}) for 30 minutes followed by a one minute wash. The contents of ^{3}HNA and metabolites in the tissue, and in the incubating medium were then determined. The data on the incubation media are described separately. The results on the tissue levels are summarised in Table 6.2. Their major features were as follows:

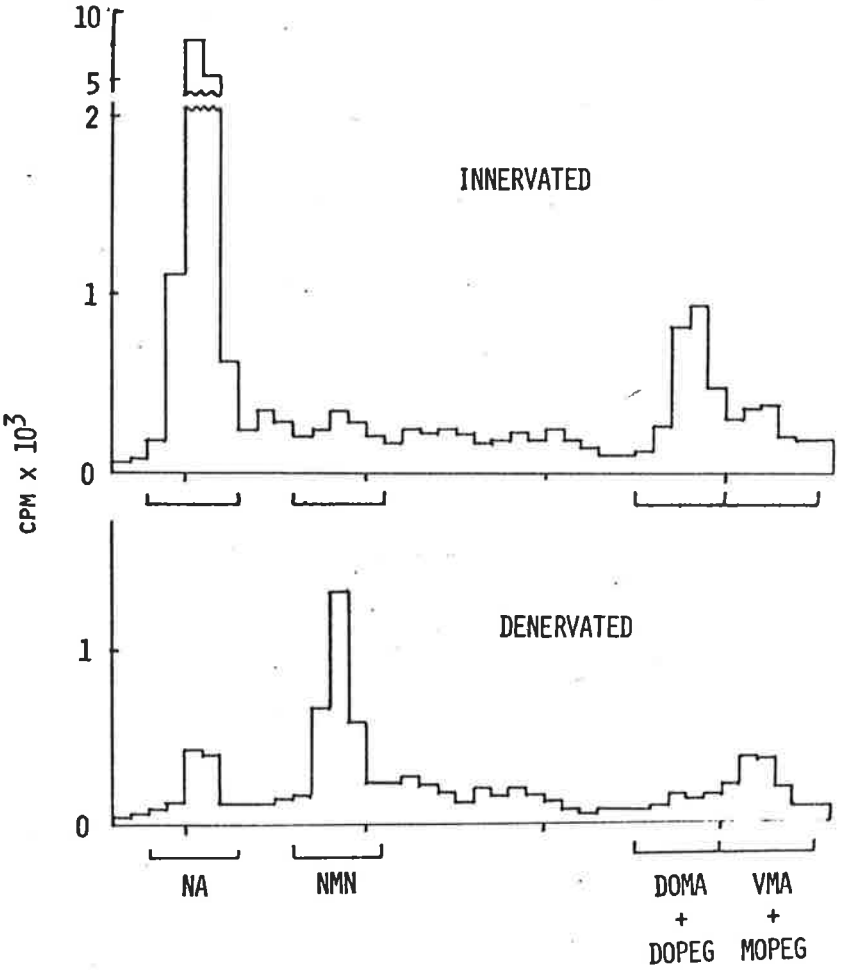


Fig. 6.1 The relative distributions of ^{3}H on paper chromatograms (Method I) for acid extracts of innervated (upper panel) and denervated (lower panel) arteries. Arteries incubated with dl^{3}HNA (3.0 μmol l^{-1}). Conditions of incubation and chromatography are outlined in the text.

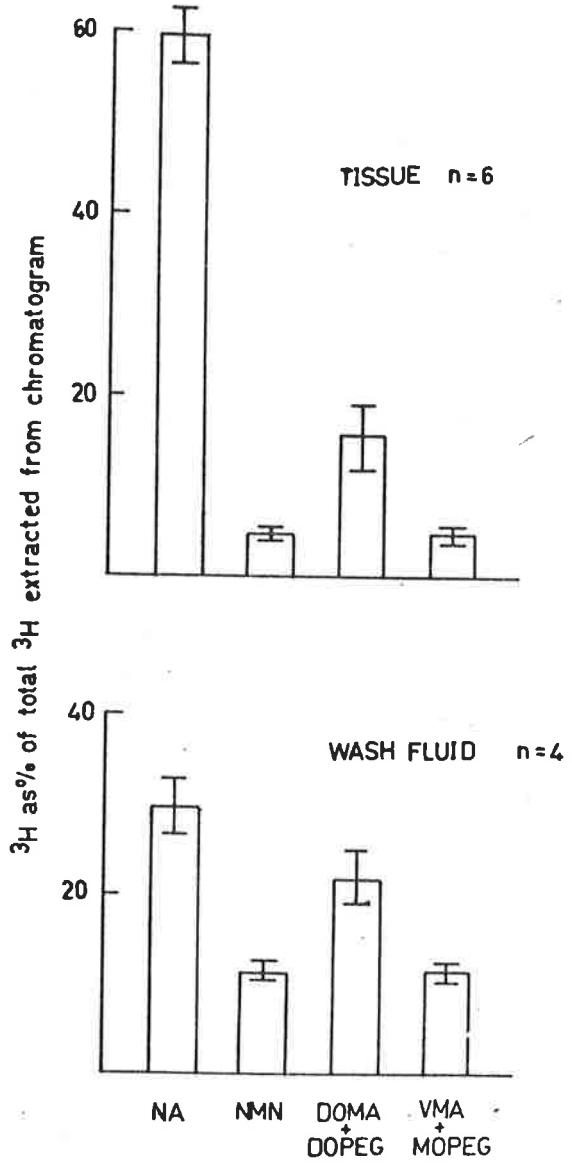


Fig. 6.2 The proportions of ^{3}HNA and its metabolites in arteries and wash solutions determined from paper chromatographic experiments (Method I).

(1) The proportion of the total ^{3}H uptake which was identified as ^{3}HNA plus metabolites varied from 100% for Method Ii to 60% for Method IV. The lower value for the latter method may be a reflection of the fact that the major proportion of ^{3}H is in the combined fraction ^{3}HNA + $^{3}HDOMA$. Since this fraction cannot be quantitated with the precision of the remaining fractions, the simplifying assumption of 100% recovery was applied to its estimate.

(2) The methods are in reasonable agreement that ^{3}HNA is the major component of the ^{3}H uptake, representing between 80% and 87% of the latter when estimated from the sum of the identified fractions.

(3) There is also good agreement between the methods that ^{3}H DOPEG is the next major component of the summed uptake of ^{3}H (5.9 - 7.7%) and this is followed by ^{3}H DOMA (2.8 - 2.9%), and the combined fraction ^{3}H MOPEG and ^{3}H VMA (2.1 - 3.2%). The analyses by Method IV shows that VMA predominates in the latter combined fraction. In contrast, the values for ^{3}H NMN show a very wide spread, from 2 to 8.8% of the summed ^{3}H uptake. As discussed in Chapter 4, there is evidence that Method II probably overestimated NMN due to crossover from the ^{3}HNA fraction. (The evidence emerged when the method was applied to the incubates, where the background concentration of ^{3}HNA against which the separation from ^{3}H NMN occurs is very much greater than in the tissue extract). The lower values of NMN (Method IV) cannot be attributed simply to an underestimate of ^{3}H NMN by this method because it will be shown later (Chapter 9) that this estimate is quantitative with this procedure.

TABLE 6.2

Tissue contents of ^{3}HNA and metabolites (in nmol g $^{-1}$) estimated by different methods

Method	n	^{3}H NA	^{3}H NMN	^{3}H MOPEG	^{3}H VMA	^{3}H DOPEG	^{3}H DOMA	TOTAL* ^{3}H	MEASURED TOTAL ^{3}H
II	6	6.4 ±1.5 (80%)	0.71 ±0.24 (8.8%)	0.17 ± 0.04 (2.1%)		0.53 ±0.06 (6.6%)	0.24 ±0.06 (2.9%)	8.04	8.0 ±1.6
III	11	4.2 ±0.39 (83%)	0.25 ±0.04 (5%)	0.16 ± 0.05 (3.2%)		0.30 ±0.07 (5.9%)	0.14 ±0.03 (2.8%)	5.05	6.55 ±0.72
IV	7	4.3 ±0.51 (87%)	0.10 ±0.02 (2%)	0.03 ±0.01 (0.7%)	0.11 ±0.04 (1.8%)	0.38 ±0.11 (7.7%)	-	4.93	8.16 ±0.52

*Total Σ^{3}H is the sum of each of the fractions.

3. Effect of enzyme inhibition

Four arteries were incubated with dl ^{3}HNA (1.2 μmol l^{-1}) in the presence of the COMT inhibitor, U0521 (55 μmol l^{-1}) for 30 minutes. A further four were preincubated with the MAO inhibitor, nialamide (350 μmol l^{-1}) for 45 minutes and washed in amine-free Krebs solution for a further 15 minutes before incubation with dl ^{3}HNA (1.2 μmol l^{-1}) for 30 minutes. The tissue levels of ^{3}HNA and the ^{3}H metabolites were analysed by Method III (Dowex → alumina).

The results (Fig. 6.3) showed that COMT inhibition markedly decreased the tissue content of ^{3}H NMN (60%), but did not produce the anticipated decrease in the content of the (VMA + MOPEG) fraction. The content of ^{3}H DOPEG was increased although not significantly; the level of ^{3}HNA was unaffected.

In contrast, nialamide did not produce a significant decrease in the contents of the deaminated metabolites, nor did it significantly increase the content of NMN. This was surprising in view of evidence subsequently presented that nialamide markedly decreased the levels of the deaminated metabolites in the incubating medium in association with an increase in NMN. However, in the present experiments, nialamide did cause a marked increase in the contents of ^{3}HNA in the tissue (Fig. 6.4). The increase was significant.

(Note: although it was tempting to attribute the lack of effect of nialamide on the deaminated metabolites to a defect of the method used to separate the metabolites (Method III), it will be noted from other results presented in this chapter (Table 6.5) that the same method indicated a profound effect of nialamide on the levels of the deaminated metabolites which effluxed from the tissue after ^{3}HNA bath washout).

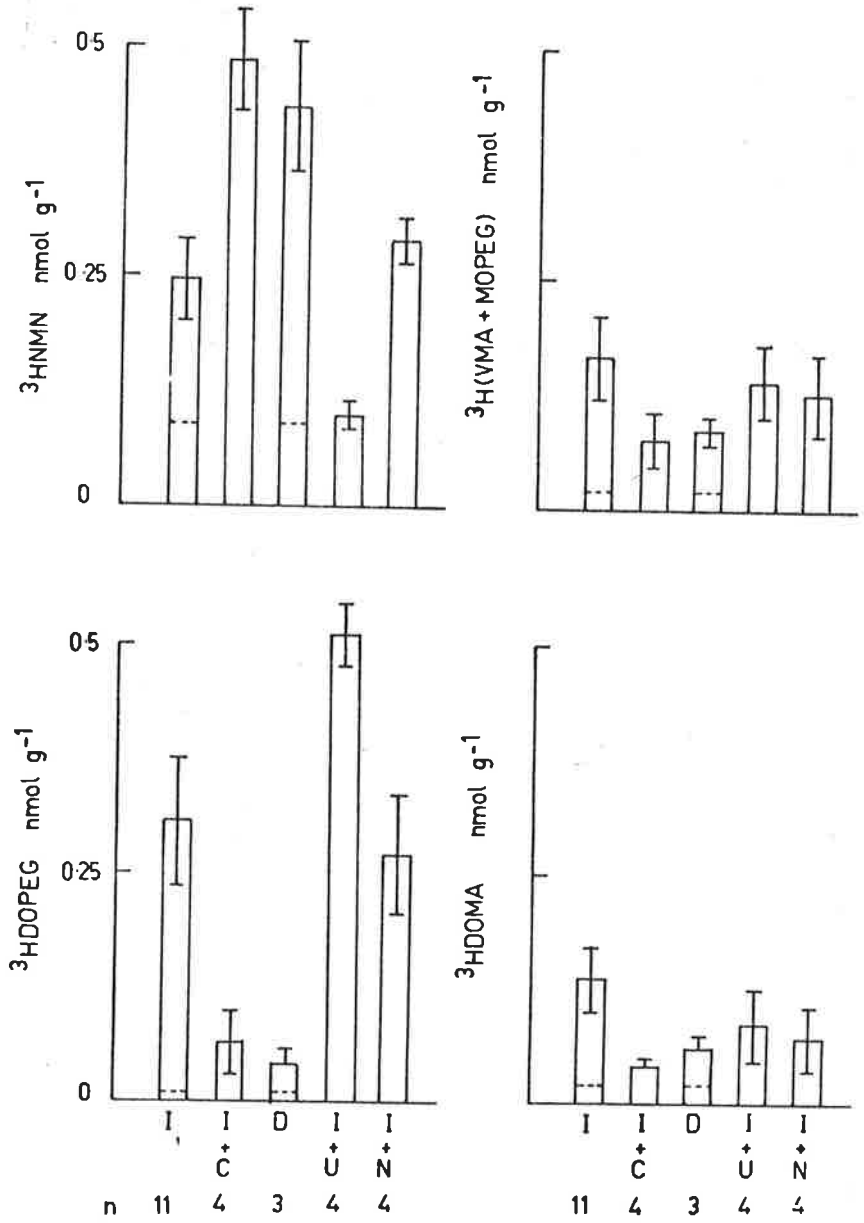


Fig. 6.3 Levels of NA metabolites in tissue after incubation with dlNA (1.2 µmol l^{-1}) determined by Method III.

I = innervated
D = denervated
U = U0521 (55 µmol l^{-1})
N = nialamide (350 µmol l^{-1})
C = cocaine (30 µmol l^{-1})

The dotted lines in the columns refer to the levels in arteries treated with PBZ 33 µmol l^{-1}.

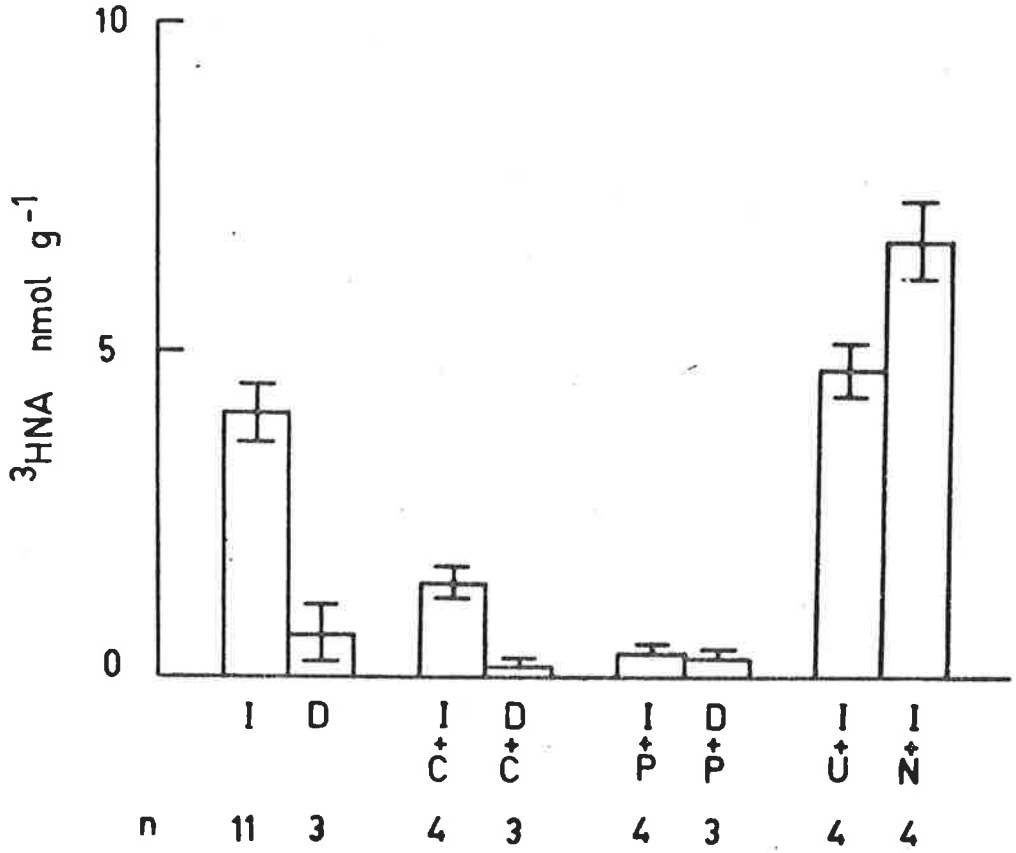


Fig. 6.4 Tissue content of ^{3}dlNA after incubation with ^{3}HdlNA (1.2 µmol l^{-1}) determined by Method III

I = innervated
D = denervated
C = cocaine (30 µmol l^{-1})
U = U0521 (55 µmol l^{-1})
N = nialamide (350 µmol l^{-1})
P = Phenoxybenzamine (33 µmol l^{-1})

4. Effect of drugs and denervation

The effects of cocaine, chronic denervation, and phenoxybenzamine were examined on the contents of ^{3}HNA and of the ^{3}H metabolites in arteries after incubation with dl^{3}HNA (1.2 μmol l^{-1}) for 30 minutes as described in Methods. The column chromatographic methods (II and III) were used. The results by both methods (Method II Fig. 6.5, Method III Figs. 6.3 and 6.4) showed essentially similar trends, in that denervation caused a large and significant decrease in the tissue content of ^{3}HNA (Method II, 78%; Method III, 84%), ^{3}H DOPEG (Method II, 87%; Method III,87%) and ^{3}H DOMA (Method I, 46%; Method II, 41%). Denervation also caused a significant increase in the tissue contents of ^{3}H NMN (Method I, 73%; Method II, 76%). These results were interpreted to mean that the major proportion of the ^{3}HNA which accumulated in this artery was in the sympathetic nerves. The decrease in catechol deaminated but not 3-O methylated metabolites was viewed as evidence for a neuronal origin of the former metabolite fraction and an extraneuronal origin of the latter fraction. Supporting evidence was provided by experiments on the effects of cocaine (30 μmol l^{-1}). The results by the two methods showed good agreement, in that cocaine, like denervation, reduced the tissue contents of ^{3}HNA (Method II, 71%; Method III, 64%), ^{3}H DOMA (Method II, 69%; Method III, 70%) and ^{3}H DOPEG (Method II, 87%, Method III, 79%). However, the effects of cocaine on the ^{3}H NMN contents were not in agreement in that the Method II series indicated that cocaine was without effect on the ^{3}H NMN contents of arteries, whereas the Method III experiments (Fig. 6.3) showed that cocaine, like denervation, significantly increased (by 86%) the ^{3}H NMN contents of the arteries. This latter finding was viewed as more reliable in view of the evidence advanced earlier that Method II may have overestimated the contents of

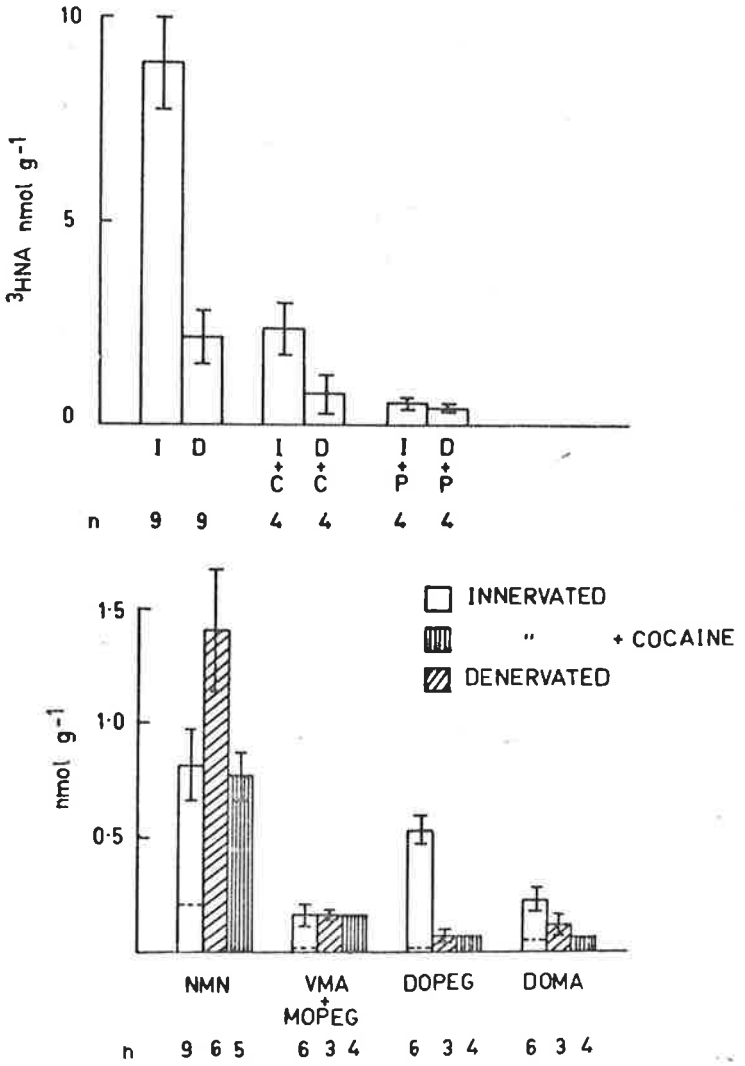


Fig. 6.5 Tissue content of ^{3}HNA (top histogram) and metabolites (bottom histogram) after incubation with ^{3}HdlNA (1.2 µmol l^{-1}) determined by Method II.

I = innervated
D = denervated
C = cocaine (30 µmol l^{-1})
P = phenoxybenzamine (33 µmol l^{-1})

The dotted lines in the columns refer to the levels of metabolites in arteries treated with PBZ 33 µmol l-1 (n=3-4).

NMN. An unexpected result was that with both methods cocaine significantly reduced (65% and 79%) the ^{3}HNA contents of denervated arteries (Figs. 6.4, 6.5), i.e. it appeared that some neuronal accumulation of ^{3}HNA persisted in the denervated arteries, suggesting that denervation may not have been complete.

The effects of PBZ (33 μmol l^{-1}) was studied in view of evidence (Iversen and Langer, 1969) that PBZ inhibits both the neuronal and extraneuronal uptakes of NA. In these experiments, the PBZ was added to the incubating medium 30 minutes prior to the ^{3}HNA and was present during the incubation. The results of the experiments (Figs. 6.3, 6.4 and 6.5) showed that PBZ, like cocaine and denervation, reduced the ^{3}HNA contents of the arteries. However, unlike cocaine and denervation PBZ decreased the contents of both the ^{3}H catechol deaminated and 3-O methyl metabolites. It did not further reduce the accumulation of ^{3}HNA in denervated arteries (Figs. 6.4 and 6.5). The estimate of the ^{3}HNA content of the arteries incubated in the presence of PBZ, obtained by pooling the data from both series of experiments was 0.42 nmol gm^{-1}. The estimate for the PBZ+ denervated arteries was 0.32 nmol gm^{-1} and for the cocaine + denervated arteries was 0.48 nmol gm^{-1}. These differences were not significant. Assuming that PBZ inhibits both extraneuronal and neuronal accumulation of ^{3}HNA, the absence of a significant difference between the content of ^{3}HNA in the denervated arteries and in denervated plus PBZ-treated arteries (Fig. 6.4) suggests that under our conditions of estimation there is no appreciable extra-neuronal accumulation of ^{3}HNA in this tissue. However, PBZ reduced the content of all the metabolites in the denervated arteries to levels approaching the limits of the assays. This suggested that the sources

of the small proportion of the catechol deaminated metabolites present after denervation were extraneuronal but intracellular in origin. The dramatic decrease in the high concentration of NMN in the denervated arteries was consistent with the intracellular formation of the NMN by extraneuronal COMT.

5. Incubating medium

Method II did not give meaningful results when applied to each of the incubating medium of six arteries. In each case the radioactivity in the NMN and DOPEG fractions in the control solutions (Krebs containing dl ^{3}HNA incubated at 37^{o}C without the tissue) was of the same order or even exceeded that of the activity in test solutions (i.e. solutions incubated with the arteries). A similar problem was encountered in some but not all subsequent experiments when Method III was employed instead of Method II. (It became apparent at a later stage that the problem was not entirely methodological but was compounded by impurity of the isotope). However sufficient results were achieved with Method III on incubating media to enable a meaningful comparison with results obtained by Method IV. These media were the solutions containing dl ^{3}HNA (1.2 μmole l^{-1}) in which the arteries had been incubated for 30 minutes. The results of four experiments are summarized in Table 6.3 from which it will be seen that both methods show reasonable agreement with respect to the proportions of DOPEG and NMN. The greatest difference is in the estimation of the VMA + MOPEG fraction, which was approximately five fold greater by Method III than by Method IV. In view of subsequent evidence that the (VMA + MOPEG) fraction in wash solutions of arteries is less sensitive to the enzyme inhibitors as estimated by Method III than when estimated by Method IV, the present

TABLE 6.3

Individual contents of metabolites (nmol g^{-1}) estimated by Method III and Method IV

METABOLITE	METHOD III	METHOD IV
NMN	0.214	0.112
	0.243	0.175
	0.009	0.138
	0.162	0.120
	0.157 ± 0.06	0.136 ± 0.02
DOPEG	1.45	1.52
	0.92	1.05
	0.21	0.44
	0.29	0.64
	0.72 ± 0.34	0.91 ± 0.28
VMA + MOPEG	2.20	0.31
	1.80	0.33
	0.56	0.21
	0.86	0.25
	1.35 ± 0.45	0.27 ± 0.03

results obtained for that fraction by Method IV were assumed to be more reliable. The relative amounts and proportions of the metabolites in the medium as compared with the tissue based on analyses of a further 5 incubating media and 7 tissues, were as set out in Table 6.4.

TABLE 6.4

Contents of metabolites (nmol g^{-1}) of ^{3}HNA in arteries and incubation media

	n	DOPEG	DOMA	VMA	MOPEG	NMN
Medium	9	2.4±0.75	0.16±0.11	0.09±0.02	0.31±0.05	0.32±0.08
Tissue	7	0.38±0.11	0.14±0.03	0.11±0.04	0.03±0.01	0.10±0.02
Total		2.78	0.30	0.20	0.34	0.42
Ratio $\frac{\text{Medium}}{\text{Tissue}}$		6.3	1.1	0.82	10.3	3.2
Relative proportion of the total metabolites						
Medium		73%	4.9%	2.7%	9.4%	9.8%
Tissue		50%	18%	14%	4%	13%

The main features are that DOPEG is outstandingly the major metabolite in both the tissue (50%) and the medium (73%). Whereas the deaminated acids DOMA and VMA are next in importance in the tissue, the reverse situation applies to the medium, where the O-methylated metabolites NMN and MOPEG are next in importance.

The effects of inhibitors of MAO and of COMT on the composition of the medium was determined in a separate series of experiments in which the efflux of metabolites was also examined. In these experiments, the

concentration of dl^{3}HNA was slightly less (0.9 μmol l^{-1}) and the period of incubation was 60 instead of 30 minutes. (Different conditions were used in order to match those in a separate study in these laboratories by D.A.S. Parker on the factors influencing stimulation-induced efflux of transmitter from sympathetic nerves). Four arteries were untreated, four were pretreated with nialamide 330 μmol l^{-1} for 45 minutes, and four were treated with U0521 55 μmol l^{-1} (the latter present before and during incubation, as well as in the wash fluids). The results are summarised in Fig. 6.6.

As with the shorter period of incubation, DOPEG was the predominant metabolite, representing 66% of the total metabolites; and MOPEG and NMN were present in much smaller proportions (6 and 9% respectively). However, the proportion of VMA (16%) appeared disproportionately much higher in the one hour than in the 30 minute incubate (Fig. 6.6; c.f. Table 6.4).

Nialamide-treatment decreased the accumulation of DOPEG, MOPEG, and VMA each by more than 90%, and at the same time increased the accumulation of NMN by a factor of 2.8.

U0521 decreased the accumulations of NMN and of MOPEG in the incubation media each by more than 90%. The decrease in VMA was smaller (40%). The level of DOPEG in the medium was greater in the U0521-treated arteries but the increase was not significant (see footnote, Fig. 6.6).

The above effects of the enzyme inhibitors were consistent with the expected roles of MAO in the formation of the deaminated metabolites, and with the anticipated role of COMT in the formation of both NMN, and the remaining O-methylated metabolites. The only qualification being that

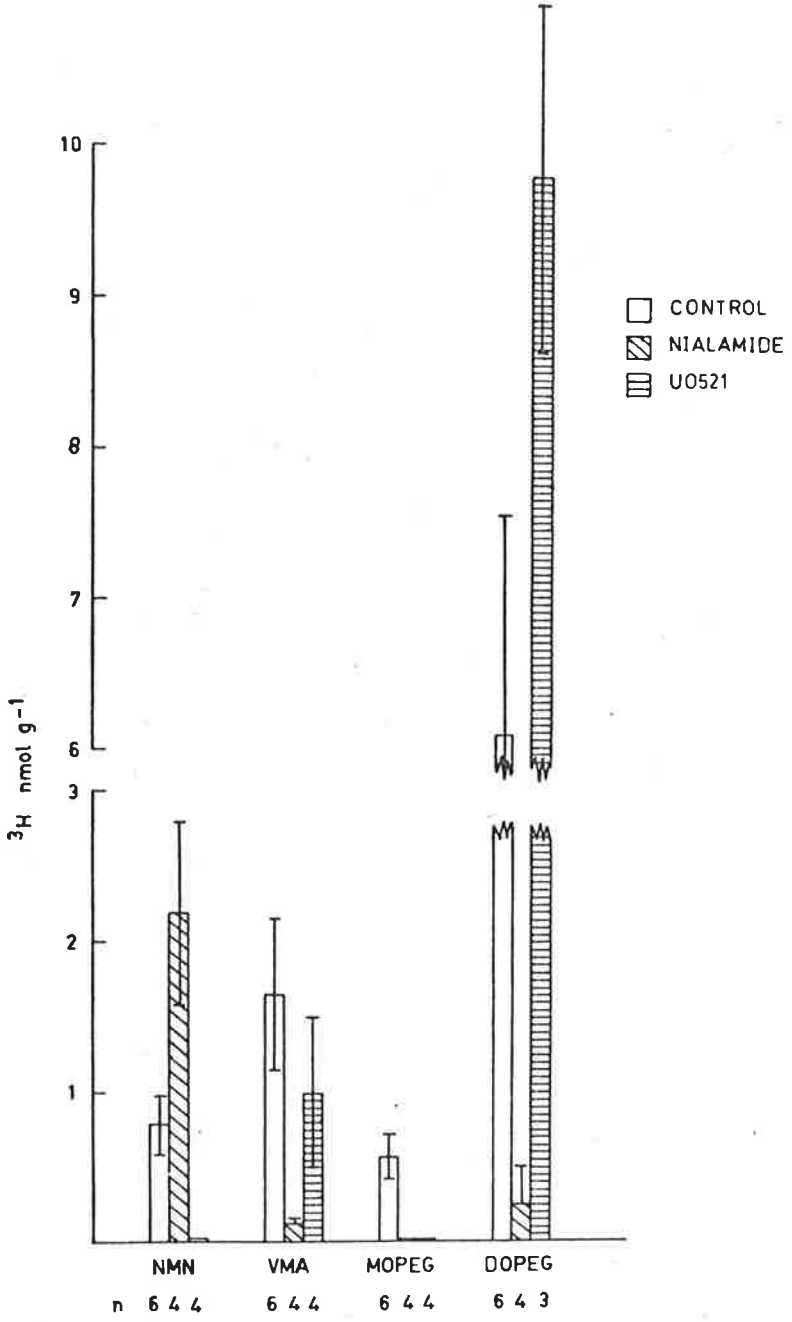


Fig. 6.6 Contents of ^{3}H metabolites in the incubation medium determined by Method IV for untreated arteries, MAO inhibited arteries (nialamide 33 µmol l^{-1}) and COMT inhibited arteries (UO521 55 µmol l^{-1}). All arteries incubated for 1 hour with dl^{3}HNA (0.9 µmol l^{-1}).

the levels of VMA in the medium may have been overestimated because the COMT inhibitor did not eliminate this fraction.

6. Efflux

Introductory note: Efflux of ^{3}H following NA washout was studied under conditions where both neuronal and extraneuronal uptake were inhibited. For the latter purpose, PBZ 33 μmol l^{-1} was added to the Krebs solution immediately after bath washout of the incubated ^{3}HNA. Two series of experiments were carried out. The first series were carried out in co-operation with D.A.S. Parker, who was examining the effects of drugs, including PBZ, on the stimulated efflux from arteries approximately one hour after their incubation with dl^{3}HNA (0.6 μmol l^{-1}) and 1NA (0.3 μmol l^{-1}). The present author took the opportunity of analysing the resting and stimulated effluxes from those arteries. It seemed that this would provide a further test of the neuronal origin of the DOPEG, and in particular whether it was formed from ^{3}HNA after the latter had been taken up and stored in neuronal vesicles. It provided also a further opportunity of testing whether any of the O-methylated derivatives were of neuronal origin.

For these experiments two arteries from each of four rabbits were cannulated and incubated for one hour with dl^{3}HNA (0.9 μmol l^{-1}) in small organ baths. To one of these arteries cocaine 30 μmol l^{-1} was present during incubation. At the end of one hour, both incubating media were removed and replaced with ^{3}H free Krebs solutions containing PBZ (33 μmol l^{-1}) and the arteries were also perfused intraluminally at a rate of 0.25 ml min^{-1} with the latter solution. The external bathing medium was replaced 3 times at 20 minute intervals. After this time, two lots of bathing medium which had been in contact with the artery for two successive

periods of 8 minutes were removed and analysed for metabolites. These samples are described as "resting efflux". During the following 4 minutes the artery was stimulated electrically by means of periarterial platinum electrodes at 5 Hz for 4.0 minutes, and after a further 4 minutes, the bathing medium was analysed by method III (column chromatography on Dower 50 followed by alumina adsorption), the efflux of ^{3}H in this solution is referred to as the "stimulated efflux".

The results on the 4 pairs of arteries are shown in Fig. 6.7 where the following features are evident, namely:

(1) The resting efflux consisted (in descending order) of DOPEG (51% of total), NA (22%) and DOMA (14%). NMN was not detected. Surprisingly, there was a significant proportion of O-methylated products (14%); however this was probably an overestimate by the method, as discussed subsequently in this section. In the arteries which had been treated with cocaine, the resting efflux was decreased by 84%, the greatest decrease being in the DOPEG and DOMA fractions (by > 94%). Surprisingly, there was a significant residual efflux of ^{3}HNA, possibly representing a non-neuronal but firmly bound component of this amine.

(2) In the stimulated efflux, ^{3}HNA was the major constituent (69%). The proportions of DOPEG and DOMA were correspondingly decreased below those in the resting efflux; the absolute rates of efflux of these two metabolites were also less during stimulation and the difference was significant for DOPEG but not for DOMA. The effluxes of ^{3}HNA and the metabolites (with the possible exception of VMA + MOPEG) did not increase during stimulation in those arteries previously treated with cocaine.

These findings provided further evidence of the almost entirely neuronal origin of both the catechol deaminated metabolites in the resting and stimulated efflux and, furthermore, suggested that their release was not dependent on the release of NA. This follows from the failure of

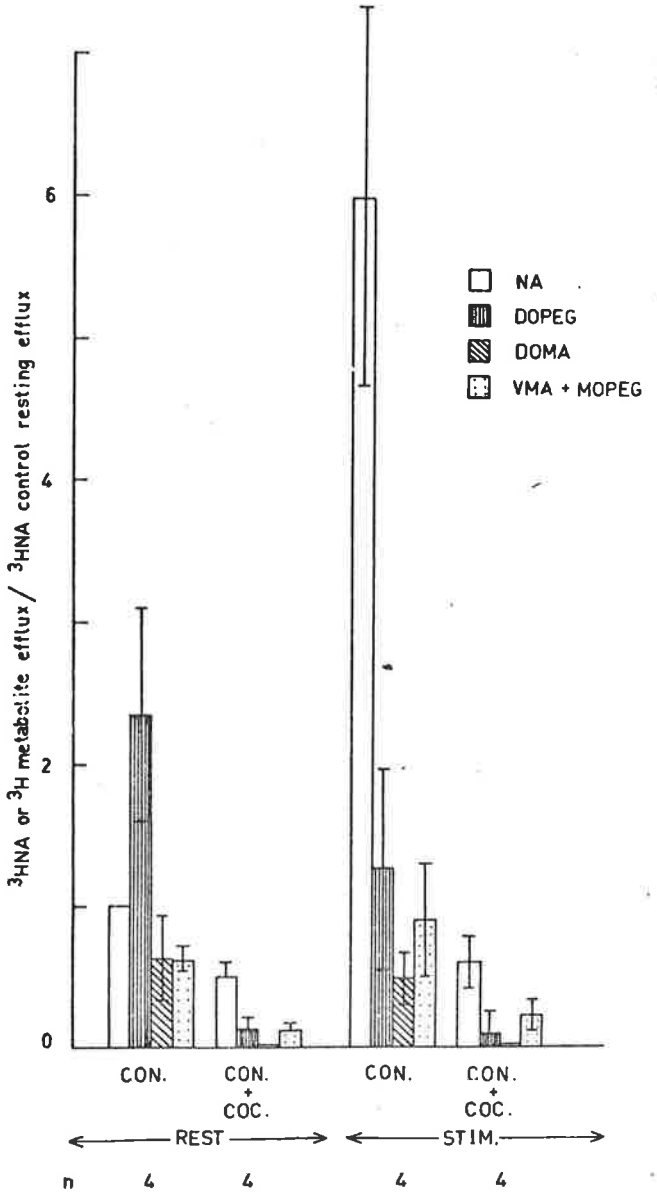


Fig. 6.7 Proportions of dl^{3}HNA and its metabolites in the bathing medium with and without stimulation. Determined by Method III.

CON = untreated
COC = cocaine (30 μmol l^{-1})

stimulation to increase the efflux of the metabolite fraction. However, an unexpected finding was the possible presence of O-methylated metabolites, as this represented the first indication that there was neuronal COMT in this artery. In view of its significance, it was decided to repeat portion of the above experiments using the TLC method (Method IV) for separating metabolites. The opportunity was taken of using an even ratio (1:1) of the dl form of NA instead of the uneven ratio (1½:1, 1:dl) which had been used in the immediately preceding experiments. To provide a further critical test, it was decided also to examine the effects on the composition of the efflux of an inhibitor of COMT (UO521) and an inhibitor of MAO (nialamide).

As in the preceding experiments, the efflux was measured in wash solutions containing PBZ to prevent re-uptake and metabolism of the ^{3}HNA occurring after its release from the storage sites within the tissue.

Six arteries were incubated for 60 minutes with dl^{3}HNA (0.9 μmol l^{-1} 10 Ci/mmol) in Krebs solution at 37^{o}C, and were washed for one minute, followed by two consecutive 30 minute periods. A further eight arteries were treated identically, with the exception that in four the COMT inhibitor, UO521 (55 μmol l^{-1}) was added to the medium 15 minutes prior to commencing incubation, and the other four arteries were first pre-incubated with the MAO inhibitor nialamide (350 μmol l^{-1}) for 45 minutes, followed by a 15 minute wash prior to incubation with NA.

The levels of ^{3}HNA and the metabolites were then estimated by TLC (Method IV), and in some cases, also by column chromatography (Method III). Method III enabled the levels of DOMA and of NA to be estimated separately. The results of the analysis of wash fluids by the two methods are in Table 6.5. All the data which was derived from Method IV is presented in

TABLE 6.5

Contents of ^{3}HNA and metabolites in wash solutions from individual arteries estimated by Method III and Method IV

Treatment	Wash period (min)	Method	NA	NMN	DOPEG	DOMA	VMA + MOPEG
Nil	0-30	IV	1.92	0.42	1.89	-	0.51
		III	1.29	0.27	1.50	0.18	2.04
	30-60	IV	0.81	N.D.	2.25	-	0.33
		III	0.66	N.D.	1.62	0.15	1.17
Nil	0-30	IV	0.87	0.21	1.05	-	0.27
		III	0.84	0.27	0.84	0.06	1.23
	30-60	IV	0.54	N.D.	1.23	-	0.18
		III	0.36	N.D.	0.78	0.06	0.78
Nialamide (350 µmol l^{-1})	0-30	IV	2.79	0.78	N.D.	-	N.D.
		III	4.05	0.90	0.18	N.D.	1.17
	30-60	IV	1.38	0.15	N.D.	-	N.D.
		III	1.59	0.36	0.15	N.D.	0.87
Nialamide (350 µmol l^{-1})	0-30	IV	1.56	0.75	N.D.	-	N.D.
		III	2.28	0.72	N.D.	N.D.	0.93
	30-60	IV	0.78	N.D.	N.D.	-	N.D.
		III	0.84	0.15	N.D.	N.D.	0.54
Nialamide (350 µmol l^{-1})	0-30	IV	4.71	0.30	N.D.	-	N.D.
		III	7.77	0.30	0.21	N.D.	0.33
	30-60	IV	3.93	N.D.	N.D.	-	N.D.
		III	4.38	0.27	0.27	N.D.	0.39
U0521 (55 µmol l^{-1})	0-30	IV	3.36	N.D.	1.65	-	N.D.
		III	2.55	N.D.	1.89	0.24	0.60
	30-60	IV	2.88	N.D.	1.77	-	N.D.
		III	1.95	N.D.	0.36	N.D.	N.D.

Footnotes

(i) Values are metabolite contents (nmol g^{-1}) estimated on portions of the same wash solution by Methods III and IV from arteries previously incubated with dl ^{3}HNA (0.9 µmol l^{-1}).

(ii) The contents shown above as NA determined by Method IV refer to NA + DOM both assumed to be recovered quantitatively by this procedure; DOMA is not estimated by Method IV (-).

(iii) N.D. refers to not detectable.

Fig. 6.8. The results indicated that the major ^{3}H components of the ^{3}H efflux during the first 30 minutes were HNA and DOPEG, with much smaller proportions of NMN, MOPEG, DOMA, and VMA. During the second 30 minutes only ^{3}H DOPEG and ^{3}H NA were detected in the efflux. The small proportion of ^{3}H DOMA meant that the ^{3}H NA + ^{3}H DOMA fraction was almost entirely ^{3}H NA. The agreement between the two methods was good for all fractions except MOPEG + VMA, as shown by the ratios of the contents measured by the two methods, namely ^{3}H NA 1.06 ± 0.1 (n=12), ^{3}H DOPEG 1.88 ± 0.6 (n=6), and ^{3}H NMN 0.94 ± 0.17 (n=6). However, as noted previously with the incubates, the level of (MOPEG + VMA) was lower by the TLC procedure, the ratio being 0.25 ± 0.02 (n=4). Hence these findings indicated that the MOPEG + VMA fraction was probably overestimated by the column chromatographic procedure and suggested that the seemingly neuronal origin of O-methylated metabolites in the preceding experiments was probably an artefact. Moreover, it was found that formation of MOPEG and of VMA were each eliminated in the arteries incubated with either nialamide or UO521 when assayed by TLC, whereas the formation of this combined fraction appeared unaffected when analysed by Method III (Table 6.5).

The efflux of ^{3}H DOPEG was undiminished during the first and second 30 minute periods, in contrast to the other ^{3}H compounds present. Thus the efflux of the other major constituent, ^{3}H NA, declined slightly from the first to the second 30 minutes, while the remaining metabolites (^{3}H NMN, ^{3}H MOPEG, ^{3}H VMA) declined to undetectable levels during the second 30 minutes.

The efflux from the COMT inhibited arteries differed only in that it did not contain any of the ^{3}H-O-methylated components during the first 30 minutes. It did not lead to an increase in the level of the catechol deaminated metabolites.

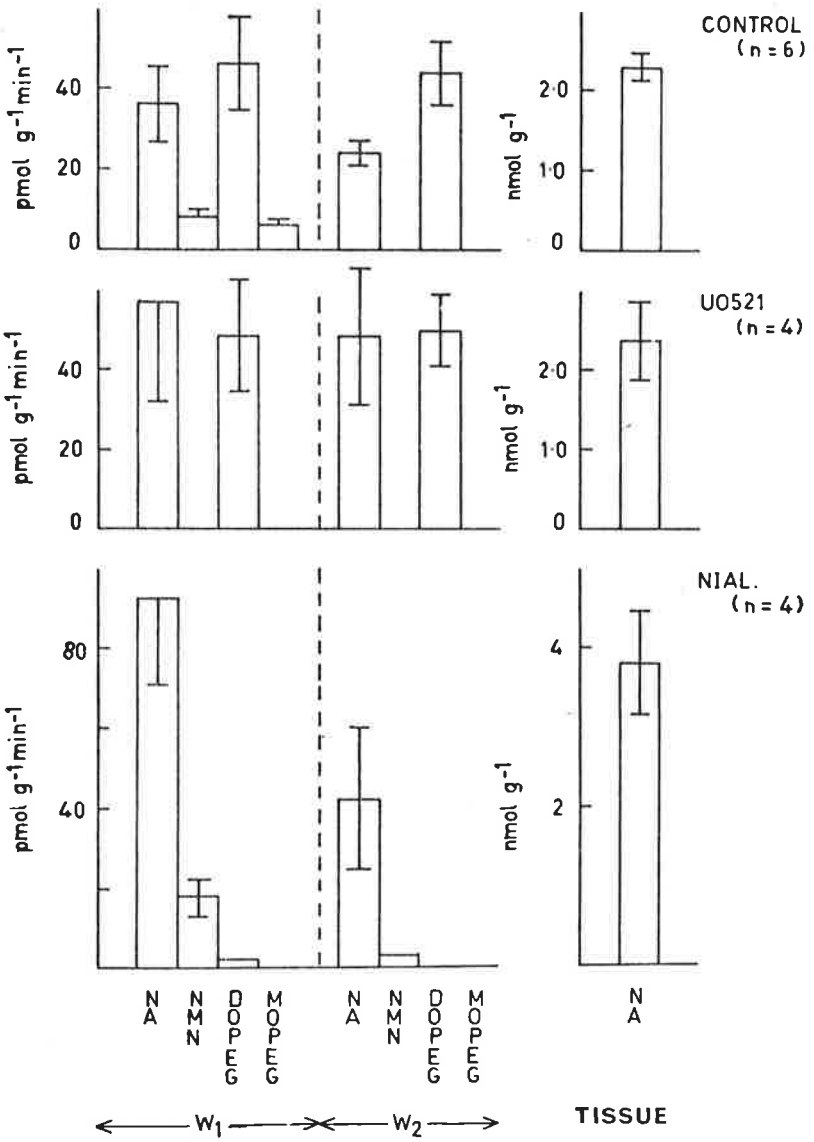


Fig. 6.8 Levels of ^{3}HNA and its metabolites in the washes and the levels of ^{3}HNA in the tissue after incubation for 60 mins with 0.9 µmol l^{-1} ^{3}HdlNA.

W1 = 1st 30 min wash
W2 = 2nd 30 min wash

UO521, 55 µmol l^{-1}; Nialamide 350 µmol l^{-1}.
Values obtained using Method IV.

The pattern of the efflux was affected to a greater degree by MAO inhibition. The effluxes of ^{3}H DOPEG, ^{3}H DOMA, ^{3}H MOPEG, and ^{3}H VMA were eliminated. This was associated with a marked increase in the efflux of ^{3}H NA during the first 30 minutes. The efflux of ^{3}H NMN was also increased, although, in absolute amounts, ^{3}H NMN still represented only 16% of the total efflux of ^{3}H. A trace of ^{3}H NMN (6% of total ^{3}H) was also detected in the second 30 minute efflux.

The results were entirely consistent with the preceding evidence for a purely neuronal origin of ^{3}H DOPEG, but provided evidence against the presence of COMT activity in the sympathetic nerves in this tissue. Otherwise it was difficult to explain the fact that the ^{3}H DOPEG efflux was well sustained over both the first and second 30 minute periods, and the fact that when efflux of ^{3}H DOPEG was eliminated by MAO inhibition, it was replaced by an increase in ^{3}H NA rather than an increase in ^{3}H NMN efflux.

The nialamide treatment was also associated with an increase in the contents of ^{3}H remaining in the tissue after the 60 minute wash. Fractionation showed that this ^{3}H was entirely the (^{3}H NA + ^{3}H DOMA) fraction. Its neuronal location was not tested, but can be assumed, in view of the evidence presented in the next section, that 91% of the tissue stores of ^{3}H remaining after a 30 minute wash of arteries which had been previously incubated with 1^{3}H NA 1 μmol l^{-1} was eliminated by cocaine 30 μmol l^{-1}.

From the preceding efflux data an estimate was made of the contents of ^{3}HNA and metabolites remaining in the tissue after a one minute wash. This was done by adding the total efflux during the 60 minutes washing to the content remaining in the tissue at the end of this period. The latter was shown to be entirely the ^{3}HNA + DOMA fraction. The results are shown diagrammatically in Fig. 6.9. It will be noted that the proportion of ^{3}H DOPEG in the total sum of (^{3}H DOMA + ^{3}HNA) plus metabolites, when estimated from the efflux data is considerably greater than the proportion when actually measured in the experiments described earlier (Table 6.2 or Fig. 6.3). Assuming that the proportion of the metabolites in the tissue after a 30 minute period of incubation is similar to the proportion after a 60 minute period of incubation (not measured in these experiments), the difference between the proportions of DOPEG is consistent with the persistence of intracellular deamination *after* bath washout of the ^{3}HNA. Such a persistence is readily explained by the exposure of the ^{3}HNA to neuronal MAO as the ^{3}HNA leaks from its vesicular storage sites into the cytoplasm of the sympathetic nerve.

7. Stereospecificity

In all the preceding studies, only dl^{3}HNA was used as substrate. At a later stage of the study, the laevo form of ^{3}HNA became available from Amersham in a state of sufficient purity to permit a limited number of observations on its uptake and metabolism in the artery. Seven artery segments were incubated with l^{3}HNA (1.2 μmol l^{-1}) for 30 minutes followed by a one minute wash. The metabolites were separated by the TLC procedure (Method IV). Fig. 6.10 presents a comparison between the results obtained with l^{3}HNA as substrate and the earlier results obtained

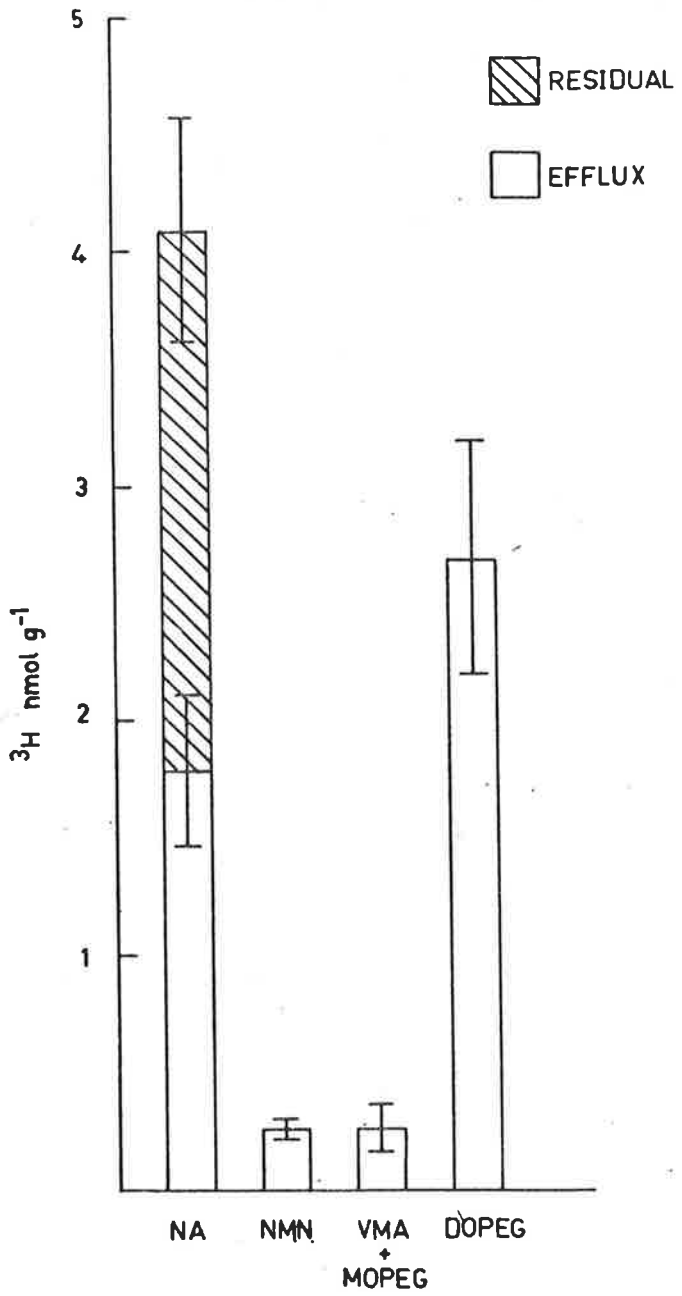


Fig. 6.9 Estimates of contents of ^{3}HNA and metabolites remaining in arteries after 1.0 min of wash, derived from efflux data shown in Fig. 6.8.

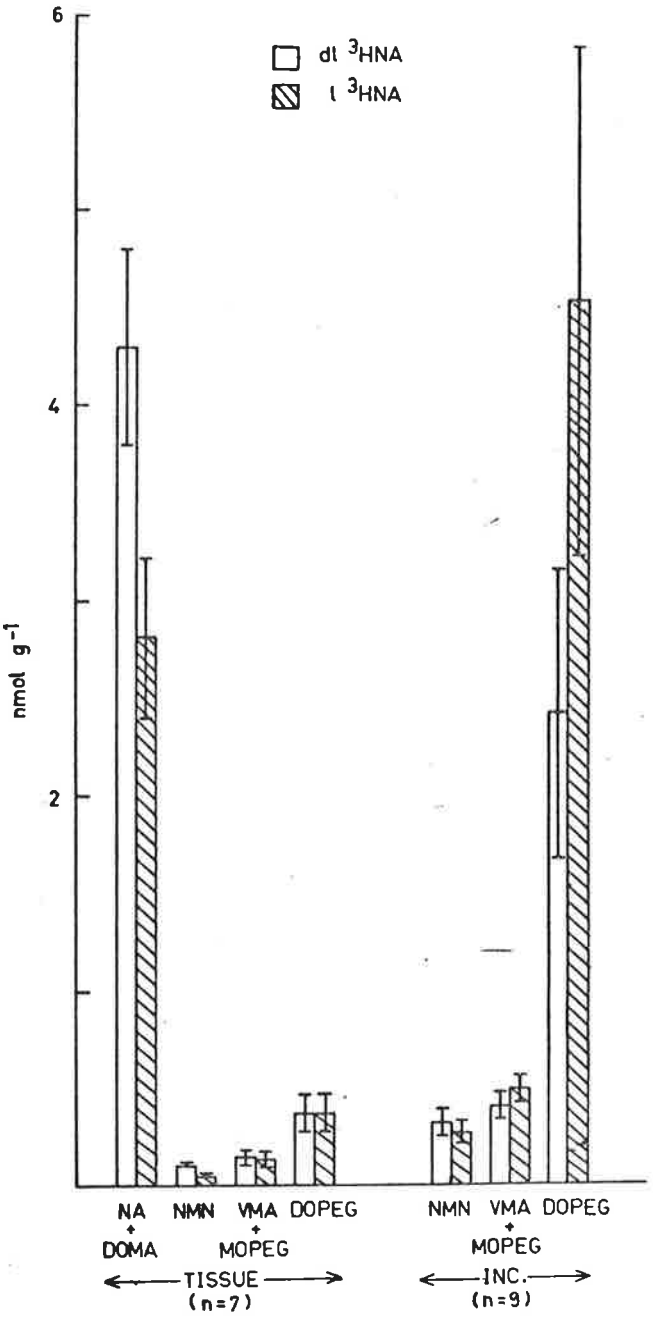


Fig. 6.10 Comparison of tissue contents of ^{3}HNA and metabolites and incubation media contents of metabolites from arteries incubated with either dl or l^{3}HNA (1.2 μmol l^{-1}). Contents determined by Method IV.

with dl^{3}HNA under identical conditions. The comparison suggests that the arteries incubated with the laevo isomer accumulate more ^{3}H DOPEG in the medium and less of the fraction (^{3}HNA + ^{3}HDOMA) in the tissue than is the case with arteries incubated with the dl^{3}HNA. These differences between the two isomers were significant for ^{3}HNA but the increase in DOPEG in the media failed to achieve significance. It is of interest that the measured accumulation of ^{3}H was less in the case of the arteries incubated with the l^{3}HNA than in the arteries incubated with dl^{3}HNA (6.4 c.f. 8.2 nmol g^{-1} ^{3}HNA equivalent, respectively). These were paired arteries; a paired t-test showed that the difference was significant.

Two further studies were carried out with the l^{3}HNA to examine the effects of inhibition of neuronal uptake (using cocaine), and of extraneuronal uptake (using deoxycorticosterone acetate - DOCA) on the efflux and tissue contents of ^{3}H. The composition of the ^{3}H material was not analysed in these experiments. In both series of experiments, the artery segments were incubated under conditions identical to those described earlier, i.e. a 30 minute period of incubation with a substrate concentration of 1.2 μmol l^{-1}. The arteries were then washed with ^{3}HNA-free Krebs solution for 31 minutes, during which the wash solutions were replaced after the first minute and the 16th minute. The ^{3}H material remaining in the arteries after

31 minutes was then determined. The results are summarised in Table 6.6.

The experiments with cocaine indicated that it produced a decrease in the accumulation of ^{3}H (78%), which is somewhat greater than that shown earlier when the dl^{3}HNA was used as substrate (66%, Table 6.1). These are based on the tissue contents after a one minute wash. These experiments also revealed that the decrease by cocaine was manifested during the first and second 15 minute wash periods, but not during the first one minute wash. In the course of this series of experiments, comparisons were also made between two substrate concentrations (l^{3}HNA 0.16 and 1.2 μmol l^{-1}) and two drug concentrations (cocaine 3.0 and 30 μmol l^{-1}). The main difference between the various treatments was the tendency of cocaine to decrease the efflux during the first 15 minutes only at the higher concentration of ^{3}HNA (1.2 μmol l^{-1}).

One qualification to the result obtained in the experiments on cocaine was the occurrence of extremely high values for the accumulation of ^{3}H in two of the untreated arteries used as controls for the cocaine-treated arteries. Excluding the one minute wash values, the accumulations amounted to 17.9 and 19.9 nmol g^{-1} of ^{3}HNA equivalence. These values

TABLE 6.6

The effect of cocaine and DOCA on the accumulation and efflux of ^{3}H from arteries

[l-^{3}HNA] μmol l-1	Treatments	n	Wash period (mins) 0-1	1-16	16-31	Tissue Residue	$\Sigma^{3}H$[a]	$\Sigma^{3}H$-1.0 min[b]
1.2	Nil	5	1.5 ± 0.4	2.47 ± 0.41	1.06 ± 0.20	8.28 ± 1.92	14.07 ± 3.11	12.48 ± 2.79
1.2	Coc (3.0 μmol l^{-1})	5	1.8 ± 0.4	1.5 ± 0.13	0.5 ± 0.07	3.5 ± 0.36	7.34 ± 0.92	5.55 ± 0.54
1.2	Coc (30 μmol l^{-1})	5	2.1 ± 0.4	1.75 ± 0.26	0.5 ± 0.13	0.88 ± 0.20	4.71 ± 0.89	2.54 ± 0.48
0.16	Nil	6	0.28 ± 0.06	0.37 ± 0.09	0.21 ± 0.05	1.97 ± 0.29	2.72 ± 0.59	2.49 ± 0.51
0.16	Coc (3.0 μmol l^{-1})	5	0.28 ± 0.06	0.33 ± 0.06	0.09 ± 0.02	0.66 ± 0.15	1.34 ± 0.29	1.06 ± 0.23
0.16	Coc (30 μmol l^{-1})	5	0.31 ± 0.05	0.33 ± 0.05	0.06 ± 0.006	0.18 ± 0.02	0.88 ± 0.10	0.57 ± 0.08
1.2	Nil[c]	4	0.44 ± 0.04	1.39 ± 0.19	0.48 ± 0.08	5.0 ± 0.38	7.32 ± 0.23	6.88 ± 0.22
1.2	DOCA[c] (27 μmol l^{-1})	4	0.49 ± 0.08	1.31 ± 0.15	0.54 ± 0.04	5.0 ± 0.55	7.35 ± 0.35	6.86 ± 0.34

Footnotes

(i) Values are ^{3}H contents (nmol gm^{-1}) of wash solutions and tissues at the completion of wash, from arteries previously incubated with ^{3}Hl-NA (1.2 or 0.16 μmol l^{-1}).

(ii) For treated arteries the drugs cocaine and DOCA were present during incubation with ^{3}Hl-NA and in the subsequent wash solutions.

(iii) (a) the sum of the ^{3}H contents of all wash solutions plus the ^{3}H contents of arteries at the completion of wash.

(b) the sum of the ^{3}H contents of wash solutions excluding contents of the 1.0 minute wash, plus the ^{3}H contents of arteries at the completion of wash.

(c) these arteries were incubated in the presence of ethanol.

contrasted with the mean values of 6.3 ± 0.9 (n=7) obtained in the first series of experiments described in this section, with the values of 7.0 ± 0.54 (n=4) obtained in the DOCA experiments, and with the individual values of ^{3}H accumulation of 6.3, 7.1 and 10.8 obtained in the remaining untreated segments used in the cocaine experiments.

The experiments with DOCA failed to reveal a significant effect on the effluxes during any stage of the wash period, or on the residual ^{3}H content of the tissue. This finding contrasted with the marked effect of cocaine and again indicated that extraneuronal uptake and inactivation is of minor importance compared with the neuronal mechanism in this tissue.

The efflux of ^{3}H during the one minute wash was estimated in these experiments by multiplying the 50 second efflux from the period 10 seconds to 60 seconds by a factor of 1.2. The measured efflux during the first 10 seconds was not included owing to the probable presence of ^{3}H from the incubating medium. Excluding the values from the cocaine-series of experiments, the values in 8 arteries where the one minute efflux was determined amounted to 0.49 ± 0.03 nmol g^{-1} ^{3}HNA equivalence (n=8). These values corresponded to a mean percentage of 7.2 ± 0.72% of the total measured accumulation of ^{3}H in the arteries. The relatively smaller standard error of the absolute values compared with the percentage values suggests that this efflux, or part of this efflux at least, is independent on the preceding accumulated efflux. This suggestion is in accord with the probability that the one minute efflux consists primarily of the components of ^{3}HNA which is distributed in the extracellular compartment at the conclusion of incubation, and

which efflux rapidly immediately the ^{3}HNA is washed from the incubating medium. If the specific gravity of the artery is assumed to be one, this efflux corresponded to 41% of the ^{3}HNA concentration in the incubating medium.

The size of the extracellular compartment was not determined in the present series of experiments. However, the concentration of ^{14}C sorbitol which was present in the tissue following a one minute wash was determined in four artery segments which had been incubated with ^{14}C sorbitol (8.1 μmol l^{-1}) for 30 minutes. Assuming that the specific gravity of the artery was 1.0, the concentration of sorbitol corresponded to a compartment size of 0.06 ± 0.005 nmol g^{-1}(n=4). Such a compartment would account for 0.075 ± 0.01 nmol g^{-1} of ^{3}HNA. It has been noted earlier in this chapter that the residual ^{3}HNA remaining in the tissues after some treatments approached this value, for example, the contents of ^{3}HNA in 6 of the 7 denervated plus cocaine treated arteries included in Figures 6.2 and 6.3 ranged between 0.11 and 0.24 nmol g^{-1}.

DISCUSSION

1. DI^{3}HNA

Although there are quantitative differences between the results of the different separative procedures, all agree that the major proportion (80-87%) of the ^{3}H material which accumulated in the artery during incubation was unchanged ^{3}HNA. That the artery has accumulated the amine against a concentration gradient is evident from the relative concentrations of unchanged amine in the artery and medium (4.2-6.4 nmol g^{-1} cf. 1.2 nmol ml^{-1}). Since the concentration of amine in the tissue was

determined after a one minute wash, it is probable that very little of the amine is present in the extracellular compartment. The latter is approximately 0.6 ml per gram in this artery, so that the maximum contribution which might be expected from this source to the total tissue content is 0.6 x 1.2 nmol g^{-1} of the artery. As it will be shown in Chapter 8, more than 80% of the sorbitol space in the artery is cleared of ^{14}C sorbitol in one minute, and the residual sorbitol after one minute corresponds to a concentration of NA in the tissue of 0.075 nmol g^{-1}. Hence the tissue content of ^{3}HNA represents an accumulation of the amine which is (a) approximately 3 fold greater than can be attributed to a uniform distribution of the amine in the tissue in the same concentrations as in the bathing medium; (b) approximately 6 fold greater than if distributed uniformly in the extracellular compartment; and (c) at least 60 fold greater than the concentration to be expected to be present in the extracellular compartment after a one minute wash.

That the major site of accumulation of the NA in the sympathetic nerves was indicated by the decrease of 80% or greater in the accumulation of unchanged amine which occurred during cocaine treatment or after chronic sympathetic denervation of the artery. From this decrease it can be estimated that the neuronal stores accumulated between 3 and 4 nmol g^{-1} of the amine. The efflux data on the $dl^{3}HNA$ also gives an indication of the size of the neuronal store, since it was shown that approximately 62% and 30% of the ^{3}H which had accumulated in the artery during incubation with $dl^{3}HNA$ was still present after washing for 30 minutes and 60 minutes, respectively. It is reasonable to assume that the residual ^{3}H material was almost entirely neuronal, since it was

shown in the experiments using the laevo isomer of ^{3}HNA that more than 90% of tissue content of ^{3}H which remains after a 30 minute wash, is decreased by cocaine.

The cocaine or chronic denervation insensitive ^{3}HNA which is present in the tissue is assumed to be contained in or associated with extra-neuronal structures. This ^{3}HNA amounted to approximately 30% of the total unchanged ^{3}HNA when estimated by Method II for both cocaine and denervated arteries. By Method III, the estimates were still approximately 30% for cocaine, but were less (approximately 20%) for chronic denervation. However, in both series of experiments the residual NA was further (significantly) reduced by the two procedures in combination so that this residual value (<10% of total ^{3}HNA) is probably the best indication of the tissue content unchanged extra-neuronal ^{3}HNA. This content amounted to 0.7 nmol g^{-1} by Method II and 0.2 nmol g^{-1} by Method III, and in both cases was not significantly different from the residual values prevailing in PBZ-treated arteries (0.5 nmol g^{-1} Method II, and 0.3 nmol g^{-1} Method III). The last finding suggests that this relatively small amount of extraneuronal ^{3}HNA is not located in the smooth muscle cells. This conclusion follows from Gillespie's evidence that PBZ (164 µmol l^{-1}) inhibits uptake of ^{3}HNA into the smooth muscle cells of the rabbit ear artery (Avakian and Gillespie 1968). His evidence implies that the PBZ-insensitive ^{3}HNA must be located in the artery wall in structures outside the smooth muscle cells and the sympathetic nerves. This ^{3}HNA is not entirely due to amine which has not been washed from the extracellular space, since the latter value corresponds to only 0.075 nmol g^{-1} NA. The difference may represent a

residual contribution from the collagen and elastin bound ^{3}HNA described by Gillespie. The half time of efflux from the latter ($t_{\frac{1}{2}}$ 0.6-1.8 min., Avakian and Gillespie 1968) would not exclude such a possibility. A more remote possibility is that there may be a firmly bound extracellular and non-neuronal source of ^{3}HNA. The only evidence in support of such a possibility is the observation (see Fig. 6.7) that a small but definite efflux of ^{3}HNA was detected one hour after bath washout of ^{3}HNA in arteries where neuronal uptake of ^{3}HNA during incubation had been inhibited by cocaine, and in which cellular uptake of ^{3}HNA during the washout period had been inhibited by PBZ. Although it might be argued that this represents incomplete blockade of uptake of ^{3}HNA by cocaine, such an argument seems refuted by the failure of subsequent nerve stimulation to increase the efflux of this ^{3}HNA. However it should be noted that Henseling *et al.* (1976), in a recent study on the rabbit aorta, found that the firmly bound ^{3}HNA in that tissue appears entirely neuronal (representing vesicular and cytoplasmic compartments). Perhaps more important is that the validity of the evidence presented here must be questioned simply because the activity of the ^{3}HNA which was measured in the 8 minute efflux of the cocaine-treated arteries was close to background.

2. Metabolism and MAO

DOPEG appears as the major metabolite both in the tissue and in the medium during incubation, as well as in the efflux of ^{3}H into the medium after bath washout of the ^{3}HNA. Its origin from neuronal MAO was indicated by its virtual elimination in the tissue by cocaine or chronic denervation, and its virtual elimination from both the incubation medium and from the subsequent ^{3}H efflux by preincubation

of the artery by nialamide. The apparent resistance of the DOPEG and the other deaminated metabolites in the tissue to nialamide remains puzzling. A possible explanation is that the enzyme was not completely inhibited, so that metabolites were found in quantities which were sufficient to a tissue compartment, but not to overflow into the medium. This explanation is not at variance with the later observation that the nialamide treatment eliminated the appearance of DOPEG in the efflux from arteries after both washout of the ^{3}HNA since the latter DOPEG may have been formed from NA after its binding by vesicles, whereas the tissue DOPEG may have been formed by a different mechanism, for example, by the activity of neuronal MAO on ^{3}HNA as it was transported into the cytoplasm from the extracellular medium. However, a contradiction is posed by the absence of DOPEG in the ^{3}H material remaining in the artery after a one hour wash period; clearly, the latter observation implies that the apparently nialamide-resistant DOPEG in the tissue after a 30 minute incubation must have effluxed from the tissue during the wash period. Related to the possibility that MAO may not have been completely inhibited in our experiments, Tarlov and Langer (1971) found that parygline pretreatment failed to affect the concentration of VMA + MOPEG in the spontaneous efflux from the guinea pig atria, and decreased the concentration of DOPEG by only 33%, i.e. the latter metabolite still formed 21% of the efflux of ^{3}H compared with 33% of the efflux of ^{3}H in MAO-intact atria. In contrast the appearance of DOMA was eliminated by the MAO inhibition. They also noted that in the rat vas deferens pargyline pretreatment failed to eliminate the increase in DOPEG efflux which occurred during nerve stimulation, although again DOMA formation was prevented. They concluded

"DOMA and the glycol DOPEG are made in different locations to which drugs may not have equal access." There does not appear to be definite evidence of differing locations, although the aldehyde dehydrogenase and the aldehyde reductase which are responsible for DOMA and DOPEG formation respectively have been shown to have different distributions in the cell. The former enzyme associated with the mitochondria and the latter present in the cytoplasm of cells from rat brain (Erwin and Deitrich 1966; Tabakoff and Erwin 1970). There is also a recent report (Henseling *et al.* 1976) that pargyline (500 μmol l^{-1}) *in vitro* (plus a COMT inhibitor) inhibited the formation of deaminated metabolites by 95% during incubation of the rabbit aorta with dl-^{3}HNA, but this treatment did not prevent the appearance of deaminated metabolites up to a level of about 50% of that of l-^{3}HNA during the late stage of efflux (Eckert *et al.* 1976). Thus the inability of pargyline to abolish the formation of deaminated metabolites was seen when dl^{3}HNA and l^{3}HNA were used and is probably not related to the use of these different sterioisomers of NA. However in view of the finding of Eckert *et al.* (1976) that pretreatment of rabbits with pargyline, in addition to *in vitro* treatment, decreased the formation of the late-effluxing deaminated metabolites it is more likely that the effectiveness of pargyline is related to the intracellular concentration of this drug achieved in these studies. Consistent with this possibility is the observation that in homogenates pargyline selectively inhibits type B MAO when this drug is present at low concentrations and inhibits both type A and type B MAO when present in high concentrations (Squires 1972). Thus it is possible that *in vitro* treatment of tissues with pargyline results in the inhibition of type B MAO and incomplete inhibition of type A MAO, the latter form of the enzyme deaminates NA to a greater extent than the former (Goridis and Neff 1973). This line of reasoning

although consistent with the conclusions of Tarlov and Langer (1971) requires the presence of type A and type B MAO in sympathetic nerves and the possible selective formation of DOMA by the type B enzyme and formation of DOPEG by the type A enzyme. Whilst there is strong evidence indicating the presence of a neuronal type A MAO (Goridis and Neff 1973) there is also support for the existence of at least two forms of MAO in adrenergic neurons (Jarrott 1971). Unfortunately the relationship between aldehyde dehydrogenase, aldehyde reductase and the type A and type B forms of MAO has not been determined. Although not an argument in itself that nialamide treatment was not completely effective in the rabbit ear artery, the observation of Eckert serves as a reminder that the effectiveness of inhibition of deamination will be influenced by factors such as duration of treatment. Concentration may also be important, for example, the substrate concentration to which the intracellular (inhibited) enzyme is exposed is likely to be very different during incubation than during efflux.

The remaining metabolites are present in much smaller proportions than DOPEG; for example, in terms of the metabolites which are next in importance (quantitatively), MOPEG and NMN represent about one-eighth of the proportion of DOPEG in the medium, while DOMA represents about only one-third of the proportion of DOPEG in the tissue (Table 6.4). In view of this result, it is a reasonable assumption that the major enzymes involved in the inactivation of ^{3}HNA are neuronal MAO and aldehyde reductase. The site of the aldehyde reductase is assumed to be neuronal, but the possibility is not excluded in this study that DOPEG was formed from the intermediate aldehyde after the latter had diffused

from the nerve into the surrounding non-neuronal cells. However, Levin (1974) has shown that DOPEG is the main metabolite formed by the adventitia of the rabbit aorta, where the smooth muscle cells are absent, and hence there is strong presumptive evidence that the reductase enzyme is also present in the sympathetic nerves of the rabbit ear artery.

Another feature of the action of nialamide in these studies was that it tended to increase the accumulations of both ^{3}HNA and ^{3}H NMN in the tissues. The effect on ^{3}H NMN was also apparent in the incubation medium. The increase in NA observed in the tissue also occurred in the ^{3}H which effluxed from the tissue after bath washout. This result is explicable in terms of decreased intraneuronal inactivation of the NA after its uptake into the cytoplasm of the nerve so that the level in the nerve is increased, and this in turn leads to a greater efflux of the unchanged amine from the nerve. The increase in the incubation medium levels of NMN almost certainly reflects the availability of a greater substrate concentration of unchanged amine to this enzyme consequent on the elimination of neuronal MAO as a major pathway for inactivating NA.

That the source of the increased NMN is extracellular NA is strongly indicated by the observation that the tendency for the metabolism to be diverted towards NMN is virtually absent in the efflux of nialamide-treated arteries under conditions where the cellular re-uptake (neuronal and extraneuronal) of the NA which effluxes from its neuronal storage sites is prevented by PBZ.

3. Metabolism and COMT.

The results in this chapter highlight the probability that COMT plays a minor role compared with MAO in the inactivation of NA. Thus

the proportion of O-methylated metabolites (approximately 20%) to the total metabolites is small both when the dl or l isomers of ^{3}HNA are used. However, the probability that COMT exerts some influence on the availability of substrate to the MAO pathway was suggested by the observation that COMT inhibition, while eliminating the accumulations of the O-methylated metabolites in the incubating medium, did tend to increase the level of DOPEG in the medium. The earlier experiments indicated that this effect was also associated with an increase in the content of ^{3}HNA remaining in the tissue. The results of the efflux experiments also suggested that the levels of ^{3}HNA in the wash fluids of arteries tended to increase under conditions of COMT inhibition. These trends may be explained if inhibition of COMT caused a small but definite increase in the concentration of extracellular ^{3}HNA available to the neuronal uptake system. It should be noted that the data strongly confirms the conclusion from the enzymic studies (Chapter 5) that the enzyme is almost entirely extraneuronal in distribution. This followed from the evidence that chronic denervation (and in one experiment cocaine also) not only failed to decrease, but significantly increased the tissue content of ^{3}H NMN. As indicated already, such an increase can be explained by an increase in the availability of substrate to the COMT pathway once the major (MAO) pathway is eliminated. The possibility that this effect may have masked a decrease consequent on the elimination of some neuronal COMT activity was virtually excluded by the further result that inhibition of MAO diverted the metabolite pattern in the ^{3}H efflux from arteries under conditions where cellular re-uptake of ^{3}HNA was prevented, from DOPEG to NA, rather than to NMN. The word "virtually" is included in the preceding statement to take into account the fact that a "trace" of NMN did appear in the efflux during the second 30 minute wash period

from the nialamide-inhibited arteries (Fig. 6.8).

4. Stereospecificity and metabolism

Comparison between the metabolism of the l and the dl form of ^{3}HNA (Fig. 6.10) pointed to a similar distribution of the metabolites, with the possible exception that the accumulation of DOPEG in the incubating medium tended to be greater in the case of the l form. Although the difference was not significant, it is tempting to relate this observation to the finding of Eckert *et al.* (1976) that there is a pronounced stereospecificity for the leavo isomer of DOPEG formation in the rabbit aorta. These workers referred to evidence that the laevo form of the product of noradrenaline metabolism by monoamine oxidase (1-3,4 dihydroxyphenylglycoldehyde) is bound more firmly to aldehyde reductase than to aldehyde dehydrogenase. Eckert *et al.* (1976) have also presented evidence favouring greater binding of the neuronal vesicles for the l than for the dl form of NA in the rabbit aorta. Hence it is a puzzling finding that the level of l^{3}HNA tended to be less, rather than greater than, that of the dl^{3}HNA in the rabbit ear artery at the conclusion of the 30 minute period of incubation with ^{3}HNA. From the results obtained by the other methods used in this study, it seems likely that the content of DOMA (assayed with NA by the method used) is too low to explain the smaller accumulation of l^{3}HNA. Nevertheless, it should be emphasised that the estimated contents of the combined fractions of ^{3}HNA and ^{3}HDOMA by the TLC method used are approximate only, and almost certainly an underestimate of the true levels since the data is based on the simplifying assumption of 100% recovery of this fraction. This qualification does not apply to the estimations of the remaining metabolites by this method.

5. Comparison with other tissues

DOPEG has been shown to be the main metabolite in the efflux of ^{3}H from the following tissues after their incubation with ^{3}HNA or, in the case of isolated heart, during their perfusion with ^{3}HNA; rat vas deferens (Langer, 1970, dl^{3}HNA; Graefe *et al.* 1973, l^{3}HNA), guinea pig atrium (Tarlov and Langer, 1971), cat nictitating membrane (reported in Graefe *et al.* 1973, c.f. however, the catechol deaminated fraction was reported to be only a minor component in the cat nictitating membrane -(Langer *et al.* 1972), cat spleen (Cubeddu *et al.* 1974), cat heart (Graefe, 1975, l^{3}HNA), and rabbit heart and rat heart (Bonisch *et al.* l^{3}HNA, to be published; reported by Graefe *et al.* at the 6th International Congress on Pharmacology, Helsinki, 1975).

In the preceding studies the neuronal origin of the DOPEG in the cat heart and spleen, and in the rabbit heart, was shown by its elimination by cocaine, with the qualification that in the rat heart approximately 10% of the efflux was cocaine insensitive. In the guinea pig atria, the neuronal origin of DOPEG was suggested by the ability of bretylium to inhibit its formation (Adler-Graschinsky *et al.* 1972). Bretylium is considered to be a selective inhibitor of neuronal MAO as a result of its accumulation in the sympathetic neurone (Furchgott and Sanchez Garcia 1968). The results on the rabbit aorta also provided striking evidence of the neuronal origin of the DOPEG, since the adventitia contains the sympathetic nerves in this region. Levin showed that DOPEG was no longer the major metabolite when the aorta without the adventitia was incubated with ^{3}HNA. (Comparative data on the rabbit aorta will be considered as a separate section of this discussion).

Hence the present evidence that DOPEG is the main metabolite and is neuronal in origin in the rabbit ear artery means that this tissue conforms to a pattern common to a relatively large number of different types of sympathetic nerve-effector organs.

Another common feature may be the preferential oxidation of the l form of ^{3}HNA to the glycol; as discussed already, this is suggested by the observation that the proportion of DOPEG formed was greater from l^{3}HNA than from dl^{3}HNA. Although the increase was not significant, it is consistent with the same trend described in the rat vas deferens by Graefe *et al.* (1973) and in the rabbit aorta by Levin (1974).

One of the questions which has been examined in several of the above studies is whether the metabolites (mainly DOPEG) present in the wash solutions are formed from ^{3}HNA prior to its efflux from the nerve or whether they are formed from ^{3}HNA which has been recaptured by the nerve while in the process of effluxing from the tissue. One approach to this question has been to examine the effects of neuronal uptake inhibition (cocaine, or desipramine) added during efflux on the pattern of the metabolite distribution in the efflux. In this way, Tarlov and Langer (1971) and Cubeddu *et al.* (1974) showed that the DOPEG in the resting efflux in the guinea pig atria, and in the cat spleen, respectively, was not formed by recapture of the effluxing ^{3}HNA. This conclusion did not apply to the metabolites in the stimulation-induced efflux. Since their formation was prevented by cocaine, it was concluded these represented metabolism of the ^{3}HNA after its release from the nerves and subsequent recapture. Although the same question was not specifically examined in the present study, a relevant finding

was that DOPEG constituted the main metabolite in the efflux from PBZ-treated arteries. As indicated already, this implies that the DOPEG was formed from the ^{3}HNA which had accumulated in the nerves during the wash period. Hence this finding points to a further feature in common between the artery and at least two other tissues. This similarity may also extend to the stimulation-induced efflux, despite the limited relevant data on the ear artery since the content of DOPEG in the stimulation-induced efflux was measured only in the PBZ-treated artery. However, the limited conclusion can be drawn from the results that stimulation itself does not mobilise or facilitate in any way the intraneuronal metabolism of ^{3}HNA prior to its release. In fact, rather the reverse applied in the ear artery insofar as both the absolute and relative amounts of DOPEG declined during stimulation as though the mobilisation of NA storage vesicles may have actually decreased the availability of substrate (presumably ^{3}HNA leaking from granules) to the intraneuronal MAO.

An important qualification may apply to the data on efflux of ^{3}H from the rabbit ear artery in the presence of PBZ, namely, that in the concentration used (30 μmol l^{-1}) PBZ was shown by Cubeddu *et al.* (1974) to increase the spontaneous efflux of ^{3}H from the cat spleen. The increases amounted to approximately 30% and 100%, respectively, for the concentration of 30 μmol l^{-1} and 100 μmol l^{-1} concentration. The increases were due to an increase in the efflux of DOPEG, indicating that this drug was able to exert a reserpine-like action at the above concentrations. Although the author's data does not indicate whether a similar effect may have occurred in the rabbit ear artery, there is data available from a separate study in the candidate's laboratory by D.A.S. Parker which shows that PBZ increased the resting efflux of ^{3}H in the rabbit

ear artery. However, the conditions used were not quite comparable, since the arteries in Parker's study were treated with PBZ for 30 minutes only, and the efflux was not measured until a further 30 minutes of washing with PBZ-free Krebs solution. Hence, extrapolating from the data on the cat spleen, the possibility cannot be completely excluded at this stage that 20-30% of the efflux of ^{3}H DOPEG measured in the rabbit ear artery may have reflected a direct effect of PBZ on the neuronal storage of ^{3}HNA which facilitated its metabolism by the neuronal MAO. Despite this possibility, it should be stressed that such an action on the part of the PBZ would not violate the major reason for studying the efflux in the presence of PBZ, namely, to provide a further test for the presence or absence of neuronal COMT. The failure to identify the sympathetic nerves as a source of O-methylated metabolites indicates another feature common to the rabbit ear artery and other organs, in particular the rabbit, cat, rat and guinea pig atria preparations, as well as the cat spleen. Only in the case of the cat nictitating membrane is there clearcut evidence of a neuronal source (as well as the extraneuronal source) of the O-methylated metabolites. The rat vas deferens may be an exception in that it does not normally release NMN spontaneously (Graefe *et al.* 1973; Langer 1970) or in response to stimulation, but does do so once MAO is inhibited (Tarlov and Langer 1971). Whether the NMN formed under these conditions arises from ^{3}HNA after its release does not seem to have been reported.

6. Comparison with the rabbit aorta

This comparison is considered separately, partly because of the common nature of the tissues and partly because Levin (1973, 1974) has provided detailed investigations on this tissue under conditions

closely resembling those used in the studies on the rabbit ear artery. The conditions are summarised in Table 6.7. In addition, there have been a number of relevant studies on this preparation reported recently by Trendelenburg and his colleagues (Eckert *et al.* 1976; Henseling *et al.* 1976). In Table 6.7 the absolute amounts of metabolites formed in the rabbit ear artery are compared with those in the rabbit aorta (intact, adventitia, and media). Since the concentrations of ^{3}HNA were not identical, their relative proportions of the metabolites are shown in Table 6.7. The implications of the comparison are quite striking. The comparison shows that the distribution pattern in the intact ear artery closely resembles that of the *adventitia* of the rabbit aorta, where DOPEG is the main metabolite, and the proportion of NMN is only one-fifth of the total. In marked contrast, the pattern in the media of the aorta shows that NMN is much in excess of the remaining metabolites representing 76% of the total metabolites. The distribution of metabolites from the intact aorta is intermediate between the two. Since the sympathetic nerves are localised in the adventitia in the rabbit aorta, as in many other arteries (Burnstock *et al.* 1970), it is apparent that the above comparison is a further indication that the pattern of the metabolites of NA in the rabbit ear artery is determined by neuronal MAO. It is of interest to consider why this should be the case. An obvious explanation is that the ear artery has a much denser sympathetic innervation that the rabbit aorta (Bevin *et al.* 1972).
This was shown histochemically, and by direct assay of their NA contents, by Bevan *et al.* (1972). It is also manifested by a much greater neuronal uptake of ^{3}HNA by the ear artery (Bevan *et al.* 1972). However, there is a second factor to be considered. In the present

TABLE 6.7

Comparison of metabolite contents of the rabbit ear artery and rabbit aorta

Tissue	Metabolite DOMA	DOPEG	MOPEG	VMA	NMN	Σ metab
Ear artery (whole)	0.38(9.2)	2.82(67.6)	0.34(8.2)	0.20(4.8)	0.42(10.2)	4.16
Aorta (adventitia)	0.16(11.0)	0.80(55.0)	0.10(6.9)	0.09(6.2)	0.29(20.0)	1.44
Aorta (media)	0.03(1.8)	0.11(6.8)	0.18(11.0)	0.06(3.4)	1.23(76.0)	1.62
Aorta (whole)	0.06(4.7)	0.38(30.0)	0.13(10.0)	0.07(5.5)	0.62(49.0)	1.27

Footnotes:

(i) Values are total amounts of metabolite formed (tissue plus medium) expressed as nmol g^{-1}.

(ii) Values in brackets refer to the amount of each metabolite expressed as a percentage of the total amount of metabolites formed by that tissue.

(iii) The rabbit ear artery data is based on 7 or more determinations (except for DOMA where n=4) for which tissues were incubated with dl^{3}HNA (1.2 µmol l^{-1}) for 30 minutes and arteries washed for 1.0 minute.

(iv) The rabbit aorta data is based on the published findings of Levin (1974) in which 11 or more tissues were incubated with dl^{3}HNA (0.3 µmol l^{-1}) for 30 minutes, (tissues not washed after incubation).

(v) Σ metab refers to the sum of all metabolites for that tissue.

study, segments of arteries were used so that the amine gained access to the tissue previously by diffusing from the adventitia surface. The combination of a small intraluminal diameter (<0.5 mm), plus its probable further occlusion due to the vasoconstrictor action of NA, means that it is unlikely that significant amounts of NA were able to diffuse into the tissue from the lumen. This means that most of the ^{3}HNA which reached the underlying smooth muscle was first exposed to the sympathetic nerve terminals which are located in the adventitia. It was appreciated at the outset that the technique would favour primarily a neuronal metabolism, and at the time it was thought this would assist rather than hinder the first aim of this study which was to test the possible neuronal localisation of some of the COMT activity in this artery. However, the preceding considerations draw attention to an extremely interesting question, namely, whether, if NA were applied intraluminally only, the metabolic pattern would then correspond more to that seen in the isolated media of the aorta. An answer to this question would indicate whether, as seems likely, the pattern of NA metabolism in the two vessels is essentially the same, and the differences may reflect only (1) the different routes of application of the amine to the vessels (intraluminal *plus* extraluminal in the case of the aorta), and (2) the different morphologies of the vessel insofar as the synaptic gap between the nerves and the smooth muscle extends over much greater distances in the aorta, and the relative proportions of structural protein to smooth muscle cells are much greater in the conducting-type of artery represented by the aorta than in the muscular-type represented by the rabbit ear artery.

Another interesting comparison between the data of Levin on the rabbit aorta and that on the rabbit ear artery is shown in Table 6.8. This includes the relative proportions of the metabolites in the tissue and in the incubating medium. As pointed out in the Results, in the rabbit ear artery the medium to tissue ratios of DOPEG and of MOPEG are high (6.3 and 10.3 respectively), whereas those of DOMA and VMA are extremely low (both <1.1). The ratio for NMN is intermediate in value (3.2). There is a striking parallel both qualitatively and quantitatively between these ratios and the ratios for the intact rabbit aorta. From Levin's later study (Levin, 1974) it is evident that the ratios quoted are little different for isolated media and for isolated adventitia, with the possible exception that proportionally more of the VMA effluxes into the incubating medium from the isolated adventitia. Levin has pointed out that the high medium to tissue ratios for the glycols are consistent with their unchanged nature (compared with the remaining metabolites) which would permit their ready diffusion from cells. Besides the preceding considerations, the effects of cocaine or denervation, of inhibition of MAO, and also differences between the metabolism of the laevo form compared with the vaecemic form of ^{3}HNA also emphasise similarities rather than differences between the two vessels. Thus in both tissues, inhibition of MAO increased the accumulation of unchanged ^{3}HNA within the tissue and increased the accumulation of NMN. Unfortunately, the total accumulation only of NMN was shown by Levin (1973) and it is not known whether, as in the ear artery, an increase was observed in the accumulation in the medium but not in the tissue. The same considerations apply to the decreases in DOPEG and DOMA seen in their accumulation in the medium but not in the tissue. Also, in both tissues inhibition of COMT eliminated the O-methyl metabolites but did not increase the tissue

TABLE 6.8

Comparison of the relative proportions of metabolites in the rabbit ear artery and rabbit aorta

Metabolite	Relative proportions of metabolites (%) Tissue (a)		Medium (b)		Ratio (c) $\frac{\text{Medium}}{\text{Tissue}}$	
	REA	RA	REA	RA	REA	RA
NMN	13	48.8	9.8	40.23	3.2	3.67
DOPEG	50	12.9	73.1	42.62	6.3	14.7
DOMA	18	17.2	4.9	3.5	1.1	0.89
MOPEG	4	5.2	9.4	11.1	10.3	9.52
VMA	14	16.0	2.7	2.6	0.82	0.72

Footnotes

(i) (a) the amount of metabolite in the tissue expressed as a percentage of the total metabolites present in that tissue.

(b) the amount of metabolite in the incubation medium expressed as a percentage of the total metabolites present in the incubation media.

(c) the ratio of the content of metabolite in the medium to the content of the same metabolite in the tissue.

(ii) The values for the relative proportions of metabolites and the medium to tissue ratios are taken from Table 6.4 and are based on the results of experiments in which 7 or more ear arteries (REA) were incubated with dl^{3}HNA (1.2 μmol l^{-1}) for 30 mins.

(iii) The values for the rabbit aorta (RA) are based on the published results of Levin (1973) for which 14 segments of aorta were incubated with dl^{3}HNA (0.3 μmol l^{-1}) for 60 mins.

levels of NA. Finally, it should be noted that, in both tissues, the metabolite patterns vary in the same way when l^{3}HNA is used instead of dl^{3}HNA, i.e. the formation of DOPEG tends to increase and the formation of NMN to decrease. Unfortunately, it was not possible in the ear artery to distinguish between the levels of NA and DOMA, so that it is not clear whether the decrease in the combined level in the tissue incubated with l^{3}HNA reflected a decrease in one or both. In the rabbit aorta, there was no difference between the tissue levels of NA, but there was a decrease in the formation of DOMA when the laevo isomer was used.

In summary, the comparison between the two preparations emphasizes that, despite their different physiological functions, their biochemical mechanism of inactivating NA show a close similarity, the only real difference being a predominance of neuronal metabolism in the more highly innervated muscular (ear) artery.

CHAPTER 7

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

CHAPTER 7

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

INTRODUCTION

In the preceding experiments some of the properties of the neuronal uptake system for NA in the REA were characterized, as well as evidence for extra-neuronal accumulation and O-methylation of NA. Essentially the evidence was the failure of either cocaine or chronic denervation to decrease the accumulation of NA or its O-methylated metabolites to the same extent as did PBZ. The present chapter provides further information on the properties of the extra-neuronal uptake and O-methylation system in the REA. Isoprenaline was selected instead of noradrenaline for this purpose. The selection of isoprenaline was based on the following considerations:- (1) it has a lower affinity for neuronal uptake and a higher affinity for extraneuronal uptake (Iversen, 1967) and (2) it is a substrate for COMT but is not deaminated by MAO (Hertting, 1964). Hence, in theory, the use of isoprenaline offered a simpler approach to the study of extraneuronal uptake and inactivating mechanisms. That this reasoning was not entirely justified will be apparent from the results and hence will be considered again in the Discussion. The plan of the experiments was as follows:-

(1) The amount of unchanged ^{3}HISO present in the artery following a period of incubation with ^{3}HISO was measured, together with an investigation of the nature of the metabolites present in both the tissue and the incubation media. The latter investigation was considered important to test the assumption (mentioned earlier) that only O-methylation occurred, since there was evidence of the formation of a deaminated metabolite of isoprenaline in mice (Ross 1963) and further evidence from a recent study that, after oral administration of ^{3}HISO to dogs, ^{3}HVMA was present in their urine (Conway et al., 1968). There was evidence also that in man, ISO was excreted in part as a sulphate conjugate (Conolly et al. 1972).

(2) The effects of an inhibitor of COMT (U0521), and of an inhibitor of extraneuronal uptake (DOCA) on the uptake and O-methylation of ^{3}HISO were studied in an attempt to define the extraneuronal processes involved and their interrelationship. Here it should be noted that the selection of U0521 and of DOCA was based not only on biochemical evidence of the inhibitory effects of these classes of compounds on the two processes involves (Giles and Miller 1967; Salt 1972), but also in the light of the considerable experience which had accumulated in the author's laboratory on the pharmacological interaction between these two agents. As mentioned in the Discussion (Chapter 10), each of these agents separately caused an increase in activity of the REA to catecholamines (adrenaline mainly studied), but these increases were not additive. Thus the aim in this part of the study was to some extent circular, i.e. by using these agents or tools to explore extra-neuronal uptake processes it was hoped at the same time to shed further light on the mechanisms underlying their pharmacological actions, particularly that of DOCA.

Towards the latter part of this study it became apparent that there might also be a neuronal component of the uptake and inactivation of ^{3}HISO, again despite the simplifying assumption which led to its use in the first place. Accordingly, there was a third aspect of the study, namely determination of the effects of chronic sympathetic denervation on the uptake and metabolism of ^{3}HISO.

It should be noted that the techniques used in this study for the estimation of ^{3}HMeOISO are different than those that were currently available, in particular the procedures of Hertting (1964) and Bönisch and Trendelenberg (1973). The reasons for their preference and their justification have already been discussed in Chapter 4.

METHODS

Arteries weighing from 10 to 30 mgm were equilibrated for 60 minutes in Krebs bicarbonate solution gassed with 95% O_2, 5% CO_2 and containing ascorbic acid (290 µmol l^{-1}) and EDTA (10.8 µmol l^{-1}). (The composition of the Krebs solution and the type of incubation apparatus used is described in Chapter **2**). After equilibration the segments were incubated for 30 minutes at 36^{o}C with purified dl^{3}HISO (0.81 µmol l^{-1}), 5 Ci(mmol)$^{-1}$). (The purification and preparation of ^{3}HISO prior to incubation is described in Chapter 3). At the end of the incubation period the arteries were placed in one mil aliquots of amine-free gassed Krebs solution for one minute. The amines (^{3}HISO and ^{3}HMeOISO) were then extracted from the artery segment (without homogenisation) in a solution of HCl (100 mmol l^{-1}) containing EDTA (22 µmol l^{-1}). (The efficiency of this procedure was documented in Chapter 4).

The uptake of ^{14}C-sorbitol was used as a measure of the extracellular space. For this purpose arteries were incubated with ^{14}C-sorbitol (8.1 µmol l^{-1}) in the presence of non-labelled ^{3}HISO (0.81 µmol l^{-1}), washed for one minute in Krebs solution, and the radioactive contents of arteries and wash solutions determined as described in the general methods chapter (Chapter 2).

Treatments

(1) Drugs: Unless otherwise stated, drugs were added to the incubation media throughout the incubation with ^{3}HISO. Drugs were not added to wash solutions unless specifically stated in the text. The drugs used were UO521, DOCA, cocaine and PBZ (prepared as stock solutions as described in Chapter 2).

(2) Chronic denervation: Either one ear artery, or both ear arteries, of rabbits were denervated by surgical removal of the homolateral or both superior cervical ganglia 14 days prior to the removal of arteries. The surgical techniques and the fluorescence histochemical procedures used for determining the effectiveness of denervation are described in the general methods chapter (Chapter 2).

Separation techniques

The nature of the metabolites of ^{3}HISO were determined using the ion exchange paper chromatographic technique described in Chapter 4, the non-labelled reference compounds used being ISO, MeOISO, DOMA, VMA, DOPEG and MOPEG. As indicated in Results, the analysis revealed only ^{3}HMeOISO as a metabolite of ^{3}HISO. In most of the subsequent studies,

the ^{3}HISO and ^{3}HMeOISO were separated on borate impregnated thin layer silica gel plates as described in Chapter 4. In a smaller number of arteries ^{3}HISO and ^{3}HMeOISO were separated using the solvent extraction procedure described in Chapter 4. Since there were some puzzling variations in recovery values with the solvent procedures, results based on this method are distinguished from those based on the superior thin layer chromatographic method.

RESULTS

1. Metabolism of ^{3}HISO

The distributions of ^{3}H from incubates, and from concentrated tissue extracts, (the latter concentrated by the lypholysation procedure described in Chapter 4), were determined on paper chromatograms and compared with the distribution of non-labelled "putative" metabolites. (Chromatograms illustrating these comparisons are shown in Fig. 7.1, where it can be seen that there were only two significant regions of ^{3}H for both tissue extracts and incubates and that these regions corresponded to the migration of non-labelled ISO and MeOISO. In contrast, test solutions of ^{3}HISO in Krebs solution (i.e. not exposed to arteries) and of ^{3}HISO in the acid medium used for extracting the artery segments, both gave single peaks of ^{3}H only, corresponding to that of non-labelled ISO. There was no accumulation of ^{3}H in the regions of the chromatograms that corresponded to the migrations of the deaminated (DOMA, DOPEG) or 3-O-methyl deaminated (VMA, MOPEG) derivatives of ISO.

It was concluded from these findings that the principal metabolite of ^{3}HISO in the REA was ^{3}HMeOISO.

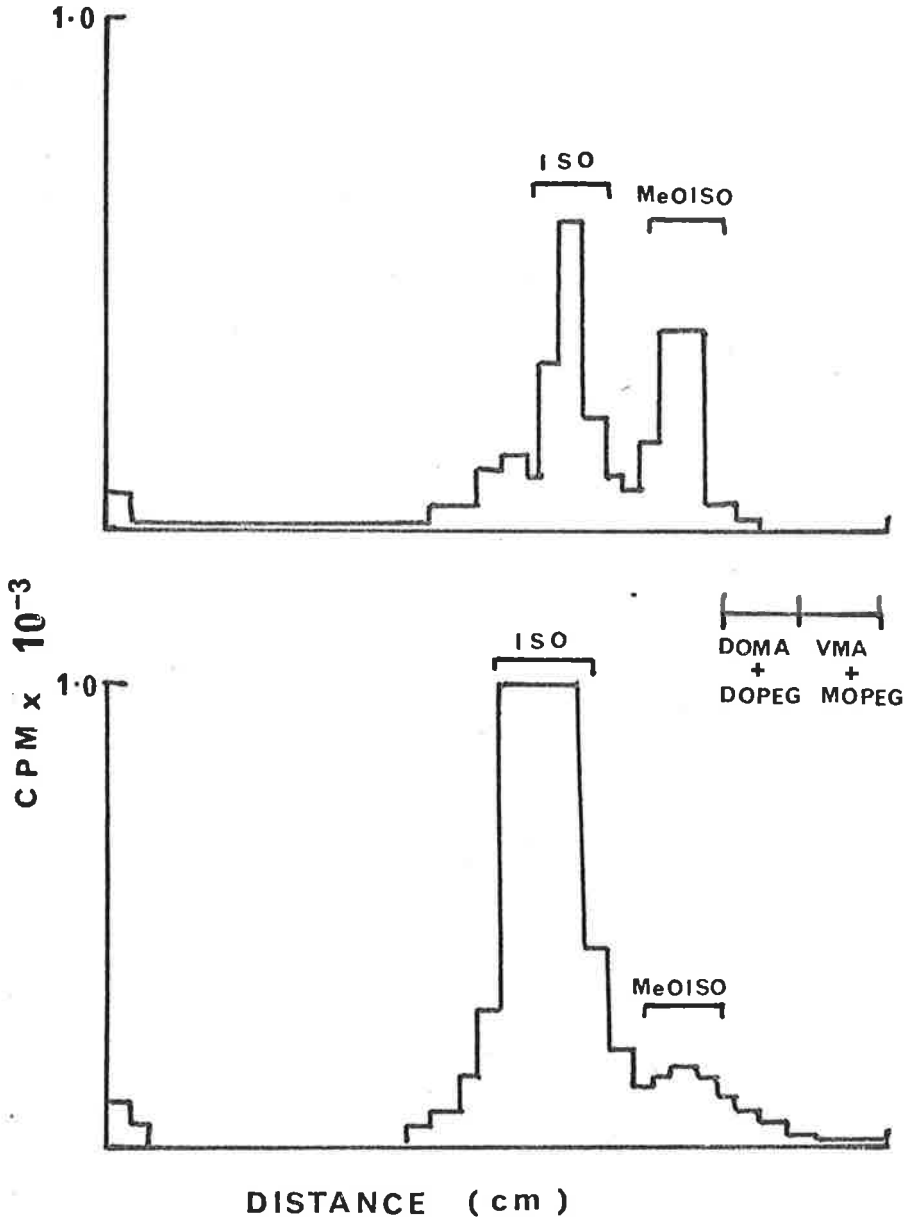


Fig. 7.1 The distributions of ^{3}H on paper chromatograms (Method I) for acid extracts of arteries (upper panel) and incubates (lower panel). Arteries incubated with ^{3}HSIO (0.81 µmol l^{-1}).

2. Accumulation of ^{3}HISO and ^{3}HMeOISO

The contents of ^{3}HISO and ^{3}HMeOISO in the tissue, and of ^{3}HMeOISO in the incubating media, were determined in 11 arteries which were incubated with ^{3}HISO (0.81 μmol l^{-1}) for 30 minutes and then washed for one minute in amine-free Krebs solution. The results are summarised in Fig. 7.2 and show the following features:

(i) The content of unchanged ^{3}HISO in the tissue (1.06 ± 0.19 nmol g^{-1}) is significantly greater than the ^{3}HISO content of the incubating medium.

(ii) The tissue content of ^{3}HMeOISO is 0.97 ± 0.11 nmol g^{-1}, i.e. only a little below that of unchanged ^{3}HISO.

(iii) The amount of ^{3}HMeOISO released into the medium is about 4 fold greater than that which is present in the tissue. (This estimate takes no account that a proportion of ^{3}HMeOISO is lost in the one minute wash. This has been estimated precisely from efflux data described in the following chapter as 0.35 ± 0.02 nmol g^{-1} (n=4), which means the tissue:medium ratio for MeOISO is closer to 1:3).

The relatively small difference between the tissue and medium concentration of unchanged ^{3}HISO suggested that the ^{3}HISO may have been uniformly distributed through the tissue water, rather than actively accumulated. To test this possibility, the sorbitol space present after a one minute wash was estimated in another group of 12 arteries. These arteries were incubated with ^{14}C sorbitol 8.1 μmol l^{-1} under the same conditions as in the studies on isoprenaline. The mean sorbitol space was 0.072 ± 0.012 cm^{3} g^{-1} (n=15) which corresponded to a ^{3}HISO content of only 0.088 nmol g^{-1}, i.e.< 10% of the experimentally

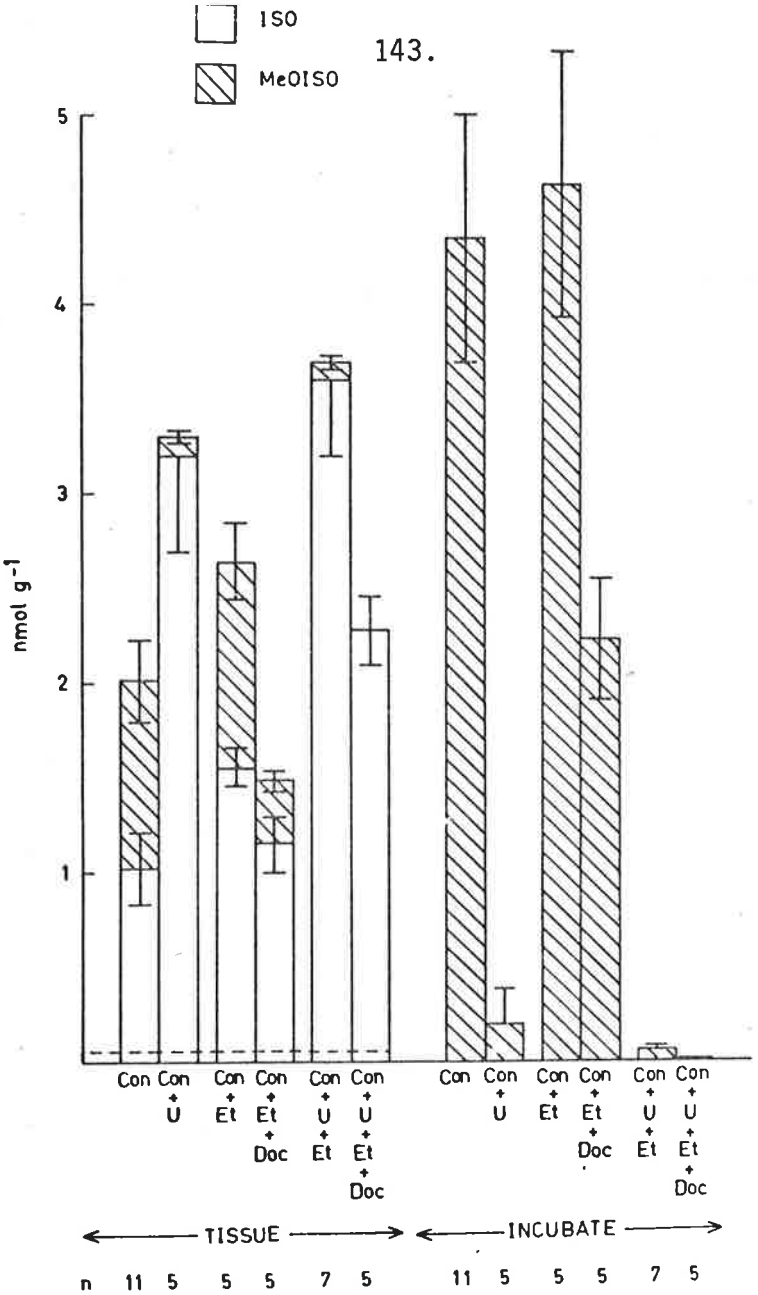


Fig. 7.2 Levels of ^{3}HISO and ^{3}HMeOISO (nmol g^{-1}) in tissues and Krebs incubates. Arteries were incubated for 30 minutes with ^{3}HISO (0.81 µmol l-1).

Con = untreated
U = U0521 (27 µmol l^{-1}
Et = Ethanol (173 mmol l^{-1})
Doc = DOCA (27 µmol l-1)

Significance ISO
Con vs U $p < 0.001$ unpaired
Con + Et vs Con + Et + Doc $p > 0.05$ paired
Con + Et + I vs Con + Et + Doc $0.05 > p > 0.02$ unpai

MeOISO (Tiss)
Con vs U0521 $p < 0.001$ unpaired
Con + Et vs Con + Et + Doc $p < 0.001$ paired

MeOISO (Incubate)
Con vs U0521 $p < 0.001$ unpaired
Con + Et vs Con + Et + Doc $0.05 > p < 0.02$ paired

determined value. Hence it was concluded that at least 90% of the ^{3}HISO that was present after the one minute wash was not contained in the extracellular space. This latter content corresponds to about half the tissue mass since the true extracellular space is approximately 0.6 cm^3 g^{-1}. (The value of 0.6 cm^3 g^{-1} was determined in experiments on the efflux of sorbitol described in the following chapter).

During the experiments on sorbitol, it was established that the sorbitol space of tissues measured after a 10 minute incubation (0.09 ± 0.01 cm^3 g^{-1}, n=6) was not significantly different from that after 30 minute incubation (0.08 ± 0.01 cm^3 g^{-1}, n=6), indicating that the sorbitol had equilibrated throughout the extracellular space within 30 minutes. In addition, the precaution was taken of estimating whether significant loss of material occurred on to the filter paper when the artery was rapidly blotted prior to extraction with acid. The loss of activity represented 16.4 ± 4.1% (n=6) of the ^{14}C activity of the tissue.

3. COMT inhibition

The effects of incubating 5 arteries with U0521 (55 μmole l^{-1}) are summarised in Fig. 7.2. These effects comprised:

(i) Inhibition of ^{3}HMeOISO formation, as indicated by decreases in both the tissue and medium levels of this amine of more than 90% and 95%, respectively. Traces of ^{3}HMeOISO (0.078 nmol g^{-1}) were detected in all of the arteries, but could not be detected in one of the incubating media of the 5 arteries.

(ii) A 3 fold increase in the ^{3}HISO content of the tissue. The mean value of 3.19 ± 0.49 nmole g^{-1} was now about 4 fold greater than the concentration in the medium. This difference represented unequivocal evidence that the free amine accumulated within the tissue. A noteworthy

feature was that the extra accumulation occurring in the COMT-inhibited artery (c.f. 2.13 nmole g^{-1}) was significantly greater than could be accounted for by simple stoichiometric replacement of the ^{3}HMeOISO.

4. DOCA

The effect of DOCA (27 μmole l^{-1}) on the accumulation and O-methylation of ^{3}HISO was studied in 10 arteries, including 5 that were treated with U0521 (27 μmole l^{-1}). Since the DOCA was dissolved in ethanol, the control arteries (n=12) in these experiments were incubated with ethanol (173 mmole l^{-1}). The results are summarised in Fig. 7.2, where it can be seen that DOCA caused 69% and 52% decreases in the ^{3}HMeOISO contents of the tissues, and of the media. Surprisingly, DOCA had little effect on the content of unchanged ^{3}HISO in the tissue, the difference between the mean values of 1.56 ± 0.13 and of 1.16 ± 0.15 nmole g^{-1} (corresponding to DOCA absent and DOCA present) being not significant ($p > 0.05$, unpaired t-test).

However, when COMT was inhibited, DOCA caused a more marked (38%) and significant decrease ($p < 0.05$ unpaired t-test) in the tissue content of ^{3}HISO. It is of interest that the small amounts of ^{3}HMeOISO still present in the tissue were also decreased by the DOCA.

When compared with values obtained in other experiments on untreated arteries, the vehicle for DOCA (ethanol) was without significant effect on the levels of ^{3}HMeOISO in the tissue or media. Also, it was without effect on the accumulated levels of ^{3}HISO in COMT-inhibited arteries. An exception was provided by the tissue levels of ^{3}HISO in the COMT-intact arteries, which were significantly greater (47%) in the ethanol-treated group.

In summary, DOCA selectively inhibited O-methylation of ISO. It inhibited accumulation of unchanged ^{3}HISO when COMT was inhibited, but did not do so when COMT was intact. The effects of DOCA contrasted with those of U0521 in that the decrease in O-methylation produced by the latter agent was accompanied by an increase in the accumulation of unchanged ^{3}HISO.

5. Denervation and cocaine

To test the possibility that the DOCA insensitive components of ^{3}HISO accumulation (and also O-methylation) may have reflected uptake of ^{3}HISO into nerves, the effects of chronic denervation and of cocaine on this accumulation and O-methylation were investigated.

The effects of chronic sympathetic denervation (by removal of the homolateral superior cervical ganglia 14 days earlier) are summarised in Fig. 7.3. The paired contralateral innervated arteries from the same animals were used as controls. All arteries were incubated for 30 minutes with ^{3}HISO (0.81 μmole l^{-1}) followed by a one minute wash. It will be seen that denervation did not influence to a significant extent the accumulation of ^{3}HMeOISO in the tissue or medium. However, there was a significant decrease (36%) in the accumulation of ^{3}HISO in the denervated arteries.

The effects of cocaine (30 μmol l^{-1}) were examined in 6 arteries in which COMT was intact, and in a further 5 arteries in which COMT was inhibited by U0521 55 μmol. The conditions of incubation were otherwise identical to those employed in the denervation experiments. It should be noted that the data on the COMT-intact arteries are less reliable, since the separations of ^{3}HISO and ^{3}HMeOISO were achieved

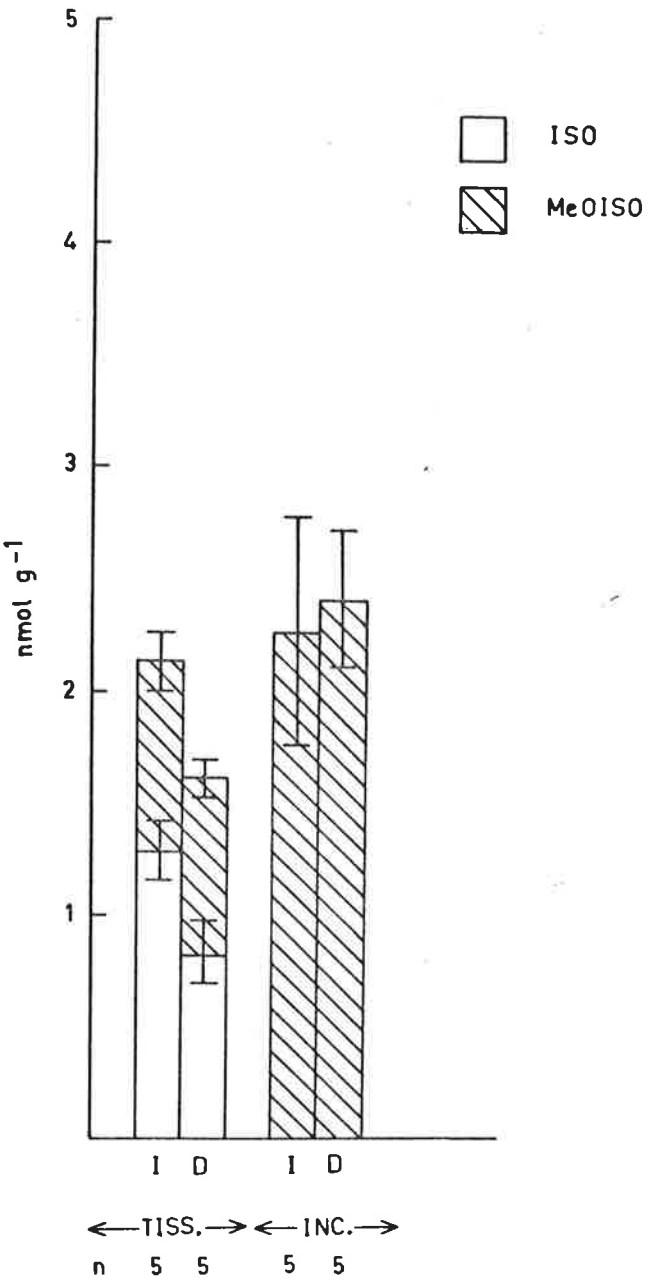


Fig. 7.3 Levels of ^{3}HISO and ^{3}HMeOISO (nmol g^{-1}) in tissues and Krebs incubates: Arteries were incubated for 30 minutes with ^{3}HISO (0.81 µmol l^{-1}).

I = innervated arteries
D = denervated arteries

Significance

ISO I vs D $p < 0.05$ paired

MeOISO Tissue I vs D $p > 0.5$ paired

MeOISO Incubate I vs D $p > 0.5$ paired

with the solvent extraction technique at a time when there were variations in the efficiency of recovery. However, it was obtained in the one group of experiments, so that the comparisons between untreated and cocaine-treated arteries are probably valid. For this reason, data is summarised in Table 7.1. It will be seen that it illustrates precisely the same trend as observed in the denervation experiments, namely, a decrease in the accumulation of unchanged ^{3}HISO, but no effect on the tissue or media levels of ^{3}HMeOISO.

The effect of cocaine (30 μmol l^{-1}) on the accumulation of ^{3}HISO in COMT-inhibited arteries is summarised in Fig. 7.4. (This data is not subject to the qualifications expressed in the preceding paragraph, since it did not involve separation of ^{3}HISO and ^{3}HMeOISO). The main feature is the lack of significant effect of cocaine on the accumulation of ^{3}HISO under the conditions.

Since the preceding results pointed to a significant neuronal accumulation of unchanged ^{3}HISO, the possibility existed that this process may have "masked" in some way an inhibitory effect of DOCA on the extra-neuronal component of accumulation. In one series of experiments, the arteries of both ears in each of 6 rabbits were sympathetically denervated by removal of both superior cervical ganglia 14 days previously. Both arteries from each animal were then incubated with ^{3}HISO, both with and without DOCA, under the same experimental conditions as used in the earlier experiments described in this section. The results are summarised in Fig. 7.4, where it can be seen that the effect of DOCA is qualitatively similar to that in the innervated arteries, i.e. it still fails to cause a significant decrease in the accumulation of unchanged ^{3}HISO, although decreasing the levels of ^{3}HMeOISO in the tissue and medium. Although the decrease

TABLE 7.1

Artery contents of ^{3}HISO and ^{3}HMeOISO, and incubation media contents of ^{3}HMeOISO (nmol per gm artery) determined by the solvent extraction procedure

Treatment	n	^{3}HISO Tissue	^{3}H-MeOISO Tissue	^{3}H-MeOISO Medium
Nil	9	0.43 ± 0.05	1.0 ± 0.08	4.07 ± 0.45
Cocaine (30 μmol l^{-1})	6	0.23 ± 0.03*	0.95 ± 0.07	3.23 ± 0.43
Phenoxybenzamine (33 μmol l^{-1})	6	0.29 ± 0.03*	0.12 ± 0.01*	0.92 ± 0.10*

Footnotes

(i) Results are the mean values (± S.E.M.) for 9 untreated, 6 cocaine treated and 6 phenoxybenzamine treated arteries incubated with dl^{3}HISO (0.81 μmol l^{-1}) and washed for one minute in dl^{3}HISO-free Krebs.

(ii) The ^{3}HISO and ^{3}HMeOISO contents determined by the solvent extraction procedure described in Chapter 4.

(iii) * $P < 0.05$ when compared with controls (unpaired t-test).

in the tissue levels appears less than observed in the innervated arteries, the decrease in ^{3}HMeIOSO accumulation in the media is equally prominent.

The effects of DOCA were also examined in 6 COMT-inhibited, cocaine-treated arteries. The results are summarised in Fig. 7.4. They show that DOCA continued to exert its normal inhibitory action on unchanged ^{3}HISO accumulation despite the presence of cocaine.

In summary, the results in this subsection pointed to a significant accumulation of ^{3}HISO in sympathetic nerves when COMT was intact, but not when COMT was inhibited. The failure of denervation and of cocaine to influence ^{3}HMeIOSO formation provides strong evidence against the possibility that the neuronal ISO undergoes O-methylation. The results also highlight the fact that the above effects of cocaine and denervation are opposite to those of DOCA, which depresses O-methylation but does not influence ^{3}HISO accumulation when COMT is intact. The latter effects of DOCA are little affected by chronic denervation (in COMT-intact arteries) or by cocaine (in COMT-inhibited arteries).

One qualifying feature of the results deserves mention. The preceding conclusions, i.e. the effects of chronic denervation, were based on the use of the contralateral innervated arteries as the controls. A surprising feature is that the accumulation of ^{3}HMeOISO in the media in those controls is significantly less than was measured in the commonly used controls in these studies, i.e. untreated controls from rabbits which had not been subjected to any prior treatments or operations before removal of arteries. The difference can be seen by comparing the data in Figs. 7.2 and 7.3. The same difference between

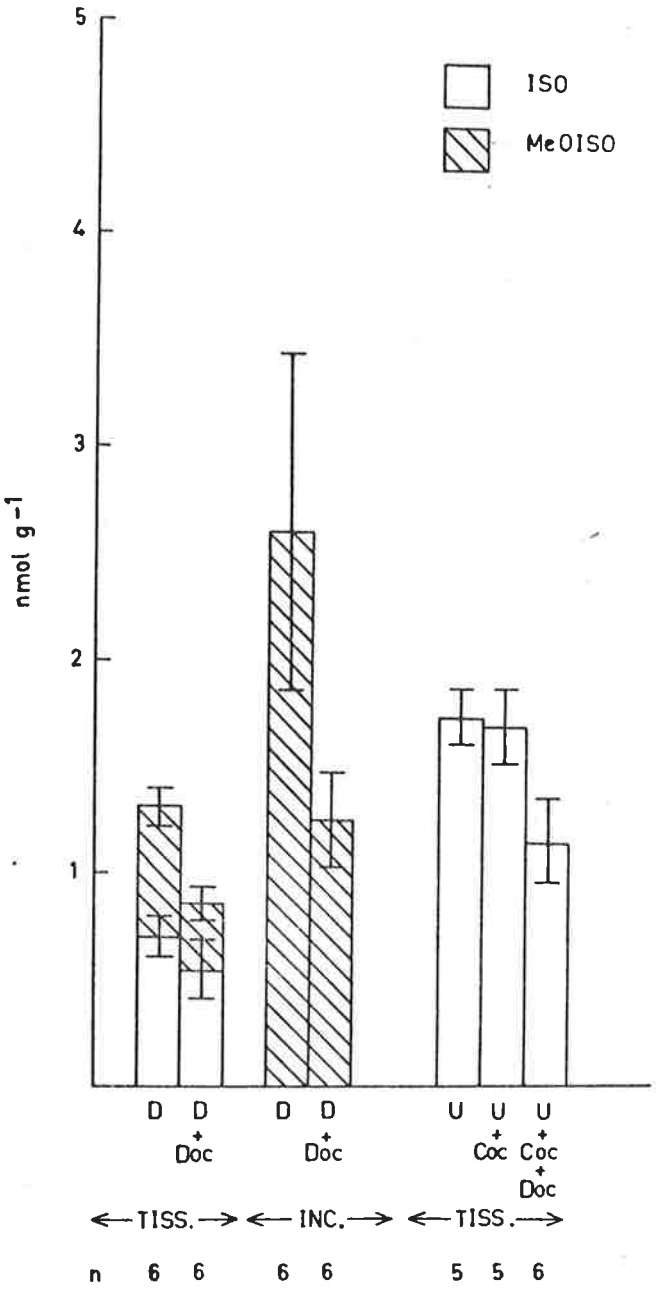


Fig. 7.4 Levels of ^{3}HISO and ^{3}HMeOISO (nmol g^{-1}) in tissues and Krebs incubates: Arteries were incubated for 30 minutes with ^{3}HISO (0.81 µmol l^{-1})

D = denervated
Doc = DOCA (27 µmol l^{-1})
U = U0521 (27 µmol l^{-1})
Coc = cocaine (30 µmol l^{-1})

Significance ^{3}HISO D vs D + Doc $p > 0.05$
U vs U + Coc $p > 0.05$
U vs U + Coc + Doc $0.05 > p > 0.02$
^{3}HMeOISO Tis. D vs D + Doc $0.1 > p > 0.05$
^{3}HMeOISO (incubate) D vs D + Doc $0.05 > p > 0.02$

the two types of control is apparent in the case of the arteries from the experiments where both ears were denervated. These differences suggest that either the preceding surgery, or the denervation of one artery, may have altered the activities of COMT or extraneuronal uptake in the opposite innervated artery.

DISCUSSION

1. Metabolism

The results in this section have provided a useful indication of the extent, and localisation of the accumulation and O-methylation of ^{3}HISO in the REA.

The first experiments described in this section provided evidence that 3-O-methylation was the major metabolic pathway for ISO in the artery. The possibility that there may have been oxidative deamination was excluded by the failure to detect significant peaks of radioactivity in the region of the chromatogram corresponding to the catechol or 3-OMe catechol acids and glycols. Unfortunately, sulphate or glucaromide conjugates of ISO were not available for comparison. However, it is unlikely that these highly water soluble derivatives would behave on the chromatogram like the more lipid soluble ISO or its 3-methoxy derivative and the identity of ^{3}MeOISO as the major metabolite will be assumed for the remainder of this discussion. It should be noted that another confirmation of identity of this metabolite was its virtual disappearance when arteries were incubated with the COMT inhibitor UO521.

The appearance of the ^{3}MeOISO in both tissue segments and incubating media was not surprising in view of (i) the earlier evidence that COMT activity was present in the homogenates and (ii) subsequent evidence that normetanephrine was a significant metabolite of NA in this artery, indicating that the amine still gained access to the COMT when the artery's cellular structure was intact. Furthermore, these earlier studies had pointed to a largely extraneuronal site of O-methylation of NA and the present results show that this argument applies equally well to O-methylation of ISO. This followed from the failure of chronic denervation or of cocaine to appreciably influence the tissue or bath levels of the methoxy compound. This failure was complemented by the sensitivity of these levels to inhibition by DOCA, which has been shown previously to be one of a number of steroids which inhibit extraneuronal uptake in rat heart (Salt 1972). The sensitivity to DOCA can thus be construed as additional evidence for the extraneuronal localisation of O-methylation, or alternatively, can be viewed as an argument to confirm that the site of action of DOCA in the REA, as in the rat heart, is extraneuronal. This action of DOCA could be explained in one of two ways, namely, either as a direct effect on the enzyme, similar to that of other agents such as U0521, or as an indirect effect resulting from inhibition of access of substrate or cofactors to the enzyme. The former possibility seems excluded at the outset by the evidence that, unlike U0521, DOCA does not cause an associated increase in the tissue levels of unchanged ISO. The second possibility is also in accord with the failure of various investigators (including this author and others in his supervisor's laboratory) to demonstrate inhibition by DOCA of O-methylation of catechols using partially purified rat liver COMT preparations, (S. Byrne, B.Sc. Hons. thesis (1975); R.J. Head, unpublished observations). Other evidence

supporting the role of steroids in inhibiting uptake rather than direct inhibition of COMT has also been presented in the Introduction (Chapter 1).

The factors controlling the accumulation of ^{3}HISO, and its localisation, appear more complex. This complexity arises from the evidence (i) that this process is partly neuronal in origin when COMT is intact (ii) that there is a large increase in the accumulation of ^{3}HISO when O-methylation is prevented; this accumulation is greater than expected if the ISO simply replaced the MeOISO in the tissue, (iii) that this increased accumulation of ISO when COMT is inhibited is entirely extraneuronal (since it is now unaffected by cocaine), and (iv) that, in contrast to procedures which eliminate neuronal uptake of catecholamines, DOCA only affected the uptake of unchanged ^{3}HISO when COMT is inhibited.

The simplest explanation of the immediately preceding findings under (i), (ii) and (iii) is that when COMT is intact, only the amine which is taken up extraneuronally undergoes O-methylation. Presumably the neuronal ISO is taken up by the mechanism responsible for the transport of catecholamines into the cytoplasm of the nerve. Here it remains intact as a result of the absence of intraneuronal COMT and its inability to be deaminated by MAO. The evidence that it is transported by this mechanism is, firstly, that cocaine exerts a similar effect to chronic denervation on the uptake of unchanged amine (Table 7.1), and, secondly if one compares the neuronal contents of dl^{3}HNA (i.e. the difference between innervated and denervated artery contents of dl^{3}HNA (viz. 6.8 nmol g $^{-1}$, seen in Fig. 6.5 of the previous chapter), with the neuronal contents of dl^{3}HISO (i.e. the difference

between innervated and denervated artery contents of dl^{3}HISO (viz. 0.46 nmol g $^{-1}$, seen in Fig. 7.3), the ratio of these contents (NA:ISO, 15:1), is not dissimilar to the ratio (33:1) of the relative affinities of dlNA and dlISO for neuronal uptake (Uptake$_1$) in the rat heart (Burgen and Iversen 1965). The qualification being that NA and not ISO is metabolized neuronally in this artery and hence the ratio (15:1) is probably underestimated.

The failure to detect a neuronal component when COMT is inhibited is puzzling. This failure was based on the evidence that the increased accumulation of ^{3}HISO in the COMT-inhibited artery was unaffected by cocaine. The latter finding, plus the evidence that DOCA's action was unaffected by cocaine, provides presumptive evidence that the additional ISO which accumulates when COMT is inhibited is taken up into extraneuronal structures. However, it is not clear why this additional uptake should have decreased the amount of ISO in the synaptic cleft available for neuronal uptake (over and above the rate of removal prevailing when COMT was intact). It is possible, of course, that the magnitude of the neuronal uptake has remained unchanged, but is now relatively small compared with the total uptake so the effects of inhibition of neuronal uptake are harder to detect. Clearly, more experimental data is required before this discrepancy can be further analysed.

Perhaps the most interesting feature of these findings is the greater inhibitory effects of DOCA on the uptake of unchanged amine in COMT inhibited compared with COMT intact arteries. The simplest explanation is that this level is normally kept low by the rapid

O-methylation of the ISO, i.e. uptake is rate limiting. Hence a decrease in uptake of ISO into the COMT-containing compartment in the tissue will be manifested primarily as a decrease in the rate at which O-MeISO is formed. Under conditions of COMT inhibition, ISO accumulates in this compartment at a rate determined now more directly by the rate of uptake. Under these conditions, the inhibitory effect of DOCA on extraneuronal uptake is more clearly manifest as a decrease in the rate of accumulation. This explanation is clearly oversimplified and is presented at this stage solely to provide some background to the studies presented in the following chapter.

Comparison with other tissues

While this study was in progress, the first of the extensive studies of Trendelenburg and co-workers on the uptake of catecholamines in rat heart appeared. There are many points of resemblance between the properties of the ISO uptake and O-methylating system in the heart preparation, and in the REA. This includes the greater accumulation of ISO following COMT inhibition, and differences between the magnitude of steroid inhibition of this uptake in COMT intact and COMT inhibited preparation.

These workers also compared the medium and tissue levels of ^{3}HMeOISO and ^{3}HISO in a variety of tissues following 30 min incubation with ^{3}HISO at a concentration (0.95 μmol l^{-1}) close to that employed in the present study (0.81 μmol l^{-1}). Their summary is shown in Table 7.2, which also includes comparative data from the present study. It is noteworthy that the capacity of the REA for OMe formation greatly exceeds all the rat tissues shown with the exception of the rat heart. Another point of resemblance between the rat heart and REA is the equally prominent tendency for the extra accumulation of ^{3}HISO following COMT inhibition.

TABLE 7.2

A comparison of the accumulation and O-methylation of ^{3}HISO (shown as the means ± SE (nmol gm^{-1}) of n experiments) in the rabbit ear artery and various tissues of the rat

Tissue	Treatment	n	^{3}HISO Tissue	Tissue	^{3}HMeOISO Medium	Ratio $\frac{\text{Medium}}{\text{Tissue}}$(a)	$\frac{\Sigma^{3}\text{HMeOISO}}{^{3}\text{HISO}}$(b)
Rabbit ear artery	Nil	11	1.063 ± 0.193	0.971 ± 0.113	4.350 ± 0.327	4.48	5.00
" " "	U0521 (55 μmol l^{-1})	5	3.185 ± 0.494	0.078 ± 0.024	0.195 ± 0.098	2.50	0.09
Rat ventricle	Nil	11	0.912 ± 0.117	0.185 ± 0.029	0.174 ± 0.025	0.94	0.39
" "	U0521 (100 μmol l^{-1})	7	1.147 ± 0.148	0.019 ± 0.007	0.016 ± 0.015	0.84	0.03
Rat aorta	Nil	5	1.077 ± 0.431	0.258 ± 0.083	0.254 ± 0.091	0.98	0.48
Rat heart	Nil	4	2.813 ± 0.116	1.122 ± 0.044	15.284 ± 2.500	13.62	5.83

Footnotes

(i) (a) the ratio of the amount of ^{3}HMeOISO in the medium to content of ^{3}HMeOISO in the tissue.

(b) the ratio of the total amount of ^{3}HMeOISO formed (i.e. tissue plus medium) to the content of ^{3}HISO in the tissue

(ii) The results for the rabbit ear artery are taken from Fig. 7.1 and are based on experiments in which arteries were incubated with dl^{3}HISO (0.81 μmol l^{-1}) for 30 min. For arteries treated with U0521 (55 μmol l^{-1}) this drug was added 30 mins before (and present during) incubation with ^{3}HISO.

(iii) The values for all other tissues are based on the published results of Bönisch *et al.* (1974) for which segments of these tissues were incubated with dl^{3}HISO (0.95 μmol l^{-1}) for 30 min. The only exception being the values for the rat heart for which tissues were perfused with dl^{3}HISO (0.95 μmol l^{-1}) for 10.0 minutes.

Trendelenberg and co-workers themselves have speculated that the coronary vessels may represent the main compartment in the rat heart in which O-methylation occurs (Bönisch *et al.* 1974). The points of similarity between the REA and rat heart do not detract in any way from their suggestions. However it is not possible, from the limited information obtained in this present study, to deduce whether the REA possesses more than one compartment for the extra-neuronal uptake and O-methylation of ^{3}HISO as has been described in rat and guinea pig hearts and in the cat nictitating membrane by Trendelenburg and his associates. The possible nature of ISO compartments and their relationship to those described in other tissues will be considered in greater depth in the following chapter.

The findings from other laboratories suggest that additional similarities exist between the REA and other vascular tissues, in respect to their ability to accumulate and O-methylate ISO. Included in this comparison is the study of Bevan *et al.* (1972) who have provided evidence for a predominantly medial distribution of ^{3}H in segments of rabbit aorta previously incubated with ^{3}HISO (0.1 μmol l^{-1}), that was reduced, but not abolished, in ^{3}H content by prior treatment with PBZ (100 μmol l^{-1}). This finding is in accord with the predominantly extra-neuronal origin of the accumulation of ^{3}HISO in the REA and consistent with the observation (Table 7.1) that PBZ decreased, but not abolished, the ^{3}H contents of ear arteries. (The possibility of a tissue bound non intracellular origin of part of the accumulation of ^{3}HISO in arteries will be discussed in the following chapter).

A recent study, based in part on a histochemical autoradiographic analysis of the distribution of ^{3}HISO in dog mesenteric artery and saphenous vein, has provided direct evidence for the extra-neuronal (smooth muscle) accumulation of ^{3}HISO in arteries and veins (Osswald and Guinmaraes, to be published; reported at the 6th International Congress on Pharmacology, Helsinki, 1975). The similarity between these tissues and the REA is most striking, for like the REA, treatment of these arteries and veins with U0521 produced an increase in the accumulation of unchanged amine (^{3}HISO). Furthermore these authors found that treatment of these tissues with a steroid (Cortexone) reduced the tissue content of ^{3}H and the O-methylation of ^{3}HISO.

Although the findings from this study have suggested that at least 36% of the accumulation of ^{3}HISO in the REA is of neuronal origin, similar findings for other vascular tissues are lacking. One exception being the recent report of Cornish *et al.* (1976), these authors have provided evidence for a neuronal uptake of ^{3}HISO in the kitten coronary artery. In that study they suggest that about 30% of the ^{3}HISO accumulation in arteries can be attributed to neuronal uptake. However it must be pointed out that these authors appeared to have used a concentration of ^{3}HISO (20.3 μmol l^{-1}) much greater than that used in this present study (0.81 μmol l^{-1}).

Comparisons between the results of this present study and those reported for other vascular tissues in which the extraneuronal accumulation and metabolism of catecholamines other than ISO have been examined, will be considered in the General Discussion (Chapter 10).

In summary it was concluded that many of the properties of extra-neuronal accumulation and O-methylation of ISO seen for this artery are shared by a variety of vascular tissues.

CHAPTER 8

KINETIC ASPECTS OF THE METABOLISM AND EFFLUX OF ^{3}HISO

CHAPTER 8

KINETIC ASPECTS OF THE METABOLISM AND EFFLUX OF ^{3}HISO

INTRODUCTION

This section comprises a heterogenous group of experiments designed to answer specific questions arising from the results of the preceding section. These questions were:

(1) Was the inhibitory potency of DOCA on O-methylation of ISO influenced by the period of incubation?

(2) Was the inhibition competitive or non-competitive?

(3) Did DOCA influence the efflux of ^{3}HISO from this artery?

The first two questions resulted from the possibility that the relative magnitudes of the DOCA-resistant and DOCA-sensitive components of ISO accumulation and O-methylation may have been influenced by the particular conditions of incubation, in particular the time courses of accumulation of MeOISO in the untreated and DOCA-treated arteries may have differed at different times during incubation. For example, the efflux of MeOISO from the untreated preparations may have been initially rapid, while that from the DOCA-treated preparations initially slow. If so, estimation after 30 minutes would underestimate the effects of DOCA during the initial efflux. The second question involved the

relationship between the relative concentrations of DOCA and ISO, since it was conceivable that the DOCA-insensitive components of O-methylation may have simply reflected competition between ISO and the drug for structures controlling access to COMT. The third question was based on the consideration that analysis of the efflux curves might shed further light on the number and size of the tissue compartments involved in ISO accumulation and metabolism. This approach was prompted by the extensive analysis of ISO efflux by Uhlig *et al.* (1974). At the same time, it was considered important to assess the nature of the events occurring in the first minute of washout.

METHODS

The procedures used for incubation of arteries with dl ^{3}HISO (0.81 μmol l^{-1}) and the estimation of ^{3}HISO and ^{3}HMeOISO contents of arteries and ^{3}HMeOISO contents of incubates were the same as those described in the previous chapter (Chapter 7).

The ^{3}HMeOISO and ^{3}HISO contents of wash solutions were determined by the solvent extraction procedure described in Chapter 4. Briefly this method involved the addition of a saturated solution of sodium borate (pH 10.0) to Krebs wash solutions and extraction of ^{3}HMeOISO (and non labelled MeOISO) with the solvent mixture Toluene:isoamylalcohol (3:2). The ^{3}HMeOISO was recovered in an aqueous solution by extraction of the organic mixture with a solution of HCl (300 mmol l^{-1}). The ^{3}H content of the aqueous solution and the content of non labelled MeOISO (used to estimate recovery) were determined by liquid scintillation spectrometry and colourimetric assay. The ^{3}HISO content of the original wash solution was estimated by the difference procedure described in Chapter 4.

Efflux experiments

The washout of ^{3}H amines from artery segments previously incubated with $^{3}HISO$ (0.81 μmol l^{-1}) were measured. These arteries were cannulated prior to incubation so that, at the commencement of washout, they were perfused (0.3 ml min^{-1}) with amine-free Krebs solution. This perfusion was continued throughout the washing procedure. Washing involved transferring the segment into fresh 2.0 ml aliquots of Krebs solution at 10 second intervals for the first 60 seconds and thereafter at 2 minute intervals for the remaining 30 minutes. The wash solutions were analysed for $^{3}HISO$ and $^{3}HMeOISO$ contents after separation of these amines by the solvent extraction technique described briefly above and in more detail in Chapter 4.

To help determine the origins of the $^{3}HISO$, which effluxed from the *artery*, these effluxes were compared with those of ^{14}C-sorbitol from arteries previously incubated with ^{14}C-sorbitol (8.1 μmol l^{-1}) in the presence of unlabelled $^{3}HISO$ (0.81 μmol l^{-1}). The effluxes of sorbitol were expressed in the same units as those used for $^{3}HISO$ (i.e. n mol g^{-1} min^{-1}).

Analysis

The effluxes of $^{3}HISO$ or $^{3}HMeOISO$ were expressed either as rates (i.e. n mol g^{-1} min^{-1}) or as the total amount which had effluxed from zero time (i.e. cumulative efflux, i.e. n mol g^{-1}). When relevant, the efflux of ^{14}C-sorbitol was subtracted from that of $^{3}HISO$ so that the efflux of $^{3}HISO$ was corrected to exclude efflux from ^{14}C-sorbitol space. The ^{14}C-sorbitol space will be referred to subsequently as the

extracellular space or compartment. In experiments where the rates of efflux were not linear when plotted as log rate vs time, the assumption was made that the curve consisted of at least two exponential components, and these components were then distinguished by the curve "peeling" technique.

The "peeling" technique was applied as follows:

(I) Since a single exponential decline is a straight line on a semilogarithmic plot, the last stage of the efflux curve was examined for linearity (using a ruler). If approximately linear, a mono-exponential rate was then calculated from the regression line of best fit and the values calculated from this regression line were subtracted from the experimental curve over its entire length. This indicated whether the earlier part of the efflux curve also approximated to a single monoexponential decline. If this appeared to be the case, again a line of best fit was determined. From these two (at least) monoexponential rates of decline the total amounts of amine (cumulative) that would have effluxed at these rates were calculated (i.e. compartment size) as well as the $t_{\frac{1}{2}}$ values for these compartments, i.e. the time required for one half of the cumulated ^{3}H to efflux from the tissue. This analysis was performed on the mean values for the various rate determinations.

(II) The second form of analysis was similar to that described above except the line of best fit (for the mean of various rate determinations) was obtained for the last stage of efflux by determining the correlation coefficients (r^2) between combinations of the experimentally derived data and an exponential curve. The method employed was that described

in the Hewlett-Packard Applications and Programs manual for use with the HP-25 programmable calculator. This method permitted calculation of the values a and b for the linearized equation ($\ln y = \ln a + bx$) describing an exponential function of the form $Y = ae^{bx}$ ($a > 0$). In this way the regression line of best fit was calculated (i.e. by estimating a $\ddot{y}$ value for a given x, i.e. $\ddot{y} = ae^{bx}$) and the values for this regression line subtracted from the experimental curve over its entire length. The compartment size (i.e. the area under the curve) was obtained from the value $\frac{a}{b}$ and the time for the compartment to half empty ($t_{\frac{1}{2}}$) from the value $\frac{0.693}{b}$.

(III) The final form of analysis was the same as II above except that all calculations were performed on individual (and not mean) rate data. This then permitted statistical analysis to be obtained for both compartment sizes and corresponding $t_{\frac{1}{2}}$ values.

(IV) Tissue Residue. For these experiments the ^3H present in arteries at the completion of wash (i.e. ^3H in the tissue that did not efflux) was determined experimentally. The amount of ^3H that would be expected to remain in the tissue, due to the termination of wash at a time corresponding to slow phase of efflux, was calculated from the regression lines (individuals and not means) for the rates of efflux describing compartments with the largest half times. Any difference between experimentally determined tissue residue and derived (i.e. calculated) residue was thought to reflect (1) a further compartment characterised with a very large half time or (2) ^3H bound to the tissue that did not in any way contribute to efflux (i.e. bound ^3H).

RESULTS

1. Accumulation

The time courses of the accumulation of ^{3}HMeOISO in the bathing solutions of arteries during incubation with ^{3}HISO in the absence, and in the presence of DOCA for 60 minutes are shown in Fig. 8.1. Two concentrations of ^{3}HISO were used (0.48 and 3.75 μmol l^{-1}), and both the control and DOCA solutions also contained ethanol (173 mmol l^{-1}). It will be noted that the rate at which MeOISO accumulated increased during the first 5 to 10 minutes, after which it was approximately linear with time to 60 minutes. A similar trend is apparent in the DOCA-treated preparations, i.e. there was little difference between DOCA's inhibitory potencies throughout the 60 minute period of incubation.

The time course of accumulation of ^{3}HMeOISO in the incubation media and the corresponding time courses of accumulation of ^{3}HISO and ^{3}HMeOISO in arteries that were incubated with dl^{3}HISO (0.81 μmol l^{-1}) for 5, 15 and 30 min are shown in Fig. 8.2. It may be seen that the rate of accumulation of ^{3}HMeOISO in the incubation media for these arteries was qualitatively similar to that seen (Fig. 8.1) for arteries incubated with a smaller and larger concentration of ^{3}HISO. The rate of accumulation of ^{3}HMeOISO in the medium for the period 0 to 5 minutes of incubation (estimated by extrapolation from the value at 5 min to zero min) was much smaller than that seen after 5 min. A similar trend for the time course of formation of MeOISO (i.e. tissue MeOISO plus incubate MeOISO) was only just evident (Fig. 8.2), i.e. the initial rate of formation of MeOISO (i.e. 0-5 min) was not markedly different from that seen between 5 and 15 min. The reason for this difference

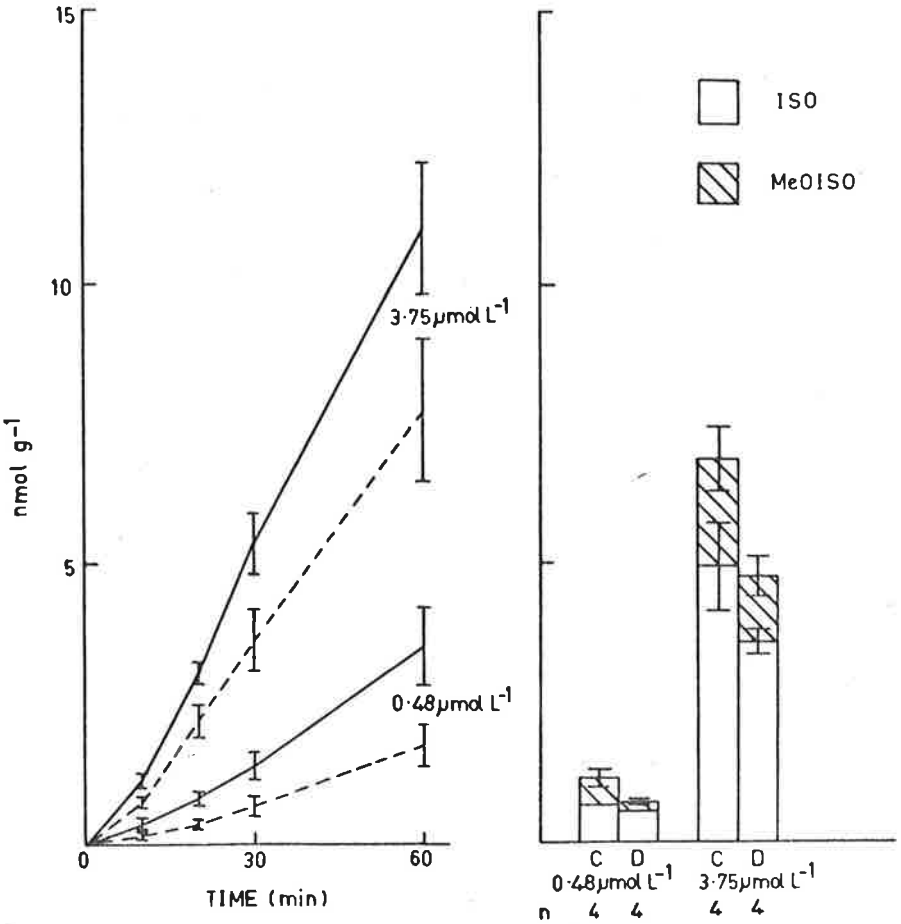


Fig. 8.1 The effect of DOCA (27 μmol l^{-1}) on the rate of accumulation of ^{3}HMeOISO in incubation media. Shown are the means (± SEM) ^{3}HMeOISO contents (in nmol g^{-1}) of incubation media analysed at 10, 20, 30 and 60 min of incubation for (i) 4 arteries incubated with dl^{3}HISO (0.48 μmol l^{-1}) in the presence of DOCA and 4 arteries incubated with dl^{3}HISO (0.48 μmol l^{-1}) with ethanol (173 mmol l^{-1}); (ii) 4 arteries incubated with dl^{3}HISO (3.75 μmol l^{-1}) in the presence of DOCA and 4 arteries incubated in the presence of ethanol. All DOCA values significantly smaller ($p < 0.05$) than controls except for ^{3}HISO (3.75 μmol l^{-1}) at 60 mins.

The solid lines show the rate of accumulation of ^{3}HMeOISO in ethanol treated arteries, the broken line shows the corresponding rates for DOCA treated arteries. The contents (nmol g^{-1}) of ^{3}HISO and ^{3}HMeOISO (mean ± SEM) prevailing in tissues at the end of incubation and after a 1.0 minute wash are also shown.

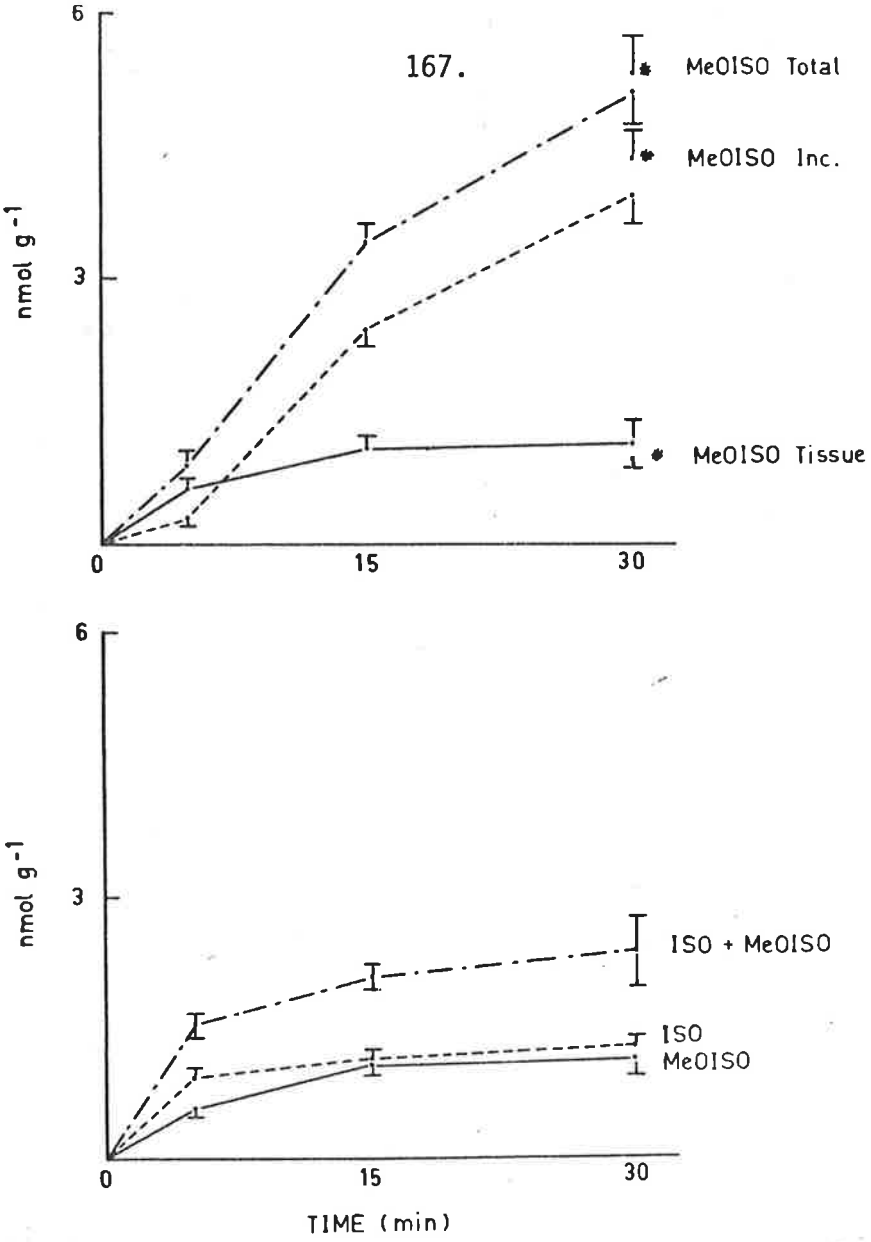


Fig. 8.2 Upper panel: the rates of accumulation of ^{3}HMeOISO in arteries (tissue) and incubates (Inc) and the rate of formation of ^{3}HMeOISO (Total).

Lower panel: the rate of accumulation of ^{3}HMeOISO, ^{3}HISO and ^{3}HISO + ^{3}HMeOISO in arteries.

Each point represents the mean (± SEM) of 4 observations at 5, 15 and 30 minutes of incubation. Arteries incubated with ^{3}HISO (0.81 μmol l^{-1}).

can be seen in Fig. 8.2 where it is apparent that the content of ^{3}HMeOISO in the artery at 5.0 min (unlike the contents at 15 and 30 min) is greater than the corresponding ^{3}HMeOISO content of the medium. After 5.0 min the ^{3}HMeOISO content of arteries is relatively constant and smaller than the ^{3}HMeOISO contents of the incubate, and under these conditions the rate of accumulation of ^{3}HMeOISO in the medium parallels the rate of total formation of ^{3}HMeOISO (see Fig. 8.2). These findings were thought to reflect upon a process within the arteries that required an attainment of a particular tissue content of ^{3}HMeOISO before the rate of efflux of ^{3}HMeOISO into the medium became constant with time, i.e. artery accumulation ^{3}HMeOISO precedes accumulation of ^{3}HMeOISO in the medium. The results of these experiments point to a rapid accumulation and O-methylation of ^{3}HISO in the artery. It may be seen (Fig. 8.2) that the ^{3}HISO content of tissues at 5 mins represents 78% of that seen at 30 mins of incubation. Similarly the ^{3}HMeOISO contents at 5 mins represent 52% of that seen at 30 mins.

In Fig. 8.3, the rates of accumulation of ^{3}HMeOISO have been plotted against three concentrations of ^{3}HISO. Since the data at the third concentration, 0.81 μmol l^{-1}, is derived from the 30 minutes incubation experiments in the preceding chapter, the rates in Fig. 8.3 have also been calculated at 30 minutes from the data in Fig. 8.1. An important feature of the curve for the untreated artery in Fig. 8.3 is the evidence of saturation of the O-methylating system at the highest concentration of ^{3}HISO. A similar tendency to saturation, although less marked, is apparent also for the relationship between rate of O-methylation and concentration of ^{3}HISO in the DOCA treated preparations. The important feature here is that DOCA's inhibitory

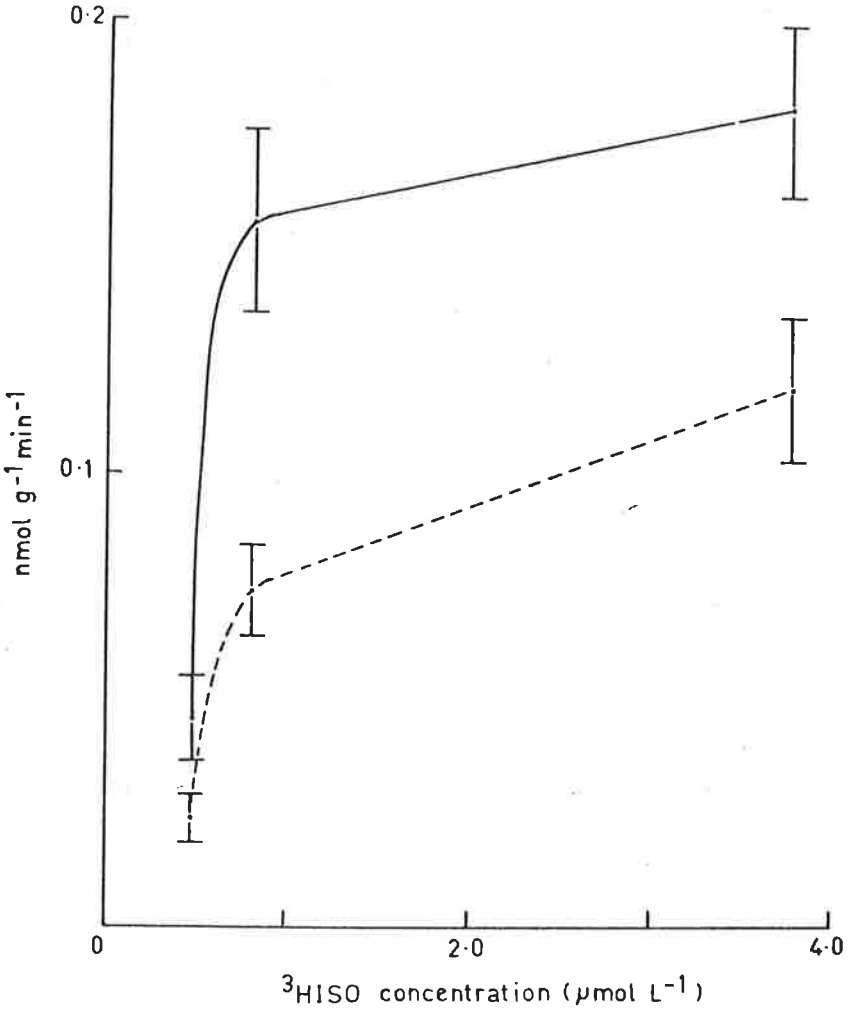


Fig. 8.3 The effect of DOCA (27 μmol l^{-1}) on the rate of appearance of ^{3}HMeOISO (based on values at 30 mins of incubation) for three concentrations of ^{3}HISO (0.48, 0.81 and 3.75 μmol l^{-1}).

Ordinate: rate of accumulation of ^{3}HMeOISO (nmol g^{-1} min^{-1})

Abscissa: concentration of ^{3}HISO (μmol l^{-1})

Values represent the mean (± SEM) of 4 or more determinations, solid line refers to control arteries, the broken line to DOCA treated arteries. Rate values for ^{3}HISO concentrations of 0.48 and 3.75 μmol l^{-1} taken from data shown in Fig. 8.1 and values for ^{3}HISO 0.81 μmol l^{-1} taken from data shown in the previous chapter.

action is markedly reduced at the highest concentration of ^{3}HISO. The same trend is apparent in the case of ^{3}HMeOISO contents of the tissues estimated after the 60 minute period of incubation (Fig. 8.1), i.e. the decrease in these contents produced by DOCA is much less at the highest concentration of ^{3}HISO. It should be noted that, as is the case with the 30 minute periods of incubation, the MeOISO remaining in the tissue after 60 minutes incubation represents only a small proportion (approx. 14-18%) of the total MeOISO generated during this period. In view of the finding in the previous chapter suggesting that DOCA inhibited the accumulation of ^{3}HISO only in COMT inhibited arteries, information as to the competitive or non-competitive nature of this inhibition was obtained by determining the effect of DOCA on the accumulation of ^{3}HISO in COMT inhibited arteries at four different substrate (^{3}HISO) concentrations. For these experiments arteries were incubated with dl^{3}HISO (0.08, 0.81, 8.1 and 81 μmol l^{-1}) for 30 min in the presence of U0521 (55 μmol l^{-1}) and ethanol (173 mmol l^{-1}) and the ^{3}H contents of these tissues compared with those seen for arteries incubated under the same conditions but in the presence of DOCA (27 μmol l^{-1}). The results of these experiments are summarized in Table 8.1 where it may be seen that the magnitudes of inhibition of the accumulation of ^{3}HISO caused by DOCA were similar for all concentrations of ^{3}HISO. It can also be seen from Table 8.1 that the ratio of the tissue contents of ^{3}H (in nmol gm^{-1}) to the bath concentration of ^{3}HISO (in nmol ml^{-1}) tended to decrease as the substrate concentration increased. The latter observation being consistent with a decrease in the ability of arteries to concentrate ^{3}HISO at the higher substrate (^{3}HISO) concentrations. It was concluded from these experiments that

Footnotes:

(i) shown are the ^{3}H contents (mean ± S.E.M., nmol g^{-1}) of arteries incubated for 30 min with dl^{3}HISO (0.08, 0.81, 8.10, and 81.0 µmol l^{-1}) and washed for 1.0 min in amine free Krebs solution.

(ii) Treatments. U0521 (55 µmol l^{-1}) was present in all incubation media and added before (15 min) incubation with dl^{3}HISO. DOCA (final concentration 27 µmol l^{-1}) was added (in ethanol) to arteries at the commencement of incubation with ^{3}HISO. Ethanol (final concentration 173 mmol l^{-1}) was added to control arteries at the commencement of incubation.

(iii) (a) refers to the molar ratio of ISO to DOCA prevailing in the incubation media.

(b) refers to the ratio of the ^{3}H contents of arteries (nmol g^{-1}) to the content of ^{3}H originally present in the incubation medium (nmol ml^{-1}).

(c) the mean ^{3}H content of DOCA treated arteries expressed as a percentage of the ^{3}H content of control (ethanol) arteries.

(iv) Significance: * these values are significantly ($p < 0.05$, unpaired comparison) smaller than the corresponding control values.

TABLE 8.1

The effect of DOCA on the accumulation of ^{3}HISO in COMT inhibited arteries

Treatment	^{3}HISO concentration µmol l^{-1}	$\frac{[ISO]^{a}}{[DOCA]}$	n	^{3}H content nmol g^{-1}	$\frac{^{3}H_{tiss}{}^{b}}{^{3}H_{bath}}$	DOCA as[c] % control
U0521 + EtOH	0.08	0.003	2	0.304 ± 0.018	3.8	52.3
U0521 + EtOH + DOCA			2	0.159 ± 0.039	1.9	
U0521 + EtOH	0.81	0.03	9	3.05 ± 0.044	3.8	73.8
U0521 + EtOH + DOCA			9	2.25 ± 0.27	2.8	
U0521 + EtOH	8.10	0.3	4	18.7 ± 1.99	2.3	69.2
U0521 + EtOH + DOCA			4	12.95 ± 1.66	1.6	
U0521 + EtOH	81.0	3.0	4	144.7 ± 26.3	1.8	67.4
U0521 + EtOH + DOCA			4	97.58 ± 0.89	1.2	

DOCA's inhibitory effect on the accumulation of unchanged amine was relatively independent of substrate concentration and hence reflected a non-competitive mode of inhibition.

2. Efflux curves

(i) Efflux of ^{3}HISO and of ^{3}HMeOISO, and of ^{14}C sorbitol were compared by measuring the ^{3}H and ^{14}C contents of the wash solutions from arteries following their incubation with either ^{3}HISO (0.81 μmol l^{-1}) or ^{14}C sorbitol (8.1 μmol l^{-1}) for 30 minutes. The relative rates of effluxes of ^{3}H and of ^{14}C over a 30 minute period of washing are presented in Fig. 8.4. The difference between the two is that the efflux of ^{14}C declined rapidly so that it was completed by 6 minutes, whereas the efflux of ^{3}H, although also declining rapidly at first, was still evident at 30 minutes. The results of this comparison suggested that the bulk of the ^{3}H which comprised ^{3}HISO in the extracellular space had also effluxed within the first few minutes.

(ii) The latter suggestion was further studied in a second group of arteries by analysing the nature of the effluxes which occurred during the first minute of washout (at 10 second intervals). The results are summarised in Fig. 8.5, and in Fig. 8.6, which shows the plot of the curves, log rate of efflux vs time of washing, during the one minute period.

The experimental curve contained 3 components; an initial very rapid efflux (termed I) a second slower but well sustained efflux (II), a third slower efflux which approached the steady state by the final time of measurement (about 6 minutes). When analysed by the curve peeling technique, only the second phase could be defined unequivocally by a monoexponential rate decline, with a $t_{\frac{1}{2}}$ of 0.2 min and a compartment size of 0.53 nmol g^{-1}. This was assumed to represent efflux from the

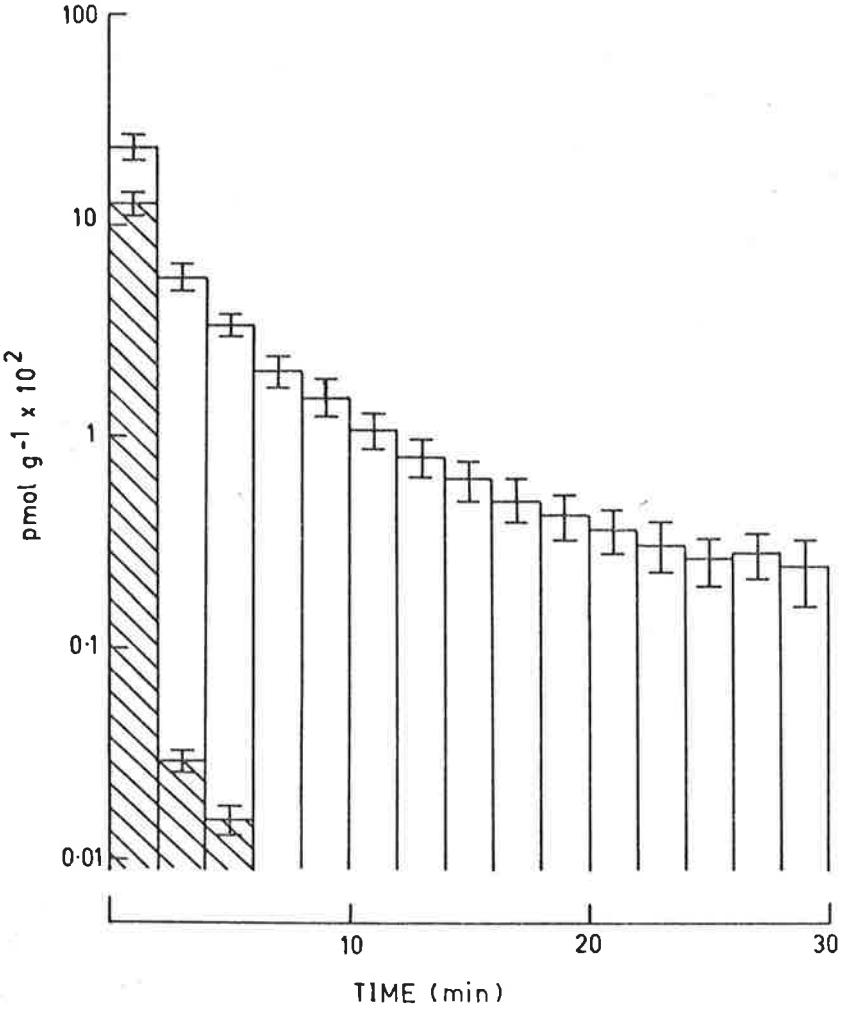


Fig. 8.4 Efflux of total radioactivity during washout of segments of arteries after an initial incubation with ^{3}HISO (0.81 μmol l^{-1}) and ^{14}C sorbitol (8.1 μmol l^{-1}). Sorbitol space expressed as equivalent ^{3}HISO (pmol g^{-1}).

Ordinate: contents (pmol g^{-1})
Abscissa: time (min)

Shown are the means (± SEM) for 4 arteries previously incubated with ^{14}C sorbitol (hatched histograms) and 4 or more arteries previously incubated with ^{3}HISO (open histograms).

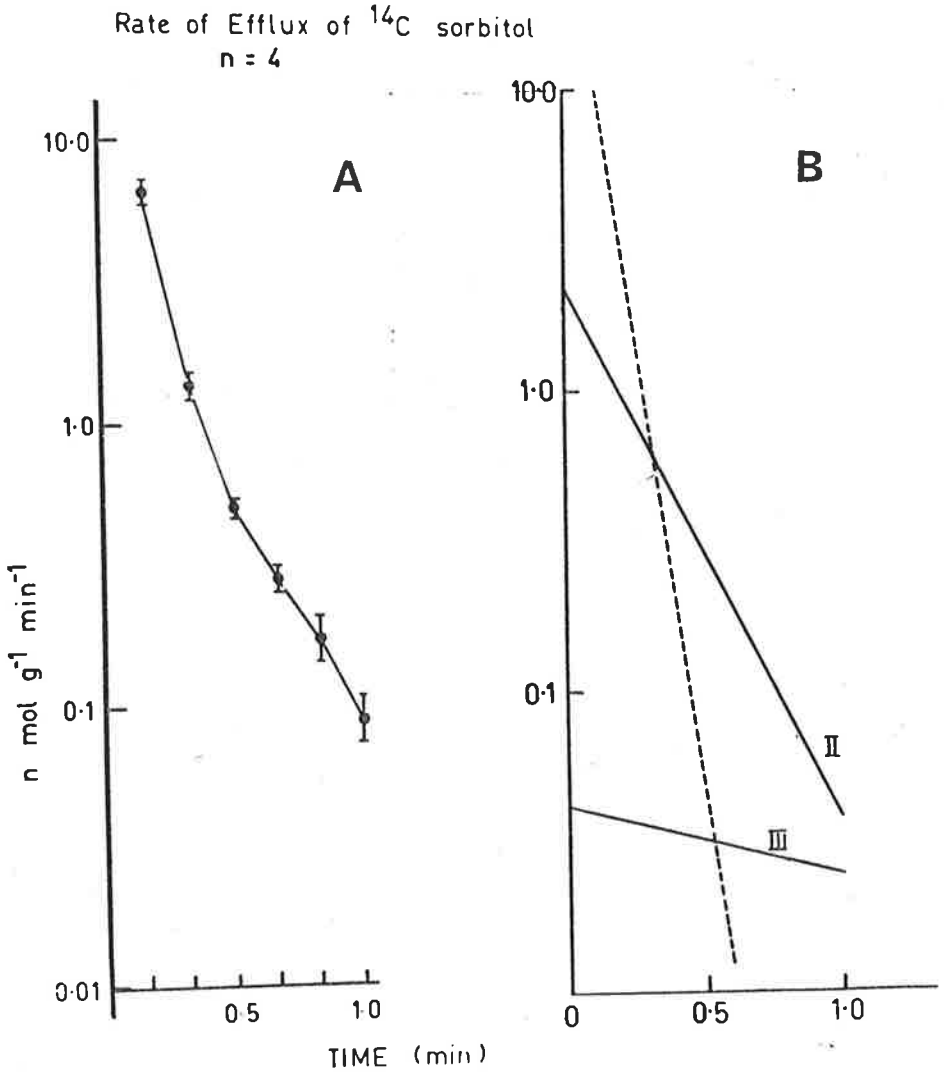


Fig. 8.5A. Efflux of radioactivity during washout of segments of arteries after an initial incubation with ^{14}C sorbitol (8.1 nmol g-1) for 30 min.

Ordinate: rate of efflux (expressed as ^{3}HISO nmol g^{-1} min^{-1} log scale)
Abscissa: time (min) after onset of washout.

Shown are the means (± SEM) for 4 arteries.

B. Shown are the individual rates of efflux (corresponding to Phase I, dotted line; Phase II and III)after curve peeling by Procedure III.

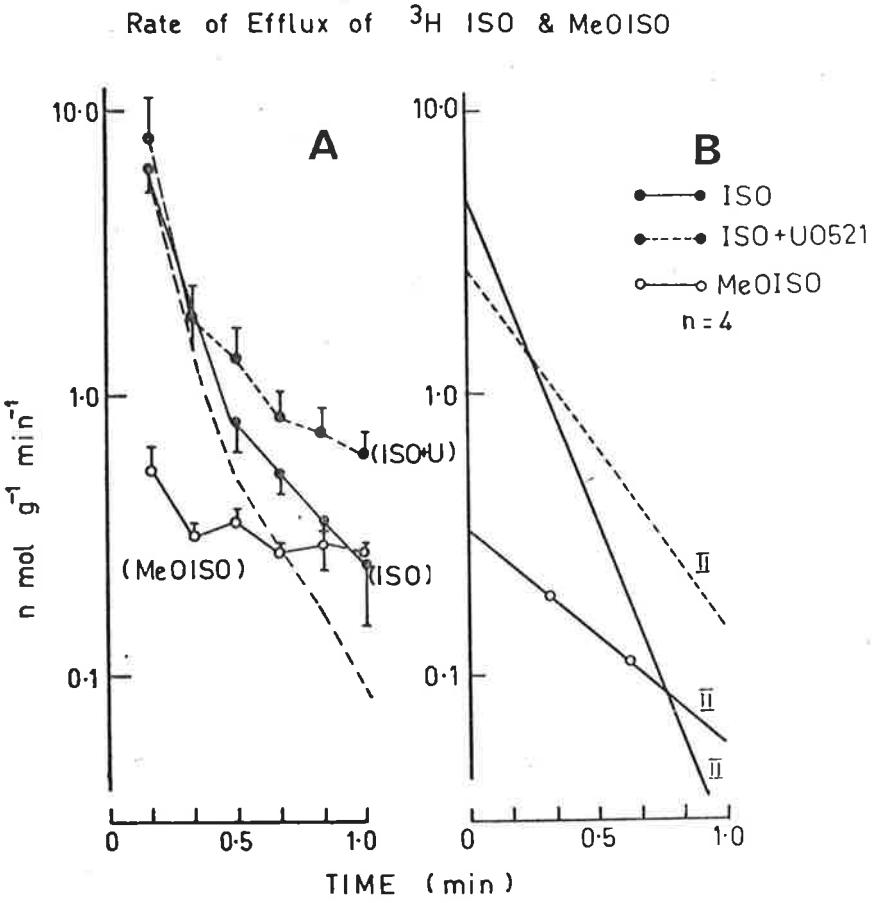


Fig. 8.6A. Efflux of radioactivity during washout of segments of arteries after an initial incubation with ^{3}HISO (0.81 µmol l^{-1}) in the absence (solid line) and presence of U0521 (55 µmol l^{-1}, dotted line).

Ordinate: rate of efflux (nmol g^{-1} min^{-1}, log scale)
Abscissa: time (min) after onset of washout.

Shown are values (± SEM) for ^{3}HISO and ^{3}HMeOISO for 4 arteries, dotted line represents efflux of ^{14}C sorbitol seen previously in Fig. 8.5.

B. Shown are the individual rates of efflux (corresponding to Phase II of efflux) after curve peeling by Procedure III.

extracellular compartment. The efflux from this compartment that occurred within the first minute of wash was equivalent to 0.49 nmol g^{-1} or about 92% of the size of the compartment determined above. The extracellular space estimate derived from this one minute content was 0.65 cm^3 g^{-1} and agrees well with the estimate of 0.61 cm^3 g^{-1} by Bevan and Waterson (1971) for this artery.

Phase I was attributed to ^{14}C sorbitol which adhered to the tissue from the incubating medium and also to the presence of some of the latter in the lumen. It should be mentioned that the arteries were not blotted or perfused during transfer from the incubate to the first wash solution. Phase III corresponded to a small compartment (0.09 nmol g^{-1}) with a $t_{\frac{1}{2}}$ of 1.05 min. However its significance is doubtful because the compartmental analysis was based on rates of efflux at only three intervals of time. Fig. 8.5 includes only Phase II and Phase III monoexponential rate declines.

The separate rates of efflux of ^{3}HISO and of ^{3}HMeOISO during the first one minute were determined in both untreated and in UO521 (55 µmol l^{-1}) treated arteries. The UO521 was present 10 minutes before commencing incubation with ^{3}HISO (0.81 µmol l^{-1}), throughout incubation and throughout washing. The individual rates of efflux of ^{3}HISO from UO521 treated arteries were significantly greater than the rates of efflux of ^{14}C sorbitol at 0.5, 0.66, 0.83 and 1.0 minutes. The rates of efflux of ^{3}HISO from untreated arteries were not significantly greater than the rates of efflux of sorbitol. The amounts of ^{3}HISO corresponding to the differences in rates of ^{3}HISO and ^{14}C sorbitol efflux for the first one minute of wash were 0.32 nmol g^{-1} for untreated and 0.69 nmol g^{-1} for UO521 treated arteries.

The curves of the ^{3}HISO rates of efflux from untreated and U0521 treated arteries were similar in shape to those of ^{14}C sorbitol, i.e. biphasic and best described by two phases of efflux (tentatively called Phase I and Phase II). Phase I was attributed to ^{3}HISO which adhered to the tissue from the incubating medium. The Phase II curves for ^{3}HISO (for both untreated and U0521 treated arteries) were greater than the Phase II curve for ^{14}C sorbitol (see Fig. 8.6). It was thought that this may have been due to the efflux of ^{3}HISO which had been present in the extra cellular space at the end of incubation together with the efflux of ^{3}HISO from additional sites within the tissue (the presence of such sites not indicated by ^{14}C sorbitol efflux).

In contrast to the effluxes of ^{3}HISO and ^{14}C sorbitol, the efflux of ^{3}HMeOISO declined only slightly during the one minute period (Fig. 8.6). The total amount which effluxed was 0.35 ± 0.18 nmol g^{-1}, i.e. about 36% of that which is normally present in the artery at the end of incubation and after a one minute wash (Table 7.2, Chapter 7). Assuming that the ^{3}HMeOISO content of the extra cellular space fluid is similar to the ^{3}HMeOISO concentration for incubation media after 30 minutes of incubation (i.e. 4.4 nmol g^{-1}, Table 7.2), then it can be shown that for an average size artery (i.e. 20 mgm of artery) with an extra cellular space of 0.65 cm^{3} g^{-1} that the content of ^{3}HMeOISO in the extra cellular space would be 0.06 nmol g^{-1}. Presumably this amount of ^{3}HMeOISO would efflux into wash solutions within the first minute of wash. However this value may be underestimated if in fact a local high concentration of ^{3}HMeOISO prevailed in the extra cellular space at the end of incubation due to the close proximity of this fluid to sites of formation of ^{3}HMeOISO. It is noteworthy that the ratio of the contents of ^{3}HISO (not attributable to extra cellular ^{3}HISO) to ^{3}HMeOISO (corrected

for a calculated extracellular ^{3}HMeOISO content) is 1.1:1.0 (i.e. 324:0.293) and almost identical to the ratio of the contents of ^{3}HISO and ^{3}HMeOISO prevailing in the tissue after 1.0 minute of wash i.e. 1.09:1.0 (Table 7.2).

(iii) In another series of experiments, the effluxes of ^{3}HISO and of ^{3}HMeOISO were measured during 30 minutes of washout. The cumulative effluxes are shown in Fig. 8.7. (This includes the data shown earlier in Fig. 8.6). The same data is plotted as the logarithm of the rates of efflux versus time of washout in Fig. 8.8. The rates of efflux of ^{3}HISO from COMT-inhibited arteries are also shown. It will be seen that curves showing the rates of decline in the effluxes of ^{3}HISO from both COMT-intact and COMT-inhibited arteries resemble each other in shape and each comprises an initially rapid followed by a slower phase. As was evident during the first one minute, the rates of efflux are greater in the COMT-inhibited arteries. This is in accord also with the earlier evidence of greater accumulation of ^{3}HISO in the COMT-inhibited arteries. When analysed by the curve peeling technique, (Procedure 1, Methods section) these rates of efflux were well described by two single exponential functions, curves for which are shown in Fig. 8.8. These two functions will be referred to as Phase 3 (the initially faster phase and Phase 4 (the final slower phase). In contrast to the curves for ^{3}HISO, the curve showing the log rate of efflux of ^{3}HMeOISO versus time indicated a rapidly declining rate which was monophasic, in that it closely approximated to a single exponential function. Its shape was closer to that of Phase 3 than of the Phase 4 decline in rate of ^{3}HISO.

(iv) DOCA. To examine the action of DOCA (27 μmol l^{-1}) on efflux of amines, the drug was added (in ethanol) to the incubating medium 5 minutes before washout was commenced, and was present in all the wash

Fig. 8.7 Cumulative efflux of total ^{3}H and $^{3}HMeOISO$ from arteries previously incubated with $^{3}HISO$ (0.81 μmol l^{-1}) for 30 mins.

Ordinate: cumulative efflux in nmol g^{-1}
Abscissa: time (mins) after commencement of wash

Shown are means (± SEM) for 4 to 8 arteries.

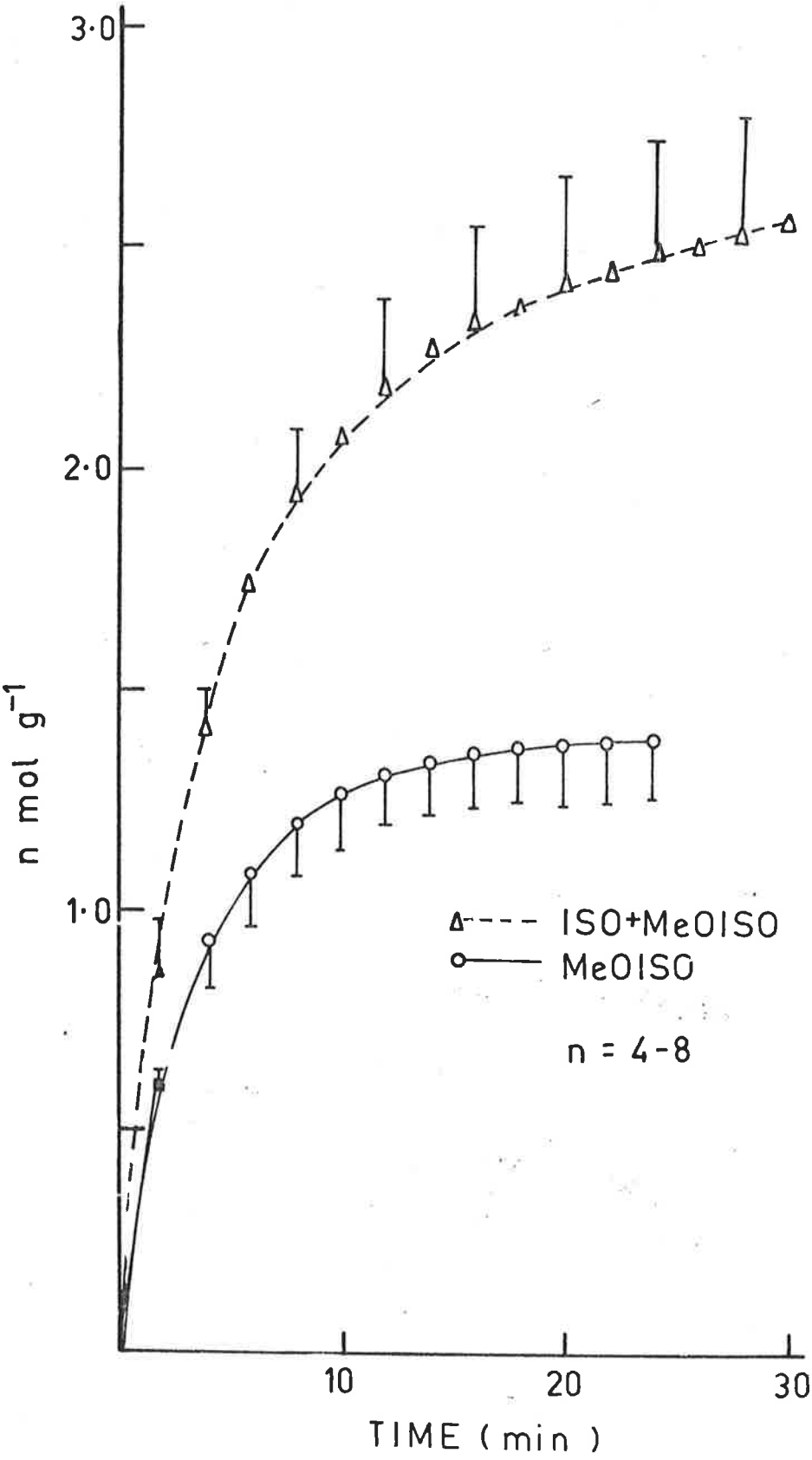
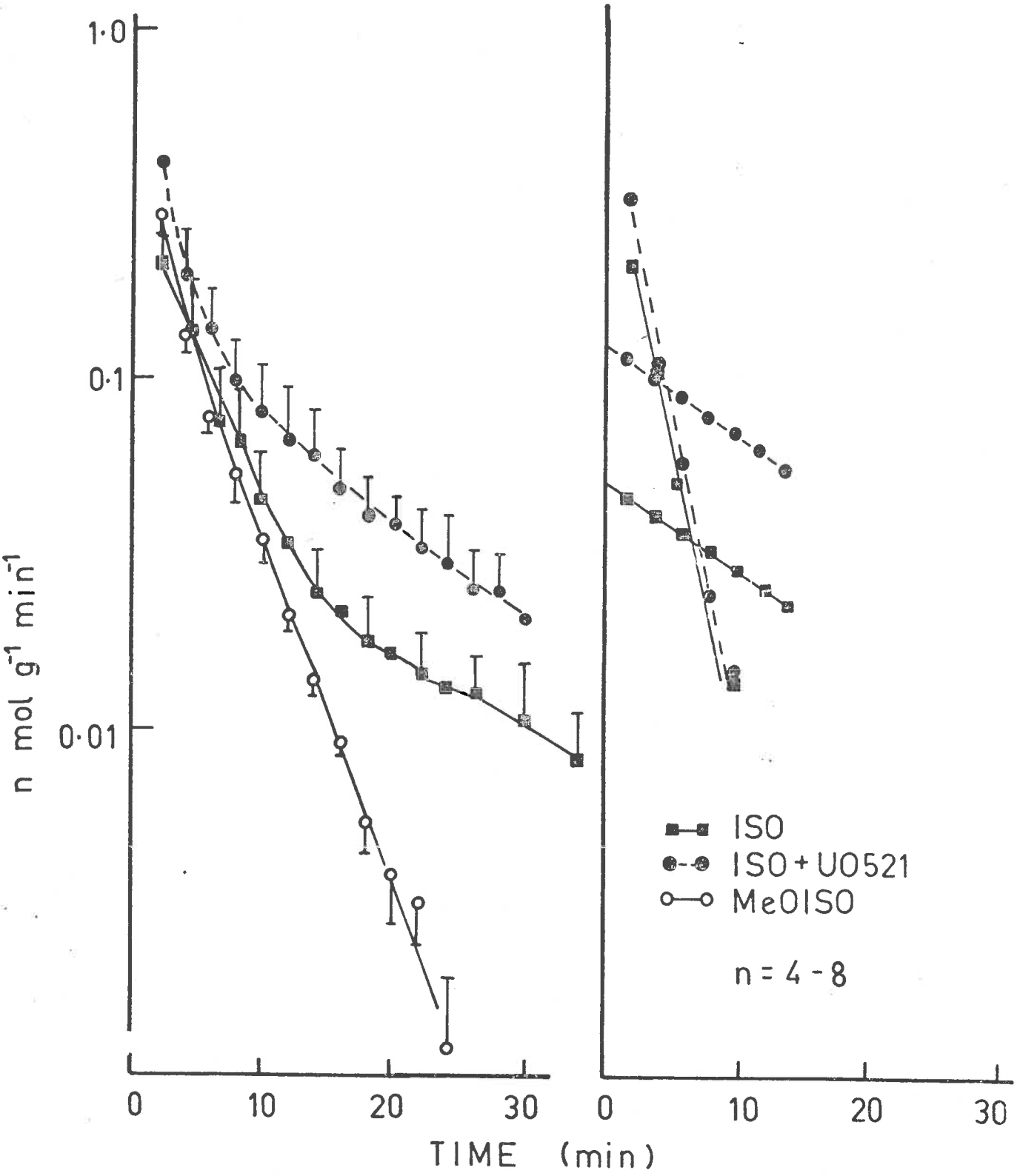

3·0
2·0
1·0
n mol g⁻¹
ISO+MeOISO
MeOISO
n = 4-8
10
20
30
TIME (min)

Fig. 8.8 A. Efflux of radioactivity during washout of segments of arteries after an initial incubation with ^{3}HISO (0.81 µmol l^{-1}) in the absence (solid line) and presence of UO521 (55 µmol l^{-1}, dotted line).

Ordinate: rate of efflux (nmol g^{-1} min^{-1} log scale
Abscissa: time (min) after onset of washout

Shown are values (± SEM) for ^{3}HISO and ^{3}HMeOISO for 4 to 8 arteries.

B. Shown are the individual rates of efflux (corresponding to Phase III and IV of efflux) after curve peeling by Procedure I


1·0
0·1
0·01
n mol g⁻¹ min⁻¹
0
10
20
30
TIME (min)
ISO
ISO + UO521
MeOISO
n = 4 - 8

solutions. The control preparations were treated in an identical fashion with ethanol alone (173 mmol l^{-1}). U0521 (55 μmol l^{-1}) was present in all incubating and wash solutions to ensure inhibition of COMT. (The selection of 5 minutes before rather than at the end of incubation, was based on pharmacological evidence (Johnson, Ph.D. thesis (1976), University of Adelaide) that the potentiating effect of DOCA on the constrictor response of the REA to adrenaline required 5 minutes for maximum development).

The rates of efflux of ^{3}HISO in the DOCA-treated and control arteries are summarised in Fig. 8.9. The efflux curves for both DOCA and control arteries showed the diphasic declines described earlier (i.e. Phases III and IV). The only difference between the two was the tendency for the rates to be significantly less from the DOCA-treated artery during the first part of the efflux period. Analysis of the curve for DOCA showed that DOCA's effect was primarily on the Phase III efflux of ^{3}HISO. A more detailed description of DOCA's action on the efflux of ^{3}HISO appears in the following subsection.

(v) Compartments. The properties (capacities and $t_{\frac{1}{2}}$ values) of the individual compartments from which the various phases of efflux occurred were also estimated by the standard mathematical procedures as described in the methods section. The results are summarised in Tables 8.2 and 8.3. Their main features were as follows:

1. Untreated arteries:

(a) a compartment (corresponding to Phase IV of efflux) containing mainly ^{3}HISO the content of which (0.7 to 0.97 nmol g^{-1}) is similar to the ^{3}HISO content of compartment III (below) but associated with a much larger $t_{\frac{1}{2}}$ value (8 to 25 min).

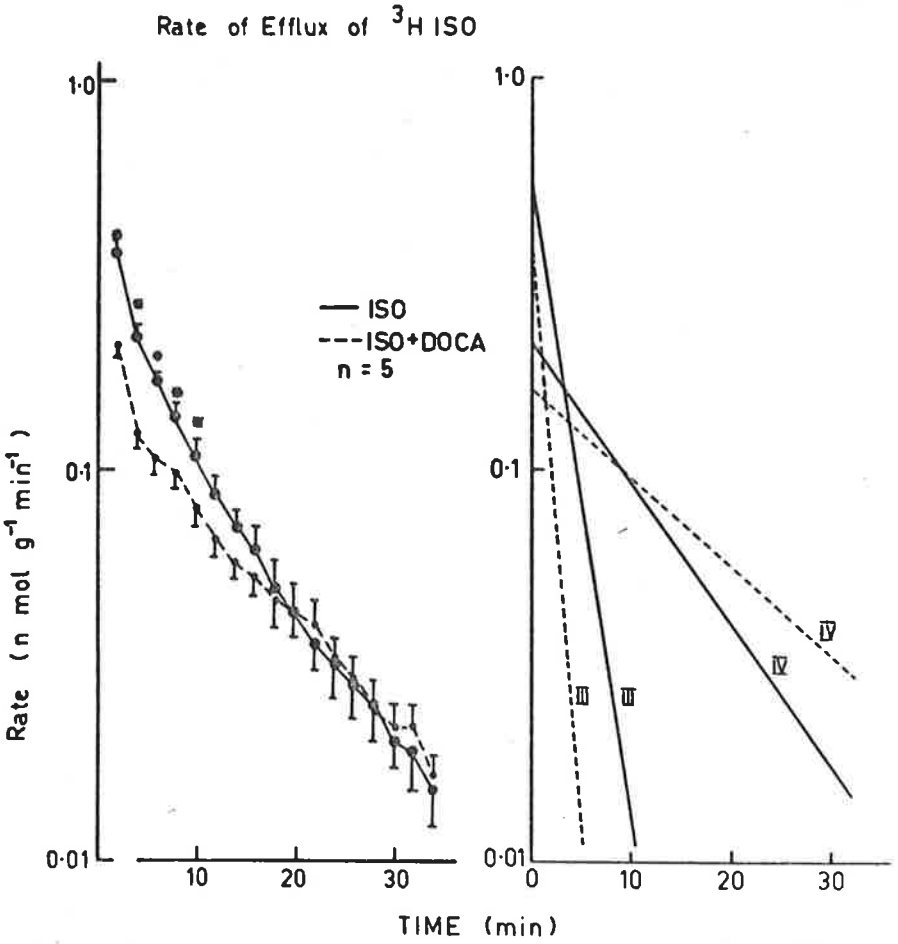


Fig. 8.9 A. Efflux of radioactivity during washout of segments of arteries after an initial incubation with ^{3}HISO (0.81 µmol l^{-1}) in the presence of UO521 (55 µmol l^{-1}). DOCA (27 µmol l^{-1}) added to arteries 5 min before end of incubation and present during incubation (shown as dotted line).

Ordinate: rate of efflux (nmol g^{-1} min^{-1}, log scale)
Abscissa: time (min) after onset of washout.

Shown are values (± SEM) for 5 DOCA treated and 5 control arteries. Values shown as * are significantly greater ($p < 0.05$ paired test) than corresponding values for DOCA treated arteries.

B. Shown are the individual rates of efflux (corresponding to Phase III and IV of efflux) after curve peeling by Procedure III.

Footnotes:

(i) The curve peeling procedures used were described in the methods section and comprised: procedure I, curve fitting by eye; procedure II, single curve fitted to mean values; procedure III, curves fitted to individual experiments.

(ii) Shown are values for compartment size (in nmol g^{-1}) and half time ($t_{\frac{1}{2}}$) values of efflux.

(iii) ^{14}C sorbitol values represent sorbitol space expressed in nmoles of isoprenaline per gram of artery.

(iv) Significance. For ^{3}HISO untreated vs. U0521 treated, $p < 0.05$ for size and $t_{\frac{1}{2}}$ for compartment IV.

TABLE 8.2

Compartmental analysis of efflux curves obtained with arteries that were incubated with either ^{14}C sorbitol (8.1 μmol l^{-1}) or dl^{3}HISO (0.81 μmol l^{-1}) for 30 mins prior to wash

Treatment	Compound effluxed	Proc.	II Size nmol g^{-1}	II $t_{½}$ min	n	III Size nmol g^{-1}	III $t_{½}$ min	n	IV Size nmol g^{-1}	IV $t_{½}$ min	n	Residue nmol g^{-1} Calc'd	Residue nmol g^{-1} Expt.
		I	0.53	0.20	-	-	-	-	-	-	-		
Nil	^{14}C Sorbitol	II	0.67	0.20	4	0.09	1.05	4	-	-	-		
		III	0.72±0.05	0.19±0.03	4	0.11±0.04	0.78±0.07	4	-	-	-		
		I	-	-	-	0.7	2.1	4	0.7	8.0	4		
	^{3}HISO	II	1.02	0.14	4	0.85	0.8	3	0.87	22.3	4		
		III	-	-	-	1.24±0.40	1.37±0.32	4	0.97±0.42	24.8±1.75	4		
Nil		III	-	-	-	1.68±0.58	1.45±0.27	7	0.93±0.28	19.9±2.9	7	0.18±0.05	0.23±0.0
		I	-	-	-	1.3	2.3	7	-	-	-		
	^{3}HMeOISO	II	0.28	0.57	4	0.82	3.45	4	-	-	-		
		III	0.34±0.12	0.61±0.18	2	1.01±0.19	3.34±0.13	4	-	-	-		
		III	-	-	-	1.06±0.12	3.24±0.15	7	-	-	-		
		I	-	-	-	1.0	2.0	4	1.6	7.8	4		
U0521	^{3}HISO	II	0.99	0.25	4	0.78	1.42	4	2.01	10.04	4		
		III	1.49±0.38	0.26±0.08	4	0.74±0.17	1.49±0.13	4	1.97±0.49	10.5±1.01	4	0.22±0.09	0.38±0.1

TABLE 8.3

Compartmental analysis of efflux curves obtained with COMT inhibited arteries incubated with dl ^{3}HISO (0.81 μmol l^{-1}) for 30 mins prior to wash in Krebs solution containing ethanol (173 mmol l^{-1}) or DOCA (27 μmol l^{-1})

Treatment	Compound Effluxed	Proc.	II Size nmol g^{-1}	II $t_{½}$ min	n	III Size nmol g^{-1}	III $t_{½}$ min	n	IV Size nmol g^{-1}	IV $t_{½}$ min	n	Residue nmol g^{-1} Calc'd	Residue nmol g^{-1} Expt.
U0521		I	-	-	-	1.4	2.0	5	2.6	8.5	5		
+	^{3}HISO	II	-	-	-	1.51	1.98	5	3.03	9.9	5		
EtOH		III				1.77±0.50	1.81±0.26	5	3.02±0.44	9.9±0.78	5	0.13±0.01	0.35±0.08
		I	-	-	-	0.07	2.0	5	2.0	9.5	5		
U0521		II	-	-	-	0.66	3.20	5	3.20	11.0	5		
+	^{3}HISO	III	-	-	-	1.12±0.40	2.62±0.47	2	2.62	11.7±1.13	5		
DOCA		III	-	-	-	not detectable		3	-		-	0.28±0.06	0.39±0.09

Footnotes: as for Table 8.1 except:

(i) based on efflux rates determined 2 minutes after commencement of wash

(ii) ethanol added to control incubates 5 minutes before completion of incubation and present throughout wash

(iii) DOCA added to incubates 5 minutes before completion of incubation and present throughout wash

(b) a compartment (III) with a capacity of 0.7 to 1.6 nmol g^{-1} of ^{3}HISO and a $t_{\frac{1}{2}}$ value in the range 0.8 to 2.3 minutes. ^{3}HMeOISO is included in this compartment because its $t_{\frac{1}{2}}$ value is only slightly greater (2.3 to 3.5 min); the capacity (0.9 to 1.3 nmol g^{-1}) is similar to that of ^{3}HISO.

(c) a compartment corresponding to Phase II of efflux which contains ^{3}HISO. As discussed already this compartment is probably the extra cellular space in the tissue. Since the ^{3}HMeOISO efflux during the first two minutes (viz. 0.58 nmol g^{-1}) was greater than could be attributed to simple efflux from compartment III it is tempting to place a small proportion of ^{3}HMeOISO efflux into a separate compartment (i.e. compartment II).

2. COMT inhibited arteries:

(a) the capacity of compartment IV was significantly greater than in untreated arteries by a factor of 2, but the $t_{\frac{1}{2}}$ value was decreased.

(b) compartment III appeared unchanged in capacity with no change in $t_{\frac{1}{2}}$ value.

(c) the capacities and $t_{\frac{1}{2}}$ values of compartment II appeared to be unchanged (based on mean values only).

3. DOCA treated arteries:

(a) a decrease (shown by procedures I and II) in the ^{3}HISO contents of compartment III (i.e. when compared to the ^{3}HISO contents of compartment III for ethanol treated arteries). Analysis of the individual curves by procedure III showed that for three arteries there was no correlation between the observed rates and an exponential decline in rate corresponding to Phase III of efflux, i.e. the analysis suggested the presence of only phase IV of efflux.

(b) no change in the $t_{\frac{1}{2}}$ values of compartment IV (i.e. when compared to the $t_{\frac{1}{2}}$ values of compartment IV for ethanol treated arteries).

4. Tissue residues:

The mean ^{3}H contents of arteries determined experimentally at the conclusion of the wash period were greater than the mean values calculated from the regression lines for the last phase (IV) of efflux, but only in one comparison (U0521 treated arteries) was the increase significant. It was noted however that values of ^{3}H content in some individual experiments were close to those obtained by calculation.

DISCUSSION

Accumulation experiments

(i) MeOISO:

The curves of rate of accumulation of ^{3}HMeOISO vs time in the medium containing substrate concentrations of 0.81 and 3.75 μmol l^{-1} show a pronounced increase in rate a few minutes after incubation was commenced. At the lower substrate concentration (0.48 μmol l^{-1}) the initial accumulation of ^{3}HMeOISO appeared to take a longer period of time to reach its steady state. However in the one group of experiments where the tissue contents of MeOISO were also measured at different times it was apparent that the formation of ^{3}HMeOISO (tissue plus medium) at substrate concentration 0.81 μmol l^{-1} was relatively constant from the commencement of incubation. This was because a major proportion of the ^{3}HMeOISO formed by the artery after 5 minutes of incubation had accumulated in the tissue and a smaller proportion had effluxed into the medium.

After 15 or 30 minutes of incubation the reverse was the case, i.e. the relative proportions of ^{3}HMeOISO in the medium was larger than that in the tissue. These findings are consistent with the presence of a low capacity compartment within the artery in which ^{3}HMeOISO accumulated after its formation. It is assumed that ^{3}HMeOISO is stored in one compartment only namely the compartment III described in the results. The evidence for this compartment is discussed later in this section. The filling of such a compartment in the early stages of incubation would decrease the amount of ^{3}HMeOISO which effluxes into the medium; when this compartment was saturated the rate of efflux into the medium would correspond to steady state rate of formation of ^{3}HMeOISO. The formation and accumulation of ^{3}HMeOISO in the artery must be rapid since the ^{3}HMeOISO contents of arteries after 5 minutes of incubation were approximately 50% of that of arteries incubated for 30 minutes. However it should be noted that the levels of MeOISO in the arteries that were incubated with ^{3}HISO at a concentration of 0.81 μmol l^{-1} do not reflect the maximum capacity of the MeOISO containing compartment. This follows from the evidence that the tissue levels of ^{3}HMeOISO increased with increasing substrate concentration; for example the ^{3}HMeOISO contents of arteries incubated at a substrate concentration of 3.75 μmol l^{-1} were about twice those seen in arteries incubated at the substrate concentration of 0.81 μmol l^{-1}.

The possibility of substrate saturation of the COMT in this compartment was also evident from these results. This follows from the shape of curves seen for the plot of rate of appearance of ^{3}HMeOISO in the media vs substrate concentration. This curve plateaus, which indicates saturation of the enzyme may have occurred at a substrate concentration of 3.75 μmol l^{-1}, i.e. assuming that the uptake of ^{3}HISO

into the artery was not itself rate limiting. The latter assumption is tenuous and based only on the observation that the ratio of ^{3}HISO contents in arteries to substrate concentration are similar at high (3.75 μmol l^{-1}) and low (0.81 μmol l^{-1}) concentrations (i.e. 5:3.75 (Fig. 8.1) or 1.33:1 and 1.063:0.81 (Table 7.2) or 1.3:1 respectively).

(ii) ISO:

The accumulation of ISO appeared to be more rapid than that of MeOISO since the time content at 5 minutes was about 90% of that prevailing at 15 or 30 minutes. This finding suggests that the substrate gains rapid access to the COMT. The data (Table 8.1) showed that the storage capacity of the artery increased over a range of ^{3}HISO concentration in the medium. This follows from the finding (in COMT inhibited arteries) that the tissue levels of ^{3}HISO, after adjustment for the amount present in the extracellular space continued to exceed the medium concentration of ^{3}HISO even when the concentration of ^{3}HISO was varied 1,000 fold. This implies that at least one of the compartments which stores ^{3}HISO following COMT inhibition has a high capacity for this amine.

(iii) DOCA:

The accumulation of MeOISO in the medium of DOCA treated arteries possessed a similar time course to that in untreated arteries, ie.. the inhibitory potency of DOCA was about the same at each time interval measured. This finding thus excluded the possibility (mentioned in the introduction) that the measurements based at 30 minutes may not have estimated the full extent of DOCA's inhibitory action. The most likely explanation for the failure of DOCA treatment to completely eliminate MeOISO formation in arteires is competition between DOCA and the substrate for the O-methylating compartment in the artery. This

follows from the observation that DOCA's inhibitory effect on the accumulation of ^{3}HMeOISO in the incubating medium is less pronounced at high substrate concentrations. The possibility that DOCA reduced the efflux of ^{3}HMeOISO from these arteries seems unlikely in view of the evidence presented in the previous chapter showing that DOCA significantly reduced both artery and incubate contents of ^{3}HMeOISO. Furthermore there is evidence that in rat heart another steroid (corticosterone) was without effect on the efflux of MeOISO from that tissue (Uhlig *et al.* 1974). In contrast to the apparently competitive inhibition by DOCA of O-methylation, DOCA's inhibition of the accumulation of ^{3}HISO (in COMT inhibited arteries) was similar in magnitude over a wide range (1,000 fold) of substrate concentration. It seems likely that DOCA's action is directed toward only one part of this accumulation and its mode of inhibition is non-competitive. In view of histochemical evidence indicating steroids prevented the accumulation of NA into the smooth muscle cells of the rabbit ear artery (Nicol and Rae 1972) it is tempting to suggest that the DOCA sensitive component of the accumulation of ^{3}HISO reflects uptake into the cytoplasm of the smooth muscle cells of this artery. Nicol and Rae also showed that steroids did not affect the binding of NA to collagen nor did they decrease the accumulation of NA into nerve terminals. Thus in view of the evidence presented in the previous chapter showing that there is a neuronal component of the accumulation of ^{3}HISO in this artery and that PBZ does not abolish the ^{3}HISO content of arteries, it is possible that the steroid insensitive component of accumulation may in part be accounted for by ^{3}HISO being taken up by sympathetic nerves or by tissue elements such as collagen or elastin.

Efflux experiments

(i) MeOISO:

Analysis of the efflux of ^{3}HMeOISO in the first one minute of wash showed that the amount of ^{3}HMeOISO that effluxed during this period, represented about 36% of that seen for arteries (seen in the previous Chapter) after a one minute wash. Furthermore it was shown that the ratio of the one minute wash contents of ^{3}HISO (corrected for extra cellular ^{3}HISO present at the end of incubation) to the corresponding ^{3}HMeOISO contents were similar to those prevailing in the tissue after a one minute wash. Thus it is reassuring that the artery contents of amines shown in the previous chapter (Table 7.2) do reflect the bulk of the amines present in the cells at the end of incubation and that the tissue ratio of unchanged amine to metabolite was not distorted by exclusion of the 1.0 minute wash data.

The total amount of ^{3}HMeOISO that effluxed from these arteries, 1.38 ± 0.13 nmol g^{-1} (shown in the cumulative plot in Fig. 8.7) when corrected for the amount of ^{3}HMeOISO that effluxed in the period 0 to 1.0 minute of wash, i.e. 1.04 nmol g^{-1}, is similar to the ^{3}HMeOISO contents of arteries (shown in the previous chapter, Table 7.2) that were washed for 1.0 minute. It seems unlikely that any significant O-methylation of unchanged amine (^{3}HISO) occurred during the period of efflux. This finding contrasts markedly with that seen for the efflux of ^{3}HNA (Chapter 6) where significant metabolism of ^{3}HNA, i.e. conversion of DOPEG had occurred during 30 and 60 minutes of efflux.

Analysis of the rates of efflux of ^{3}HMeOISO in the period 0 to 30 minutes of wash showed that the efflux of ^{3}HMeOISO could be described by a single exponential decline in rate. The $t_{\frac{1}{2}}$ for this compartment

(3.3 mins), although greater than the corresponding time for efflux of material from extracellular spaces, was still much smaller than the $t_{\frac{1}{2}}$ for compartment IV. Thus in view of the finding that about 50% of the steady state tissue content of ^{3}HMeOISO was present after 5 minutes of incubation, it is probable that in this artery there is a single compartment which rapidly accumulates ^{3}HMeOISO and from which ^{3}HMeOISO rapidly effluxes.

(ii) ISO:

In contrast to the efflux of MeOISO, the efflux of ISO was characterised by two phases, one of which (III) had a $t_{\frac{1}{2}}$ comparable with that of the efflux of MeOISO, the other (IV) possesses a much longer $t_{\frac{1}{2}}$. It is likely that the rapid phase of efflux reflected loss of unchanged amine from the compartment from which ^{3}HMeOISO effluxes.

(iii) UO521:

COMT inhibition was associated with an increased rate of efflux of ^{3}HISO from arteries. This observation is consistent with the findings from the previous chapter which showed that COMT inhibition was associated with an increased accumulation of unchanged amine. Surprisingly the results of the compartmental analysis, based on these rates of efflux, showed that COMT inhibition caused an increase in the content of compartment IV, i.e. the compartment not described by efflux of ^{3}HMeOISO. It was anticipated that COMT inhibition would have caused a stoichiometric replacement of the ^{3}HMeOISO in compartment III with unchanged amine (^{3}HISO). The fact that this did not occur suggests that the activity of COMT in compartment III regulates in some way the entry and accumulation of ISO into compartment IV. Possibly compartment III has only a limited capacity for unchanged ISO.

(iv) DOCA:

The presence of DOCA in wash solutions decreased the rate of efflux of ^{3}HISO from the COMT inhibited arteries. DOCA's effect seemed primarily directed toward the efflux of ^{3}HISO corresponding to Phase III of efflux, i.e. the compartment from which both ^{3}HISO and ^{3}HMeOISO efflux. The latter observation is in accord with the findings from the previous chapter which showed that DOCA decreased only part of the accumulation of ^{3}HISO in COMT inhibited arteries.

(v) General:

All of these findings point to the possible involvement of two compartments in this artery for the accumulation and O-methylation of ^{3}HISO. These are summarized as follows:

1. Compartment III which is characterized by:

(a) COMT activity which is saturated at relatively low substrate concentration and leads to

(b) a rapid formation and accumulation of ^{3}HMeOISO, and a rapid efflux of ^{3}HMeOISO and unchanged amine

(c) ability to store both unchanged amine and MeOISO (with a limited capacity for both)

(d) sensitivity to DOCA, with respect to both entry and efflux of ISO.

2. Compartment IV which is characterized by:

(a) the ability to store unchanged amine including the increased ^{3}HISO content resulting from COMT inhibition

(b) absence of MeOISO activity

(c) a relatively slow efflux of ^{3}HISO when COMT is intact but a faster efflux when COMT is inhibited; the latter efflux is not affected (in content or $t_{\frac{1}{2}}$ value) by DOCA.

Some possible relationships between the present findings and the previously reported (Johnson and de la Lande 1973) sensitizing action of DOCA will be treated in depth in the general discussion chapter (Chapter 10).

(iv) Relationship to other tissues:

1. Cat nictitating membrane

From their studies on the cat nictitating membrane in which neuronal uptake was eliminated by a combination of chronic sympathetic denervation and treatment with cocaine, and metabolism was inhibited by pargyline and U0521, Graefe and Trendelenburg (1974) concluded that dl^{3}HNA distributed rapidly into a quickly equilibrating compartment with a small distribution volume, followed by a further slow transfer into a second extraneuronal compartment with a high distribution volume. They also concluded that hydrocortisone blocked the entry or accumulation of the ^{3}HNA in the quickly equilibrating compartment. The supporting evidence included (a) the shape of the time course of accumulation of ^{3}HNA, which showed appreciable accumulation of the amine at zero time. This suggested that the first compartment had filled within the first period of measurement (about 2 minutes from their Fig. 8). Tested at various amine concentrations the extra neuronal uptake could not be saturated and the inhibition produced by hydrocortisone was small and unrelated to the amine concentration. In contrast when O-methylation occurred in COMT intact membranes the accumulation of normetanephrine was saturable so that its level in the tissue achieved equilibrium with the concentration in the medium within several minutes. They also showed that the Michaelis-Menten analysis of the plot of ^{3}HNMN formation against

^{3}HNA concentration was consistent with the presence of two methylating systems, one with a high affinity and low capacity for ^{3}HNA the other with a low affinity and high capacity. The analysis also suggested that the high affinity O-methylating system was sensitive to hydrocortisone and even then the effect of the steroid was non competitive, i.e. relatively independent of the concentration of the substrate. In contrast to normetanephrine accumulation, the accumulation of ^{3}HNA was negligible, being accounted for by filling of the extracellular space.

Qualitatively, the accumulation and O-methylation of isoprenaline resembled that of noradrenaline, the main difference was a higher V_{max} for the filling of the high affinity compartment. This made it simpler to analyse the selectivity of the effect of hydrocortisone on the two compartments since O-methylation in the high affinity system comprised most of the observed O-methylation (measured for one minute in the range of substrate concentration, 0 to 50 μmol l^{-1}). Their resultant kinetic analysis was again consistent with the view that hydrocortisone inhibited the high affinity O-methylation of catecholamines. As with noradrenaline the accumulation of unchanged isoprenaline appeared to represent only isoprenaline in the extracellular space of the tissue.

Although the data in the present study is limited by comparison, it shows some possible similarities and differences between the properties of the accumulation in the two tissues (i.e. cat nictitating membrane and rabbit ear artery). One difference is that when COMT is intact in the artery the accumulation of ^{3}HISO is greater than can

attributed to the filling of the extra cellular space. However this may not be a genuine difference and may simply reflect different times of incubation. Graefe and Trendelenberg (1974) have pointed out that in an earlier study (Trendelenberg *et al.* 1971) they were able to detect significant extra neuronal accumulation of unchanged ^{3}HNA in isolated membranes which were incubated with ^{3}HNA for longer periods of time (i.e. 20 minutes). Twenty minutes is comparable with the occasional 15 minute and more commonly 30 minute periods of incubation employed in this study.

A point of similarity is represented by the presence in the artery of a limited capacity for storing ^{3}HMeOISO, as shown by the lag in the time course of its appearance in the incubation medium, in association with a failure of the ^{3}HISO to accumulate further in the tissue after 15 minutes of incubation. It is not clear from their study, whether, as in the ear artery, inhibition of COMT is associated with a marked increase in the accumulation of unchanged ^{3}HISO. One might interpret their data on ^{3}HNA accumulation in the MAO and COMT inhibited membranes as evidence in favour of a similar accumulation of ^{3}HISO, and their finding that hydrocortisone exerts a small but definite non-competitive inhibition of this accumulation of unchanged ^{3}HNA as a further argument of a similarity between the properties of the ^{3}HISO accumulating systems in the two preparations. The latter follows from the evidence in this chapter showing that the inhibitory action of DOCA is approximately the same over a thousand fold range of concentration of substrate. Further as in the membrane the accumulation of the unchanged amine in the COMT-intact arteries was unaffected by this steroid.

2. Isolated hearts

A further intensive analysis of the accumulation of ^{3}HISO and ^{3}HMeOISO has been reported in the rat and guinea pig heart by Trendelenberg and colleagues (Bönisch and Trendelenberg, 1974; Bönisch *et al.* 1974; Uhlig *et al.* 1974).

In their first study, they showed the O-methylation of ^{3}HISO reached steady state conditions much more rapidly than the accumulation of unchanged ^{3}HISO (i.e. 10 minutes cf. 30 minutes) and that inhibition of COMT, increased both the accumulation of unchanged amine and the time required to reach steady state conditions. They showed also that the rate of O-methylation under steady state conditions was identical with the initial rate of O-methylation. The lack of parallelism between the ^{3}HISO content of the heart and the rate of O-methylation led them to suggest a two compartment model to explain their findings, i.e. (i) a COMT containing compartment which equilibrated quickly with the extracellular concentration of ^{3}HISO and in which the rate of O-methylation depended exclusively on the concentration of unchanged amine in the extracellular fluid and (ii) a more slowly equilibrating compartment which stored unchanged amine. In their second report they provided further information on the compartments by showing that the rate of removal of ^{3}HISO was increased with increasing substrate concentration to a much greater extent than could be accounted for by an increase in ^{3}HMeOISO formation (i.e. 30 fold cf. 3 fold) and was associated with a marked earlier attainment of the steady state conditions in the case of ^{3}HMeOISO.

In contrast to the results on the nictitating membrane the accumulation of unchanged amine was decreased by corticosterone (non-competitively) while the kinetics of O-methylation could be accounted for by a single compartment system which was competitively inhibited by corticosterone. The efflux curves of ^{3}HISO from hearts in which COMT was inhibited showed at least two compartments (other than extracellular space, and heart cavity filling). These were termed compartments III ($t_{\frac{1}{2}}$ = 10.1 mins) and IV ($t_{\frac{1}{2}}$ = 22.6 mins). Both were markedly decreased (to 12-23%) by the presence of corticosterone in the incubation medium (surprisingly the compartment termed II and attirbuted to extracellular space by Bönisch *et al.* appeared also to have been markedly decreased). When corticosterone was present in the wash solution only, the separate efflux from III and IV appeared to be now from one compartment only resembling IV in $t_{\frac{1}{2}}$. They suggested that corticosterone had delayed efflux from III.

Finally (Uhlig *et al.* 1974) they examined the effect of corticosterone on the efflux of ^{3}HMeOISO from a compartment corresponding to III in the rat heart. They showed also that the steroid inhibited the appearance of the ^{3}HMeOISO when present during perfusion with ^{3}HISO. However when added during efflux, it decreased the efflux of unchanged amine but not that of ^{3}HMeOISO. Instead it delayed the appearance of a peculiar convexity of the ^{3}HMeOISO curve. This convexity was attributed to the presence of sufficient unchanged ^{3}HISO in the compartment III to maintain the enzyme in a saturated state after perfusion with ^{3}HISO had ceased. In view of the failure of corticosterone to modify efflux of ^{3}HMeOISO, Uhlig *et al.* (1974) suggested that the two compartment model which best explained their results consisted of the two compartments in parallel, both of which

was able to store unchanged amine, but only one of which possessed COMT activity. The ability of corticosterone to efflux of ^{3}HMeOISO when present during infusion with ISO, but not when added subsequently, was explained in terms of its ability to inhibit influx of unchanged ^{3}HISO into compartment III (containing COMT) but to be without effect on the activities within compartment III once it had accumulated unchanged amine. They postulated also that corticosterone decreases efflux as well as influx of ^{3}HISO to account for the ability of the steroid to prolong the appearance of the plateau of ^{3}HMeOISO efflux.

As with the case of the analogy with the nictitating membrane, the data on the ear artery is limited by comparison with that on the isolated hearts, but does offer interesting points of comparison:

(i) The first point is, the artery resembles the heart in its ability to store unchanged amine in excess of that predictable from stoichiometric requirements once COMT is inhibited. This implies that as is in the heart, COMT acts normally to limit storage of unchanged amine.

(ii) A second similarity is that the compartment analysis also favours two compartments for storing unchanged amine. The half times are shorter in the artery than in the heart (see Table 8.4), but as in the rat heart there is a suggestion that the efflux of ^{3}HISO from compartment III is more sensitive to a stercid (DOCA) than is compartment IV. This is apparent from the experimentally derived efflux curves. The mathematically derived monoexponential do not appear to show this, but it should be pointed out that the correlation of goodness of fit of the derived curves for compartment III was much poorer for the curve with DOCA present than the curve with DOCA absent.

(iii) A third point of similarity is that the efflux of ^{3}HMeOISO occurs from a compartment in the artery with a $t_{\frac{1}{2}}$ closer to compartment III than IV as is the case with the heart.

(iv) An important difference is that, in the artery DOCA did not decrease the accumulation of unchanged amine in artery except when COMT was inhibited. This implies that unlike the heart, the compartment possessing COMT activity does not normally store unchanged amine in excess of that which can be methylated. This agrees with the failure to observe any evidence of convexity in the ^{3}HMeOISO efflux curve in the rabbit ear artery.

(v) Another difference was the failure to observe in the ear artery O-methylation of the accumulated unchanged amine during efflux as has been suggested for the rat and guinea pig hearts (Uhlig *et al.* 1974) based on the observation that the cumulated ^{3}HMeOISO contents of wash solutions from these tissues were greater than the ^{3}HMeOISO contents of hearts measured at the end of incubation.

It is apparent from the above discussion that whilst there are similarities between the rat heart and the ear artery several pronounced differences exist. In view of the possibility suggested by Bönisch *et al.* (1974) that the compartments in the rat heart may represent morphologically separate tissues, one of which may involve vascular smooth muscle; and in view of the evidence presented in the previous chapter showing that there was a neuronal component of ^{3}HISO accumulation, it is possible that the differences seen in the above comparison may be a result of an interplay of separate tissue types within both tissues. Similar studies, but less extensive than those reported for

the rat heart, have been described for the rabbit aorta and a comparison between this vessel and the ear artery is treated in the following subsection.

3. Rabbit Aorta

The studies of Levin (Levin, to be published; reported at the 6th International Congress on Pharmacology, Helsinki, 1975) and Henseling *et al.* (1976) have shown that in nerve-free segments of aorta (in which metabolizing enzymes were inhibited) l^{3}HNA and dl^{3}HNA is accumulated by a rapidly equilibrating process, with a $t_{\frac{1}{2}}$ value for accumulation of about 10 minutes. Furthermore the accumulation of ^{3}HNA in the enzyme inhibited and enzyme intact preparations is inhibited by corticosterone (Levin, to be published; Trendelenberg 1973). In an earlier report Levin (1974) investigated the relationship between the time course of accumulation of l^{3}HNA in nerve free aortic segments and the time course of formation of metabolites. It may be seen from a summary of these findings (Levin 1974, Fig. 2) that while the formation of ^{3}HNMN was linear with time the ^{3}HNMN content of the tissue increased initially and then plateaued at a steady state value. The initial rate of accumulation of l^{3}HNA was more rapid than that of ^{3}HNMN and the tissue content significantly greater than ^{3}HNMN when the latter achieves steady state accumulation. Thus the findings of Levin for the aorta parallel closely the results of this present study concerning the time course of accumulation and O-methylation of ^{3}HISO in the ear artery.

The distribution of dl^{3}HNA into extra neuronal compartments in the enzyme inhibited nerve free aorta has been described recently by Henseling *et al.* (1976). The experimental design and the compartmental

analysis of efflux curves used by the latter authors are similar to those used for this present study. A comparison of the properties of compartments seen for the aorta and ear artery together with those described for the rat and guinea pig hearts is shown in Table 8.4. The main feature in this comparison is the similarity in compartment sizes and $t_{\frac{1}{2}}$ values for the two blood vessels. Although the comparison is restricted to the nature of compartments in COMT inhibited preparations, additional information concerning the effect of corticosterone on compartments in the aorta has been briefly reported by Trendelenberg (1973). Corticosterone reduced the formation of ^{3}HNMN in enzyme intact segments of nerve free aorta and in UO521 treated segments reduced the efflux of ^{3}HNA from compartment III, only when the steroid was present in wash solutions (Trendelenberg 1973).

It is concluded that many of the properties of extra neuronal accumulation and O-methylation of ^{3}HISO seen for this artery are shared by a variety of vascular tissues.

Neuronal ^{3}HISO and efflux

Unfortunately there does not appear to be any information available relating to a possible contribution of neuronal ^{3}HISO to efflux. In as much as the detailed studies of Trendelenberg and colleagues have been concerned with innervated, denervated and nerve free preparations, detailed comparisons between these preparations are not evident. The study of Henseling *et al.* (1976) has indicated that axoplasmic accumulated ^{3}HNA may efflux in part, with a rate corresponding to compartment IV of efflux for the rabbit aorta. Thus in view of evidence presented in the previous chapter that part of the accumulation of ^{3}HISO in COMT intact arteries was neuronal in origin, a contribution

Footnotes:

(i) Treatments

U UO521 (55-100 μmol l^{-1}) present throughout entire experiment

C cocaine (30 μmol l^{-1}) present throughout entire experiment

P pargyline (500 μmol l^{-1}); tissues incubated with pargyline prior to incubation with dl^{3}HNA

Cort. corticosterone (20 μmol l^{-1}) added to wash solutions only

Doc. DOCA (27 μmol l^{-1}), added to incubates 5 minutes before washout and present in wash solutions

EtOH (173 mmol l^{-1}), added to solutions as described for DOCA above

(ii) *These effluxes gave poor correlations with the monoexponential decline in rate of efflux corresponding to compartment III.

TABLE 8.4

A comparison of the proposed extra neuronal compartments in COMT inhibited vascular tissues of the rabbit, rat and guinea pig

Reference	Species	Tissue	Amine [μmol l^{-1}]	Treatment	COMPARTMENT III content (nmol g^{-1})	COMPARTMENT III $t_{½}$ (min)	COMPARTMENT IV content (nmol g^{-1})	COMPARTMENT IV $t_{½}$ (min)
Henseling *et al* 1976	rabbit	aorta (media)	dl^3HNA (1.18)	U+C+P	0.97 ± 0.09	2.9 ± 0.3	1.15 ± 0.10	11.3 ± 0.3
present study	rabbit	ear artery	dl^3HISO (0.81)	U	0.74 ± 0.76	1.5 ± 0.13	1.97 ± 0.49	10.5 ± 1.01
Bönisch *et al* 1974	guinea pig	heart	dl^3HISO (0.95)	U	0.75 ± 0.24	3.1 ± 1.0	0.93 ± 0.05	15.2 ± 0.8
Bönisch *et al* 1974	rat	heart	dl^3HISO (0.95)	U	4.84 ± 1.05	10.1 ± 0.7	5.32 ± 0.79	22.6 ± 1.3
Bönisch *et al* 1974	rat	heart	dl^3HISO (0.95)	U+Cort.	-*	-*	9.40 ± 0.85	29.4 ± 1.2
present study	rabbit	ear artery	dl^3HISO (0.81)	U+DOC	-*	-*	2.62 ± 0.47	11.7 ± 1.13
present study	rabbit	ear artery	dl^3HISO (0.81)	U+EtOH	1.77 ± 0.50	1.81 ± 0.26	3.02 ± 0.44	9.4 ± 0.78

of neuronal ^{3}HISO to efflux cannot be ignored. It would seem unlikely that the efflux of ^{3}HISO from COMT inhibited arteries reflects efflux of ^{3}HISO from neuronal sites. This follows from the finding that cocaine does not decrease the accumulation of ^{3}HISO in COMT inhibited arteries.

The relationship between COMT activity and neuronal accumulation of ^{3}HISO may simply reflect the following:

(1) in COMT intact arteries the ^{3}HISO contents are much smaller than those for COMT inhibited arteries and the neuronal component of accumulation represents a significant proportion of the accumulated ^{3}HISO in COMT intact but not COMT inhibited arteries.

(2) Furthermore if in COMT intact arteries, ^{3}HISO was also bound to tissue elements (e.g. elastin and collagen) the proportion of neuronal ^{3}HISO to accumulated extra neuronal ^{3}HISO would be even greater than (1) above. Under these conditions cocaine and not DOCA would decrease the accumulation of ^{3}HISO in COMT intact arteries. The latter assumes that COMT serves to minimize the extra neuronal accumulation of unchanged amine. When this enzyme is inhibited extra neuronal accumulation of ^{3}HISO occurs and this uptake is sensitive to DOCA but not cocaine.

The relationship between O-methylation and accumulation may also explain why Nicol and Rae (1972) in their histochemical study observed inhibition of the smooth muscle accumulation of NA by steroids in ear arteries not treated with a COMT inhibitor. The concentrations of NA used for the histochemical studies were far greater than those shown (in this present study) to elicit substrate saturation of COMT. Thus it is possible that at high substrate concentrations accumulation of unchanged amine occurs in a similar fashion to that seen at lower

substrate concentrations in the presence of a COMT inhibitor; i.e. accumulation of unchanged amine is inhibited by DOCA when extra neuronal COMT does not serve to significantly decrease the content of unchanged amine.

CHAPTER 9

THE UPTAKE AND METABOLISM OF ^{3}H NORMETANEPHRINE

CHAPTER 9

THE UPTAKE AND METABOLISM OF ^{3}H NORMETANEPHRINE

INTRODUCTION

In the preceding study evidence was presented in support of a functional role of catechol-O-methyltransferase in limiting the extra-neuronal accumulation of the catecholamine isoprenaline. Isoprenaline was selected because it was not a substrate for MAO. In the present study, the influence of extraneuronal MAO on the accumulation and inactivation of an amine which is not a substrate for COMT, normetanephrine (NMN), has been studied. ^{3}HNMN was selected in part because it is a substrate for MAO (Tipton 1972) which possesses a low affinity for neuronal uptake and a high affinity for extraneuronal uptake (Burgen and Iversen 1965). However, it was also selected because it is a metabolite of NA in the artery (Chapter 6), and it was of interest to ascertain whether the further deamination of NMN may have contributed to the formation from NA of the O-methyl deaminated glycol (MOPEG) and the O-methyl deaminated acid (VMA).

The plan of the study was to first examine the accumulation of NMN and its metabolites in the medium and tissue following 30 minutes incubation of ear artery segments with dl^{3}HNMN. The influence on these processes of an extraneuronal uptake inhibitor (DOCA), a neuronal

uptake inhibitor (cocaine), and an inhibitor of MAO (nialamide) was then examined. In this way it was hoped to determine whether, as in the case of isoprenaline and COMT activity, the activity of extraneuronal MAO influenced the accumulation of the NMN, and whether this accumulation was also sensitive to DOCA.

This study also includes some comparative observations on the metabolism of NMN by the rabbit aorta. It is apparent from a comparison of the data of Levin on the rabbit aorta (Levin 1973) and the data on the rabbit ear artery in Chapter 6 that NMN represented a relatively major metabolite of NA in the former tissue when compared with the latter.

METHODS

Incubation

The same procedure as that commonly used in the studies on ISO and NA was employed. Segments of artery were incubated for 30 minutes with Krebs solution gassed with 95% O_2 - 5% CO_2 containing dl ^{3}HNMN (1.03 µmol l^{-1}). The segments were then washed, first for 10 seconds, then for 50 seconds, after which they were extracted without homogenisation in a solution of acetic acid (1 mol l^{-1}) containing EDTA (22 µmol l^{-1}). The contents of ^{3}HNMN and the metabolites were then determined on TLC-borate impregnated plates in an identical fashion to that described for NA (Chapter 4, Method IV), with the qualification that only non-labelled NMN, VMA and MOPEG were added as carrier compounds.

In a few experiments, the identities of the metabolites in artery extracts were further tested by ion exchange paper chromatography using the same procedure as described in Chapter 4 for NA (Method I). In the studies on the rabbit aorta, segments were incubated under the same conditior used for the ear artery except that the ^{3}HNMN concentration was 6.0 µmol l^{-1}. However, the extraction and separative procedures were different. The aorta segments were extracted with HCl (100 mmol l^{-1}) containing EDTA (21.5 µmol l^{-} and the metabolites separated by alumina adsorption and column (Dowex-50 (NA^+)) chromatography as described in Chapter 4 (Method II).

Purification of ^{3}HNMN

The results of preliminary experiments (outlined in Chapter 3) had suggested that the manufacturer's stock solutions of ^{3}HNMN contained ^{3}H material that behaved like NA on paper chromatograms and thin layer chromatograms. Accordingly the solutions used for this study were purified before use using the procedure described in Chapter 3 and briefly outlined below.

The manufacturer's stock solutions of ^{3}HNMN were treated with alumina after which the effluent was adjusted to pH 6.5 and passed through a Dowex 50 (Na^{+}) column. The column was eluted with 1.0 N HCl and the fractions corresponding to NMN were pooled, freeze-dried, and redissolved in 2 ml of 0.01 N HCl containing EDTA. Samples of this solution were added directly to the Krebs solution.

RESULTS

In the first series of experiments, four artery segments from each of five rabbits were incubated with dl^{3}HNMN (1.03 μmol l^{-1}) for 30 minutes and washed for one minute in amine-free Krebs solution. The drug treatment of each segment was as follows: one was untreated; one was incubated with nialamide (350 μmol l^{-1}) for 60 minutes and then washed for 15 minutes prior to commencing incubation with ^{3}HNMN; one was incubated with 27 μmol l^{-1} of DOCA and 176 mmol l^{-1} ethanol; (the DOCA in ethanol being added 15 minutes prior to commencing incubation) the remaining segment was incubated with ethanol (176 mmol l^{-1}) alone. The tissues, and the incubating mediums, were then fractionated for dl^{3}HNMN and the putative metabolites ^{3}HMOPEG, and ^{3}HVMA by thin layer chromatography (Method IV, Chapter 4). The method also permitted tests for the presence of ^{3}HDOPEG and the fraction (^{3}HNA + ^{3}HDOMA).

The results of these experiments are summarised in Table 9.1. Their main features are listed below:

(1) Untreated arteries:

It will be seen that the concentration of ^{3}H in the tissue at the conclusion of incubation was approximately four-fold greater than in the medium. Unchanged $^{3}HNMN$ represented the major proportion (72%) of this ^{3}H, and it is evident from a comparison of its level in the tissue with the bathing concentration (3.11 c.f. 1.03 nmol g^{-1}) that the artery has accumulated the $^{3}HNMN$ against a concentration gradient.

The only metabolite which was consistently and unequivocally identified in the tissue and in the incubating medium in all experiments was MOPEG. In the tissue $^{3}HMOPEG$ represented 7.3% of the total ^{3}H. The amount of $^{3}HMOPEG$ which accumulated in the medium was approximately 10-fold greater than in the tissue.

$^{3}HVMA$ was consistently identified in the tissue extracts, but not in all the incubating mediums. Its percentage in the tissue (6.9%) was comparable with that of $^{3}HMOPEG$. However it was present in only two of the three incubating media which were analysed. The contents in these mediums were 2.4 and 3.0-fold greater than those in the tissues but the proportions of $^{3}HVMA$ in the medium were small compared with that of $^{3}HMOPEG$. An unexpected finding was that ^{3}H originally present in either incubation media or tissue extracts was detected in the regions of the thin layer plate corresponding to the migration of non labelled NA and DOPEG. Furthermore these contents of ^{3}H were greater than that attributable to the cross contamination of these regions by $^{3}HNMN$. (The procedures used to determine this cross contamination and the extent of contamination of the NA and DOPEG regions by $^{3}HNMN$

(ii) Medium: contents (mean ± S.E.M., nmol g^{-1}) of ^{3}HNMN and ^{3}H metabolites in incubation media from treated and untreated arteries. For some media, ^{3}H metabolites were not detected in all samples analysed (NDA); for these samples, the metabolite, the artery treatment, the number of samples detected and the contents were as follows: DOPEG (Con), n=1, 0.250 nmol g^{-1}; VMA (Con), n=2, 1.159, 1.083 nmol g^{-1}; VMA (ethanol) n=1, 0.678 nmol g^{-1}, VMA (DOCA) n=1, 1.153 nmol g^{-1}. N.D. refers to not detectable in all samples analysed.

(iii) Wash: ^{3}H contents (mean ± S.E.M. nmol g^{-1}) of wash solutions for the period 0.16 to 1.0 min of wash.

(iv) Significance (p values, unpaired comparisons).

	Tissue		Medium	
	EtOH vs DOCA	Con vs Nialamide	EtOH vs DOCA	Con vs Nialamide
Total ^{3}H	$0.02 > p > 0.01$	$p > 0.1$	-	-
MOPEG	$0.01 > p > 0.002$	$p < 0.001$	$p > 0.1$	$0.002 > p > 0.001$
NMN	$0.01 > p > 0.002$	$p > 0.1$	-	-
VMA	-	$0.01 > p > 0.002$	-	-
DOPEG	$p > 0.1$	$p > 0.1$	-	-

TABLE 9.1

Accumulation and metabolism of ^{3}HNMN in the rabbit ear artery

	Tissue				Medium				Wash			
	Con	EtOH	Nial	DOC	Con	EtOH	Nial	DOC	Con	EtOH	Nial	DOC
Total ^{3}H	4.33± 0.073	3.94± 0.252	4.30± 0.373	3.25± 0.056	-	-	-	-	1.09± 0.124	0.97± 0.056	0.96± 0.050	0.98± 0.087
MOPEG	0.315± 0.026	0.288± 0.036	0.039± 0.000	0.155± 0.000	3.09± 0.77	2.02± 0.30	0.113± 0.077	1.73± 0.180	-	-	-	-
NMN	3.114± 0.236	3.160± 0.232	3.532± 0.426	2.251± 0.125	0.848± 0.075	0.862± 0.044	0.806± 0.139	0.865± 0.114	-	-	-	-
VMA	0.298± 0.051	0.209± 0.025	0.080± 0.000	0.239± 0.000	NDA	NDA	1.39± 0.075	NDA	-	-	-	-
DOPEG	0.242± 0.211	0.055± 0.025	0.133± 0.093	0.044± 0.000	NDA	ND	ND	ND	-	-	-	-

Footnotes

(i) Tissue: shown are tissue contents (mean ± S.E.M., nmol g^{-1}) of ^{3}H, MOPEG, VMA and the contents of ^{3}H based on the estimates of ^{3}H in the fraction DOPEG, from 5 untreated arteries; 4 ethanol (174 mmol l^{-1}), 5 nialamide (350 µmol l^{-1}), 5 DOCA (27 µmol l^{-1}) treated arteries; all incubated with dl^{3}HNMN (1.04 µmol l^{-1}) for 30 mins. Values for VMA and DOPEG are based on 3 determinations.

have been described in Chapter 4). ^{3}H corresponding to NA was not detected in any incubates (i.e. from untreated or treated arteries) but was present in one untreated artery segment and 4 of the remaining segments used for this study. In all cases the ^{3}H contents represented less than 2% of the total ^{3}H present in arteries. ^{3}H corresponding to DOPEG was present in the media from 3 of the 4 untreated arteries but not present in any of the remaining 16 incubates (i.e. in media from treated arteries). However ^{3}H corresponding to DOPEG was present in extracts from 3 untreated arteries (comprising 5.5% of the total ^{3}H content) and all of the remaining artery segments used for this study (see Table 9.1).

Whether the results seen for artery extracts could be regarded as evidence for the formation of ^{3}HNA and $^{3}HDOPEG$ from $^{3}HNMN$ is difficult to say and will be treated in greater depth in the discussion to this chapter. However it was mentioned previously (Methods) and will be reiterated here, that the $^{3}HNMN$ used for this study was purified with a view to removing ^{3}H catechol impurities from these solutions.

(2) Nialamide:

Data on five arteries which were pretreated with nialamide (350 $\mu mol\ l^{-1}$) for 60 minutes prior to incubation with $^{3}HNMN$ are included in Table 9.1. It can be seen that nialamide decreased the mean contents of $^{3}HMOPEG$ and $^{3}HVMA$ in the tissue by 88% and 73%, respectively, and in the incubating medium by 96% and 100% respectively. (The 100% decrease simply means that $^{3}HVMA$ was not detected in any medium in contrast to its presence in two of the control media). The content of $^{3}HNMN$ in the tissue was increased by 13%. Although this was not

significant (see Table 9.1) it is of interest that the absolute increase (0.42 nmol g^{-1}) is of the same order as the decrease in the ^{3}HMOPEG + ^{3}HVMA fraction (0.49 nmol g^{-1}). It should be noted also that the accumulation of total ^{3}H in the artery was unaffected by nialamide. Hence the data indicates that there has been an approximately stoichiometric replacement of the deaminated metabolites by ^{3}HNMN following MAO inhibition.

(3) Ethanol and DOCA:

Ethanol was used as the vehicle for preparing stock solutions of DOCA and therefore the control arteries in the experiments on DOCA were always incubated with ethanol (176 mmol l^{-1}) under identical conditions. The results are summarized in Table 9.1. Comparison with the data in the untreated arteries (using the unpaired t-test, since it is not based on pairs of arteries from the same animals), showed that there was a small, and just significant, decrease in the accumulation of total ^{3}H in the tissue (see Table 9.1). Each of the metabolites in the tissue and in the medium tended to be less in the ethanol-treated preparations, but the differences were not significant. The largest decrease was in the DOPEG in the medium, but this was significant only at the $p = 0.1$ level. Other data was also available on the effect of ethanol in nialamide-treated arteries (Table 9.2). The results showed that ethanol tended to increase the accumulation of ^{3}H in the nialamide-treated arteries, the increase being just significant ($p = 0.05$).

Although these results pointed to a possible marginal effect of ethanol on NMN uptake and metabolism, the numbers on which they were based are small and the effects appeared insufficient in magnitude to vitiate interpretation of the effects of DOCA, described subsequently.

(4) DOCA and cocaine

DOCA's action was characterised by a small but significant decrease (18%) in the accumulation of ^{3}H in the artery (Table 9.1). This reflected a significant decrease in the tissue contents of ^{3}HNMN and ^{3}HMOPEG (29% and 46% respectively); the tissue content of VMA, and of DOPEG were unaffected. DOCA also caused a small but not significant decrease (14%) in the accumulation of MOPEG in the medium. VMA was detected in only one of each of the ethanol, and ethanol plus DOCA mediums; DOPEG was not detected in any.

In a second series of experiments, the effect of DOCA (27 μmol l^{-1}) on the uptake of ^{3}H in four nialamide-treated (350 μmol l^{-1}) arteries incubated with ^{3}H NMN was examined. The control (paired) arteries were treated with ethanol alone. The results are summarised in Table 9.2. Compared with untreated arteries, ethanol caused a small (20%) increase in total uptake of ^{3}H, which was significant at $p < 0.05$ (unpaired t-test). DOCA decreased the total uptake by 49%. This was almost twice as great as the decrease produced by DOCA in the MAO-intact arteries (see Table 9.1) and suggested that approximately 50% of the ^{3}H (presumably ^{3}H NMN) had accumulated in an extraneuronal compartment. To test whether there was also accumulation in a neuronal compartment, the effect of cocaine (29 μmol l^{-1}) on the uptake of ^{3}H was examined in another four nialamide treated segments which had been incubated with ^{3}H NMN. The results are also included in Table 9.2. It will be seen that cocaine also decreased the accumulation of ^{3}H; the decrease (30%) was less than that produced by DOCA. Although this finding pointed to a significant neuronal contribution to the accumulation of ^{3}H NMN in the artery, in consideration

Footnotes:

(i) Tissue: shown are the tissue contents (mean ± S.E.M., nmol g^{-1} for n arteries) of ^3H, MOPEG, VMA and the contents of ^3H based on the estimates of ^3H in the fraction DOPEG, from cocaine (29 μmol l^{-1}) treated, nialamide (350 μmol l^{-1}) treated, DOCA (27 μmol l^{-1}) treated and ethanol (174 mmol l^{-1}) treated arteries; all incubated with dl^{3}HNMN (1.04 μmol l^{-1}) for 30 mins. Values in brackets refer to mean contents for corresponding treatments in the absence of cocaine shown previously in Table 9.1.

(ii) Medium: contents (mean ± S.E.M., nmol g^{-1}) of MOPEG in incubation medium from cocaine and cocaine plus DOCA treated arteries. VMA was not detected in any of the incubation media and ^3H corresponding to DOPEG found only 2 media (from arteries Coc + EtOH (0.195 nmol g^{-1}) and Coc + Doc (0.042 nmol g^{-1})).

(iii) Wash: contents (mean ± S.E.M., nmol g^{-1}) of ^3H in wash solutions for the period 0.16 to 1.0 mins of wash.

(iv) Significance (p values, paired comparisons)

Total ^3H		Tissue ^{3}HNMN	
Nial vs Nial + Coc	$0.02 > p > 0.01$	Coc + EtOH vs Coc + Doc	$p < 0.01$
Nial + EtOH vs Nial + Doc	$p < 0.001$		
Coc + EtOH vs Coc + Doc	$p < 0.01$		

TABLE 9.2

The effect of DOCA on the accumulation and metabolism of ^{3}HNMN in cocaine treated arteries and the effect of DOCA and cocaine on the accumulation of ^{3}HNMN in MAO inhibited arteries

		T I S S U E					Medium	Wash
		Total ^{3}H	MOPEG	NMN	VMA	DOPEG	NMN+metab.	MOPEG	Total ^{3}H
Coc + EtOH	4	3.04±0.24 (3.94)	0.12±0.03 (0.29)	1.34±0.26 (3.16)	0.335±0.085 (0.21)	0.044±0.013 (0.055)	1.84 (3.72)	1.03±0.311 (2.02)	1.125±0.303
Coc + DOC	4	1.85±0.16 (3.25)	0.098±0.017 (0.16)	0.623±0.067 (2.25)	0.223±0.020 (0.24)	0.084±0.38 (0.044)	1.03 (2.69)	0.38±0.087 (1.73)	0.820±0.137
Nial	4	5.16±0.44	-	-	-	-	-	-	1.19 ±0.11
Nial + Coc	4	3.61±0.34	-	-	-	-	-	-	1.10 ±0.01
Nial + EtOH	4	6.17±0.32	-	-	-	-	-	-	1.56 ±0.063
Nial + DOC	4	3.12±0.13	-	-	-	-	-	-	1.53 ±0.089

of the finding that extracts of arteries contained ^{3}H that behaved chromatographically like DOPEG, and that these ^{3}H contents decreased with nialamide treatment (Table 9.1), the remote possibility that the action of cocaine seen above reflected a decrease in the accumulation of ^{3}HNA formed from ^{3}H NMN was explored. Accordingly acid extracts of control arteries and cocaine treated arteries were analysed for ^{3}HNA contents using the paper chromatographic procedure (Method 1) described in Chapter 4. These radio-chromatograms showed that there was no significant amounts of ^{3}H corresponding to the region of migration of non labelled NA. In view of this finding together with the observation that ^{3}H NMN is the major ^{3}H compound present in nialamide treated arteries (Table 9.1) it was concluded that cocaine's action in decreasing ^{3}H contents of arteries reflected a decrease in ^{3}H NMN in these tissues. Hence, in a final experiment, the effect of DOCA on the accumulation and metabolism of ^{3}H NMN was examined in four pairs of segments which were incubated in a medium containing cocaine (29 μmol l^{-1}) to inhibit neuronal uptake. The results are summarised in Fig. 9.1.

It will be seen that $^{3}HMOPEG$ and $^{3}HVMA$ were still formed in the presence of cocaine. The amount of $^{3}HVMA$ formed was comparable with that prevailing in the absence of cocaine in earlier experiments (ethanol treated), but the amount of $^{3}HMOPEG$ was considerably less (0.12 c.f. 0.29 nmol g^{-1}, respectively). Since there was a discrepancy in this last series of experiments between the measured total uptake and the uptake estimated by the sum of the ^{3}H NMN plus ^{3}H metabolite fractions (the latter summed uptake being only 60% of the former), it should be noted that the proportion of $^{3}HMOPEG$ in the 'summed' uptake (6.6%) is also much lower than in the absence of cocaine (9%), so that the effect of cocaine

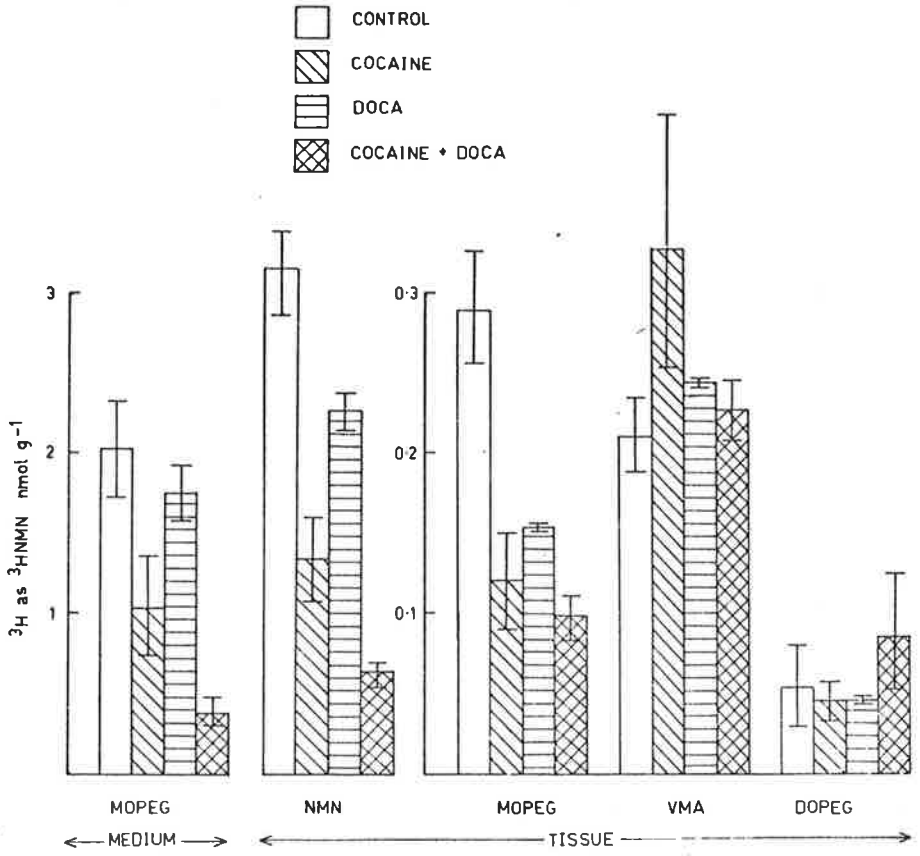


Fig. 9.1 A summary of the effects of cocaine and DOCA on the metabolite contents of arteries and incubation media, for arteries incubated with dl^{3}HNMN (1.03 µmol l^{-1}). Contents of ^{3}HNMN and metabolites determined by Method IV.

on ^{3}HMOPEG formation is probably a genuine one. Cocaine also exerts an inhibitory effect on the accumulation of ^{3}HMOPEG in the medium. The amount of ^{3}HNMN accumulated in the tissue was also less in the presence of cocaine, both in absolute terms and when expressed as a proportion of the summed uptake (72% c.f. 85%).

Compared with its action in the absence of cocaine, in the presence of cocaine DOCA caused a greater decrease in the total uptake of ^{3}H, (25% c.f. 39%) mainly as a result of a greater inhibitory effect on the accumulation of ^{3}H NMN. Its inhibitory effect on the accumulation of ^{3}HMOPEG in the medium was also more marked in the presence of cocaine. In contrast, it had little effect on the tissue content of ^{3}HMOPEG, although tending to decrease that of ^{3}HVMA.

(5) One minute wash:

In the preceding experiments, all the arteries were washed first for 10 seconds, then for 50 seconds, to provide conditions comparable to those used in the studies on NA and ISO. The amount of ^{3}H lost in the one minute period was estimated by multiplying the efflux of ^{3}H in the 50 second period by 1.2. This estimate is approximate in the sense that it assumes linearity of the rate of efflux in the whole of the 60 second period. The values are shown for each experiment in the relevant tables. It will be seen that they are all in the region of 1.2 nmole g^{-1} tissue, with the exception of the experiments on the effect of DOCA in the nialamide treated arteries where the values are (inexplicably) significantly higher (1.5 nmol g^{-1}). This value is higher than that estimated for the one minute efflux of ^{3}H of arteries

after incubation with ^{3}HNA (0.44 ± 0.04 nmol g^{-1}, Table 6.6). As will be seen from Table 9.2, the estimates range from 19 to 33% of the estimate of the total ^{3}H which would have accumulated in the artery at the end of the incubation, but prior to the one minute wash. The highest % estimates apply to the DOCA-treated arteries where the accumulation of total ^{3}H was least. The composition of this one minute wash efflux was determined only in the last experiment in 5 segments, namely three from the cocaine- and DOCA-treated arteries, and two from their cocaine-alone-treated controls. The analyses were carried out by ion exchange column chromatography on Dowex 50 (Na^{+}), i.e. the first part of Method III, Chapter 4. The proportions of NMN in the two control wash solutions were 86 and 87%; in the three wash solutions from the DOCA treated segments were 89, 94, and 95%. The remainder in each case was in the fraction containing all the acids and glycols (i.e. approximately 0.1 nmol gm^{-1}). The corresponding mean proportions of NMN in the control and DOCA-treated tissues were 73 and 61%. The high proportion of NMN in the wash solution is consistent with its presence in the extracellular space and rapid efflux following bath washout. However, if it is assumed that approximately one-half of the tissue can be represented as extracellular space which is cleared of drug in one minute, it follows that approximately $\frac{1.2 - 0.1}{2} \simeq 0.55$ nmol g^{-1} of the NMN in the one minute wash has effluxed from structures other than the extracellular space. This means that a very considerable proportion (30 to 40%) of the total tissue uptake of NMN at the conclusion of a 30 minute period of incubation (1.34 + 0.55, control; 0.62 + 0.55, DOCA-treated, nmol g^{-1}) had effluxed in the first minute in the cocaine (ethanol and DOCA (ethanol) treated arteries (Table 9.2).

Since the small proportion of acids and glycols in the one minute wash must have effluxed from structures other than the extracellular compartment, it is of interest that the quantity involved (10% of 1.2 nmol g^{-1}) represents a ratio to NMN (1:3.9) which is considerably smaller than that prevailing in the tissues (1:2 for the control tissues and 1:1.7 for the DOCA treated tissues).

6. Rabbit aorta

The tissue contents of ^{3}H in 3 segments of rabbit thoracic aorta incubated with dl^{3}HNMN (5.4 μmol l^{-1}) for 30 minutes and washed for 1.0 minute, are shown in Table 9.3. The results show that the ^{3}H contents of these segments were not significantly greater than the concentration of ^{3}HNMN in the incubation medium. Inhibition of MAO activity by nialamide (350 μmol l^{-1}) in a further 3 segments of aorta did not significantly alter the contents of ^{3}H. Unfortunately the ^{3}H contents of wash solutions were not estimated and hence the total ^{3}H contents of these tissues at the completion of incubation was not known.

Tissue extracts and incubation media were subjected to alumina and Dowex 50 chromatography and the ^{3}H contents in the fractions corresponding to VMA + MOPEG, NA + DOPEG and DOMA determined. The levels of ^{3}H in all of these fractions for samples of incubation media were similar to ^{3}H contents due to cross contamination of these fractions by ^{3}HNMN (Table 9.3), the latter observation indicating that the ^{3}H metabolite contents of these media were so small as to be not detectable by this procedure. In contrast analysis of tissue extracts showed that the ^{3}H present in all fractions was greater than that attributable to the cross contamination of these fractions by ^{3}HNMN. The contents of ^{3}H O-methylated deaminated metabolites (^{3}HVMA + ^{3}HMOPEG) ranged from 13 to 18% of the total ^{3}H accumulated in the aorta (Table 9.3). MAO inhibition decreased these contents of deaminated metabolites in three segments of aorta by

TABLE 9.3

Accumulation and metabolism of ^{3}HNMN in the rabbit aorta

Rabbit	Aorta treatment	Tissue contents (nmol g^{-1})				Medium ($\frac{^{3}H_{fr}}{^{3}H_{inc}}$ %)		
		Total ^{3}H	MOPEG + VMA	DOMA	DOPEG + NA	MOPEG + VMA	DOMA	DOPEG + NA
1	Nil	5.84	0.79	0.12	0.17	4.60 (4.45)	0.35 (0.15)	1.83 (1.39)
2	Nil	4.44	0.77	0.12	0.15	4.76 (4.45)	0.18 (0.15)	1.52 (1.39)
3	Nil	6.84	1.25	0.17	0.20	4.23 (4.45)	0.16 (0.15)	1.37 (1.39)
1	Nialamide ($350\ \mu mol\ l^{-1}$)	4.89	0.40	0.076	0.20	4.3 (4.45)	0.16 (0.15)	1.22 (1.39)
2	Nialamide ($350\ \mu mol\ l^{-1}$)	4.24	0.34	0.078	0.15	3.51 (4.45)	0.20 (0.15)	1.44 (1.39)
3	Nialamide ($350\ \mu mol\ l^{-1}$)	5.88	0.53	0.126	0.23	3.62 (4.45)	0.18 (0.15)	1.39 (1.39)

Footnotes

(i) Tissue: shown are the individual contents (nmol g^{-1}) of ^{3}H, ^{3}H O-methylated deaminated metabolites (MOPEG + VMA) and the ^{3}H contents of catechols (based on estimates of ^{3}H in the fractions DOMA and DOPEG + NA) for untreated and nialamide treated segments of aorta, incubated with dl^{3}HNMN ($5.4\ \mu mol\ l^{-1}$) for 30 mins.

(ii) Medium: shown are the proportions of the total ^{3}H in fractions (corresponding to MOPEG + VMA, DOMA and DOPEG + NA) to the total ^{3}H added to the incubation media ($\frac{^{3}H_{fr}}{^{3}H_{inc}}$ %). Also shown (in brackets) are the proportions of ^{3}H in fractions, from incubation media containing ^{3}HNMN but no tissue.

49%, 44% and 58% respectively.

The presence of ^{3}H catechols derived from $^{3}HNMN$ was also indicated from the results of these experiments. The ^{3}H contents in the DOMA fraction were greater (3.9 to 4.9 fold) than that expected on the basis of cross contamination of this fraction by $^{3}HNMN$ and decreased in all 3 MAO inhibited segments of aorta (Table 9.3). Similarly the ^{3}H contents of the DOPEG + NA fraction were greater (2.2 to 2.6 fold) than that attributable to the cross contamination by $^{3}HNMN$, but surprisingly were increased in two of the nialamide treated arteries (Table 9.3). Although the reason for this increase was not apparent from the data it was thought to reflect the possibility that DOPEG contributed minimally to the contents of ^{3}H in the NA + DOPEG fraction, and as such under conditions of MAO inhibition the decrease in tissue contents of $^{3}HDOMA$ and $^{3}HDOPEG$ was offset by an increase in the tissue content of ^{3}HNA. The total ^{3}H catechol contents (NA + DOMA + DOPEG) in untreated segments amounted to 0.31 ± 0.04 nmol gm^{-1} or approximately 5% of the total ^{3}H present in the aorta.

DISCUSSION

The results show that the artery has the capacity to accumulate NMN and metabolise it to MOPEG, VMA, and possibly DOPEG. In untreated arteries the percentage of the metabolites in the tissue was 20% of the total (metabolites plus NA). This estimate is similar to the corresponding percentage of total metabolites derived from ^{3}HNA in untreated arteries (20, 17 and 13% Table 6.2, Chapter 6). NMN comprised between 2 and 5% of the latter; assuming that about 20% of the NMN has been further metabolised during incubation of the NA, one might expect that this

further metabolism has contributed at most about 0.4-1% (i.e. one-fifth of 2 and 5%) to the accumulated deaminated metabolites of NA in the artery. Alternatively expressed, one fifth of the NMN would have yielded between 0.02 and 0.05 nmol g^{-1} of VMA plus MOPEG, i.e. between one-seventh and one-third of the amount of the VMA + MOPEG found in the tissue after its incubation with ^{3}HNA. It is also conceivable that the MOPEG which accumulated in the medium of arteries that were incubated with ^{3}HNA was partly derived from ^{3}HNMN as its immediate precursor. This possibility follows from the observation that MOPEG accumulated in the medium of the arteries incubated with ^{3}HNMN to yield a content of approximately 3 nmol g^{-1} tissue. This is approximately equal to the concentration of ^{3}HNMN retained in the tissue. Hence it is possible to argue from the data in Table 6.2, Chapter 6 that, since the metabolism of ^{3}HNA results in a tissue level of ^{3}HNMN of between 0.1 and 0.25 nmol g^{-1}, an equivalent amount of MOPEG in the medium may have resulted from the metabolism of NMN. The total amount of MOPEG in the medium was 0.31 ± 0.05 (n=7) mmol g^{-1} (Chapter 6), so that, as a minimum estimate, about one-third of this may have stemmed from the metabolism of NMN. However, it is apparent from the preceding extrapolations that the proportion of NMN which is metabolised is too small to suggest that this represents a major pathway in the inactivation of NA.

The data showed that the tissue has a considerable capacity to accumulate NMN. This was approximately three-fold, i.e. only slightly below that of ^{3}HNA (3-5 fold) and greater than that commonly observed with ^{3}HISO (1.3 fold).

The experiments with nialamide showed that, while inhibiting the accumulation of MOPEG in the tissue and medium, and of DOPEG in the tissue, nialamide itself did not increase the amounts of tritiated material which accumulated in the tissue or the amounts which effluxed during the one minute wash. Although it tended to increase the accumulation of unchanged NMN, the effect was small (13%) and not significant. In this respect, the effect of inhibiting the inactivation of NMN differs markedly from that of ISO, where COMT inhibition increased the accumulation of total ^{3}H by a factor of 1.6. This increase included a three fold increase in the accumulation of unchanged ISO which was greater than could be attributed to stoichiometric replacement of the tissue by MeOISO by ISO. Presumably, then, the inactivation of NMN does not act to limit the accumulation of unchanged NMN to a significant degree. This conclusion accords well with unpublished observations of de la Lande and Campbell in the author's laboratory. These workers showed that the contractile response to NMN on the isolated rabbit ear artery helical strip was not enhanced by preincubating the segment with nialamide (350 $\mu mol\ l^{-1}$) for 40 minutes followed by a 15 minute washout. The results of similar experiments on the rabbit aorta, although limited in numbers, were not in contradiction to those described above for the ear artery. In particular the lack of increase in the accumulation of ^{3}H in MAO inhibited segments of aorta together with the relatively small formation of O-methyl deaminated metabolites suggests that this tissue behaves in a similar fashion to the ear artery.

The remaining experiments described in this section offer evidence as to the site of accumulation of ^{3}HNMN and its metabolites. Assuming that cocaine and DOCA selectively inhibit neuronal and extraneuronal uptake, their inhibitory effects on uptake of ^{3}H in the nialamide treated

arteries indicate that 30% of uptake may be neuronal and a greater proportion (50%) is taken up extraneuronally into a DOCA-sensitive compartment. Unfortunately, the only comparison involving cocaine and untreated (MAO-intact) arteries was unpaired and marred by the discrepancy between the summed uptakes of metabolites plus ^{3}HNMN, and the experimentally determined value of total uptake in the cocaine-treated group. However, even if the values in the cocaine-treated arteries are increased in proportion to the measured total uptake, the tissue and medium levels of NMN remain significantly below those generated in the control (ethanol treated) arteries. Hence it is reasonable to extrapolate from this data to suggest that at least ($\frac{3.16 - 2.22}{3.16}$ = 30%) of the accumulated NMN, and of the accumulated MOPEG, approximately $\frac{.29 - 0.21}{.29}$ = 31% in the tissue and 49% in the medium represent minimum estimates of the proportion of these components which are neuronal in origin. This result is surprising when considered in the light of the extremely low affinity of dlNMN for neuronal (U_1) as compared with extraneuronal uptake (U_2). Thus the relative affinities of NMN for U_1 and U_2 quoted by Iversen (1967) are 0.55 and 1785, respectively, and the ratios of the ID_{50}'s for $\frac{U_2}{U_1}$ are 0.02 for NMN as compared with 374 for NA. There are several possible explanations for the apparent discrepancy between the low affinity for neuronal uptake and the effect of cocaine which suggests that approximately one-half of the MOPEG which effluxes into the medium is neuronal in origin. One is that this action of cocaine is extraneuronal. Other than that the earlier parts of this study have not revealed any unequivocal evidence to support such an action, this possibility cannot be excluded. However, de la Lande (1975), in a review of adrenergic mechanisms in the rabbit ear artery, could find little evidence from pharmacological studies on

this preparation to support an extraneuronal action of cocaine. A second possibility is that the relative affinities of NMN for neuronal uptake compared with extraneuronal uptake is considerably greater in the ear artery than in the rat heart, on which the data of Iversen (1967) is based. Again, this possibility cannot be excluded.

However, a third possibility is that the neuronal uptake (i.e. U_1) of NMN is small but is facilitated by the subsequent oxidation of NMN to MOPEG by neuronal MAO in the cytoplasm of the nerve. In support of this possibility there is evidence that NMN is a relatively specific substrate for type A MAO together with NA and 5-hydroxy-tryptamine (Goridis and Neff 1971). It is a reasonable assumption that extraneuronal MAO in the rabbit ear artery is predominately the type B form of MAO. This follows from the pharmacological evidence of de la Lande and Jellett (1972) that the extraneuronal MAO has little capacity to metabolize NA. Furthermore earlier evidence in this thesis (Chapters 5 and 6) argues; (i) against a high affinity of extraneuronal MAO for NA in the rabbit ear artery (a characteristic of type B enzyme, Neff *et al.* 1973); (ii) for a high affinity of extraneuronal MAO for tyramine in homogenates of the rabbit ear artery (a characteristic of type A and type B enzymes, Johnston 1968). Hence the situation emerges that in the case of NMN (i) a high affinity for extraneuronal uptake may be associated with (ii) a low affinity for extraneuronal MAO while (iii) a low affinity for neuronal uptake is associated with (iv) a high affinity for the intra-neuronal MAO. Hence it is possible to explain MOPEG formation as the interplay of factors (i) (ii) (iii) and (iv). Unfortunately the problem remains that cocaine decreases the accumulation of NMN. This effect of cocaine is difficult to explain except in terms of accumulation of unchanged NMN within the nerve, which in turn is scarcely compatible

with a rate of oxidation sufficiently fast enough to facilitate diffusion of unchanged amine into the cytoplasm. It is possible that the extent of neuronal accumulation of NMN in this study is overestimated for precisely the same reasons offered as explanation for the predominantly neuronal metabolism of NA in this artery (Discussion, Chapter 6) viz; most of the NMN which reaches the smooth muscle of this preparation is probably first exposed to the sympathetic nerve terminals which are located in the adventitia. That is the presentation of the amines to the artery in this manner may in some way favour neuronal rather than extraneuronal processes.

Of the metabolites which were insensitive to cocaine, about one-third of the tissue VMA and approximately two-thirds of the MOPEG in the medium were sensitive to DOCA, i.e. these represent proportions of the particular metabolites which are taken up extraneuronally into compartments where access is limited by the steroid. It is tempting to suggest that these compartments are identical with the compartment(s) which accumulate unchanged NMN, since it is evident that about 50% of the unchanged amine is also accumulated in a DOCA-sensitive compartment. If so, it is possible that the compartment has not reached saturation with respect to any of its constituents, since elimination of one fraction (metabolites) resulted in replacement by, but not further accumulation of, unchanged amine. However, much more intensive analysis will be needed to define such a possibility, preferably along the lines used by Trendelenberg and colleagues in the elucidation of the inter-relationship between amine storing compartments for NA and ISO in various tissues. However for such an analysis due consideration should be paid to the fact that NMN (like DOCA) is a potent extraneuronal uptake inhibitor in contrast to the catecholamines NA and ISO whose O-methylated metabolites are potent inhibitors of extraneuronal uptake (i.e. NMN and

MeOISO respectively, Mireylees and Foster 1973). The point being that extraneuronal metabolism of NMN (by MAO) would tend to decrease the tissue content of this extraneuronal uptake inhibitor whereas O-methylation of the catecholamines would tend to increase the contents of extra-neuronal uptake inhibitors.

Catechol formation

Limited evidence supporting the formation of ^{3}H catechols from $^{3}HNMN$ came from the results of studies on the rabbit ear artery and the rabbit aorta. The evidence supporting demethylation of $^{3}HNMN$ in the ear artery was restricted to the finding that ^{3}H material, co-chromatographing with non labelled DOPEG, was present in most artery extracts. The occasional appearance of ^{3}H material, corresponding to NA, in these acid extracts may well have reflected the rapid deamination of the intermediate ^{3}HNA. However there was no evidence of ^{3}HNA formation in MAO inhibited arteries although in MAO inhibited segments of aorta there was an increase in the ^{3}H content of the fraction corresponding to NA + DOPEG (thought to be mainly ^{3}HNA). In view of the fact that ^{3}H catechols were detected primarily in extracts of tissues previously incubated with $^{3}HNMN$, it is possible that demethylation of $^{3}HNMN$ occurred subsequent to incubation (and prior to chromatography) and was catalysed by the acid extracts of tissues. Consistent with this possibility is the evidence (to be presented in Appendix 3) showing that demethylation of NMN may occur in aqueous solutions under specific conditions and that this process is oxidative in nature. If this process of demethylation did occur in the tissue during incubation, the fact that the amounts of ^{3}H catechols formed represented only a small proportion of the total ^{3}H in these tissues, suggests that such a process would be of minor biochemical

significance in terms of the molecular rearrangement of NMN. However a similar conclusion might not apply to pharmacological investigations for the formation of the catecholamine (NA) may be in part responsible for the responses elicited by solutions of NMN on pharmacological preparations.

CHAPTER 10

GENERAL DISCUSSION

CHAPTER 10

GENERAL DISCUSSION

It is the purpose of this discussion to summarize the results of the author's studies and to comment on their significance in relation to the pharmacological properties of the rabbit ear artery.

1. MAO

It was shown in Chapter 5 that approximately 90% of the MAO activity, with tyramine as substrate, was extraneuronal in distribution in the rabbit ear artery. This accorded well with earlier histochemical evidence that MAO activity, with tryptamine as substrate was extraneuronal, and also with biochemical evidence of de la Lande and Johnson (1972). These authors measured the concentrations of NA in wash solutions from denervated arteries that were previously incubated with NA in a high concentration (118 μmol l^{-1}) and showed that the NA concentrations were much greater when MAO was inhibited than when it was intact. They concluded that extraneuronal deamination represented the major pathway for the inactivation of NA when the amine concentration in the bathing solution was high. However, the pharmacological study of de la Lande and Jellet (1972) failed to show an influence of extraneuronal MAO on the sensitivity to extraneuronal NA. These authors concluded that neuronal rather than extraneuronal MAO plays an important role in the response of this

artery to NA. The subsequent studies in this thesis, particularly in Chapter 6, bear out their conclusion.

The experiments on ^{3}HNA showed that DOPEG was the major metabolite of NA present in both tissues and incubation media as well as in the efflux of ^{3}H into media after washout. That the major proportion of this metabolite was derived from neuronal MAO, was indicated by its decrease in tissue contents from denervated or cocaine treated arteries and its elimination from the incubation medium and efflux in nialamide treated arteries. In view of the latter, the increased accumulation of NA seen in nialamide treated arteries suggests that neuronal MAO serves to decrease the axoplasmic contents of NA. Consistent with this view was the observation that the efflux of ^{3}HNA was greater for MAO inhibited than for MAO intact arteries.

The results also suggested that MAO was virtually the only mechanism for the neuronal metabolism of NA. This followed from the failure of MAO inhibition to divert a significant proportion of the ^{3}HNA which effluxed from arteries, to a COMT pathway as indicated by an increase in the efflux of ^{3}HNA and not ^{3}HNMN. The experiments on ^{3}HNA also provided evidence for a firmly bound component of NA accumulation. This evidence will be considered later in this discussion. All of these findings point to the possibility that neuronal uptake of NA in this artery was followed by retention (presumably in storage vesicles) and deamination but not O-methylation.

The presence of extraneuronal MAO was indicated by the observation that both ^{3}HNA and ^{3}HNMN were deaminated in cocaine treated arteries and ^{3}HNA was metabolized by MAO in denervated arteries. The results

of these experiments suggested that the extent to which extra-neuronal MAO contributed to the inactivation of NA was minor compared with that of neuronal MAO. This conclusion was based on the relatively small proportions of deaminated metabolites which persisted in cocaine treated and denervated arteries. However, these proportions do not provide a precise estimate of the contribution of extraneuronal MAO because the data does not include the effects of denervation and cocaine treatment on the levels of the metabolites in the medium.

It was seen that denervation did not lead to a significant increase in the tissue contents of O-methylated deaminated metabolites in arteries incubated with dl^{3}HNA, which contrasts with the increase of ^{3}HNMN in these arteries. This is an argument for a less important role of extraneuronal MAO than that of extra-neuronal COMT in limiting the extraneuronal accumulation of NA. The studies on ^{3}HNMN were consistent with this view because inhibition of MAO caused a small, but not significant increase in the artery contents of ^{3}HNMN.

Although the effects of cocaine and denervation on the tissue levels of the metabolites have provided some insights into the role of MAO in the inactivation of NA in this artery, there are obvious deficiencies in the data presented in Chapter 6. There is a need for further experimentation to determine:

(i) the effects of cocaine or chronic denervation on accumulation of metabolites of dl^{3}HNA in the medium

(ii) the tissue and medium contents of metabolites following incubation of denervated or cocaine treated arteries with the laevo isomer of NA.

(iii) the metabolites present in the one minute wash solutions.

In regard to (iii), the metabolite contents in wash solutions from two arteries were determined during the preparation of this manuscript. The results showed that the metabolite contents of the wash solutions represented one sixth of the total content of metabolites in arteries after the one minute wash and their relative proportions were as follows: NA 0.55, DOPEG 0.20, MOPEG 0.09, NMN 0.08, VMA 0.08 (Kennedy, J., private communication).

There is an apparent paradox between the results of the enzyme and histochemical studies which suggest that the MAO activity was largely extraneuronal and the results of the pharmacological and biochemical studies which have emphasised the importance of neuronal rather than extraneuronal MAO for the inactivation of NA in this artery. One possible explanation is that the rate of neuronal uptake of NA is sufficiently greater than that of extraneuronal uptake at low concentrations of NA, and this serves to make relatively more substrate available to the neuronal enzyme. A second possibility, mentioned in the discussion section of Chapter 6, was that the predominantly neuronal metabolism of NA seen in those experiments may have reflected an extraluminal exposure of arteries to NA. It follows that there is a need to examine the metabolism of ^{3}HNA applied intraluminally since one of the arguments in favour of the importance of extraneuronal MAO, advanced by de la Lande and Jellet (1972), was the failure of responses to intraluminal NA to be potentiated by inhibition of MAO. A third possibility reflected a difference in the neuronal and extraneuronal distributions of the type A and B

forms of MAO described by Goridis and Neff (1973). Tyramine (the substrate used for enzyme assays) and tryptamine (the substrate used for histochemical procedures) are deaminated by type A and type B forms of MAO (Squires 1973). Because NA is deaminated by the type A form the possibility arises that extraneuronal MAO in the ear artery comprises mainly type B MAO. It was thought that the experiments on the deamination of ^{3}HNMN described in Chapter 9 might provide further information concerning these two forms of MAO in the artery. This was because NMN is a relatively specific substrate for type A MAO (Goridis and Neff 1972) with a high affinity for extraneuronal uptake (Burgen and Iversen 1965). The results pointed to the presence of some type A within the nerves since there was a significant decrease in the formation of MOPEG by cocaine. On the other hand it was not known whether the extraneuronal metabolism represented a small portion of extraneuronal type A MAO or a small degree of metabolism of NMN by the predominating type B form. This point might be resolved by further investigations of the effects of the specific inhibitors, clorgline (type A inhibitor) and deprenyl (type B inhibitor) on the formation of deaminated metabolites of NA and of NMN in the intact artery.

2. COMT

The experiments in Chapter 5 indicated that COMT activity in artery homogenates was not decreased by sympathetic denervation and therefore it was concluded that the COMT activity was mainly extra-neuronal. This conclusion proved to be in accord with the distribution of metabolites described in Chapters 6 and 7. The predominantly

extraneuronal origin of COMT was indicated by:

(i) the finding that the content of ^{3}HNMN in the tissue after incubation with dl^{3}HNA was increased by chronic sympathetic denervation and by cocaine treatment.

(ii) the failure of MAO inhibition to divert neuronal metabolism to a COMT pathway.

(iii) the finding that O-methylation of ^{3}HISO was not decreased by sympathetic denervation or cocaine treatment in contrast to the decrease in accumulation of unchanged amine.

The small proportion of ^{3}HNMN compared with DOPEG in both the medium and in arteries that were incubated with ^{3}HNA suggested that the extraneuronal COMT pathway is less important functionally than the neuronal pathway for the inactivation of NA. Johnson (Ph.D. thesis, 1975) showed that inhibition of COMT in the rabbit ear artery potentiated responses to NA, A and nerve stimulation and that this potentiation was not decreased by cocaine. Besides confirming that extraneuronal COMT was physiologically important in the inactivation of catecholamines, the findings of Johnson accord well with a predominantly extraneuronal origin of this enzyme (this pharmacological evidence has been presented in part in the study of Head *et al.* 1975).

3. UPTAKE AND ACCUMULATION

The data presented in Chapter 6 emphasised the importance of neuronal uptake of NA in the artery. The results showed that the tissue contents of dl^{3}HNA were decreased by sympathetic denervation and by cocaine treatment. Besides confirming that the sympathetic nerves were the major sites of accumulation of ^{3}HNA in the artery, this finding supports the pharmacological evidence that neuronal

uptake is functionally important when NA is applied extraluminally (de la Lande *et al.* 1967).

The effects of denervation and of cocaine were additive in decreasing the artery contents of ^{3}HNA so that these contents now approached those prevailing in PBZ treated arteries. Since PBZ prevents both the neuronal and extraneuronal accumulation of NA in sympathetically innervated tissues these results implied that:

(i) there was no significant extraneuronal accumulation of NA

(ii) that either cocaine or chronic sympathetic denervation possessed an additional extraneuronal action

(iii) cocaine treatment was not completely effective in preventing neuronal uptake of ^{3}HNA or that chronic sympathetic denervation was incomplete.

Evidence supporting (iii) above came from the observation that approximately 10% of the tissue content of ^{3}H present in arteries after 30 minutes of wash was insensitive to cocaine. The possibility that cocaine in a concentration of 30 μmol l^{-1} did not completely prevent the neuronal uptake of NA cannot be ignored. It is of interest that the magnitude of the potentiation to extraluminal NA by cocaine observed in the study of de la Lande *et al.* (1967) was about ten-fold. This implies that the nerves had extracted about 90% of the applied NA before it reached the underlying smooth muscle. This value is not too different from the estimate in the present study that nerves account for at least 80% of the NA taken up by the artery. The finding that cocaine caused a further decrease in the accumulation of ^{3}HNA is in accord with the observation of de la Lande (1975) that cocaine caused a small increase in sensitivity to

NA in sympathetically denervated arteries. Regardless of the explanation, the evidence for extraneuronal accumulation of NA was less precise than that for neuronal accumulation. There is a discrepancy between these findings and the results of previous histochemical studies on this artery which showed an accumulation of NA in the smooth muscle cells of arteries after incubation with NA (Avakian and Gillespie 1968). However, as pointed out previously, the substrate concentration in the histochemical studies was far greater than that used in the present studies. The possibility exists that accumulation occurred at the high substrate concentration because the extraneuronal metabolizing enzymes were saturated and as a consequence did not significantly decrease the cytoplasmic concentration of unchanged amine.

The pharmacological evidence suggests that the extraneuronal uptake system is less important than that of the neuronal uptake system in the control of sensitivity to NA. It was found by Johnson (Ph.D. thesis 1976) that the extraneuronal uptake inhibitor DOCA enhanced the sensitivity to NA less than two-fold in innervated arteries and about two-fold in cocaine treated arteries. This contrasts with a ten-fold increase in sensitivity produced by cocaine (de la Lande *et al.* 1967).

4. ^{3}HISO and ^{3}HNMN

In Chapter 8 evidence was presented to favour both neuronal and extraneuronal accumulation of ISO and extraneuronal O-methylation by COMT. Although the O-methylation was sensitive to DOCA, the accumulation of ^{3}HISO was only sensitive to DOCA under conditions of

COMT inhibition. These findings and the observation that COMT inhibition markedly increased the accumulation of ^{3}HISO suggested that there was an intimate relationship between the activities of COMT and that of DOCA in the artery.

The kinetics of efflux of ^{3}HISO was studied in an attempt to shed further light on the relationship. The results (Chapter 9) pointed to the presence of at least two compartments for the accumulation and O-methylation of ^{3}HISO. One of these appeared to be responsible for the formation of MeOISO, and possibly for storage of unchanged amine. The access to this compartment was sensitive to DOCA. The second compartment was less sensitive to DOCA, and only stored unchanged amine. Unfortunately time did not permit the logical conclusion to this study, namely to examine the efflux of ^{3}HISO from denervated arteries. This may have indicated the compartment associated with the neuronal accumulation of ^{3}HISO.

The studies on ^{3}HNMN are of interest because they indicated that DOCA also inhibited access of amine to an extraneuronal compartment containing MAO. Whether this is the same compartment which is responsible for the O-methylation of ^{3}HISO remains to be determined.

The pharmacological studies which are most relevant to the present studies on ^{3}HISO are those of Johnson (Ph.D. thesis 1976). He showed that the vasodilator action of ^{3}HISO on the rabbit ear artery was potentiated 3 to 4-fold by DOCA, but this effect was manifested only at low substrate concentrations (range 0.16 to 0.23 nmol l^{-1}). At high substrate concentrations (6.1 µmol l^{-1}) when ^{3}HISO was vasoconstrictor, the potentiating effect of DOCA was

greatly decreased. These findings were interpreted as evidence that the uptake system which influenced the concentration of ^{3}HISO at its receptors, possessed only a limited capacity for ^{3}HISO, so that it became saturated at the higher substrate concentration. Johnson also examined the interaction between the sensitising actions of DOCA, and of a COMT inhibitor (UO521) on the vasoconstrictor response of the artery to adrenaline. He showed that the maximum sensitising actions (3 to 4 fold) of these agents were not additive, i.e. neither agent potentiated in the presence of the other. These findings were similar to those of Kaumann (1972) on cat heart. Kaumann proposed the concept of a high affinity low capacity compartment for catecholamines which is of major importance in removing the amine from the vicinity of the receptors. This concept has subsequently received support from the extensive biochemical and pharmacological studies of Trendelenburg and his colleagues (Bönisch and Trendelenburg 1974). Their studies were discussed in relation to those on the ear artery in Chapter 8.

A possible relationship between the present findings and the pharmacological sensitising actions of UO521 and DOCA is represented in Fig. 10.1. It is assumed that extraneuronal uptake and subsequent O-methylation of the catecholamine decreases the concentration of the latter at the receptors in the smooth muscle. By interrupting uptake and hence O-methylation, DOCA diverts amine to the receptors and thus enhances the response. The potentiating effect of UO521 on the response to a low concentration of catecholamine can be explained as follows; in the absence of COMT activity, the unchanged

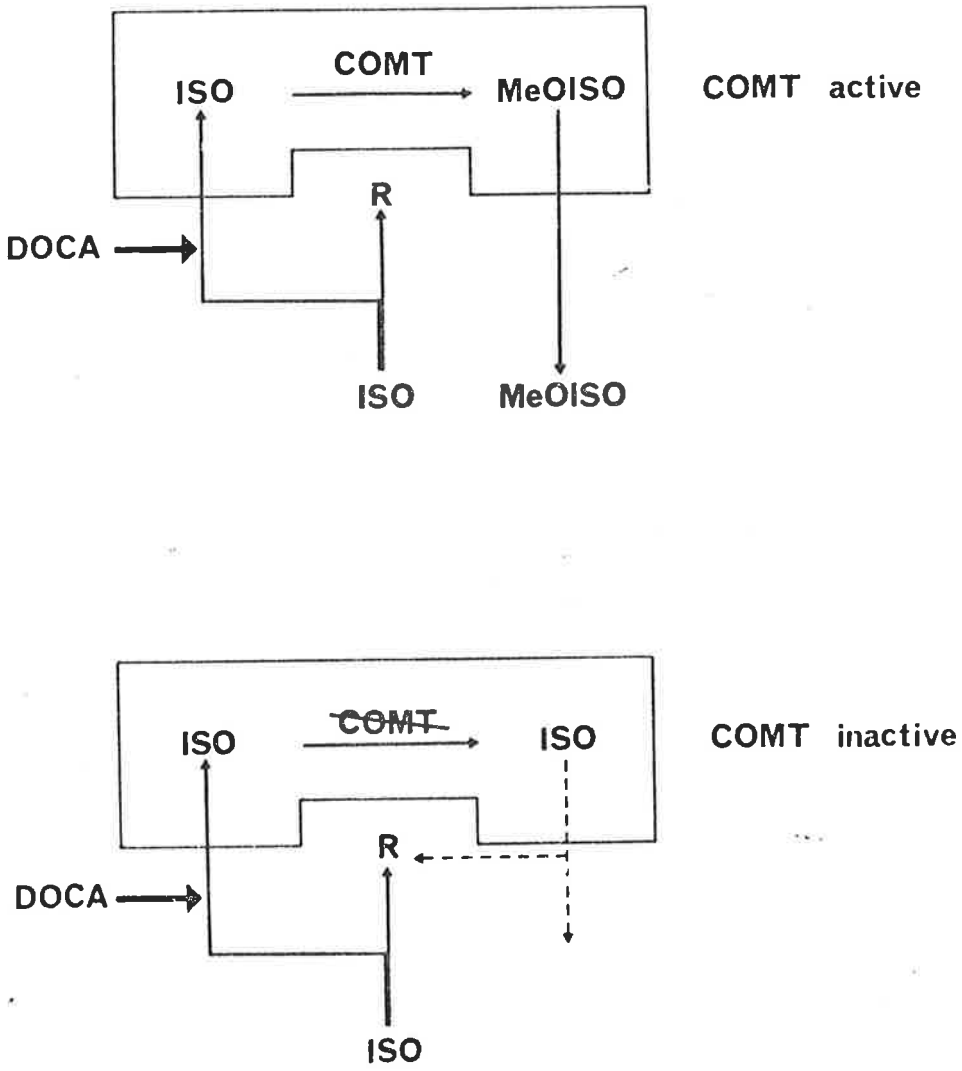


Fig. 10.1 Diagrammatic representation of the influence of uptake and O-methylation on the concentration of isoprenaline at the receptors in the smooth muscle of the rabbit ear artery. The directions of the arrows indicate the directions of isoprenaline fluxes.

amine accumulates in its storage sites to a level which results in a considerable increase in the efflux of the amine back into the region of the receptors. This explanation assumes (a) that the amine which accumulates in the absence of COMT activity has little, if any, influence on the concentration of amine which reaches the receptors and (b) that the physiologically important component of extraneuronal uptake has very little capacity for storing unchanged amine. The second assumption (b) is necessary to explain the failure of DOCA to sensitise the artery in the presence of a COMT inhibitor. If unchanged amine which accumulated after its uptake was of physiological importance (i.e. in increasing the concentration of amine at the receptors), then inhibition of this uptake should enhance the response to the amine. The fact that this does not occur in the COMT-inhibited arteries is readily explained if, under these conditions, DOCA caused a decrease in efflux of unchanged amine from low capacity (readily saturable) storage sites as a secondary consequence of inhibiting uptake.

The morphological localization of the high affinity low capacity COMT containing compartment may well be associated with cellular membranal structures of the smooth muscle cells. Although there is no direct evidence for such a location so that its existence is highly speculative, it is consistent with the finding that part of the COMT activity of the rabbit aorta is associated with the microsomal fraction (Verity *et al.*, 1972). Such a location is illustrated in figure 10.2 and may help to explain some of the results of this thesis. At low concentrations of catecholamines COMT activity or

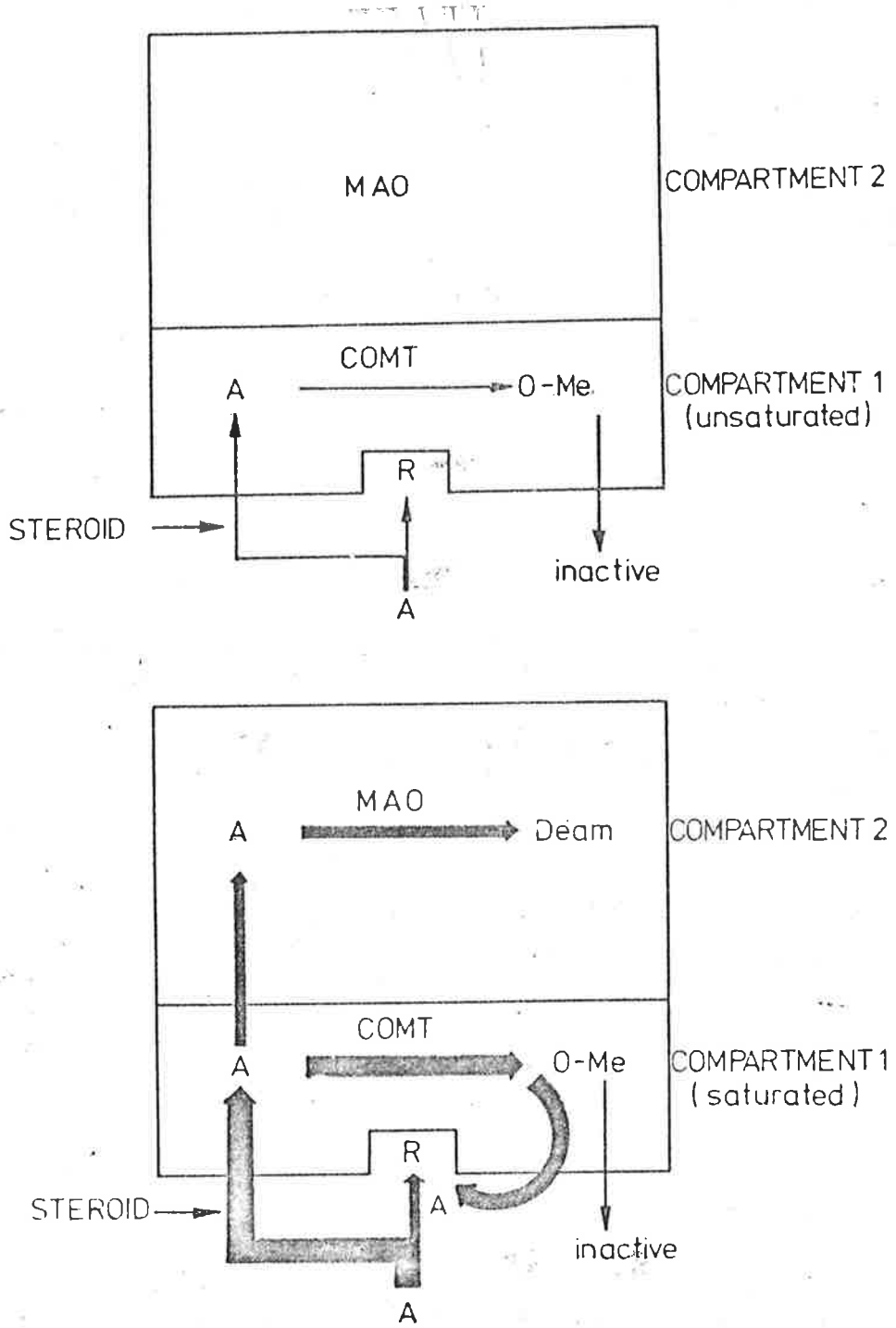


Fig. 10.2 Possible arrangement of a two compartment extraneuronal system in the rabbit ear artery. Concentrations of amines (A) are represented by the thickness of the arrows, the directions of which indicate fluxes into and out of the smooth muscle cell. A = adrenaline; R = receptor; O-Me = inactive O-methylated product; Deam = inactive deaminated product

the product of this activity would serve to reduce entry and hence accumulation of unchanged amine into the cytoplasm of smooth muscle cells. Under these conditions catecholamines would be prevented access to the mitochondrial MAO and this would account for the minimal extraneuronal accumulation and deamination seen in Chapter 6. At higher substrate concentrations COMT would be inhibited and no longer serve to prevent access of amine into the smooth muscle cells. Under these conditions catecholamines may accumulate and be deaminated by MAO. The possibility that the COMT products of catecholamines regulate this entry into the cytoplasm of smooth muscle cells comes from two sources. Firstly, the O-methylated derivatives of catecholamines are non-competitive inhibitors of extra-neuronal uptake. Secondly, the COMT inhibitor UO521 is not an amine and unlike O-methylated catecholamines causes an increased accumulation of unchanged amine. If the concentration of catecholamines at the receptors is regulated by the content of O-methylated catecholamine in the high affinity low capacity COMT containing compartment, then this would be characterized by an initial decrease in catecholamine concentration at the receptors due to uptake of unchanged amine into this compartment. This would be followed by a time dependent increase in catecholamine content at the receptors due to the filling of the compartment with O-methylated catecholamine. This leads to the possibility that this compartment may well be part of a membranal receptor-complex located on the smooth muscle cells of the tissue.

In conclusion this study has emphasized the importance of the processes for the uptake and metabolism of catecholamines in vascular tissues, and has attempted to provide insights into the biochemical mechanisms responsible for the pharmacological properties of these tissues.

APPENDICES

INTRODUCTORY NOTE

This section comprises a heterogenous series of investigations related to the studies described in the main body of this thesis. The studies included in this appendix are concerned primarily with methodological aspects of experiments involving catecholamines, and although considered important, these investigations were not directly concerned with the inactivation of catecholamines in arteries and for this reason have been treated separately. These studies include:

(1) The application of the semi-automated THI assay to the measurement of catecholamines in biological samples.

(2) A study of the purity and stability of high specific activity tritiated catecholamines.

(3) A description of an electrolytic oxidative O-demethylation process for the formation of catecholamines from 3-O-methyl catecholamines.

APPENDIX 1

SEMI-AUTOMATED CATECHOLAMINE ASSAY

INTRODUCTION

A convenient and sensitive assay for catecholamines (CA's) was a major requirement for many of the investigations undertaken for this thesis and for many of the clinical and pharmacological experiments conducted in this laboratory. Because of the relatively large number of samples required for analysis, attention was focused on the use of automated analytical procedures for the assay of CA's. Most of these procedures are based on the fluorometric tri-hydroxyindole (THI) assay first described by Ehrlen (1948) and Lund (1949 a, b, c) and later modified for automatic analysis by Merrills (1962).

Despite the fact that automation of the THI assay represents an improvement over the existing manual assays in reproducibility (Fiorica 1965) its usefulness is limited by the procedures required for the purification of the catecholamines. These procedures can be divided into two classes:

(a) those based on the analysis of CA's in acetic acid eluates from alumina columns, and

(b) those based on hydrochloric acid eluates from cation exchange resins.

However in preliminary experiments it was noted that:

(1) neither chromatographic procedure (and hence automated method) was ideally suited for the analysis of CA's in the three commonly investigated biological samples, viz. urine, tissue and plasma.

(2) the eluates from either chromatographic system were only compatible with the automated procedure designed for that eluate.

(3) both alumina and ion exchange purification procedures when used alone were unsatisfactory for the determination of plasma CA's.

In view of the foregoing, the possibility of increasing the versatility of the standard Technicon Autoanalyzer was explored to permit the routine use of three automated procedures each designed to compliment those chromatographic systems which provide adequate purification of CA's with minimal sample preparation time. This present study describes the modifications employed, the use of the three different procedures, and the results of experiments in which the CA contents of urine, tissue and plasma were determined.

METHODS

Apparatus: In these experiments a Technicon Autoanalyser train was used which utilized the following modules:- sampler (Model II), proportioning pump (Model II) and fluorometer (Model II). The Technicon recorder was replaced with a more versatile multi-range potentiometric recorder (Rikadenki Model B 161). This replacement

required minor alterations to the Rikadenki recorder and these included:

(1) The insertion of transistor emitter follower circuit to match the impedance of the two systems (Fluorometer and Recorder).

(2) Insertion of a 6 pole and 2 way switch on the recorder to permit its use as either a normal or fluorometric recorder.

For the routine analysis of samples the settings on the fluorometer (Sample aperture and Full Scale Record) were set maximally and the sensitivity resulted in an increased level of detectability by permitting measurement of fluorescence in the 0-1 milliVolt (mV) attenuator setting on the recorder. In practice it was found that this control of sensitivity over a wide range of attenuator settings was extremely convenient, particularly with samples having widely divergent fluorescence contents, for it removed the need to alter the fluorometer settings as was required when used in conjunction with the fixed range Technicon recorder.

Automatic analysis: In principle the assay was identical for all three semi-automated procedures and involved fluorometric measurement of the CA contents of acid eluates from either alumina or ion-exchange columns. The autoanalyser performed the following functions:

(1) automatic sampling of unknown and standard solutions of CA's at a sample rate of 20 hr^{-1} (wash: sample 2:1).

(2) neutralization of these solutions to pH 5.0 - 6.0.

(3) oxidation of the CA's with potassium ferricyanide.

(4) formation of the fluorescent THI derivatives by the treatment of the oxidized amines with sodium hydroxide in the presence of a lutine stabilizing agent (ascorbic acid or thioglycollic acid).

(5) estimation of the fluorescence produced by THI derivatives.

The fluorescence was continuously measured at the following wave-lengths: activation 410 nM (Interference filter), emission at >485 nM (Wratten #8 sharp cut filter). The essential difference between the three semi-automated procedures was the use of three different buffering solutions (acetate, phosphate or borate) to achieve neutralisation of the three acid eluates (acetic, hydrochloric and boric acids, respectively). In the experiments where the individual CA contents noradrenaline (NA) and adrenaline (A) were determined the differential lutine stabilization procedure described by Robinson and Watts (1965) was used. The degree of quenching of fluorescence of test solutions was estimated by the internal standardization technique of Crout (1961) and non-oxidized blanks were prepared by the method described by Merrills (1963).

PROCEDURES

1. Urinary catecholamines:

Sample collection. Twelve hour urine samples were collected in plastic containers containing a solution of ascorbic acid (20 mg) in 20 ml of HCl (500 mmol l^{-1}). From each subject two twelve hour samples were collected, one representing a day collection (0900 to 2100 hours), the other a night collection (2100 to 0900 hours). The volumes were measured and a representative aliquot of the collection stored at -10^{o}C.

Batch alumina chromatography: The frozen urine samples were thawed and filtered (Whatman Filter Paper No. 1), and to a 20 ml

aliquot of the filtrate tritiated noradrenaline (^{3}HNA) (25 x 10^3 dpm) was added to permit internal recovery estimates. The sample was added to disposable polypropylene tubes (30 ml capacity) containing activated alumina (700 mg). The alumina was suspended in this solution with continuous nitrogen bubbling, and the pH adjusted to and maintained at 8.4 with Sodium Carbonate (1 mol l^{-1}) for 5 minutes. After this time the nitrogen was discontinued, the alumina particles allowed to settle and the supernatant then discarded. Distilled water (20 mls) at 4^{o}C was added to the tube, bubbled briefly with nitrogen, and the alumina again allowed to settle. The wash solution was discarded and this wash procedure was then repeated. The CA's were eluted from the alumina by the addition of 5 ml acetic acid (300 mmol l^{-1}) and bubbled with nitrogen for 5 minutes. The tubes were centrifuged (5,000 g for 10 minutes) and 4.5 ml of the acetic eluate removed. A 1.0 ml portion of the elutes was taken for estimation of the tritium (^{3}H) contents by liquid scintillation spectrometry using the method described in the general methods chapter (Chapter 2). The recovery of CA's was estimated by comparing the total ^{3}H in the acetic eluate with the amount of ^{3}H added initially to the urine sample.

Fluorescence assay: Samples of acetic eluates were added without pH adjustment to autoanalyser cups and processed at the rate of 20 per hour. The automated procedure is a modification of that described by Fiorica (1965), and is illustrated by the flow diagram showing the reagent concentrations, pump tubing sizes and manifold configuration (Fig. A.1). Standard solutions of NA and A (0.12 - 1.2 µmol l^{-1}) in acetic acid (300 mmol l^{-1}) were included with each batch of urine samples and, after assay, used to construct a calibration curve.

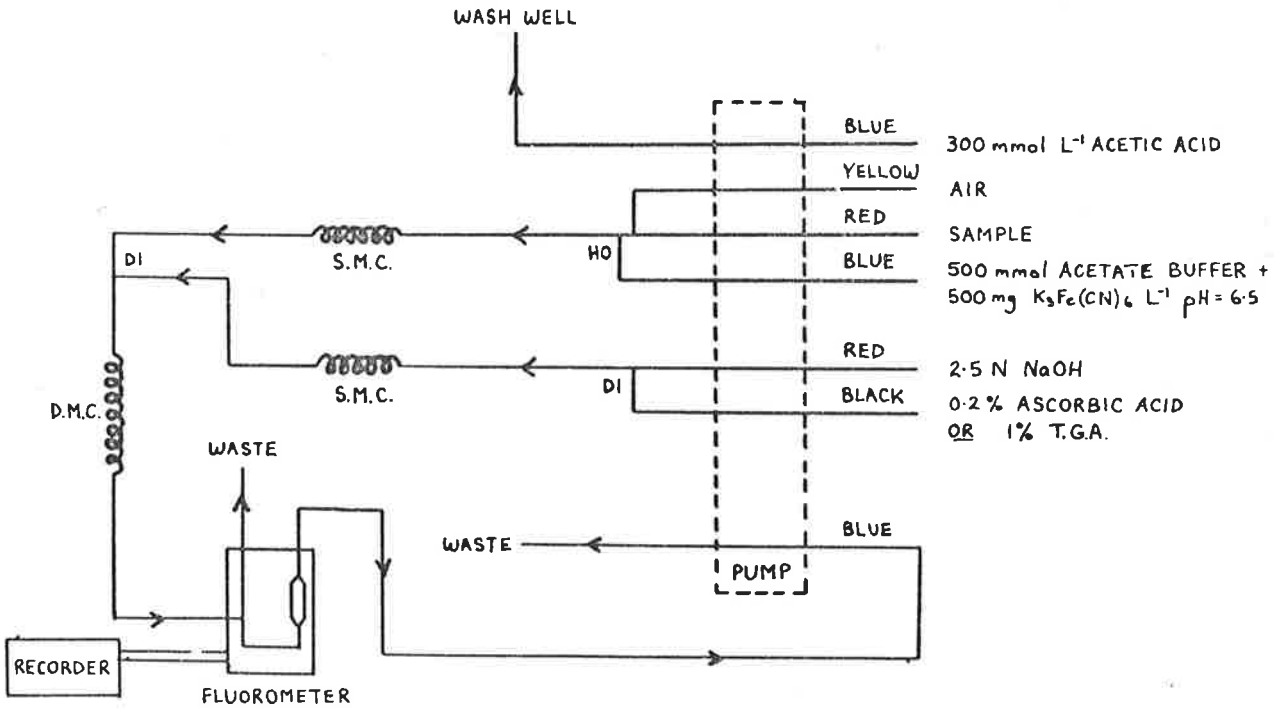


FIG. A.1. Flow diagram for the auto-analyser when used for the assay of urinary catecholamines. The reagents, their concentrations and the colour coding of the proportioning pump tubing are shown. S.M.C. and D.M.C. refer to single mixing coil and double mixing coil, respectively. Samples (standards and test solution all in 300 mmol acetic acid) were processed at 20 determination HR^{-1}. (T.G.A.= Thioglycollic Acid.

The NA contents of samples were estimated by measuring the fluorescence of samples after replacement of the ascorbic acid with thioglycollic acid. The CA contents of the urine (expressed as nmol (24 hours)$^{-1}$) were obtained after correcting for blank value, recovery from alumina and volume of original sample.

2. Tissue catecholamines:

In these experiments tissues (adrenals and vas deferentia) were taken from untreated rats and from rats treated with guanethidine (25 mg Kg^{-1}, i.p. every second day for six weeks - control animals received a corresponding injection of saline). The rats were killed by decapitation and the relevant tissues rapidly excised, and weighed. The tissues were then washed in Krebs-bicarbonate solution and placed in tubes containing 2 mls of EDTA (30 mmol l^{-1}) in HCl (100 mmol l^{-1}). These tissues were not homogenised but left for 24 hours at 4^{o}C and the CA determination made on dilutions of this clear acid extract. The samples were diluted in HCl (500 mmol l^{-1}) to permit measurement on the 0-10 mV scale of the recorder, and their CA contents determined using the manifold arrangement shown in Fig. A.2. This automated procedure is based on the method of Viktora *et al.* (1968). The A and NA contents were estimated separately for the adrenal glands but for the vas deferens the total CA measured and expressed as NA. Estimates of non-oxidized blanks and degree of quenching were performed on representative samples and the CA contents expressed as nmol g^{-1} or μmol g^{-1} after correction for blank and quench values.

3. Plasma catecholamines:

Sample collection and preparation. 25-30 mls blood were taken from an ante-cubital vein of human volunteers with a heparinized

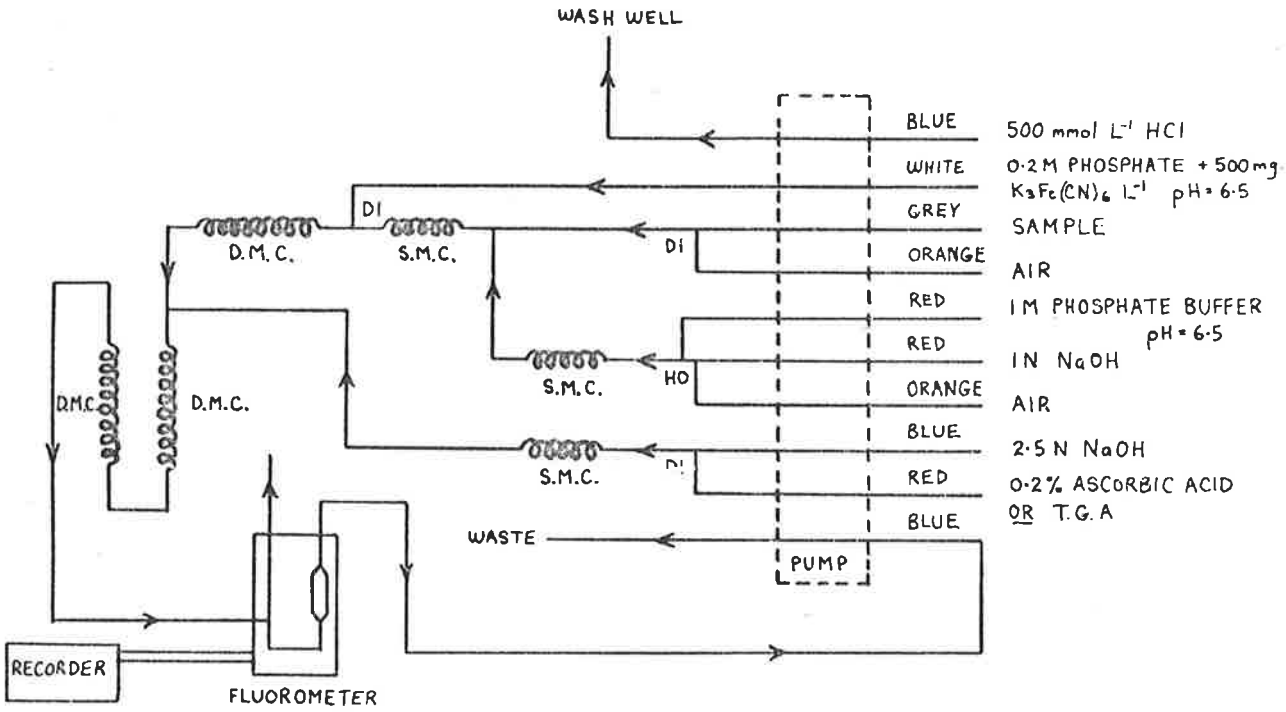


FIG. A.2. Flow diagram for the auto-analyser when used for the assay of tissue catecholamines. The diagramatic notations are the same as described in the legend of Fig.1. Samples (standard solutions of catecholamines and test solutions all in 500 mmol HCl) were processed at 20 determinations HR^{-1}.

disposable syringe. In these experiments the subjects were placed on a horizontal tilt table and blood samples taken after 30 minutes resting in the supine position. The table was then tilted to 60^{o} (feet down) from the horizontal and blood samples taken exactly 10 minutes after tilt was commenced. The blood was added to and mixed gently in chilled centrifuge tubes containing heparin and EDTA (50 mg). The tubes were centrifuged within 10 minutes of collection in a refrigerated ($4^{o}C$) centrifuge, (1,500 g for 20 minutes) and the plasma removed.

The plasma proteins were precipitated by the addition of an amount of perchloric acid (4.0 mol l^{-1}) to give a final concentration of 400 mol l^{-1}. The samples were centrifuged (10,000 g for 30 minutes) and the clear supernatant removed. The protein pellet was mixed with 3.0 mls of perchloric acid (400 mmol l^{-1}) centrifuged as above and this supernatant pooled with the first.

Alumina and amberlite chromatography: The perchloric acid extract containing the CA's was added to a polypropylene tube containing activated alumina (700 mg) and EDTA (100 mg) and treated as described for the isolation of urinary CA's, the only difference being that the CA's were eluted with 9 mls of perchloric acid (50 mmol l^{-1}). The perchloric eluate (7.5 mls) was adjusted to pH 6.0 with Na_2CO_3 (100 mmol l^{-1}) and poured into a glass column containing Amberlite CG-50 200-400 mesh (0.3 mls, Na^{+} form). The columns were washed with distilled water (7.0 mls) and the amines eluted with boric acid (600 mmol l^{-1}) in a volume of 2.0 mls. This eluate was stored at $4^{o}C$ and analysed within two hours.

Fluorescence assay: The CA (NA + A) contents of the borate eluates were determined after assay using the automated procedure schematically outlined in Fig. A.3. For these determinations the fluorescence of the eluates was estimated using the maximum sensitivity setting on the recorder. For each batch of samples a calibration curve from 3.0 to 60 nmol l^{-1} in sodium borate (660 mmol l^{-1}, pH 7.0) was constructed. Recoveries for this procedure were estimated on samples in which known amounts of NA and A (0.06 nmol) were added to plasma and the CA contents determined. Under these conditions of analysis both A + NA contribute to the fluorescence estimated. However, in these experiments the catecholamine contents of plasma samples were derived from the NA calibration curve and expressed as total CA (i.e. A + NA content). This procedure was adopted in view of the minimal contents of A in normal human peripheral plasma samples (O'Hanlon *et al.* 1970) and the difficulties involved in accurate determination of these small A concentrations.

RESULTS

General: With all three semi-automated procedures the fluorescence intensity produced by pure solutions of A and NA was linearly related to the concentration of the CA's. With the modifications described to the Technicon Autoanalyser train there was a linear relationship between the attenuator settings on the recorder and the concentration of both CA's in the range 10.0 nmol l^{-1} to 100 µmol l^{-1}. A typical recording illustrating the responses obtained for a series of NA standards is shown in Fig. A.4.

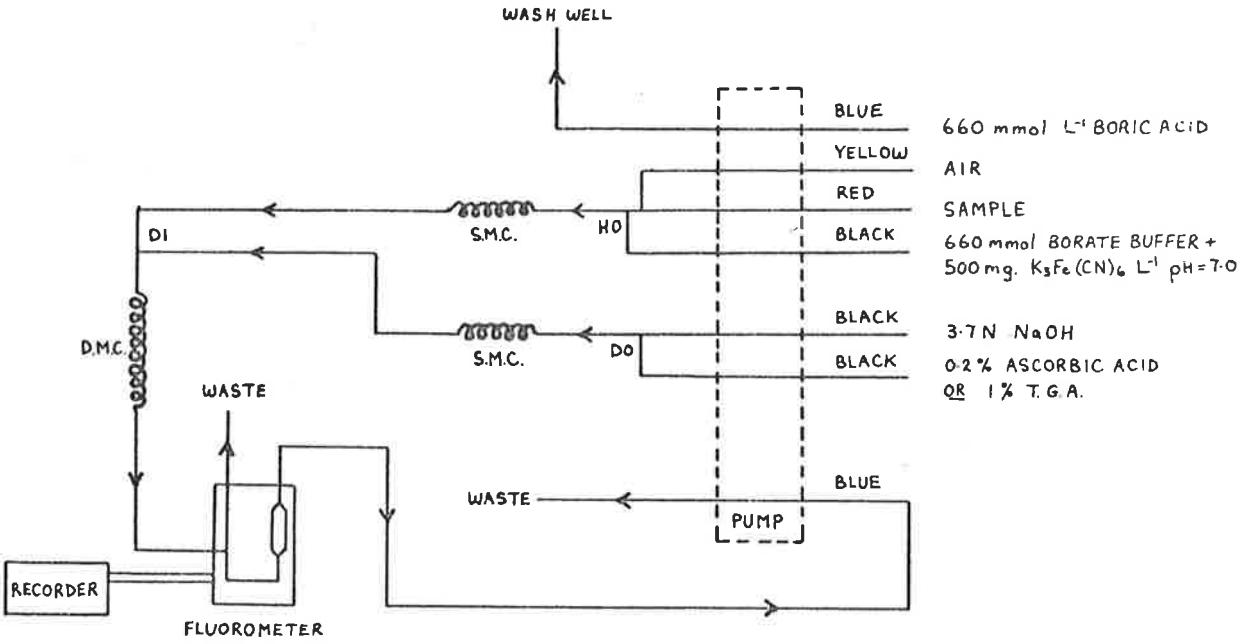


FIG. A.3. Flow diagram for the auto-analyser when used for the assay of plasma catecholamines. The diagramatic notations are the same as described in the legend of Fig. 1. Samples (standards and test solutions all in 660 mmol boric acid) were processed at 20 determinations HR^{-1}.

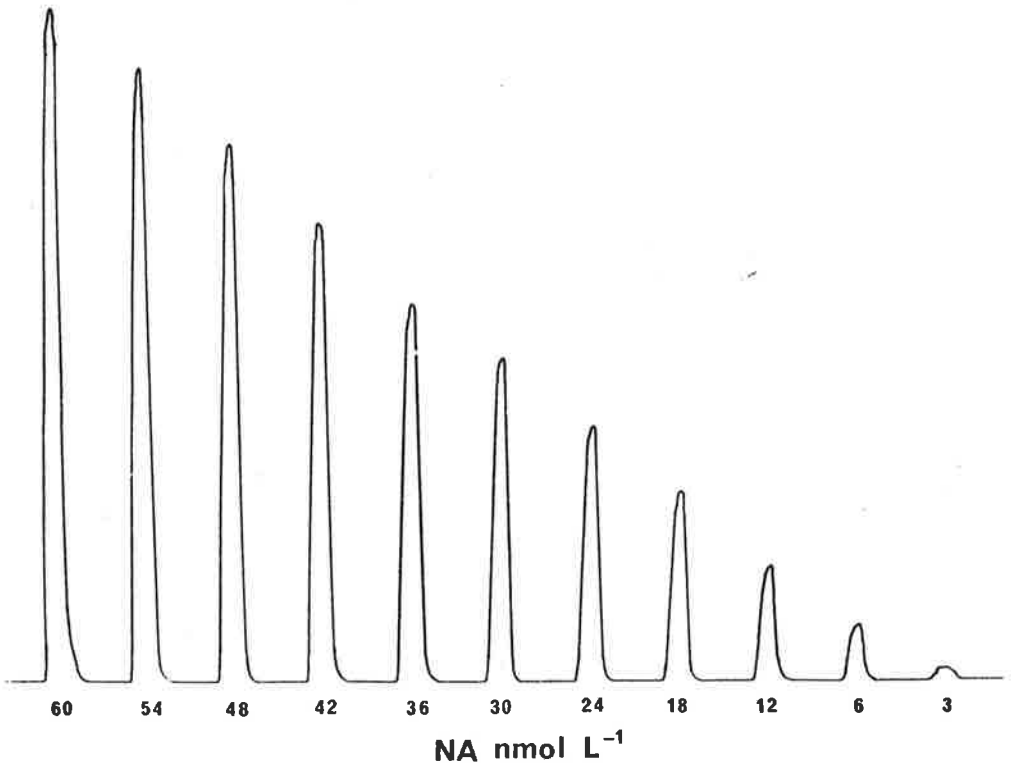


FIG. A.4. Illustrates the fluorescent responses produced by a series of pure noradrenaline (NA) standard solutions in the range of 3.0 to 60 nmol L^{-1} when using the auto-analyser manifold as shown in Fig. 3.

Specificity: The specificity of one of the semi-automated procedures was investigated by determining the contribution of the fluorescence of several analogs of NA and a selection of naturally occurring compounds. Standard solutions of these compounds were assayed directly (without chromatographic purification) using either 0.2% w/v ascorbic acid or 1% w/v thioglycollic acid as a stabilizing agent. The results of these experiments are summarized in Table A.1 where it can be seen that when ascorbic acid was used neither the O-methylated or deaminated metabolites of NA or A contributed significantly to the fluorescence estimate. Of the biochemical precursors of NA only 3,4 dihydroxy phenylalanine (L-DOPA) produced any significant fluorescence (1.9%). The structurally unrelated biogenic amines tested were also without effect. Significantly however, two synthetically prepared compounds, (3,4 dihydroxyphenyl) +2-isopropyl-aminoethanol (Isoprenaline) and 1-a-methyl-3,4 dihydroxy phenylalanine (a-methyl-DOPA) contributed significantly to the fluorescence estimate (85 and 20% respectively).

In contrast when thioglycollic acid was used as the stabilizing agent only NA produced any significant fluorescence and the compounds isoprenaline, a-methyl DOPA and adrenaline produced minimal fluorescence. A typical recording illustrating the fluorescence produced by NA, ISO and A when ascorbic acid was used as the stabilizing agent and the abolishment of this fluorescence for A and ISO with thioglycollic acid is shown in Fig. A.5.

Urine analysis: The CA contents of alumina eluates of urine samples were considerably in excess of the limit of detectability for both NA and A. In addition all samples tested produced minimal

TABLE A.1

Relative fluorescence of Adrenaline (taken as 100%) and of related compounds (all 6.0 μmol l^{-1}) using ascorbic acid and thioglycollic acid stabilisation (TGA)

Compound	Fluorescence Units	
	Ascorbic Acid	TGA
l-Adrenaline	100	< 0.5
dI-Isoprenaline	85	0.5
l-Noradrenaline	60	60
l-a-Methyl-3,4-dihydroxy phenylalanine	20	< 0.5
l-3,4 dihydroxy phenylalanine	1.94	< 0.5
a-Methyl noradrenaline	1.78	1.63
Adrenolone	1.15	< 0.1
3-Hydroxy tyramine	0.84	0.69
dl-Metanephrine	0.18	< 0.2
dl-Normetanephrine	0.1	< 0.2
Methoxy isoprenaline	0.04	< 0.1
3,4-Dihydroxy phenyl glycol	< 0.02	< 0.1
Bis-3-methoxy-4-hydroxy phenyl glycol	< 0.02	< 0.01
4-Hydroxy-3-methoxy mandelic acid	< 0.01	< 0.01
3,4-Dihydroxy mandelic acid	< 0.01	< 0.01
dl-Synephrine	0.06	< 0.01
5-Hydroxy tryptamine	0.03	< 0.01
3-Methoxy tyramine	< 0.01	< 0.01
Tryptamine	< 0.01	< 0.01
Tyramine	< 0.01	< 0.01
Tyrosine	< 0.01	< 0.01
Histamine	< 0.01	< 0.01

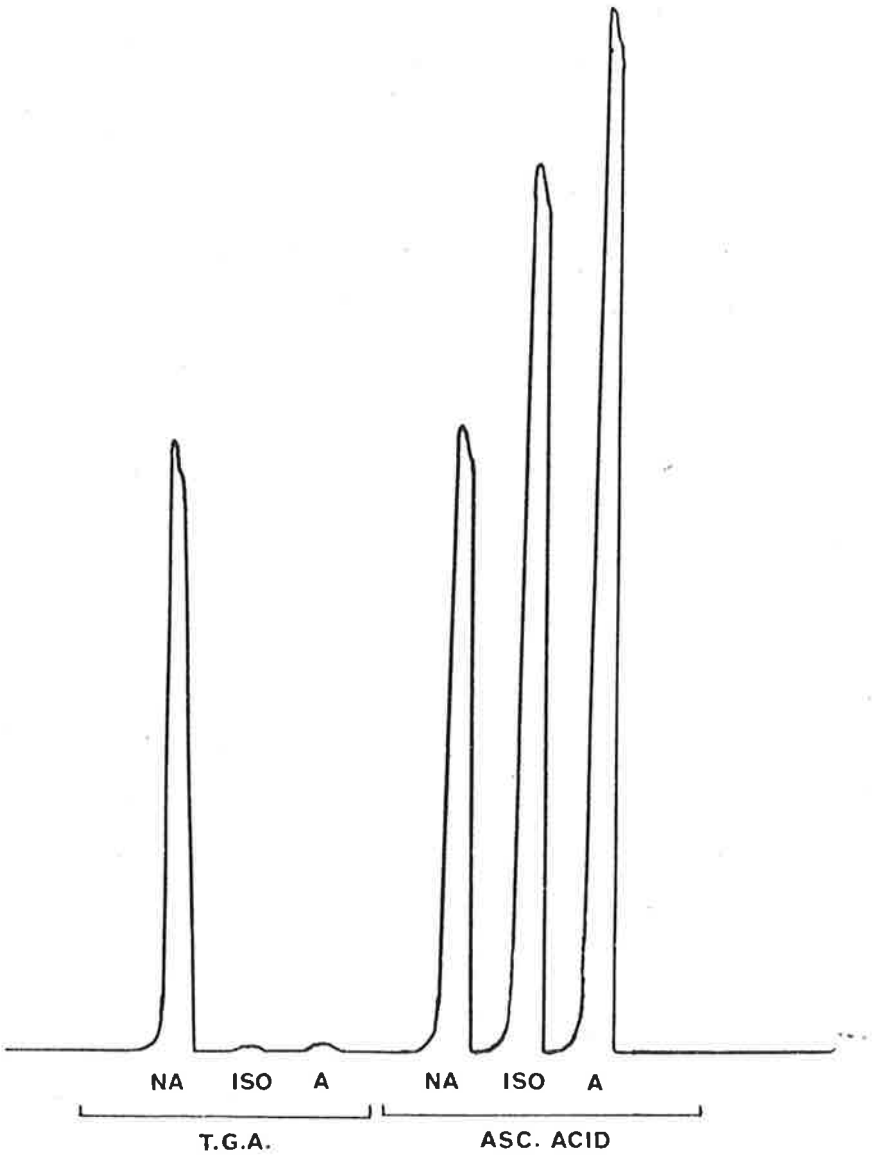


FIG. A.5. Comparison of responses to pure solutions of adrenaline (A), isoprenaline (ISO) and noradrenaline (NA); (all 0.6μmol L^{-1}) using either 0.2% w/v ascorbic acid or 1% w/v thioglycollic acid (T.G.A.) on the auto-analyser manifold shown in Fig. 2.

non-oxidized readings (< 5% of the oxidized samples fluorescent reading). For a series of 18 normal subjects the mean (± SE) excretion of NA was 112.36 ± 7.8 nmol $(24\ hours)^{-1}$ and the corresponding A excretion 26.46 ± 5.86 nmol $(24\ hours)^{-1}$. These values are similar in both CA content and the ratio of NA to A, to those reported previously for normal subjects (176.47 ± 76.4 nmol NA $(24\ hours)^{-1}$, 30.4 ± 16.8 nmol A $(24\ hours)^{-1}$ Crout (1961)). The diurnal excretion of NA and A was determined on these samples and it was found that the mean (± SE) rate of NA excretion during the night period (52.47 ± 4.98 pmol min^{-1}, 2100 - 0900 hours) was significantly smaller ($p < 0.001$) than that excreted during the day period (103.53 ± 7.92 pmol min^{-1}, 0900 - 2100 hours). A similar pattern was observed for A where the mean (± SE) rate of excreted A during the night period (10.7 ± 3.09 pmol min^{-1}, 2100 - 0900 hours) was also significantly ($p < 0.0025$) smaller than the corresponding day value (26.0 ± 6.04 pmol min^{-1}, 0900 - 2100 hours). These findings agree well with the values originally reported (Von Euler *et al.* 1955) for the diurnal CA excretion in man. In that study the excretion rates for NA (57.64 pmol min^{-1}, 2300 - 0800 hours, 135.29 pmol min^{-1}, 0800 - 2300 hours) and A (6.52 pmol min^{-1}, 2300 - 0800 hours, 25.0 pmol min^{-1}, 0800 - 2300 hours) were determined by biological assay. The mean recovery of the internal standard (^{3}HNA) in this series of experiments was 82%.

Tissue analysis: In this study the CA contents of rat adrenals and vasa deferentia were estimated after direct THI assay of acid extracts of these tissues. For both tissues the values found by this procedure agreed well with those reported in other studies where

chromatographic purification of tissue extracts preceded THI assay.

Using this method the NA content of untreated rat vas deferens was found to be 50.1 ± 10.7 nmol g^{-1} and the A and NA contents of the adrenals 2.20 ± 0.30 μmol g^{-1} and 0.50 ± 0.067 μmol g^{-1}, respectively.

The NA contents of vas deferens from rats treated with guanethidine showed a marked decrease (86%) to 6.71 ± 1.13 nmol g^{-1}. In contrast the A and NA contents of the adrenals of guanethidine treated rats were 1.63 ± 0.24 μmol g^{-1} and 1.03 ± 0.15 μmol g^{-1}, respectively.

Although these results showed no change in the total CA content of the adrenals of treated and untreated animals (i.e. 2.66 ± 0.39 μmol g^{-1} and 2.69 ± 0.36 μmol g^{-1}, respectively) a change in the individual A and NA contents was found. These findings are in accord with the reported properties of this drug viz. guanethidine treatment results in degeneration of the adrenergic neurons of the rat vas deferens but is without effect on chromaffin cells (Evans *et al.* 1972). Although direct analysis of the acid extracts of these tissues produced non-oxidized blanks that had higher values than the reagent blank, (i.e. HCl, EDTA diluted in HCl 500 mmol l^{-1}) the quenching associated with these test solutions was minimal (<7%).

Plasma analysis: The CA contents of plasma samples were determined by THI fluorometric assay of their corresponding boric acid eluates. The fluorescence produced by these purified plasma extracts in this series of experiments ranged from being equivalent to the lower limit to detectability (3.0 nmol NA l^{-1}) to thirteen

times this value (39.0 nmol NA l^{-1}). The average recovery of NA added to plasma (external standard) was 74%.

The peripheral venous plasma CA (A + NA) contents of 18 normotensive male subjects who had rested in the supine position before sampling was determined. The mean (±SE) plasma CA contents of these samples was equivalent to 1.60 ± 0.31 nmol NA l^{-1}. This value is within the range quoted for similar studies in which the CA contents were determined fluorometrically (1.48 nmol l^{-1}, O'Hanlon *et al.* 1970, 8.32 nmol l^{-1}, Anton and Sayre, 1962) and similar to those where the contents were determined radio-chemically (1.5 ± 0.27 nmol l^{-1}, Engelman *et al.* 1968). Samples taken from the same subjects ten minutes after they were tilted at 60^{o} (feet down) from the horizontal position showed a significant ($p < 0.001$) increase in peripheral plasma CA content, and the mean (±SE) values being 3.67 ± 0.69 nmol l^{-1}. This increase (2.3 fold) compares favourably with the 2.1 fold increase seen in a similar study by Fluck and Salter (1973).

DISCUSSION

In view of the fact that there is no reported automated method for the purification of catecholamines from biological extracts the only advantages offered by the semi-automated methods described for the assay of catecholamines relate to the tri-hydroxyindole determination. These advantages have been well documented (Merrills 1963, Fiorica 1965, Robinson and Watts 1965) and relate primarily to the increased reproducibility associated with automation of this assay. In contrast there has been limited investigation into the suitability of the purification procedures used in these studies and as such many of the

methods described are either too cumbersome or lack the specificity required for accurate determinations. In this study we have modified the standard Technicon Autoanalyser and this has permitted the use of purification procedures which are more ideally suited to the analysis of catecholamines in urine, tissue and plasma. To compliment these purification techniques three semi-automated systems were used and although this approach necessitated the use of three different autoanalyser manifolds it was found in practice to be relatively simple. This was due to the fact that using the conditions described, the fluorometer settings, filter combinations and sample speed were the same for all three assays and it was found that the change over time from one procedure to another took less than ten minutes. One important consideration in the design of these procedures was that for all three assays the acid extracts of eluates were added directly to the sampler tray and the neutralization of these samples processed automatically by the Technicon Autoanalyser. This step further simplified the assays and minimized the possibility of loss of CA's associated with either the manual neutralization of these acidic solutions or during the storage of samples on the sampler tray prior to assay. For the analysis of urinary catecholamines the combination of the alumina batch procedure previously described (de la Lande *et al.* 1967) with the minor modifications to the automated procedure of Fiorica (1965) proved extremely convenient. This batch analysis can be performed more rapidly and with less preparation than the existing alumina column or ion exchange techniques.

Although accurate estimations of CA's in test solutions usually require samples free from interfering material (Crout 1961) the use of the automated procedure of Viktora *et al.* (1968) enabled us to explore the possibility of direct analysis of the CA contents of tissue extracts without chromatographic purification.

This concept was based on (1) the highly specific nature of this assay and in particular the lack of interference of the precursors and metabolites of NA and other biogenic amines; (2) the previous findings from this study (Chapter 4) which indicated that tritiated noradrenaline (^{3}HNA) that is accumulated in sympathetically innervated tissues after incubation with ^{3}HNA can be quantitatively extracted by placing the tissue in small amounts of weak HCl. This procedure does not require homogenisation and the final extract is recovered free from the bulk of tissue. Although these studies were confined to estimates of the NA content of rat adrenals and vasa deferentia it is possible that this extremely simple procedure may provide an extremely rapid screening technique for determining the catecholamine content of selected tissues: the principle being that for those tissues where this method indicates a change in the NA content of the tissue the remainder of the acid extract may be purified by ion exchange column chromatography and a more accurate determination made. In contrast to the determination of the CA contents of tissues and urine the minimal amounts of NA and A present in peripheral venous plasma require the use of more exacting purification procedures. It was found that the semi-automated procedures of Fiorica (1965) and Viktora (1968) were of limited value in these studies. In both of these methods, the presence of interfering

material in column eluates and the dilution of the CA's associated with the complete elution of these amines from the columns made accurate determinations difficult. However, the development of an automated assay based on the use of sodium borate has permitted the use of the highly specific separative procedure described by Renzini *et al.* (1970). This latter technique overcomes the problems associated with the existing column procedures by producing a highly purified and concentrated eluate.

Finally, the accuracy of these procedures was determined by comparing the values obtained from samples of urine, tissue and plasma with those reported for similar studies using different techniques. These techniques were examined for their ability to show changes in the CA contents of test solutions using physiological or pharmacological regimes known to alter the concentrations of CA's in test samples. In all cases the values found in this study were within the range of values quoted for similar studies using either fluorometric or radio-chemical techniques.

APPENDIX 2

THE PURITY AND STABILITY OF TRITIATED CATECHOLAMINES

INTRODUCTION

The experiments to be described in this appendix relate to the purity of the high specific activity tritiated catecholamines that were used in the studies described earlier in this thesis.

There was strong evidence supporting the view that manufacturer's solutions of ^{3}H catecholamines were not pure and that these labelled compounds deteriorated upon storage. This evidence was based on the following:

(1) previous findings from this laboratory had indicated that the manufacturer's solutions of ^{3}H dl NA contained ^{3}H material other than NA and that the NA contents of these solutions were less than those stated (Head and de la Lande 1973).

(2) both manufacturers and users of high specific activity tritium labelled compounds had acknowledged the fact that these labelled compounds are more unstable than their non-labelled counterparts (Bayly and Evans 1968).

As regards the latter, little information was available at the time this study was commenced concerning the most suitable conditions of storage of these compounds and the type of

procedures required to remove ^{3}H impurities from these samples.

A more disturbing feature of this type of instability was shown by Persson and Waldeck (1970), who provided evidence suggesting the existence of aromatic ring hydroxylated derivative of ^{3}HDOPA in one manufacturer's stock solutions of this compound. This observation was in accord with the well documented process of hydroxyl radical formation that occurs with some high specific activity tritiated compounds (Bayly and Evans 1968). It became apparent that in addition to the now widely accepted oxidative susceptibility of catecholamines (i.e. autoxidation), additional decomposition of these amines was possible due to self radiolysis. The products of autoxidation and self radiolysis may be ^{3}H labelled and structurally related to compounds which participate in, modify, or impair adrenergic function (i.e. the products of self radiolysis are hydroxyl substituted catecholamines and those of autoxidation are indolamines).

The experimental approach adopted for this study was as follows:

(1) to estimate the NA and ^{3}H contents of manufacturer's stock solutions of ^{3}H catecholamines on arrival and to determine the optimal conditions of storage of these compounds.

(2) to examine the chromatographic behaviour of selected samples of ^{3}H catecholamines with the view to developing a purification procedure.

(3) to examine the affinity of ^{3}H labelled impurities for the neuronal uptake system.

As this investigation proceeded it became apparent that the conditions of storing ^{3}H catecholamines was an important factor in determining the stability of these compounds. Consequently, in conjunction with the manufacturer, certain samples of ^{3}HNA were prepared and stored under conditions different from those routinely used for commercial samples. Included in this study is an evaluation of the use of these different storage procedures.

METHODS

Catecholamine Assays

Chemical Assay: The catecholamine (NA and ISO) contents of samples were assayed using two semi-automated procedures, both of which have been described in the previous appendix. The semi-automated procedure adapted for the estimation of CA's in HCl solutions was used to determine:

(1) the NA and ISO contents of manufacturer's solutions of ^{3}HNA and $^{3}HISO$.

(2) the NA contents of solutions of ^{3}H dl NA stored for various periods of time in the laboratory.

(3) the THI fluorescence of fractions from Dowex 50 (Na^{+}) columns.

The semi-automated procedure adapted for the estimation of CA's in boric acid solutions was used to measure the THI fluorescence associated with fractions from Amberlite IRC-50 columns.

In experiments where the contents of CA's in manufacturer's solutions of dl^{3}HNA solutions stored in the laboratory were determined,

these estimates were performed in duplicate. It was assumed for this study (unless otherwise stated in the text) that the estimates of catecholamine contents by the manufacturer and those determined in this laboratory were both correct. On this basis the term "*percentage purity*" was used to describe these solutions of ^{3}H catecholamines, and represents the ratio of the catecholamine contents found by assay in this laboratory to those quoted by the manufacturer expressed as a percentage. Estimates of fluorescent quenching and blank determinations for test solutions were made using the procedures described in the methods section of the previous appendix.

Biological assay: For selected batches of manufacturer's solutions of ^{3}HNA, their NA contents were determined by biological assay using the isolated perfused rabbit ear preparation of de la Lande and Harvey (1965). Briefly this involved measurement of the change in perfusion pressure that occurred when solutions of NA or test solutions containing NA were injected into the perfusion stream bathing these arteries. For these assays aliquots of batches of ^{3}HNA were diluted (usually 1:100) in saline solution containing ascorbic acid (290 μmol l^{-1}) and injected into the perfusion stream in a volume not exceeding 0.3 ml. To determine the contents of lNA and dlNA the changes in perfusion pressure produced by ^{3}HCA's were compared to those of non-labelled l and dlNA.

Specific Activity Determinations:

The ^{3}H contents of manufacturer's stock solutions of ^{3}HCA's were determined by the liquid scintillation spectrometric procedure

described in the general methods (Chapter 2). Aliquots (0.10 ml) of the manufacturer's solutions and aliquots (0.1 ml) of the samples that were diluted for CA assays, were analysed for their ^{3}H contents and the values obtained, together with the estimates of their CA contents, used to calculate the specific activity of ^{3}HCA's in these solutions.

Storage of Samples of dl^{3}HNA:

The effect of different conditions of storage in the stability of NA was examined. A diagrammatic representation summarising these conditions is shown in Fig. A.6 and these conditions comprised:

(1) storage of dl^{3}HNA (0.06, 0.6 and 6.0 μmol l^{-1}) in glass vials at -10^{o}C and +4^{o}C.

(2) storage of dl^{3}HNA (0.6 μmol l^{-1}) at -10^{o}C and +4^{o}C in polypropylene tubes, to which were added solutions of ethanol and 3,4-dihydroxy phenyl acetic acid (DOPAC) to give a final concentration of 8.7 mol l^{-1} and 59 μmol l^{-1} respectively.

Additional notes:

Samples stored at -10^{o}C were not frozen with a freezing solution (e.g. acetone-dry ice mixture), but added directly to the freezer cabinet at the commencement of the study. For each particular storage regime, solutions of ^{3}dlHNA were added to multiple glass vials and polypropylene tubes. When subsequent determinations were performed on these solutions duplicate vials were removed from the freezer cabinet (-10^{o}C) thus ensuring that each of the samples frozen was only thawed once for these studies.

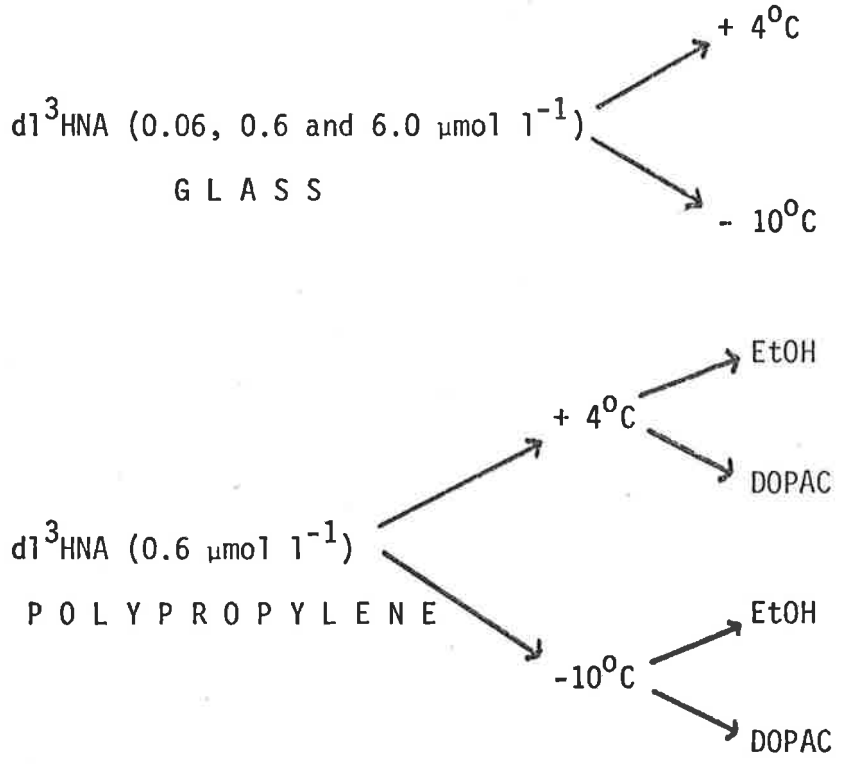


Fig. A.6 Diagrammatic summary of the conditions of storage of solutions of dl-3HNA.

Two different storage temperatures (-10°C and +4°C) were chosen because Bayly and Evans (1968) showed that the temperature at which ^{3}H compounds of high specific activity are stored is an important consideration in the stability of these compounds. These authors also showed that EtOH and aromatic alcohols retarded the deterioration of certain ^{3}H labelled compounds of high specific activity. DOPAC was chosen because it was a catechol acid and as such could be easily separated, if required, from dl^{3}HNA by either solvent extraction or ion exchange chromatography.

Chromatographic Procedures:

(1) Alumina: The procedures used in these experiments were based on those described earlier for the purification of ^{3}HCA's (Chapter 3) the only modification being the replacement of acetic acid with perchloric acid (50 mmol l^{-1}) for the elution of catechols from the alumina. To these perchloric eluates ascorbic acid was added to give a final concentration of 290 µmol l^{-1}.

(2) Dowex 50 (Na^{+}): The perchloric eluates from the alumina procedure were adjusted to pH 6.5 (with Na_2CO_3 200 mmol l^{-1}) and chromatographed on columns of Dowex 50 (Na^{+}) using the procedure described previously (Chapter 4).

(3) Amberlite IRC-50: Solutions of ^{3}HdlNA containing ascorbic acid (290 µmol l^{-1}) at pH 6.5 were chromatographed on columns (1 x 10 cm) of Amberlite IRC-50 in a similar fashion to that described for the purification of plasma catecholamines in the previous appendix. The only difference was that the columns were eluted in three successive concentrations of boric acid (0.066, 0.13 and 0.66 mmol l^{-1}).

(4) Paper chromatography: ^{3}HCA's were separated from other ^{3}H compounds present in test solutions by descending chromatography on cellulose phosphate paper (Whatman P.81). This procedure was described in detail in Chapter 4.

(5) Thin layer chromatography: In these experiments the thin layer chromatographic procedure routinely used by the manufacturers was employed. Briefly, this involved application of small volumes (0.02 ml) of test solutions together with non labelled NA (100 nmoles) to the origins of silica gel plates. These plates were chromatographed in the solvent system, n-butanol : acetic acid : water (12 : 3 : 5) air dried and the migration of non labelled NA on these plates determined after visualisation under UV light. The silica gel on the thin layer plates was scraped into tubes and the gel eluted overnight in HCl (300 mmol l^{-1}). The ^{3}H contents of these solutions were then determined.

(6) Radiochemical purity: The paper chromatographic procedures were used to obtain evidence for an association of ^{3}H with NA. The term "*Radiochemical purity*" refers to the proportion of the total ^{3}H that co-chromatographed with authentic NA in these systems.

Tissue Experiments:

Treatments: 4 rabbits and 4 rats were injected with reserpine (i.p., 2.5 mg Kg^{-1}) 24 hours before removal of the rabbit ear arteries and rat vas deferentia. 2 rabbits and 3 rats injected with saline (1.0 ml i.p.) served as controls. The effectiveness of reserpine treatment for these tissues was assessed by the fluorescence histochemical procedure (described in the general methods, Chapter 2)

and by THI assay of the endogenous NA contents of vas deferentia (using the procedure outlined in Chapter 5 for the estimation of NA contents of ear arteries). For all of these assessments a segment of ear artery was taken from each rabbit and a segment of vas deferens was taken from each rat.

Incubation: 4 segments of rabbit ear arteries (ranging from 3.0 to 7.0 mg of tissue) were individually incubated in Krebs solution containing Nialamide (350 μmol l^{-1}) for 45 minutes. They were incubated for a further 15 minutes in Krebs solution containing U0521 (55 μmol l^{-1}), then transferred to Krebs solutions (1.0 ml) containing dl^{3}HNA (0.6 μmol l^{-1}) and U0521 (55 μmol l^{-1}). For two arteries DOCA (25 μmol l^{-1}) was present in solution throughout the period of incubation. After incubation all arteries were extracted overnight in solutions of HCl (100 mmol l^{-1}) containing EDTA (21.5 μmol l^{-1}). The procedure for this has been described in detail in Chapter 4. 4 vas deferentia were treated similarly except that DOCA (27 μmol l^{-1}) was present in *all* of the incubation solutions after treatment of the tissues with nialamide. Two of these vas deferentia (combined weight of 190 mg) were added to incubation solutions (10.0 ml) containing ^{3}HNA (0.6 μmol l^{-1}) and U0521 (55 μmol l^{-1}). These vas deferentia were incubated in this solution for 45 minutes, removed, and replaced with the remaining two vas deferentia which were also incubated for 45 minutes. Both groups of vas deferentia were extracted overnight in 10 mls of HCl 300 mmol l^{-1}. In these experiments solutions of incubates and tissue acid extracts (concentrated using the lypholysation procedure described in Chapter 4) were chromatographed

on ion exchange paper. In addition the individual acid extracts of arteries and a pooled extract of vas deferentia were assayed for their NA contents (using the method described in Chapter 5) and the specific activity of ^{3}HNA in these solutions determined.

RESULTS

The purity of Manufacturer's solutions of ^{3}H:

Catecholamines: The CA and ^{3}H contents of 19 batches of ^{3}HCA's were determined immediately after the arrival of these solutions in the laboratory, (aqueous solutions containing ^{3}HCA's arrived at ambient temperature in glass containers which were sealed under nitrogen gas). The percentage purity of CA's and the ^{3}H contents of these solutions are shown in Table A.2 where it can be seen that for all but two batches (^{3}HISO samples 2 and 4) the amine contents were less than quoted. In contrast the ^{3}H contents of all samples analysed were very similar to those quoted. It can also be seen from Table A.2 that the percentage purities of ^{3}HISO were generally much greater than those for either dl or l^{3}HNA. Surprisingly the quoted specific activities for all batches of ^{3}HISO were identical (10 Ci $(mmol)^{-1}$) whereas the specific activities quoted for both dl^{3}HNA and l^{3}HNA ranged from 6.1 to 13.0 Ci$(mmol)^{-1}$ and 4.1 to 10.3 Ci $(mmol)^{-1}$ respectively. It was thought that a relationship may have existed between the quoted specific activities and the percentage purities of NA for these solutions. However there was no obvious correlation between the specific activities of either dl or l^{3}HNA and the percentage purities of these amines.

Footnotes:

(i) (a) The ratio of the catecholamine contents found by assay (chemical or biological) to those quoted by the manufacturer, expressed as a percentage.

(b) for these samples the ^{3}H contents were very similar or identical to those quoted by the manufacturer.

(ii) all samples arrived in the laboratory as aqueous solutions in glass containers at ambient temperature and assayed as soon as practicable after their arrival.

TABLE A.2

CA contents of stock solutions of ^{3}HCA's

Laboratory number	Quoted Specific Activity (Ci (mmol)-1)	Quoted ^{3}H content (m Ci ml^{-1})	Percentage Purity[a] Chemical Assay (%)	Biological Assay (%)
dl^{3}HNA				
1	12.0	1.0	12.2	9
2	7.7	1.0[b]	10.5	9
3	13.0	1.0[b]	42.1	36
4	12.0	1.0[b]	15.0	8
5	6.1	2.0	52.5	-
6	11.6	1.0	20.0	-
7	9.6	1.0[b]	55.0	-
8	12.2	1.0	40.0	-
l^{3}HNA				
1	4.1	1.0[b]	5.3	6
2	8.1	1.0[b]	65.0	-
3	10.3	1.0	65.0	-
4	8.9	1.0	60.0	-
dl^{3}HISO				
1	10.0	1.0	71.0	-
2	10.0	1.0	100.0	-
3	10.0	1.0	86.4	-
4	10.0	1.0[b]	100.0	-
5	10.0	1.0[b]	71.0	-
6	10.0	1.0[b]	98.0	-
7	10.0	1.0[b]	80.0	-

For selected samples of ^{3}HNA (Table A.2; dl^{3}HNA samples 1, 2, 3 and 4, l^{3}HNA sample 1) the NA contents were determined by chemical and biological assay. The results shown in Table A.2 indicated that the NA contents were less than those quoted by the manufacturer and the individual percentage purities agree closely with those based on chemical (THI) assay.

It was concluded that these differences in the contents of NA reflected either: (i) a decrease in the amine contents of samples after the CA's had left the manufacturer, or (ii) a discrepancy between the contents determined in this laboratory and those determined by the manufacturer.

The effect of different storage conditions on the stability of ^{3}HNA: Aliquots of one batch of dl^{3}HNA (sample 5, Table A.2) were diluted in HCl (10 mmol l^{-1}) and stored under a variety of conditions (outlined in the methods section). The NA contents of these solutions were determined by chemical (THI) assay at intervals during a period of 0 to 15 months of storage and the results of these experiments are summarised in Table A.3 and Fig. A.7.

The most striking feature of these results was that the storage of dl^{3}HNA at -10^{o}C in glass containers led to drastic decreases in the NA contents. These decreases were related to the time of storage and were observed for all 3 concentrations of ^{3}HNA studied (Table A.3). Samples stored at 4^{o}C in glass vials also exhibited a time dependent decrease in NA contents. In contrast storage of dl^{3}HNA in polypropylene tubes at either -10^{o}C or +4^{o}C resulted in much smaller decreases in NA contents, and the addition of either ethanol or DOPAC to samples of dl^{3}HNA stored in polypropylene tubes serves to reduce even further this rate of decline in NA contents (Fig. A.7).

TABLE A.3

The effect of storage temperature on the stability of of ^{3}HCA's

dl ^{3}HNA nmolL^{-1}	Storage Temperature (%)	Months of Storage			
		3	4	7	14
6.0	+4	97%	100%	77%	61%
	-10	100%	96%	58%	22%
0.6	+4	90%	54%	13%	< 1%
	-10	23%	2.2%	1.3%	< 1%
0.06	+4	94%	87%	40%	< 1%
	-10	25%	12%	13%	< 1%

Footnotes:

(i) Values represent the ratio of the NA contents determined after 3, 4, 7 or 14 months of storage to the contents determined immediately before storage, expressed as a percentage.

(ii) all samples were stored in glass vials.

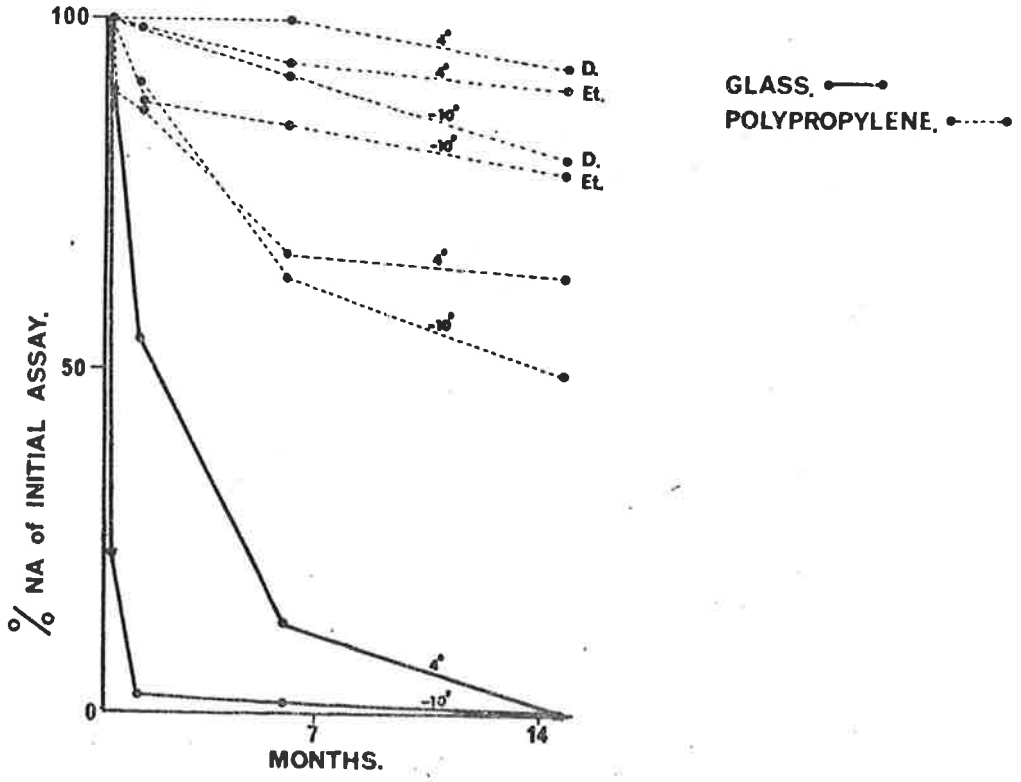


FIG. A.7 The rate of decline in the NA contents of solutions of ^{3}HdlNA (0.6μmol L^{-1}) stored under different conditions. Samples stored frozen (-10^{o}C) or as aqueous solutions (+4^{o}C) in glass vials (——) or plastic tubes (----), and in plastic tubes containing EtOH (8.7 mol L^{-1}, -----Et) or DOPAC (59μmolL^{-1}, -----D). Ordinate: the ratio of the NA contents found after storage to the contents determined before storage, expressed as a percentage. Abiscissa: Storage time in months.

In conjunction with the manufacturer 3 special batches of dl ^{3}HNA were prepared and these samples sent to this laboratory in polypropylene containers. Most of these solutions were acidified with hydrochloric acid (final concentration 100 mmol l^{-1}) before packaging and sent either at ambient temperature or frozen. Samples of dl ^{3}HNA not treated in this fashion (i.e. aqueous solutions in glass containers) sent from the manufacturer at the same time served as controls. The NA and ^{3}H contents of all samples were determined immediately after their arrival in the laboratory. The percentage purity and conditions of storage for each batch of dl ^{3}HNA are summarized in Table A.4. The results were interpreted as follows:

(1) samples that arrived in glass containers at ambient temperature had NA contents much smaller than the quoted values.

(2) samples that arrived frozen, acidified and in polypropylene tubes had NA contents that agreed more closely with the quoted values.

Despite the limited number of samples available for investigation it was possible to draw at least one conclusion from these results; viz. that deterioration of ^{3}HNA occurred for samples sent under routine conditions during transit from the manufacturer to the laboratory.

Chromatography: In the following experiments further evidence was sought regarding the possible deterioration of samples of ^{3}HNA. For these experiments solutions containing ^{3}HNA were analysed by column and paper chromatographic procedures with the view to isolate a fraction of ^{3}H material other than ^{3}HNA from these solutions.

(a) Column chromatography: For this experiment, portion of a batch of dl ^{3}HNA (sample 3, Table A.2) which was shown to contain NA

TABLE A. 4

CA contents of stock solutions of ^{3}HdlNA stored during transit from the manufacturer to the laboratory under a variety of different conditions.

Laboratory number	Quoted Specific Activity (Ci(mmol)$^{-1}$)	Storage conditions during transit	Percentage Purity [a] (Chemical assay %)
1	12.2	aqueous, glass	22
2	20.0	frozen, glass	23
3	17.5	aqueous (HCl) poly p.	43
4	17.5	aqueous (HCl) poly p.	69
5	20.0	aqueous, glass	1
6	9.7	frozen (HCl) poly p.	90
7	9.7	frozen (HCl) poly p.	76
8	9.7	frozen (HCl) poly p.	78
9	9.7	frozen (HCl) poly p.	68
10	9.7	frozen (HCl) poly p.	68
11	9.7	frozen (HCl) poly p.	70
12	8.0	aqueous, poly p.	22
13	8.0	frozen, poly p.	22
14	8.0	frozen (HCl) poly p.	71
15	18.5	aqueous, glass	45

Footnotes:

(i) (a) the ratio of the catecholamine contents found by assay (chemical) to those quoted by the manufacturer, expressed as a percentage.

(ii) Samples arrived in the laboratory as aqueous solutions (ambient temperature) or frozen (dry ice (CO_2) container) in glass or polypropylene (poly p.) tubes and assayed as soon as practicable after their arrival.

contents (by chemical assay and biological assay) smaller than that quoted by the manufacturer was chromatographed on alumina followed by chromatography on Dowex 50 (Na^+). The distribution of ^3H into the various fractions associated with these procedures were determined and the results of this analysis illustrated in Figs. A.8 and A.9. These results showed that after chromatography of the solution of dl^{3}HNA on alumina at pH 8.4, approximately 90% of the ^3H originally present in these solutions was bound to the alumina and subsequently eluted with the acid eluate (Fig. A.8). When this eluate was adjusted to pH 6.5 and passed over a column of Dowex 50 (Na^+) the bulk of the ^3H (> 90%) was bound to the resin (Fig. A.9). When this column was washed with hydrochloric acid the fractions containing ^3H also exhibited specific THI fluorescence. It was apparent from the experiments that there was no significant separation of ^3H impurities from ^{3}HNA with these two procedures.

In contrast when an aliquot of this same batch of ^{3}HNA was passed over a column containing a weak cation exchange resin (Amberlite IRC-50) a significant proportion (15%) of the total ^3H applied did not bind to this resin. This effluent fraction failed to exhibit specific THI fluorescence and when this column was eluted with increasing concentrations of boric acid a further two peaks of radioactivity, one of which showed specific THI fluorescence were obtained (Fig. A.10). Despite the fact that this procedure showed the presence of two fractions of ^3H not attributable to ^{3}HNA the sum of the ^3H in these fractions represented only 39% of the total ^3H applied to this column. This value approached, but did not achieve, the percentage difference (58%) between the NA contents

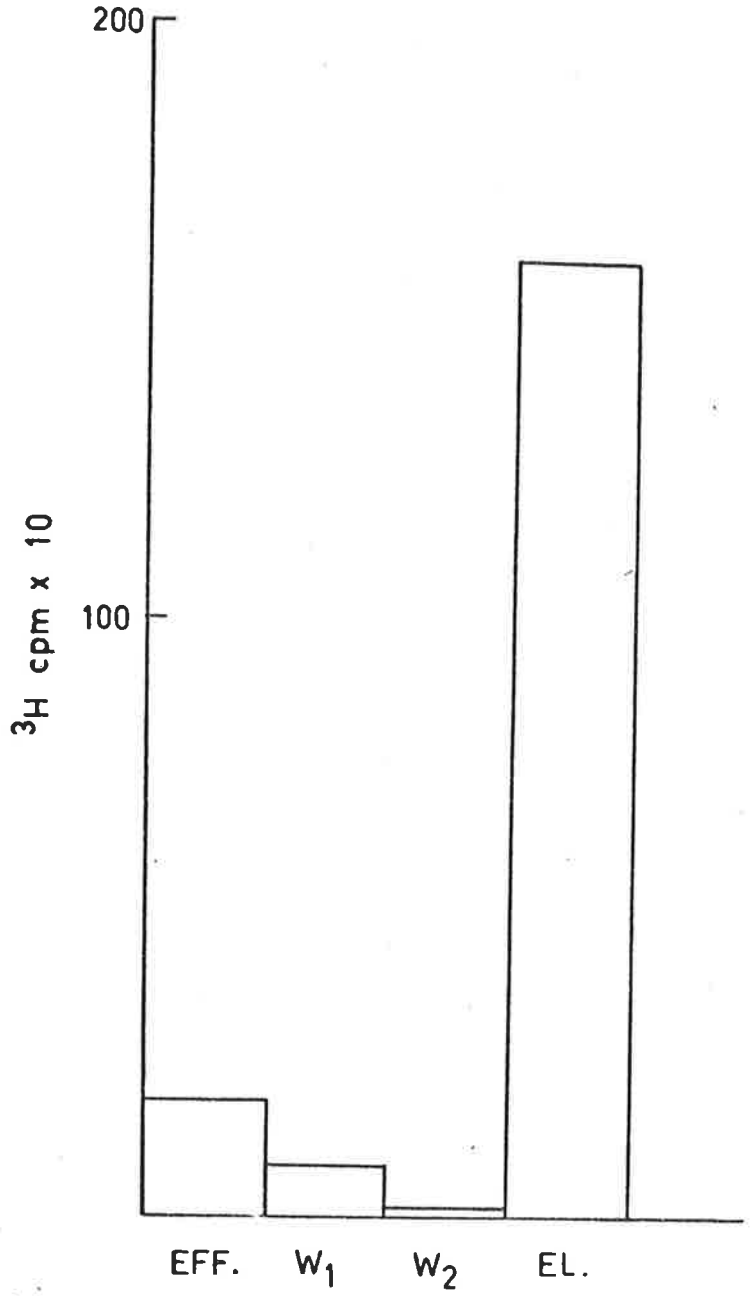


FIG. A.8 The distribution in fractions, after batch alumina chromatography of a solution of dl^{3}HNA (Sample 3 Table A.2). Note that the major proportion of the total ^{3}H is associated with the acid eluate (EL) fraction and much smaller amounts present in the effluent (EFF) fraction and wash solutions (W1, W2). Ordinate: total ^{3}H (cpm) per fraction.

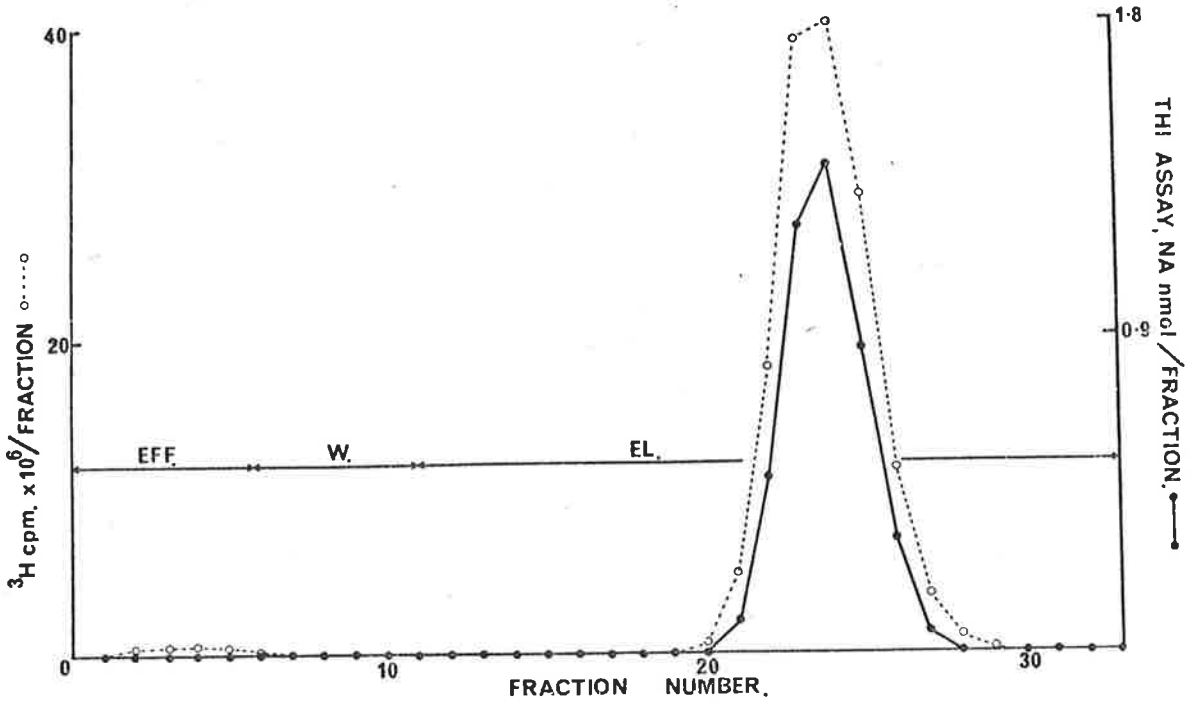


FIG. A.9 The distribution of ^3H in fractions after ion exchange (Dowex -50(Na^+)) column chromatography of the acid eluate fraction from the batch alumina fractionation illustrated in Fig. A.8. The figure illustrates the coincidence of fluorescence (determined by THI assay) and radioactivity (determined by liquid scintillation spectrometry) in the acid eluates from this column. Ordinates: the NA contents (in $nmolml^{-1}$) and ^3H contents (in cpm) of fractions. Abscissa: the number of (2.0ml) fractions.

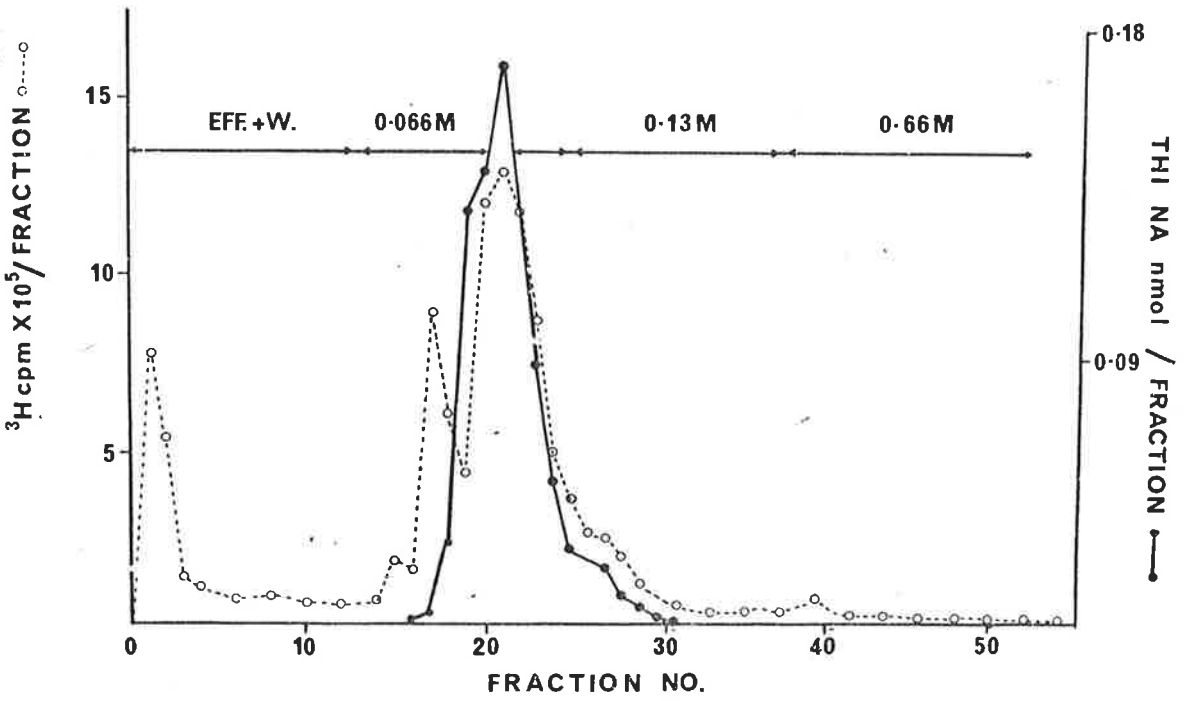


FIG. A.10 The distribution of ^3H in fractions after weak cation exchange (Amberlite I-R-C-50) column chromatography of a solution of dl^{3}HNA (Sample 3 Table A.2). The column was eluted in increasing concentrations of boric acid (0.066, 0.13 and 0.66 molL^{-1}). The figure illustrates the presence of 3 fractions of ^3H one of which exhibited specific THI fluorescence. Ordinate: NA contents (in nmolml^{-1}) and ^3H contents (in cpm) of fractions. Abscissa: the number of (2.0 ml) fractions.

determined for the ^{3}HNA in this laboratory and those quoted by the manufacturer. Unfortunately because this procedure involved the use of strong boric acid not suitable for biological investigations this line of investigation was discontinued.

(b) Paper chromatography: The nature of the ^{3}H compounds present in batches of dl^{3}HNA were determined using the ion exchange paper chromatographic technique described in the methods section. The distribution of ^{3}H on these chromatograms was qualitatively similar for all batches of ^{3}HNA analysed and was characterised by the following features:

(1) a major peak of ^{3}H that cochromatographed (rf 0.2) with non labelled NA, and

(2) two smaller peaks of ^{3}H with rf's of 0.5 and 0.8.

These features are illustrated in Fig. A.11 which shows the chromatographic distribution of ^{3}H from two batches of dl^{3}HNA (sample 8, Table A.2 and sample 1, Table A.4). Both of these samples had identical quoted specific activities (12.2 Curies $(mmol)^{-1}$) but were seen previously to differ markedly in their NA contents, (40% and 22% respectively). The proportions of ^{3}H that co-chromatographed with non labelled NA was equivalent to 89% and 75% of the total ^{3}H recovered from these chromatograms. It was again apparent that the radiochemical purity of these batches of dl^{3}HNA were greater than the apparent NA purity. However a different interpretation of these data was possible, namely that the 2 fold difference in the NA contents of the two batches of dl^{3}HNA was associated with a 2 fold increase in the amount of ^{3}H that chromatographed with an rf of 0.5, and a smaller increase (0.5 fold) in the content of ^{3}H that chromatographed with an rf of 0.8.

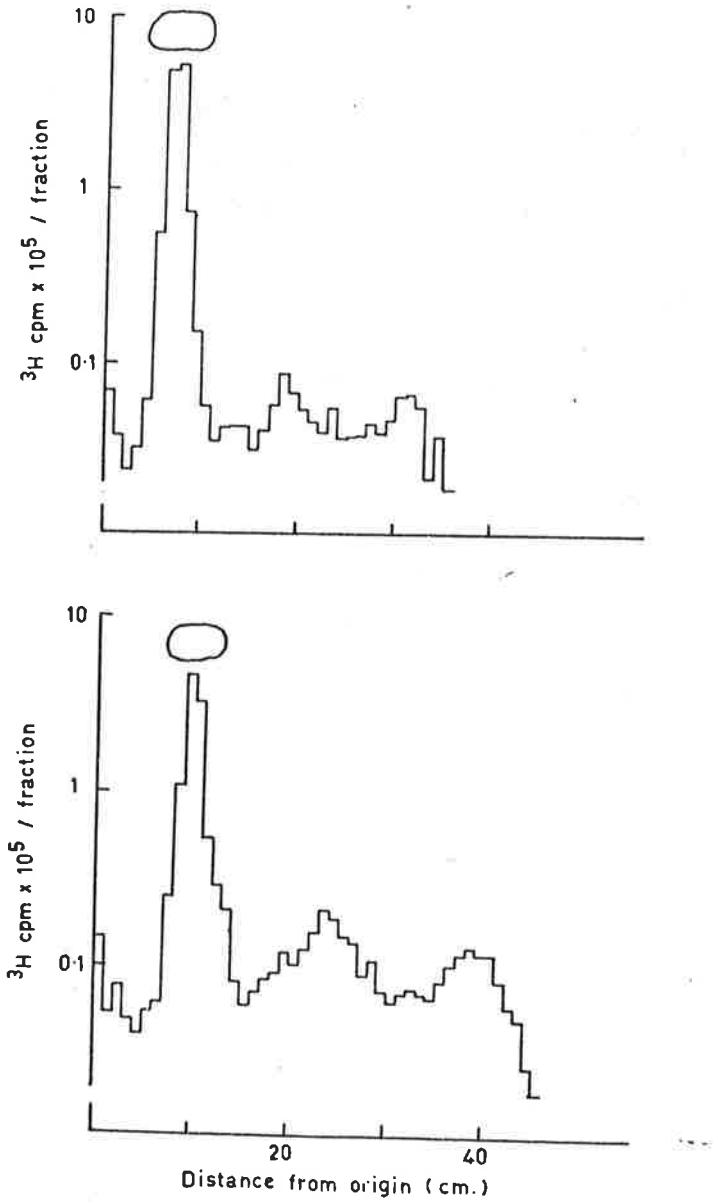


FIG. A.11 The distribution of ^{3}H on ion-exchange paper chromatograms for 2 samples of dl^{3}HNA (Sample 8 Table A.2, upper panel; sample 1 Table A.4, lower panel) illustrating 3 peaks of ^{3}H on these chromatograms. Conditions of analysis are outlined in the text and the migration of non labelled NA shown for each panel. Ordinate: ^{3}H content (cpm) of chromatogram eluates. Abscissa: distance (cm) from origin.

The following experiments were undertaken to explore the possibility that a relationship existed between the amounts of ^{3}H detected in these two regions and the NA contents of these batches of dl^{3}HNA. Samples of dl^{3}HNA which were shown to have decreased NA contents after prolonged storage (i.e. the dl^{3}HNA stored in glass vials at -10^{o}C shown in Fig. A.7) were analysed by this procedure and compared with samples that exhibited only minimal decrease in NA content (i.e. the solutions of dl^{3}HNA stored in polypropylene tubes containing DOPAC at -10^{o}C shown in Fig. A.7). The results of these experiments are shown in Fig. A.12 where it can be seen that:

(1) for the sample in which the NA contents were seen to decrease by 20%, the bulk of the ^{3}H (86%) co-chromatographed with non labelled NA and only smaller amounts of ^{3}H (4.7% and 6.3%) were present in the regions of the chromatograms with rfs of 0.5 and 0.8 respectively.

(2) for the sample in which the NA contents decreased markedly on storage (i.e. by > 99%) only 13% of the total ^{3}H recovered from chromatograms co-chromatographed with NA. The major proportions (28% and 43%) of the ^{3}H on these chromatograms being associated with the two regions of the chromatogram with rfs of 0.5 and 0.8.

These findings were confirmed by thin layer chromatographic analysis, and the distributions of ^{3}H on thin layer plates is shown in Fig. A.13. It was concluded that the time dependent decline in NA contents of samples of ^{3}HNA was accompanied by a decrease in radioactive purity of these NA solutions. Despite this conclusion, and as pointed out previously, the estimates of radioactive purity (based on chromatographic analysis) were not in agreement with the

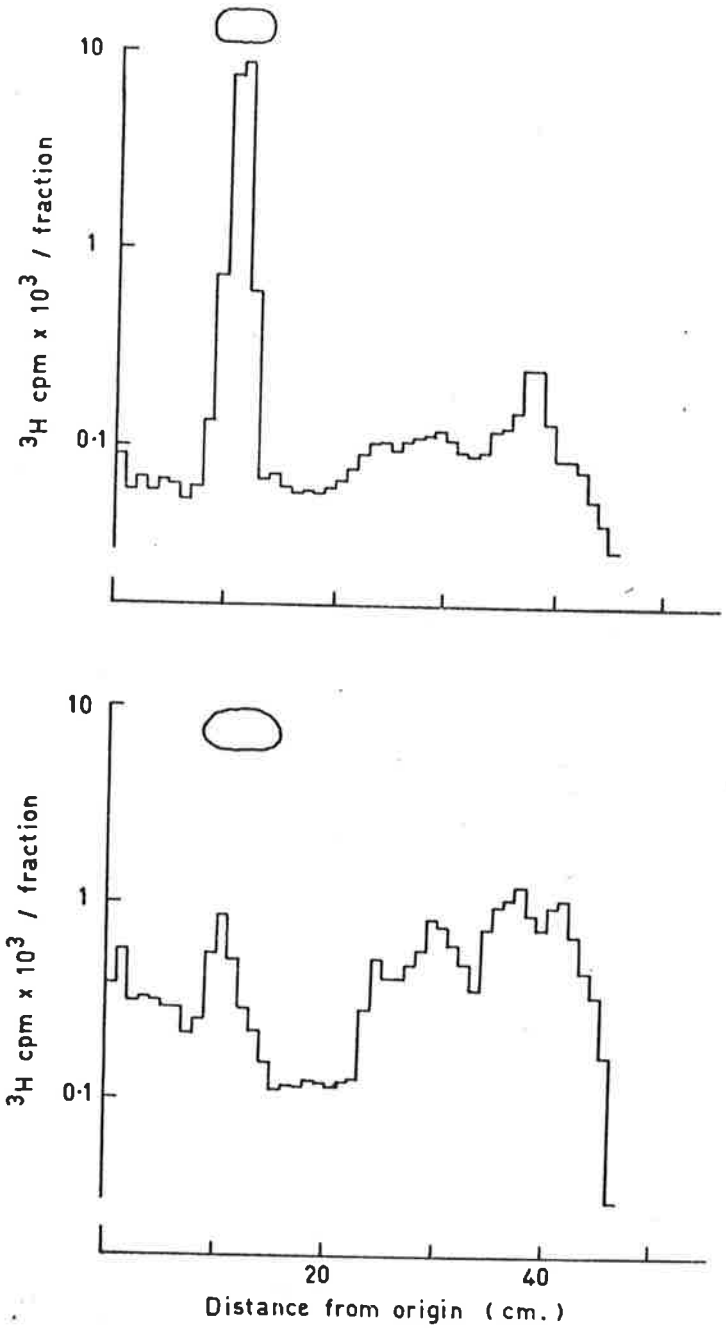


Fig. A.12 Legend the same as for Fig. A.11 except showing the ^{3}H distributions for (1) a sample of dl^{3}HNA stored for 14 months in a glass vial at 10°C (lower panel) and (2) a sample of dl^{3}HNA stored in a polypropylene tube containing DOPAC (59 μmol l^{-1}) at -10°C for 14 months (upper panel). The migration of non-labelled NA is shown for each panel.

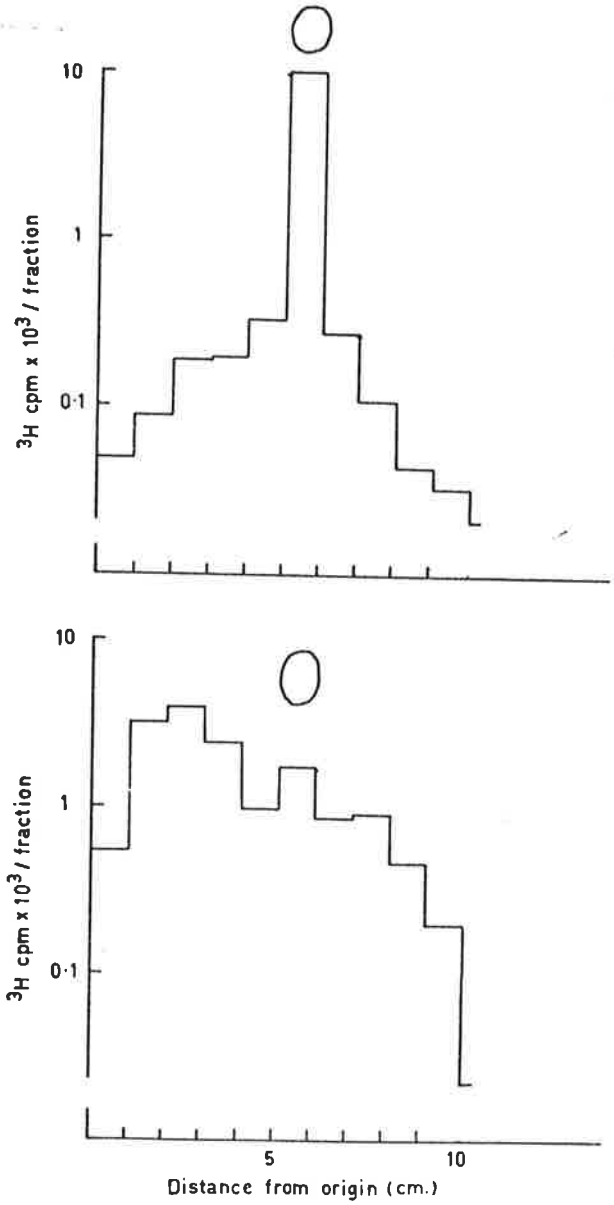


FIG. A. 13 The distributions of ^{3}H on thin layer chromatograms for the two samples shown in Fig. A.12. Conditions of analysis are outlined in the text, and the migration of non labelled NA in this system shown for each panel. Ordinate: ^{3}H contents (cpm) of chromatogram eluates. Abscissa: distance (cm) from origin.

percentage NA purity (determined by chemical assay). It was possible that these values of radioactive purity were over-estimated due to the presence of a ^{3}H impurity in solutions of dl^{3}HNA which co-chromatographed with ^{3}HNA but did not exhibit specific THI fluorescence. Support for the existence of such an impurity can be seen from the results of the paper chromatographic experiments described above and shown in Fig. A.12. It may be seen that the sample stored in glass at -10^{o}C for 14 months had only 1% on the NA content originally present, despite the fact that 12% of the total ^{3}H originally present in these samples co-chromatographed with non labelled NA. The latter observation takes into account that the total ^{3}H eluted from paper chromatograms represents greater than 70% of the ^{3}H applied to these papers and the loss of ^{3}H from these stored samples was not greater than that expected on the basis of the half life of decomposition of ^{3}H (viz < 10% in 14 months). These findings were consistent with the possibility that a ^{3}H impurity which co-chromatographed with NA but did not produce significant THI fluorescence was present in these solutions. Unfortunately it was not known whether a similar impurity existed in the original manufacturer's solutions of ^{3}HNA or to what extent this impurity, if present, contributed to the discrepancy between radiochemical purity and apparent NA purity.

Alumina purification of solutions of ^{3}HNA markedly decreased (60% and 85%) the contents of the ^{3}H material that co-chromatographed with rfs 0.5 and 0.8.

Tissue experiments: The purpose of these experiments was to determine to what extent sympathetically innervated tissues would accumulate the ^{3}H impurities known to be present in batches of dl^{3}HNA.

The experimental approach adopted involved the following:

(1) estimation of the contents of ^{3}H impurities present in isolated rabbit ear arteries and rat vas deferens that were incubated with dl^{3}HNA, and

(2) determination of the specific activities of ^{3}HNA present in acid extracts of ear arteries and vas deferentia that were incubated with dl^{3}HNA.

To simplify the approach described in detail in the methods section the following treatments were used:

(1) rabbits and rats were injected with reserpine 24 hours before removal of the tissues to decrease the endogenous NA contents in these tissues and to limit the availability of vesicular binding sites for ^{3}HNA in the sympathetic nerves of these tissues. It was hoped that this treatment would reduce the dilution of ^{3}HNA with lNA normally present in the nerve terminals of these tissues and eliminate the disparity that would occur in the binding of the d and l isomers of dl^{3}HNA to neuronal storage vesicles.

(2) All of these tissues (with the exception of two ear arteries) were incubated with dl^{3}HNA in the presence of the extra neuronal uptake inhibitor DOCA (see Chapter 7).

(3) The NA metabolizing enzymes MAO and COMT were inhibited by incubating these tissues with nialamide and UO521. It was anticipated that these treatments would reduce or eliminate the formation of ^{3}H metabolites of dl^{3}HNA, the presence of which would have rendered interpretation of the chromatographic data difficult.

Rabbit Ear Artery: The effectiveness of reserpine treatment was confirmed for the 4 rabbits treated, by fluorescence histochemical analysis and characterised by the absence of specific noradrenergic fluorescence in the ear arteries from these animals. The results of experiments in which these arteries were incubated with dl^{3}HNA (0.6 μmol l^{-1}; sample 1, Table A.4) for 30 minutes included the following observations:

(1) all artery extracts showed only a single peak of ^{3}H on paper chromatograms which co-chromatographed with non labelled NA (Fig. A.14).

(2) all incubation solutions exhibited 3 peaks of ^{3}H on paper chromatograms (Fig. A.14) with ^{3}H distributions qualitatively similar to those seen in Fig. A.11.

(3) the specific activities of ^{3}HNA in acid extracts of enzyme inhibited arteries (33.5 and 30 Ci $(mmol)^{-1}$) and enzyme inhibited DOCA treated arteries (16.4 and 22.6 Ci $(mmol)^{-1}$ were greater than the specific activity quoted for this batch of dl^{3}HNA (12.2 Ci $(mmol)^{-1}$ but less than that determined in the laboratory (55.5 Ci $(mmol)^{-1}$).

Rat vas deferens: The NA contents of vas deferens from reserpine treated rats (7.1 nmol g^{-1}) were markedly smaller than the NA contents of control (saline treated) rats (44.4 nmol g^{-1}) and provided evidence for the depletion of NA stores in the reserpine treated rats. The procedures used in these experiments in which vas deferentia were incubated with dl^{3}HNA (0.6 μmol l^{-1}, sample 2, Table A.4) have been described in the methods section and the results of these experiments are summarized as follows:

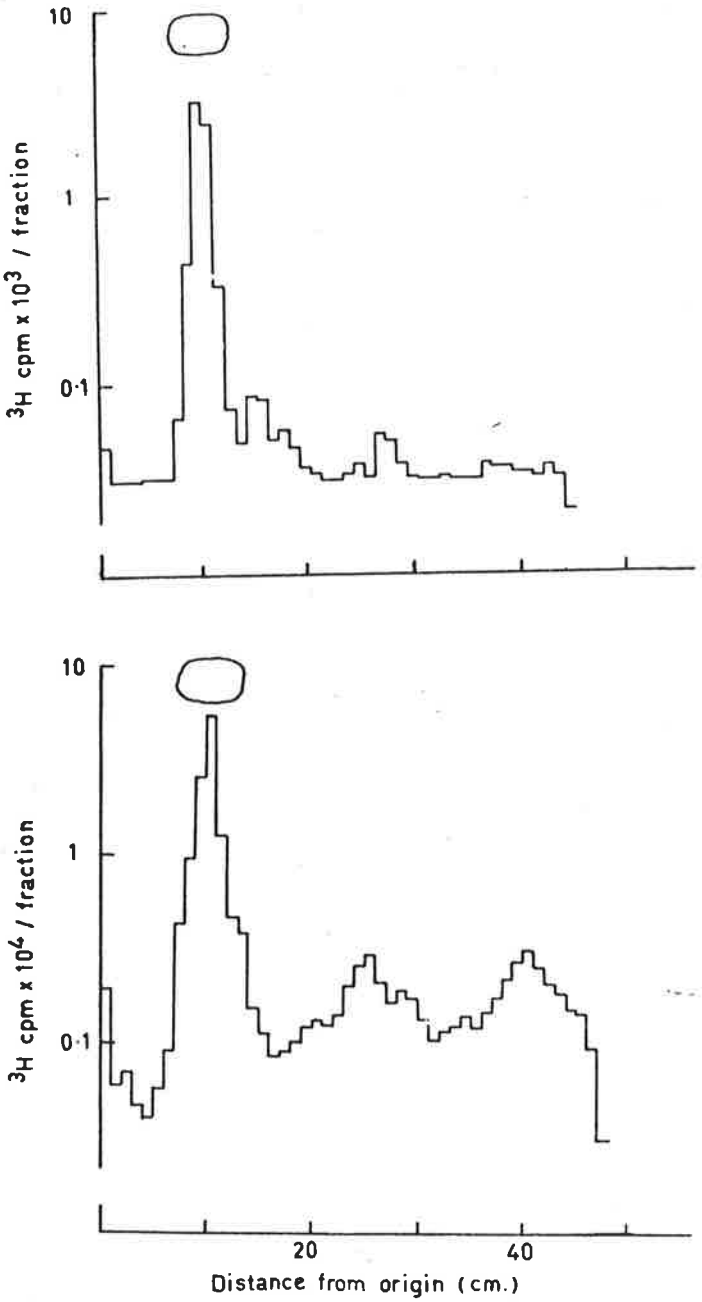


Fig. A.14 Legend the same as for Fig. A.11 except showing the ^{3}H distributions for a sample of an acid extract of an ear artery (upper panel) and its corresponding incubate (lower panel). The incubation of arteries and the preparation of solutions prior to chromatography is outlined in the text.

(1) acid extracts of all vas deferentia produced two peaks of ^{3}H on paper chromatograms (Fig. A.15). The peak containing the greatest proportion of ^{3}H, co-chromatographed with non labelled NA, the other was identical in rf value (0.8) to one of the impurities seen previously (Fig. A.11). The ^{3}H content of this latter peak represented 5.5% of the ^{3}H that was present in the peak attributable to NA.

(2) incubation solutions produced 3 peaks of ^{3}H on paper chromatograms (Fig. A.14) similar to those seen in Fig. A.11 for the manufacturer's solutions of dl^{3}HNA. Samples of incubates taken at 0, 45 and 90 minutes of incubation were identical in their distribution and contents of ^{3}H. The ^{3}H contents of the material that chromatographed with an rf value of 0.8 represented 8.9% of the ^{3}H attributable to ^{3}HNA on these chromatograms.

(3) The specific activity of ^{3}HNA in a pooled acid extract of all of these vas deferentia (5.8 Curies $(mmol)^{-1}$) was less than that quoted for this batch of all ^{3}HNA (20 Curies $(mmol)^{-1}$) and less than than determined in the laboratory (86 Curies $(mmol)^{-1}$).

It was concluded from all of these results that there was no significant accumulation of ^{3}H impurities in these tissues. This assumption takes into account the fact that the impurities characterised by rfs 0.5 and 0.8 were not detected in artery extracts and only a very small proportion of one of these impurities detected in extracts of vas deferens. It was thought that this latter observation reflected the larger amounts of tissue per volume of incubation medium (20 mg ml^{-1}) used in these experiments than used for the ear artery experiments (10 mg ml^{-1}). Thus it is possible

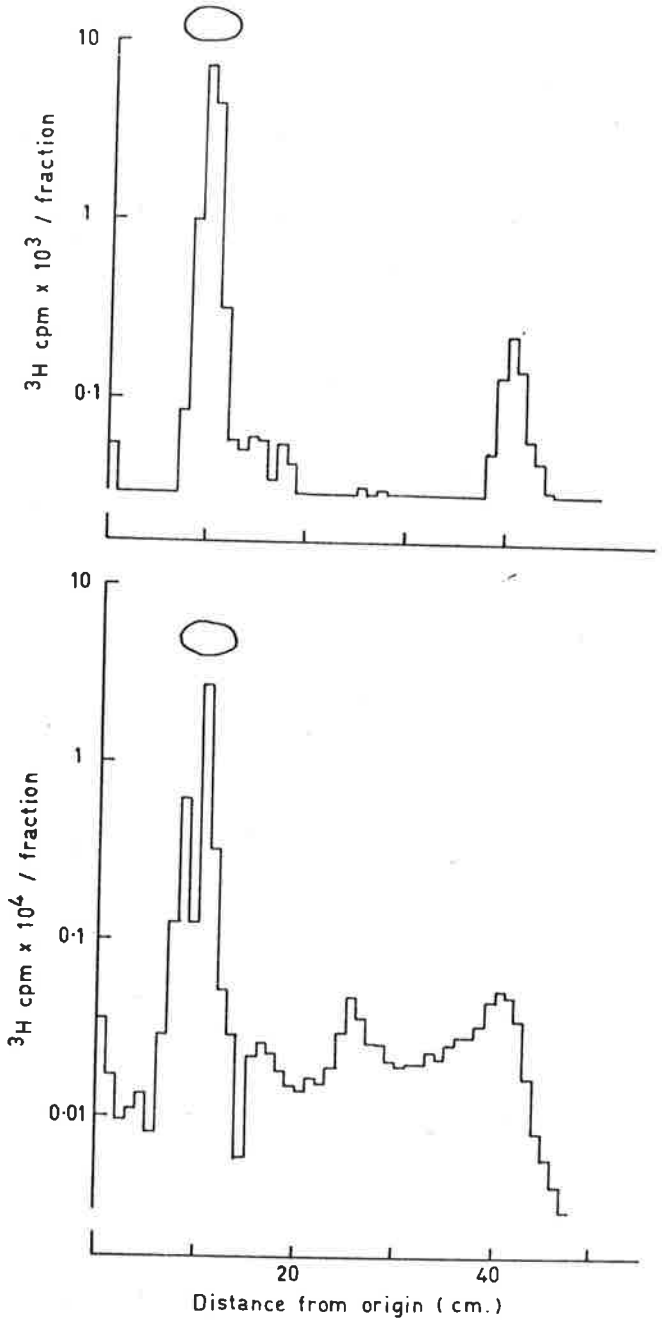


FIG. A. 15 Legend the same as for Fig. A.11 except showing the ^{3}H distributions for samples of acid extracts of vas deferentia (upper panel) and its corresponding incubate (lower panel). The conditions of incubation and sample preparation are outlined in the text.

that small amounts of these impurities may have accumulated in the sympathetic nerves of these tissues. In view of the fact that the nature of these impurities is unknown this data does not preclude a functional role of these compounds in experiments of this type.

The observation that the specific activities of ^{3}HNA seen in acid extracts of these tissues were less than those seen for the respective batches of dl^{3}HNA will be commented on in the discussion section.

DISCUSSION

The present findings have shown that the catecholamine contents of certain samples of commercially available ^{3}HNA and ^{3}HISO were consistently smaller than the contents quoted by the manufacturer. The reasons for this discrepancy were not immediately apparent but were thought to reflect one of the following possibilities:

(1) a difference between the values of CA contents measured by the manufacturer and those measured in this laboratory

(2) a decrease in the CA contents of these solutions that occurred subsequent to the manufacturer's assay and prior to the assay in this laboratory.

Unfortunately the CA contents of these solutions were determined by the manufacturer many weeks before they were measured in this laboratory thus it could not be assumed that there was a constant error in the measurement of these contents either by the manufacturer or in this laboratory. The point is that these solutions of ^{3}HCA's may have deteriorated during this period of time. Support for the validity of the CA contents of ^{3}HNA determined in this laboratory

came from two sources:

(1) the assay procedure is highly specific for the NA and ISO (see Table A.1 in the previous appendix)

(2) the manufacturer's samples of ^{3}HNA did not quench the fluorescence produced by non labelled NA and did not produce fluorescence other than that attributable to the fluorescence of NA.

The possibility that the THI assay underestimated the contents of ^{3}H labelled but not non labelled NA was also investigated. This possibility was based on the fact that formation of the THI derivative of NA necessitates the loss of the β-H (or β - ^{3}H) substituent (Pichler *et al.* 1968). Hence any differences associated with the cleavage of C-H or C - ^{3}H bond may have resulted in a difference between the fluorescent yield of ^{3}H labelled and non labelled NA. Because the results from the biological assay agreed closely with estimates based on the THI procedure, it was assumed that any differences in the fluorescence produced by ^{3}H labelled and non labelled NA were minimal.

The findings from this study have provided direct evidence for the decomposition of ^{3}HNA in solutions. It was shown that the NA contents of solutions of ^{3}HNA decreased with time of storage in the laboratory and that these decreases were related to the nature of the storage conditions. From these observations it was possible to speculate as to the type of process responsible for the decreases in NA contents. It was seen that the addition of ethanol and DOPAC to solutions of ^{3}HNA inhibited the rate of decrease of NA contents in these solutions. These observations are consistent with the proposal that ethanol and aromatic alcohols afford protection to a

wide range of ^{3}H compounds against the products of self radiolysis (Bayly and Evans 1968). Storage in polypropylene containers also served to reduce this decomposition. Whether this was due to the physical nature of the container, or to the leaching of compounds from it that afforded protection for ^{3}HNA was not known.

Further evidence supporting the decomposition of ^{3}HNA in aqueous solutions came from the results of paper chromatographic experiments. Analysis of manufacturer's stock solutions of ^{3}HNA showed the presence of two fractions of ^{3}H material, other than those attributable to ^{3}HNA.

In the absence of information as to the identity of the ^{3}H impurities it was not known whether these impurities were formed by a process of autoxidation or self radiolysis. In view of the fact that these two impurities did not bind to alumina at pH 8.4, and migrated without retardation on ion exchange paper, it is likely that these compounds are not catechols and are weaker bases than the parent catecholamines.

The significance of ^{3}H impurities in solutions of $^{3}HCA's$ relates to the possibility that these compounds may accumulate in sympathetically innervated tissues. Thus it was reassuring to find that there was no evidence for accumulation of these impurities in arteries. Experiments on the rat vas deferens permitted an estimate of the degree to which one of these ^{3}H impurities accumulated in this tissue, and the results indicated that this accumulation was extremely small. In these studies attention was focussed only on the ability of a neuronal uptake mechanism to selectively remove

^{3}HNA and not ^{3}H impurities from the bathing medium. Hence the possibility of extraneuronal accumulation cannot be ignored.

A more disturbing aspect was the evidence suggesting the presence of a ^{3}H impurity in solutions of ^{3}HNA that behaved in paper chromatographic systems like NA, but did not exhibit the THI fluorescence characteristic of this amine. This conclusion was supported by the observation that a peak of ^{3}H that did not produce THI fluorescence but eluted in a similar fashion to NA from weak ion exchange columns. A similar finding was reported by Persson and Waldeck (1970) who provided evidence for the presence of ^{3}H impurities in solutions of ^{3}HDOPA virtually indistinguishable from DOPA on paper chromatographic systems.

Whether the tissues accumulated the ^{3}H impurity that co-chromatographed with NA was difficult to ascertain. It was noted that the specific activities of ^{3}HNA in acid extracts of the tissues were smaller than those determined for the stock solutions of ^{3}HNA, which was consistent with exclusion by the tissues of ^{3}H material that did not produce specific THI fluorescence.

In summary, it was concluded from the results of this study that solutions of ^{3}HCA's are particularly unstable and require regular purification.

APPENDIX 3

ELECTROLYTIC O-DEMETHYLATION OF METHOXYCATECHOLAMINES

INTRODUCTION

During the course of experiments designed to estimate the output of noradrenaline from sympathetic nerves in the rabbit ear artery, it was observed that the application of current to Krebs bicarbonate solution containing normetanephrine or metanephrine, in the absence of tissue, considerably enhanced the vasoconstrictor activity of these solutions.

In the present communication, evidence is presented that the passage of current causes electrolytic O-demethylation of metanephrine and normetanephrine to their parent catecholamines.

MATERIALS AND METHODS

Catecholamines (noradrenaline, NA; adrenaline, A) or their 3-methoxy analogues (normetanephrine, NMN; metanephrine, MN) were dissolved in Krebs bicarbonate or 0.9% w/v saline solutions gassed with 95% oxygen and 5% carbon dioxide and maintained at 37^{o}C.

Current was passed through the solution by means of two platinum electrodes approximately 8 cm in length and positioned

1 cm apart. In most experiments the current source was a Grass model S-44 stimulator. Voltage and frequency were standardised at 70 V and 5 Hz except where otherwise indicated. Solutions treated in this way will be described as stimulated.

In those solutions containing ascorbic acid (600 mmol l^{-1}) the nomenclature ascorbic-Krebs or ascorbic-saline will be used.

Bioassay: The only modification to the method described in the previous appendix (Appendix 2) for the bioassay of catecholamines on the rabbit ear artery was the use of a double cannulated artery and the addition of cocaine (3 μmol l^{-1}) and 5-hydroxytryptamine (13 nmol l^{-1}) to the Krebs solution to enhance sensitivity. For assay, solutions were injected intraluminally in a volume not exceeding 0.3 ml.

Fluorometric analysis: The catecholamine (NA or A) contents of samples were assayed by the semi-automated trihydroxyindole (THI) method (Fig. A.1) described in detail in the first chapter of this appendix (Appendix 1). Briefly this procedure involved the automated sampling of catecholamines in acetic acid (300 mmol l^{-1}), oxidation at pH 5.8 with potassium ferricyanide (300 mmol l^{-1}) buffered with sodium acetate (1.5 mol l^{-1}) and lutine formation with alkaline-ascorbate (NaOH (2.5 mol l^{-1}), ascorbic acid (17 mmol l^{-1})). The lutines were estimated fluorometrically at the following wave lengths: activation 395 nm (interference filter), emission > 485 nm (Wratten No. 8 sharp cut filter). Under these conditions A and NA, but not MN and NMN, contribute to the fluorescence estimated. Standard solutions of A and NA were dissolved in Krebs solution or saline,

depending on the nature of the test solution, and made (300 mmol l^{-1}) with respect to acetic acid.

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

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

Paper chromatography: Catecholamines were separated from their 3-methoxy analogues by descending chromatography on cellulose phosphate paper (Whatman, P81) according to the procedure outlined in Chapter 4. For these experiments 20 μl aliquots of stimulated saline solution were applied to the paper, airdried and chromatographed in isopropanol:

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

ammonium acetate 200 mmol l^{-1}, pH 6.5, 2:1. For comparison, 20 µl aliquots of unstimulated saline solution containing the individual catecholamines and their 3-methoxy derivatives were run in parallel. After the solvent had migrated approximately 40 cm, the papers were airdried and either sprayed with freshly prepared diazotized paranitroaniline or cut into 1 cm sections. Sections were eluted overnight in 2 ml of saline (pH 5.5) containing ascorbic acid (600 mmol l^{-1}). The saline eluates were assayed either fluorometrically or by bioassay.

RESULTS

The vasoconstrictor activities of unstimulated ascorbic saline solutions containing MN (2.3 µmol l^{-1}) are illustrated in Fig. A.16. It will be noted that only the stimulated solutions elicited a response and that this response and that to NA were largely abolished by phentolamine (2.0 µmol l^{-1}). The results were identical when Krebs solution was used instead of saline.

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

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

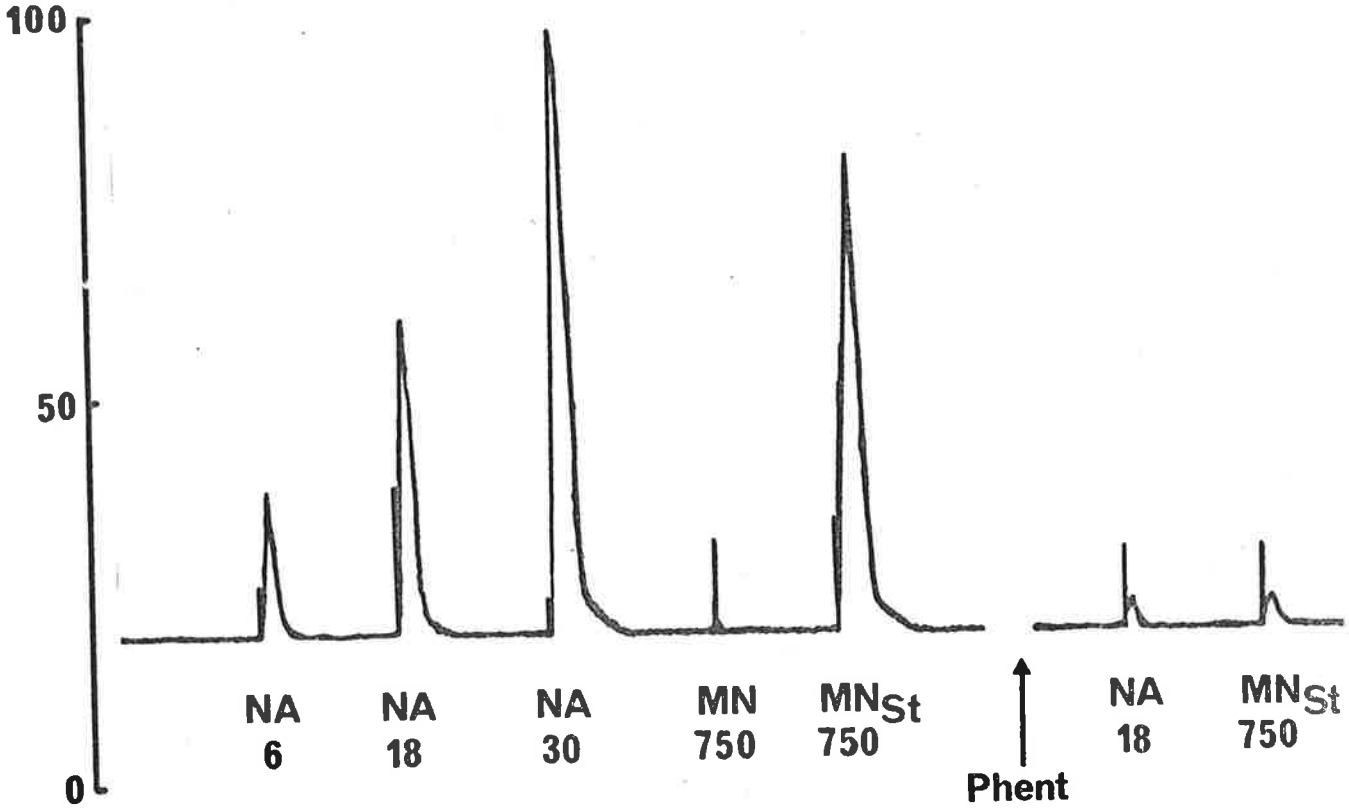

Fig. A.16 Responses to noradrenaline (NA), and unstimulated (MN_{St}) solutions of metanephrine. All doses (µmol x 10^{-6}) were injected intraluminally. At the arrow, phentolamine was perfused intraluminally in a concentration of 2 µM. Ordinate: perfusion pressure in mn Hg.

1. Measurement of the fluorescence of solutions of MN (0.5 mmol l^{-1}) or NMN (0.5 mmol l^{-1}) in ascorbic Krebs, when assayed by the THI procedure, showed that stimulation of these solutions was accompanied by an increase in fluorescence (Fig. A.17). The increase was linear over the period of stimulation (15 min.). That the fluorescence possessed identical characteristics to the corresponding catecholamine (A or NA) was indicated by its failure to appear when the order of adding potassium ferricyanide and the alkali-ascorbate solution in the THI assay procedure was reversed. In this respect, pure solutions of A, and of NA, behaved identically to the above stimulated solutions.

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

3. Diazonium sprayed ion exchange paper chromatograms of stimulated solutions of MN and NMN indicated the presence of spots which co-chromatographed with pure A and NA, respectively (Fig. A.18). These spots were not detected in unstimulated solutions. Eluates of the same regions from unsprayed papers displayed vasoconstrictor activity on the rabbit ear artery and catecholamine-like fluorescence when analysed by the THI procedure. This is illustrated for MNM in Fig. A.19. In contrast, the chromatograms of stimulated solutions

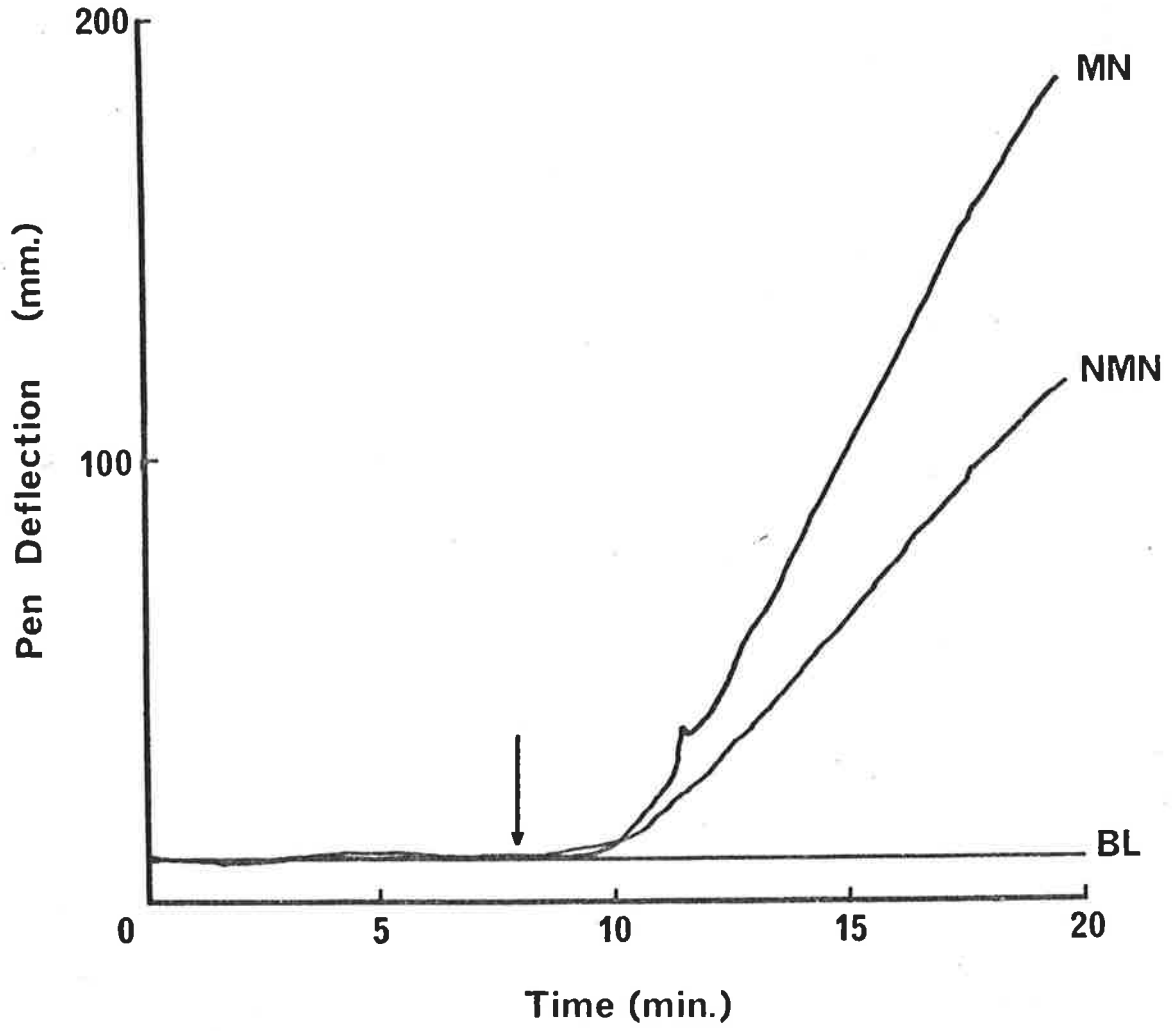

Fig. A.17 The trihydroxy indole fuorescence of solutions of MN (0.5 mM) and NMN (0.5 mM) continuously assayed before and during stimulation. The line (BL) indicates that, when the order of adding the reagents was reversed to prevent fluorescence of adrenaline, the fluorescence of the MN solution does not increase during stimulation. Ordinate: fluorescence measured in terms of pen deflection (mm).

Fig. A.18 Chromatogram of ascorbic-Krebs solutions containing one or other of NA, NMN, A or MN (all 5 mM), in the absence of stimulation (RHS) and following stimulation (LHS). The stimulated samples of NMN and MN show the presence of the catecholamines NA and A, respectively. The compounds were visualized after spraying with diazotized paranitroaniline. Stimulation parameters: 70V, 5Hz, 30 min.

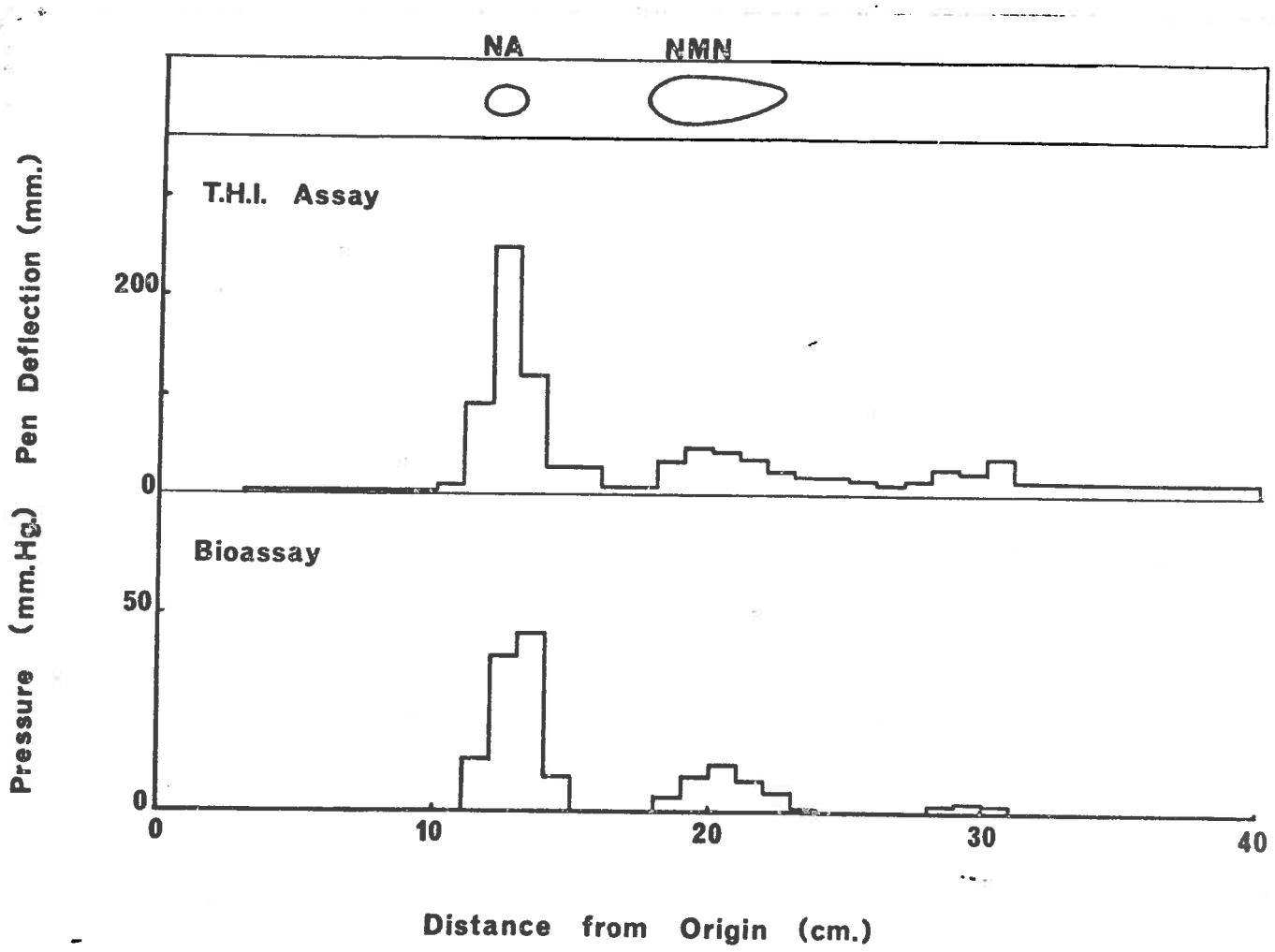

Fig. A.19 Illustrates the coincidence of fluorescence (determined by THI assay) and vasoconstrictor activity (measured by bioassay) in eluates of one cm sections of a chromatogram of a stimulated ascorbic-saline solution of NMN. The location of pure NA and NMN after visualization of the same chromatographic system is shown.

of ascorbic-Krebs or ascorbic-saline containing NA and A were identical with those of their unstimulated solutions.

4. In the absence of ascorbic acid, a red colour developed in Krebs or saline solutions of NMN and MN during stimulation. Like the vasoconstrictor activity, colour formation was restricted to the anodic compartment. Ion exchange chromatography of these solutions indicated that the spots were co-chromatographed with the catecholamines, as described in 3 above, were now greatly decreased in intensity, both when tested by diazonium spraying and by THI fluorescence assay of the paper eluates. Eluates of the same regions also failed to display constrictor activity.

Ascorbic acid-free solutions of NA or A also become coloured during stimulation. When analysed spectrophotometrically, the λmax values of the coloured solutions of NMN and of MN proved to be identical to those of the coloured solutions of NA and of A respectively (Fig. A.20). These maxima were the same as those reported for adrenochrome (487 nM) and noradrenochrome (484 nM) by Heacock and Mattock (1963). Hence the term aminochrome will be used subsequently to describe the coloured material (see Methods). It is of interest that the absorption spectrum of the aminochrome found in the stimulated MN (and also A) solution is qualitatively similar to that reported for adrenochrome by Beaudet (1951) and by Stock and Hinson (1955).

The rate of formation of aminochrome during stimulation was measured by the change in OD at 480 nM and was found to be linear with time. The rate was greater for A than for NA and greater for MN than NMN (Fig. A.21). It will be noted that some aminochrome was

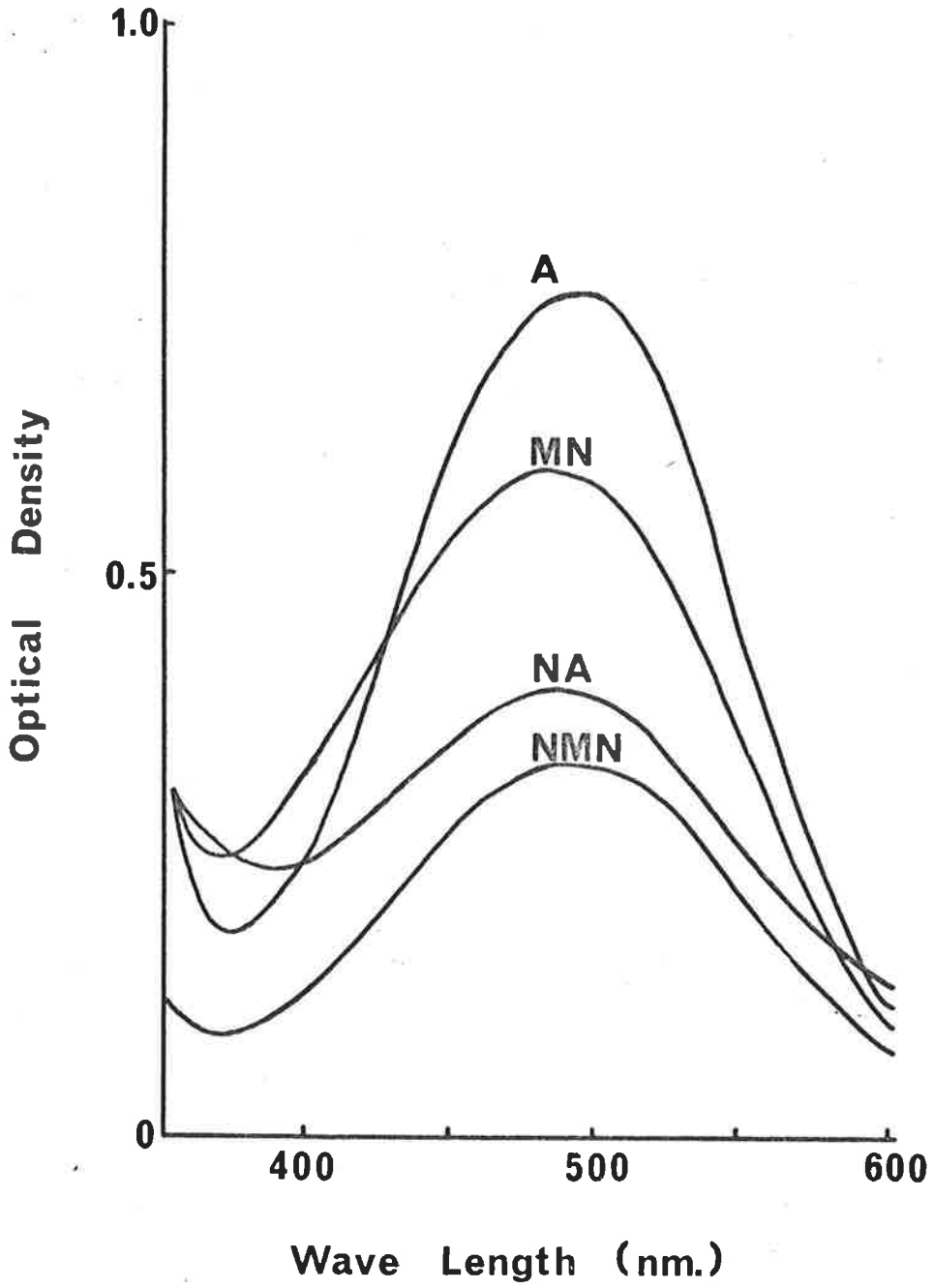

Fig. A.20 The visible absorption spectra of A, MN, NA and NMN (all 5 mM) following stimulation in ascorbic-free Krebs solution.

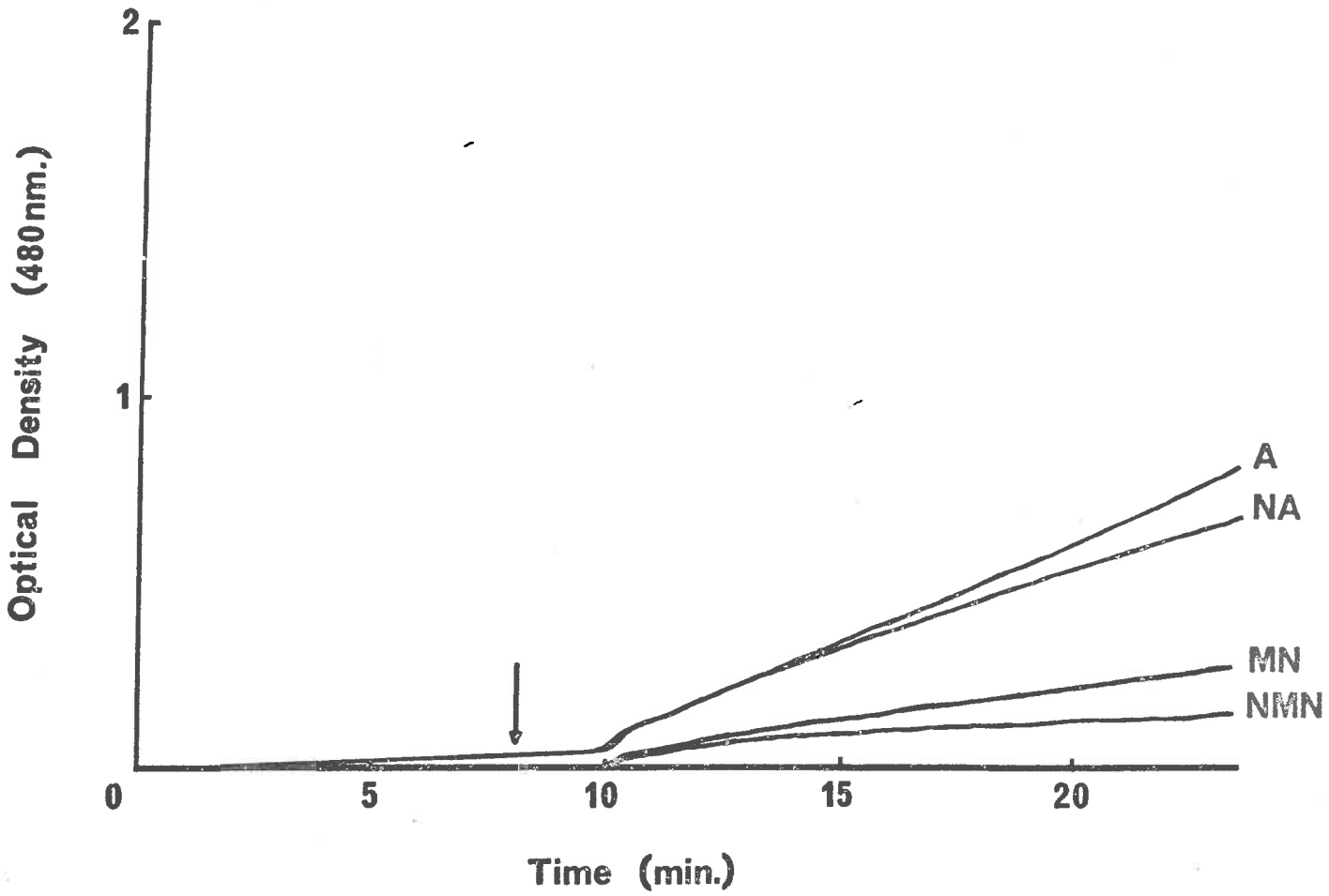

Fig. A.21 Increase in optical density at 480 nm of ascorbic-free Krebs solutions containing A, NA, MN and NMN (all 5 mM) before and during stimulation. Stimulation was commenced at 8 min (shown by arrow). Note the small increase in optical density occurring in the solutions of A and NA before stimulation.

formed in the ascorbic-free Krebs solutions prior to stimulation. This did not occur when ascorbic acid was present.

DISCUSSION

The results indicate that the vasoactive substances which are produced when current is passed through solutions of MN or NMN resemble the corresponding catecholamines in the following respects:

(a) Vasoconstriction was mediated with authentic catecholamines.

(b) The materials co-chromatographed with authentic catecholamines.

(c) The materials possessed the same THI fluorescence as the catecholamines.

A scheme which accounts for the formation of a catecholamine (II) from its parent 3-methoxycatecholamine (I) is outlined in Fig. A.22. The scheme is based on the mechanism for the oxidation of guaicol by periodate described by Adler and Magnussan (1959). Their mechanism involved oxidative demethoxylation of the methoxy group, as well as the oxidation of the phenolic hydroxyl group, to form the corresponding O-benzoquinone. The scheme shown in Fig. A.22 involves the oxidation of the 3-methoxy-4-hydroxy amine to the corresponding O-benzoquinone and the subsequent reduction of the latter to the corresponding catecholamine in the presence of ascorbic acid. In the absence of ascorbic acid, cyclisation of the O-benzoquinone to the corresponding indole occurs (adrenochrome or nor-adrenochrome) (III), thus accounting for the colour formed in the ascorbic-free solutions after the methoxy amines were stimulated.

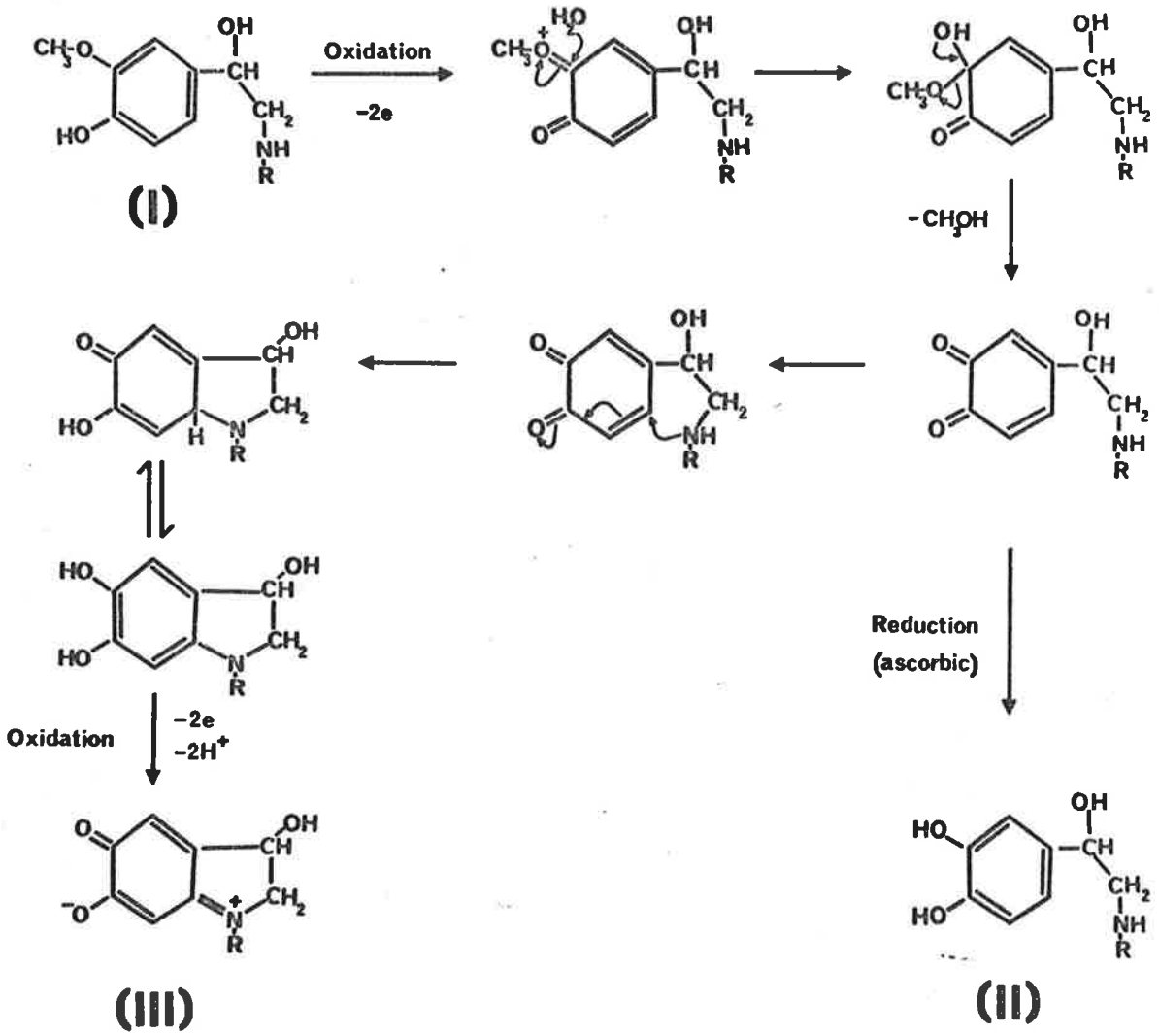

Fig. A.22 The proposed mechanisms involved in the oxidative demethylation of the 3-methoxy catecholamines (I) (normetanephrine R=H, metanephrine R=CH_3). This scheme accounts for the formation of the catecholamines (II) (noradrenaline R=H, adrenaline R=CH_3) in the presence of ascorbic acid and the aminochromes (III) (noradrenochrome R=H, adrenochrome R=CH_3) in its absence.

The significance of the electrolytic O-demethylation of NMN or MN to the normal activity of the sympathetic neuroeffector systems *in vivo* is not known. However, it has important implications for the use of the methoxy derivatives of catecholamines as pharmacological agents for inhibiting extraneuronal uptake of the latter amines. Obviously, in studies dealing with the relationships between stimulation of the nerves and response (functional or metabolic) of the tissue, the technique of field stimulation as a method of stimulating the intramural nerves is contra-indicated where the methoxy analogues are added in high concentrations to the solutions bathing the tissue. Even in the absence of added NMN the electrolytic O-demethylation may represent significant artefact in such studies, since NMN is a major metabolite of the NA released from sympathetic nerves (see Chapter 6).

DRUGS

l-adrenaline bitartrate	Koch Light Laboratories
cocaine hydrochloride	MacFarlane-Smith
diallybarbituric acid (DIAL)	Ciba
DOCA (4-pregnen-21-ol-3, 20-dione acetate)	Steraloids
d,l-7-^{3}H isoprenaline hydrochloride (specific activities listed in Appendix II)	Amersham
3-methoxyisoprenaline	Boehringer
nialamide	Pfizer
l-noradrenaline bitartrate	Koch Light Laboratories
d,l-7-^{3}H noradrenaline hydrochloride	Amersham
l-7-^{3}H noradrenaline (specific activities listed in Appendix II)	Amersham
dl normetanephrine hydrochloride	Sigma
dl-7-^{3}H normetanephrine hydrochloride (Specific activity 2.8, 3.1 Ci $(mmol)^{-1}$)	New England Nuclear
pentobarbitol (Sagatal)	May and Baker
S-adenosyl-l-methionine	Amersham
S-adenosyl-l-^{3}H methyl methionine (specific activity 5.6, 10.0 Ci $(mmol)^{-1}$)	Amersham
S-adenosyl-l-^{14}C methyl methionine (specific activity 56 mCi $(mmol)^{-1}$)	Amersham
Tyramine hydrochloride	Sigma
^{3}H Tyramine hydrochloride (specific activity 6.8 Ci $(mmol)^{-1}$)	Amersham
U0521 (3',4'-dihydroxy-2-methyl propiophenone)	Upjohn
d,l isoprenaline hydrochloride	Sigma

All other reagents were of analytical grade purity.

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