A STUDY OF THE ACTIONS OF ETILEFRINE ON SYMPATHETICALLY INNERVATED BLOOD VESSELS

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for the degree of Doctor of Philosophy

by

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C O N T E N T S

SUMMARY

1. The aim of this study was to examine the effects of etilefrine on blood vessels and to compare its actions on vascular smooth muscle with those of other sympathomimetic amines. In particular, it was proposed to determine the mode of action of etilefrine and to ascertain whether it interacted with other substances which modified noradrenaline disposition via neuronal and extraneuronal mechanisms.

2. The vascular model used in this study was the ventral caudal artery of the rat tail. This artery was used "in situ" and also as an isolated preparation. In some parts of this investigation the isolated rabbit ear artery was also used.

3. Etilefrine was found to exert a vasoconstrictor action on the ventral tail artery "in situ", which was in part mediated by noradrenaline release from sympathetic nerve endings in the vessel. The response of etilefrine was enhanced by pretreatment of the artery with noradrenaline or cocaine and abolished by phentolamine. The sympatholytic agents reserpine, guanethidine and 6-hydroxydopamine decreased the response of the artery to etilefrine. The potency of etilefrine on this vessel was less than that of noradrenaline, adrenaline and phenylephrine but similar to metaraminol.

4. In the isolated tail artery similar findings were observed as in the tail artery "in situ". Pretreatment with reserpine or guanethidine decreased the response to etilefrine and this paralleled the decrease in tissue catecholamines observed. Pretreatment with the monoamine oxidase inhibitor iproniazid elevated tissue catecholamine levels but paradoxically decreased the etilefrine response.

5. The response to etilefrine in the rabbit ear artery was analagous to that observed in the ventral tail artery. Surgical denervation of the ear artery decreased the response to etilefrine but did not alter the response to noradrenaline. Treatment of the artery with 9-alpha-fluorohydrocortisone did not affect the etilefrine response.

6. When the ventral tail artery was incubated with tritiated noradrenaline, etilefrine was found to enhance the efflux of radio-label from this vessel. This enhanced efflux was comparable with that of ephedrine but less than that induced by tyramine.

7. The metabolite content of the enhanced efflux from the ventral tail artery was also assessed. The sympathomimetic amines etilefrine, ephedrine, tyramine and REN-293 were found to increase the efflux of tritiated noradrenaline and dihydroxy-phenylglycol to different degrees. These results provided good evidence that etilefrine has an indirect sympathomimetic component to its action.

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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.

BARRY RAYMOND FROST

P U B L I C A T I O N S

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A C K N O W L E D G E M E N T S

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CHAPTER   1

INTRODUCTION

The concept of neurohumoral transmission originated in the late 1890's and early 1900's when Lewandowsky (1898) and Langley (1901) observed that injections of adrenal gland extract mimicked the action of the sympathetic nerves. The possibility that the effects of stimulating the sympathetic nerve to an organ might be due to the liberation of a chemical substance from the nerve ending was suggested by the work of Lewandowsky in 1899. In that year, Abel named the active pressor compound in adrenal gland extracts "adrenaline". In 1905, it was suggested by T. R. Elliott that stimulation of sympathetic nerves released small amounts of an adrenaline-like substance into immediate contact with effector cells. However, despite the close similarity between the sympathetic trans-mitter and adrenaline in both chemical tests and biological action, there were certain differences to be observed in most situations. These differences were commented on by Barger and Dale (1910), who investigated the pharmacological activity of a large number of synthetic amines and termed their action "sympathomimetic". They pointed out that the action of amino and ethylamino bases of the catechol group corresponded more closely with that of the sympathetic nerves than did that of adrenaline. In 1921, Loewi, and independently Cannon and Uridil, reported the release of an adrenaline-like substance from sympathetic nerves. Cannon called this substance "sympathin". This substance was similar to adren-aline in its pharmacological and chemical properties. However, when both compounds were injected into the body, adrenaline was found to have excitatory and inhibitory effects, while "sympathin"

showed only excitatory effects. Bacq, in 1934, suggested that the effects of sympathetic nerve stimulation were due to the liberation from nerve endings of a substance which behaved like noradrenaline rather than adrenaline, an observation made by Barger and Dale over twenty years previously.

In 1939, Lissak made the first extractions from sympathetic nerves and showed that such extracts had an adrenaline-like action; however, he was not able to define the precise nature of these extracted substances. In 1946, U. S. von Euler and his colleagues devised refined methods for purification and biological and chemical analysis of catecholamines and found that they could extract from the hearts of cattle, horses and cats a substance identical with noradrenaline in all its characteristics. Many workers have confirmed and extended these observations indicating that noradrenaline is the predominant .sympathomimetic substance in post-ganglionic sympathetic nerves and is liberated by their stimulation.

Work performed in concurrence with the foregoing, by Tainter and Chang (1927) and Burn and Tainter (1931), showed that cocaine or chronic denervation reduced the response of effector organs to administered tyramine and ephedrine but enhanced the effects of adrenaline. This led to the suggestion that adrenaline had a direct action on the effector cell, while ephedrine and tyramine had an indirect effect by acting on the nerve terminals. Subsequent to the discovery by Bertler et al. (1956) that reserpine depleted tissues of noradrenaline, Carlsson et al. (1957) showed that tyramine was without activity in cats treated with reserpine. In 1958, Burn and Rand extended this finding, and showed that

reserpine depleted peripheral nerve stores of noradrenaline and that the effects of indirectly acting sympathomimetic amines were abolished or decreased, but could be temporarily restored by infusion of noradrenaline (Burn and Rand, 1960).

Some years earlier, Ahlquist (1948) suggested that there were two types of adrenoreceptors on vascular smooth muscle based on the responses of a number of sympathomimetic amines. He hypothesised that alpha-receptors in smooth muscle were mostly associated with excitatory functions, e.g., vasoconstriction, while beta-receptors were mainly involved with inhibitory phenomena, e.g., vasodilatation.

Trendelenburg (1963) reviewed the actions of many sympathomimetics and showed that they could be loosely classified into direct acting, mixed acting and indirect acting amines. However, a complete range of actions is possible in different animals and tissues. Trendelenburg (1972) states that "Sympathomimetic amines should be regarded as a group of agents which share certain basic properties but which may differ quantitatively in their affinities to the various mechanisms involved in their action." It would seem that the direct effects of a sympathomimetic would be expected to be observed when the affinity of the amine for adrenoceptors is high. If an amine has a low affinity for adrenoceptors then any indirect effects should appear. He goes on to suggest that an evaluation of the actions of directly and indirectly acting sympathomimetics should take the following factors into consideration.

1. Affinity of alpha-receptor for sympathomimetic amines

In determining the relative potency of sympathomimetic

amines, two factors must be considered: firstly, the affinity of alpha-receptors for the agent and, secondly, the inactivation mechanisms responsible for its removal. Trendelenburg et al. (1970) and Trendelenburg et al. (1971), using the cat nictitating membrane, showed that the relative potencies of noradrenaline and methoxamine changed when noradrenaline inactivation mechanisms were eliminated. Since methoxamine is not a substrate for either monoamine oxidase (MAO) or catechol-O-methyl transferase (COMT) and is not taken up intraneuronally, it was found to be more potent than noradrenaline under normal circumstances. However, this situation was reversed when the inactivation of noradrenaline was prevented.
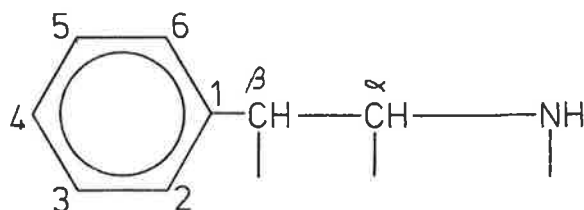
Fig. 1.1

Structural skeleton of a sympathomimetic amine.

In evaluating criteria for alpha-receptor affinity, Trendelenburg (1962) showed that the following structural changes were important. Firstly, omission of the beta-hydroxyl group or introduction of an alpha-methyl group decreases potency. Secondly, any group larger than a methyl group on the nitrogen atom or O-methylation decreases potency. Thirdly, the meta hydroxyl group is of greater importance than the para hydroxyl group. To reveal the indirect action of a sympathomimetic, Trendelenburg (1972) suggests that the amine must have a low affinity for alpha-receptors and also be a substrate for uptake (across the neuronal membrane). Evidence that amines with weak direct effects tend to exhibit strong indirect effects has been reported (Marley, 1962; Trendelenburg et

al., 1962; Schmidt and Fleming, 1963; Tsai et al., 1967).

2. Uptake of sympathomimetic amines across the neuronal barrier

For an indirect sympathomimetic to exert its action, it must possess the ability to be taken up into the nerve terminal. The drug's affinity for this uptake not only determines its capacity to release neuronal noradrenaline, but also its potential as a competitive inhibitor of noradrenaline re-uptake.

Burgen and Iversen (1965) examined a number of sympathomimetic amines for their ability to inhibit noradrenaline uptake in rat heart. Their results showed that certain structural requirements were necessary for inhibition. However, the study did not differentiate between those compounds which acted as competitive substrates for uptake and those which inhibited the uptake process but were not transported into the neurone. In addition, these amines may have had more than one site of action; for example, in competing for vesicular uptake and binding rather than just membranal uptake. Muscholl and Weber (1965) determined the arterio-venous differences in rabbit hearts perfused with alpha-methyl-noradrenaline. Their findings, using a number of sympathomimetics, agreed with the results of Burgen and Iversen (1965) and this indicated that it was membranal uptake that was measured in the latter study.

3. Uptake into vesicles containing neurotransmitter

As a requirement for vesicular noradrenaline release, the indirectly acting sympathomimetic must have the capability of

being taken up by the intraneuronal storage vesicle. This uptake system has been shown to have a high degree of stereospecificity (von Euler and Lishajko, 1964; Stjarne and von Euler, 1966) with a preference for the (-) isomer.

Two uptake systems exist for the accumulation of amines into the storage vesicle: one is dependent on $ATP-Mg^{++}$, is reserpine sensitive, and is responsible for uptake of adrenaline and nor-adrenaline (Carlsoon et al., 1963). These authors also showed that another uptake exists which is not inhibited by reserpine nor is reliant on $ATP-Mg^{++}$. In conclusion, the latter uptake is thought to be the means by which the indirectly acting sympatho-mimetics, such as tyramine, enter the vesicle and exert their action.

4. Storage in neurotransmitter vesicles

It has been shown (Musacchio et al., 1965) that storage vesicles are able to bind sympathomimetics which are catechols or have a beta-hydroxyl group. Nash et al. (1968) showed that vesicular binding may be a determinant for the drug to displace nor-adrenaline. However, Musacchio et al. (1965) and Kopin et al. (1965) showed that a high affinity for vesicular binding was not necessary for a sympathomimetic to have an indirect action. They reported that although tyramine was converted intravesicularly to octopamine, this was not a prerequisite for its indirect action.

5. Effect of monoamine oxidase

It has been suggested that alpha-methylated sympathomimetics may exert a stronger indirect action than their non-methylated

analogues (Tsai, 1967). This was postulated since alpha-methylated compounds are not substrates for MAO and so, following membranal uptake, they would achieve higher cytoplasmic concentrations. Therefore, it might be expected that MAO not only governs the inactivation of cytoplasmic noradrenaline released by the indirect sympathomimetic, but also determines the cytoplasmic levels of the releasing amine. Smith (1966) showed that with tyramine (a substrate for MAO), inhibition of this enzyme potentiated the indirect sympathomimetic action.

6. Structure of synapse

de la Lande and Waterson (1968), using the rabbit ear artery, showed that tyramine administered extraluminally was more potent as a vasoconstrictor than when it was intraluminally administered. Their findings indicated that the indirect component of tyramine's action was more evident when the drug was administered extraluminally, since denervation or treatment with cocaine tended to make the routes equipotent.

This study provided evidence that in this vessel there is an appreciable gap between the nerve endings at the medio-adventitial border and the smooth muscle cells in the media. Van Orden et al. (1967) found in the cat nictitating membrane that the adrenergic innervation is in closer proximity to the smooth muscle than it is in vascular smooth muscle.

The Vascular Model

Many different experimental model systems have been used to examine and categorise the effects and modes of action of the

various sympathomimetics. These systems may be divided into vascular and non-vascular models. The latter is represented by systems such as rat vas deferens (Barrett et al., 1969), gut (Innes and Kohli, 1969), cat iris (Marley, 1962), and cat nictitating membrane (Fleming and Trendelenburg, 1961; Draskoczy and Trendelenburg, 1970).

The vascular and cardiac models are exemplified by the isolated perfused rat heart (Iversen, 1963; Burgen and Iversen, 1965), guinea pig atrium (Furchgott and Garcia, 1968), rabbit aorta (Maengwyn-Davies and Kopponyi, 1966), rabbit ear artery (de la Lande and Waterson, 1968), and the rat tail artery (Nicholas, 1969).

The ventral caudal artery of the rat has been used by a number of workers as a vascular resistance model for examining the actions of vasoactive substances. Nicholas (1969) used the isolated rat tail artery in an organ bath and found it responded to noradrenaline, electrical stimulation, tyramine, angiotensin and vasopressin. He suggested that the rat tail artery would be a suitable preparation for the assay of sympathomimetic amines. Wade and Beilin (1970) studied the effects of a number of vasoactive drugs using the isolated perfused rat tail. The whole rat tail perfused via the ventral artery was subject to oedema and consequently an increase in resting perfusion pressure. Addition of bovine serum albumin eliminated this problem but complicated the system by introducing a potent drug binding protein. In this thesis, a proportion of the work has been carried out using a segment of the isolated rat tail (Frost et al., 1976), thereby overcoming the problem encountered by Wade and Beilin (1970). In several other experiments, the ventral artery was removed from the

tail and set up in an organ bath for perfusion as described by de la Lande et al. (1966).

It has been established by de la Lande et al. (1967a) that the perivascular nerve plexus surrounding the smooth muscle of smaller arteries (Waterson and Smale, 1967; Waterson and de la Lande, 1967) plays a substantial role in controlling the sensitivity of the artery to catecholamines. It was also reported by Frewin et al. (1971b) that there is a close similarity between the histology and innervation of the human digital artery and that of the rabbit ear artery. The rabbit ear artery has been shown by de la Lande and Rand (1965) and Waterson and Smale (1967) to be a small artery consisting of a thick smooth muscle layer which is bounded on the luminal surface by the folded intima and on its outer aspect by loose adventitial tissue. Hodge and Robinson (1972) showed that the rat tail artery in section has a smaller diameter than the rabbit ear artery but is similar in other characteristics. They showed that both rat tail and rabbit ear arteries have a zone of specific catecholamine fluorescence at the medio-adventitial border and that this fluorescence represents the site of the sympathetic nerve varicosities. The specific fluorescence in rabbit ear arteries was eliminated by cervical ganglionectomy. On this basis, the rabbit ear artery would seem to be a useful preparation to examine the effects of sympathomimetic amines.

The isolated central artery of the rabbit ear, as described by de la Lande and Rand (1965) and perfused by the method of de la Lande, Cannell and Waterson (1966), was thus used as the test vessel in some of these experiments. The isolated rat tail artery has not been as exhaustively studied as the rabbit ear artery,

but on the basis of the aforementioned work can be considered to be a similar vascular model.

Mechanisms Available for the Inactivation
of Noradrenaline in Vascular Tissue

Neuronal uptake (Uptake 1)

One of the earliest studies which indicated that adrenergic nerves could accumulate adrenaline was that of Burn (1932). He found that the response to sympathetic nerve stimulation was improved by prior infusion of adrenaline. He also suggested that the amine was stored in the tissue from which it could be subsequently released.

Early studies on neuronal uptake were carried out by Axelrod et al. (1959) and Hertting et al. (1961), who showed that tissues were able to retain infused radiolabelled noradrenaline for long periods and this ability was lost if the adrenergic nerves were destroyed. Whitby et al. (1961) found that tissues from the cat accumulated tritiated noradrenaline and that this accumulation was greatest in tissues with high sympathetic innervation, such as the heart. Hertting and Axelrod (1961) also showed that this radiolabelled noradrenaline could be released by adrenergic stimulation.

Iversen (1963, 1965b) and Burgen and Iversen (1965), using the isolated perfused rat heart, demonstrated that the neuronal uptake process had stereochemical and structural specificity. They reported that (−) noradrenaline and (−) adrenaline were taken up more rapidly than the corresponding (+) isomers. There are problems involved in determining the stereospecificity of membranal

uptake, since both vesicular uptake (von Euler and Lishajko, 1967) and MAO deamination (Blashko, Richter and Schlossmann, 1937; Giachetti and Shore, 1966a) are highly stereoselective for the (-) noradrenaline isomer. Hence the stereoselectivity obtained in accumulation experiments is influenced by these secondary mechanisms. To eliminate these problems, experiments are performed in reserpine pretreated animals and in the presence of a monoamine oxidase inhibitor which has no effect on membranal uptake. Eckert et al. (1976a, b) and Henseling and Trendelenburg (1978) showed in rabbit aortic strips that axoplasmic uptake of noradrenaline showed no pronounced stereoselectivity, whereas vesicular accumulation and retention exhibited stereoselectivity when MAO was inhibited, with preference for the (-) isomer. They also showed that noradrenaline uptake was greater than that of adrenaline, which was in turn greater than that of isoprenaline.

Muscholl (1960), Axelrod, Whitby and Hertting (1961) and Dengler et al. (1962) demonstrated both in vivo and in vitro that pretreatment with reserpine greatly reduced the accumulation of exogenously administered noradrenaline. In 1962, it was found by Kopin and Gordon that most of the tritiated noradrenaline depleted from tissues by reserpine was deaminated by MAO. This suggested that the noradrenaline was deaminated and lost from the tissues instead of being bound. Lindmar and Muscholl (1964) showed that after pretreatment with reserpine, perfused rabbit heart still removed noradrenaline from the perfusate, but no net uptake could be observed. A combination of reserpine pretreatment and inhibition of MAO by iproniazid revealed that the amine was still removed from the perfusate but that, now, a significant net uptake

occurred. They also showed that this uptake was greatly dimin-
ished by treatment with either cocaine or guanethidine. Lindmar
and Muscholl concluded that "there are two mechanisms which
concentrate noradrenaline: (1) transport across cell membranes;
(2) uptake of noradrenaline into storage sites, and pretreatment
with reserpine will block the second."

Hamberger et al. (1964), using a fluorescent histochemical
technique, confirmed that reserpine impaired vesicular noradrenaline
storage. Waterson and de la Lande (1974) and de la Lande et al.
(1974) demonstrated that the perivascular nerve plexus of the
rabbit ear artery was still capable of taking up noradrenaline in
reserpine pretreated rabbits. This is in agreement with the find-
ings of Lindmar and Muscholl (1964).

Henseling et al. (1976a, b) and Eckert et al. (1976a) have
shown that intraneuronal accumulation of noradrenaline involves
at least two compartments. These two compartments are referred
to as axoplasmic and vesicular. Hence, neuronal uptake can be
taken to mean the transmembranal influx into the neurone.

Tainter and Chang (1927) reported that cocaine administered
systemically in a variety of animals inhibited the response of
tyramine and potentiated the response of adrenaline. Burn and
Tainter (1931) extended these findings, and showed that the actions
of tyramine and ephedrine were attenuated by cocaine, whereas
adrenaline was potentiated. It was also found that chronic dener-
vation of the pupil of the cat eye sensitised this organ to adren-
aline but abolished the effects of tyramine and ephedrine. Burn
and Rand (1958) showed that tyramine was without action in reser-
pine treated animals and that tyramine's pressor response could

be restored following an infusion of noradrenaline. Trendelenburg (1959) suggested that cocaine delayed the inactivation of noradrenaline and adrenaline, leading to a higher concentration at receptor sites and hence an augmented response. Macmillan (1959), as a result of observations made in isolated rabbit atria, suggested that cocaine prevented the uptake of noradrenaline by the storage sites. Hertting et al. (1961) found that tyramine reduced the amount of tritiated noradrenaline in the heart when given either before or after a noradrenaline infusion. It was then suggested that tyramine was releasing bound noradrenaline from its storage sites.

Falck and Torp (1962), using a new histochemical technique, were the first to conclusively show that uptake of noradrenaline was directly into adrenergic nerves and not into other ancillary sites. Hillarp and Malmfors (1964), also using histofluorescence techniques, showed that pretreatment with cocaine blocked the accumulation of noradrenaline by the adrenergic nerves in the rat iris. This provided direct evidence that adrenergic nerves had an efficient uptake system in the cell membrane and that this mechanism was inhibited by cocaine. de la Lande et al. (1967a, b) also suggested a prejunctional action for cocaine in inhibiting neuronal uptake in the rabbit ear artery.

Nicholas (1969) showed in the perfused rat tail artery that cocaine increased the sensitivity to noradrenaline. Subsequently, Wyse (1976), using rat tail artery strips, suggested that both exogenous and neuronal noradrenaline are preferentially inactivated by neuronal uptake and storage. Venning (1979) found that the sensitivity of the tail artery to extraluminal noradrenaline was greatly increased in the presence of cocaine. Vanhoutte and Webb

(1979), also using rat tail strips, suggested that neuronal uptake of noradrenaline following electrical stimulation was an important disposition mechanism for the transmitter in the rat tail artery.

Extraneuronal uptake (Uptake 2)

Iversen (1965a) showed that when the isolated rat heart was perfused with high concentrations of adrenaline or noradrenaline, saturation of Uptake 1 occurred. Once saturation of Uptake 1 had occurred, a sudden and large increase in tissue uptake followed. Iversen termed this Uptake 2, and suggested that this uptake was effective only at high concentrations of adrenaline or noradrenaline. Lightman and Iversen (1969) showed that when rat heart was incubated with low concentrations of catecholamines in the presence of MAO and COMT inhibitors, the normal levels of metabolites were substituted by unchanged amine. This suggested the variability in uptake previously observed may be due to varying degrees of metabolism and influences on uptake by normetanephrine. However, in this study, metabolites were measured only in the heart tissue, not in the perfusion fluid. Iversen subsequently revised his theory and suggested that extraneuronal uptake may occur at all catecholamine concentrations. Bevan and Su (1973) also showed that uptake does occur in rat heart at concentrations of amine which would occur physiologically both at nerve terminals and in circulating blood. This uptake was also shown to be saturable, with a higher capacity and a lower affinity for noradrenaline than neuronal uptake, but a higher affinity than neuronal uptake for isoprenaline. Gillespie and Towart (1973), using the rabbit ear artery, and Lightman and Iversen (1969), in studies involving

perfused rat heart, showed that the uptake system in smooth muscle has a low affinity and a high capacity.

Iversen (1965a), Gillespie (1973) and Henseling and Trendelenburg (1978) have demonstrated that Uptake 2 has no pronounced stereoselectivity and is inhibited by various steroids, e.g., corticosterone (Iversen and Salt, 1970), haloalkylamines, e.g., phenoxybenzamine (Iversen et al., 1972), and O-methylated metabolites of catecholamines, e.g., normetanephrine (Iversen, 1965a; Mireylees and Foster, 1973). Avakian and Gillespie (1968) found accumulation of noradrenaline in smooth muscle cells of the rabbit ear artery, as well as binding by collagen and elastin following incubation with high concentrations of noradrenaline. This accumulation of noradrenaline in smooth muscle cells was found to be abolished by phenoxybenzamine and normetanephrine. On the basis of this finding, Gillespie postulated that this smooth muscle uptake was similar to Iversen's Uptake 2. Gillespie and Muir (1970) found a large variability in the ability of arterial smooth muscle from different species and different smooth muscle in one species to accumulate noradrenaline. Evidence for confirming that Uptake 2 is a very significant mechanism for transmitter inactivation in either the rabbit ear artery or rat tail artery is scarce. de la Lande et al. (1974) showed that in reserpine pretreated and MAO inhibited arteries, restoration of noradrenaline fluorescence could not be obtained by intraluminal noradrenaline perfusion unless metanephrine was present. This contrasted with rapid restoration of fluorescence without metanephrine present when noradrenaline was applied extraluminally.

Wyse (1976) suggested that the rat tail artery may have a

relatively modest extraneuronal uptake system which could only be demonstrated when high concentrations of noradrenaline and combinations of uptake and metabolism inhibitors were used. Venning and de la Lande (1979) also showed in the perfused rat tail artery that deoxycorticosterone acetate caused only a small increase in sensitivity to intraluminal adrenaline which was not significant at low dose levels. Modifications of the Uptake 2 system have been suggested. Evidence now proposes that two extraneuronal compartments may exist (Kaumann, 1972; Eckert et al., 1976a, b; Henseling et al., 1976a).

Furthermore, Powis (1973) showed that noradrenaline would bind to collagen and elastin and that this binding was sensitive to tetracyclines. He found that in the rabbit ear artery and rat tail artery (tissues with high proportions of connective tissue), oxytetracycline enhanced the response to noradrenaline ten times. He suggested that this may be of some importance in small smooth muscular arteries. In summary, it would seem from the foregoing that extraneuronal uptake operates in the rat tail artery and rabbit ear artery, but is comparatively insignificant when compared with neuronal uptake mechanisms.

Monoamine oxidase (MAO)

Jarrott (1971a) has suggested that there are at least two forms of MAO in sympathetically innervated tissues with different substrate requirements. These two types can be broadly classified as neuronal and extraneuronal. Graefe and Eckert (1972) and Henseling and Trendelenburg (1978) have shown in the rabbit aorta that both these forms (that is, neuronal and extraneuronal MAO)

are stereoselective with a preference for the (-) isomer. de la Lande et al. (1970) and Horita and Lowe (1972) demonstrated the presence of extraneuronal MAO in rabbit ear artery and rat heart, respectively. Jarrott and Langer (1971), using cat nictitating membrane, Jarrott and Iversen (1971), on rat, guinea pig and rabbit vas deferens, and Coquil et al. (1973), using rat femoral and mesenteric arteries, reported a decrease in MAO activity follow-ing surgical denervation. These findings suggest the presence of neuronal MAO.

Biochemical studies by Jarrott (1971a) on rat heart and other tissues, and histochemical studies on rabbit ear artery by de la Lande et al. (1970a) did not show a decrease in MAO activity following denervation. It was suggested by Jarrott that the amount of neuronal MAO may be much lower than extraneuronal MAO in these tissues. It would seem, on the basis of these findings, that extraneuronal and intraneuronal MAO are present in different tissues, although their relative functions and physiological roles are yet to be fully defined.

de la Lande and Johnson (1972) showed that extraneuronal MAO inactivated high concentrations of noradrenaline. It was suggested that this extraneuronal MAO activity may account for the fact that intraluminal tyramine responses in the rabbit ear artery are lower than extraluminal responses (de la Lande and Waterson, 1968; de la Lande et al., 1970a). In a later study, de la Lande and Jellett (1972) showed that inhibition of MAO caused an increase in sensitivity of the rabbit ear artery to noradrenaline but only if the noradrenaline was applied extraluminally. de la Lande et al. (1974) supported these findings histochemically and suggested

that neuronal MAO was probably more important in determining the response of the rabbit ear artery to exogenous noradrenaline. This may be attributed to higher concentrations of noradrenaline achieved intraneuronally by an efficient neuronal uptake system. These observations are in agreement with those of Furchgott and Garcia (1968) using the isolated guinea pig atrium. de la Lande (1975) suggested that the influence the neuronal uptake process has in determining the concentration of noradrenaline at the receptors is controlled by the rate of inactivation of noradrenaline by intraneuronal MAO.

Catechol-O-methyl transferase (COMT)

Studies have demonstrated that in some tissues - for example, cat heart, rabbit and rat vas deferens - COMT activity is decreased by denervation (Crout and Cooper, 1962; Jarrott, 1971b; Jarrott and Iversen, 1971). Conversely, in other tissues, such as guinea pig vas deferens and rabbit ear artery, no such decreases in COMT levels following denervation were observed (Jarrott, 1971b; Jarrott and Iversen, 1971; Head et al., 1974, 1977b). Head et al. (1975) showed that neither denervation nor cocaine pretreatment inhibited the production of the major COMT metabolite subsequent to incubation with tritiated noradrenaline. On the basis of this finding, de la Lande (1975) suggested that COMT was probably distributed extraneuronally in the rabbit ear artery. Johnson and de la Lande (1977), using the rabbit ear artery, indicated that in the presence of cocaine (inhibiting neuronal uptake), COMT inhibition by UO521 caused a potentiation of the response to catecholamines. This potentiation was blocked by the Uptake 2 inhibitor deoxy-

corticosterone acetate.

The foregoing results agree with the findings of Eisenfeld et al. (1967), Bonisch et al. (1974), Graefe and Trendelenburg (1974), and Eckert et al. (1976b), who confirmed that the rate of extra-neuronal transport appears to determine the rate of O-methylation of noradrenaline. Eckert (1976b) also showed that COMT is far more important than MAO as a determinant of extraneuronal metabolism in nerve-free rabbit aortic strips.

Kalsner (1969) had also previously shown that inhibitors of COMT or extraneuronal uptake blocking agents potentiated the responses to adrenaline and noradrenaline, and these effects were not additive. No evidence is available to show whether or not COMT is present in the rat tail artery; however, it would not seem unreasonable to assume that, with its similarity to the rabbit ear artery, COMT probably has some role in this vessel.

Scope of Study

The present study was initiated to examine in detail the mechanism of action of the sympathomimetic agent etilefrine on blood vessels. From here, the project evolved to become a comparative study, which examined the vascular effects of etilefrine and a number of other sympathomimetics, including a new sympathomimetic agent, REN-293 (2-amino-3[3,5-dihydroxyphenyl]-l-propanol). As part of the overall protocol, the interactions between etilefrine and several other agents used in treating the clinical syndrome of dysautonomia were also investigated.

Etilefrine has been used primarily in the treatment of patients with orthostatic hypotension. The beneficial effect of drug therapy in these cases has been attributed to the similar action etilefrine has to noradrenaline (Miller, Wiener and Bloomfield, 1973). Mellander (1966) demonstrated that etilefrine had a local constrictor effect which was produced by alpha-receptor occupation, and also a distant dilator effect which was mediated via the sympathetic nervous system. He further showed that etilefrine caused a pattern of response which was quantitatively similar to that of noradrenaline. An important consideration in the selection of a sympathomimetic agent to treat orthostatic hypotension is the ability the drug has to exert a direct constrictor effect on vascular smooth muscle. Parks, Sandison et al. (1961), in reviewing the drug therapy of postural hypotension, showed that in patients with autonomic degeneration, the indirectly acting sympathomimetics ephedrine and methylamphetamine had no constrictor effect on denervated blood vessels. Nanda et al. (1977) cited a case where orthostatic hypotension was due to impairment of noradrenaline release and showed that tyramine had no pressor response; however, the directly acting sympathomimetics phenylephrine and noradrenaline exhibited marked pressor responses in this situation. With etilefrine, and any other sympathomimetic having a role in treating orthostatic hypotension, it is thus important to know the extent to which the drug relies on the sympathetic nerves for its constrictor action.

The experiments to be described in this thesis seek to examine the effects of two sympathomimetics (etilefrine and REN-293) on small muscular arteries and to elucidate their possible mechanisms

of action.   In addition,   these   investigations   considered   some
aspects of the influence that neuronal uptake, extraneuronal uptake,
and metabolism by monoamine oxidase (MAO) and catechol-O-methyl
transferase (COMT) had on the actions of these sympathomimetics.

*   *   *

CHAPTER 2

GENERAL METHODS

This chapter describes the general methodology used in the perfusion and efflux experiments that were carried out during this project. The following methods and techniques are described.

1. The technique used for the perfusion of the rat tail segment via the ventral caudal artery.

2. The technique used for the perfusion of both the isolated ventral tail artery and the central artery of the rabbit ear.

3. The autoanalyser assay for catecholamines which was to confirm the success of surgical or chemical sympathetic denervation.

4. The technique of surgical denervation of the rabbit ear artery.

5. The protocol used for determining ^{3}H-noradrenaline efflux from rat tail arteries and the modification of this technique for the purpose of determining ^{3}H-noradrenaline metabolites.

6. The procedure used for separating ^{3}H-noradrenaline and its metabolites.

7. Tests of statistical significance.

All other experimental procedures are described in the appropriate sections of each chapter. A list of drugs used throughout the project is included in the Appendix.

1. Perfusion of rat tail segment

Male albino Sprague-Dawley rats (weighing 250-350 g) were stunned and exsanguinated. The tail was severed from the trunk and the proximal section of the tail then divided into two 3 cm segments. The proximal end of the ventral artery was located in each segment and cannulated under a dissecting microscope using a Sterivac No. 2 heat-drawn polythene cannula (external diameter 2 mm, internal diameter 1 mm). The cannulated segments were placed in a warming chamber at $37\,^{\circ}C$ and perfused with Krebs-bicarbonate solution (see Appendix 1a) containing EDTA (10 μM). Furchgott (1955) has reported that concentrations of EDTA of this order of magnitude neither inhibit nor potentiate the effects of adrenaline and related compounds on vascular smooth muscle. The apparatus used is shown in Figure 2.1. The perfusion fluid was gassed with 95% O_2, 5% CO_2 (Carbogen). and then pumped through a constant volume roller pump (see Appendix 1b) at a flow rate of 4-5 ml/min. Pressure changes were measured using a Statham P23GC or P23DC pressure transducer and recorded on a Rikadenki pen recorder (see Appendix 1c). The perfusion fluid flowed into the segment via the cannula in the ventral artery (Fig. 2.2) and then through the severed end of the tail, being finally removed from the warming chamber via the overflow escape. The perfusion fluid was warmed in a bath and again pre-warmed just before it entered the artery. The warming chamber (Fig. 2.3) was a per-spex box containing a brass tank heated to $37\,^{\circ}C$ by circulating water. This chamber was covered by a perspex lid during the experiment to maintain high humidity.

A five-channel sequential timer (see Appendix 1d) was used
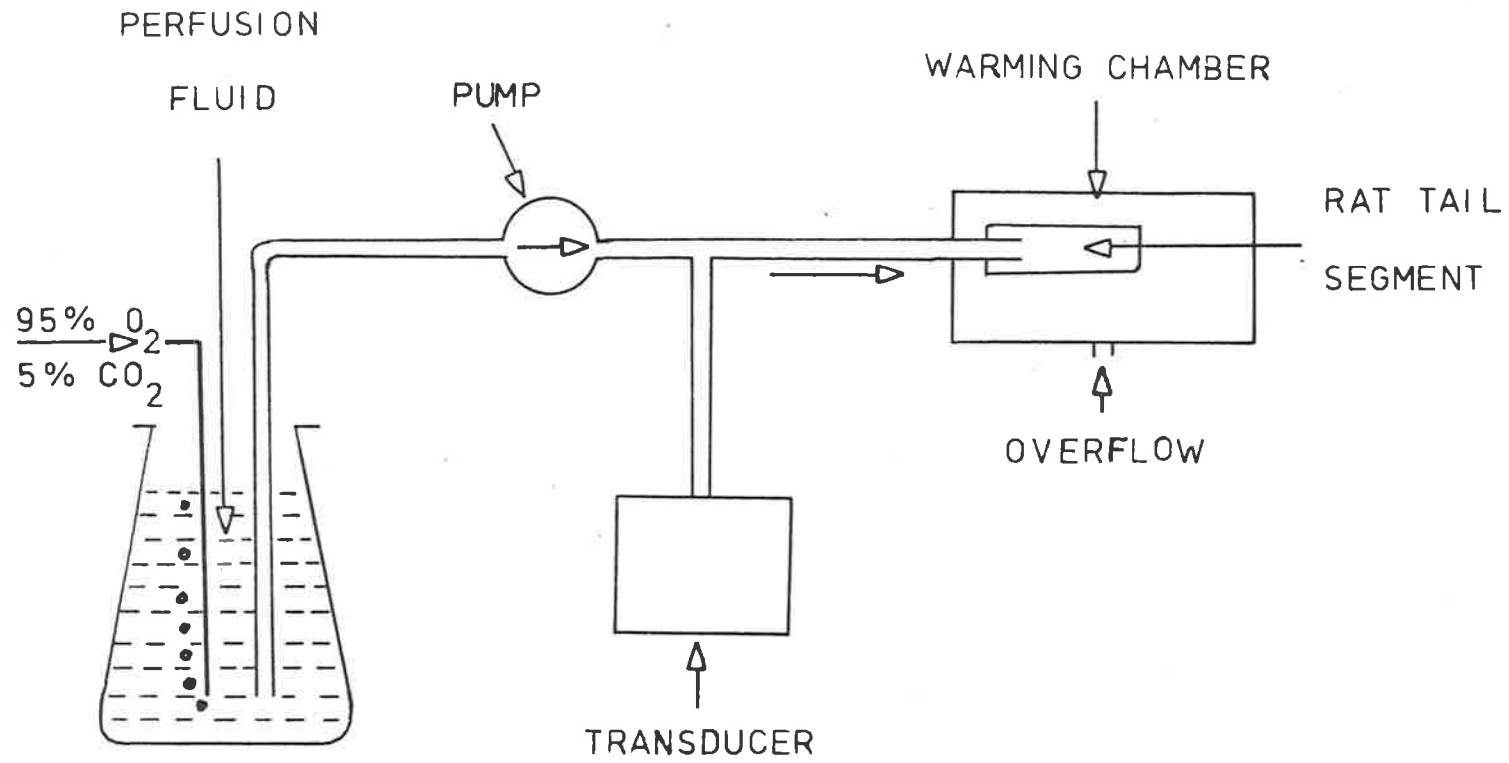
Fig. 2.1

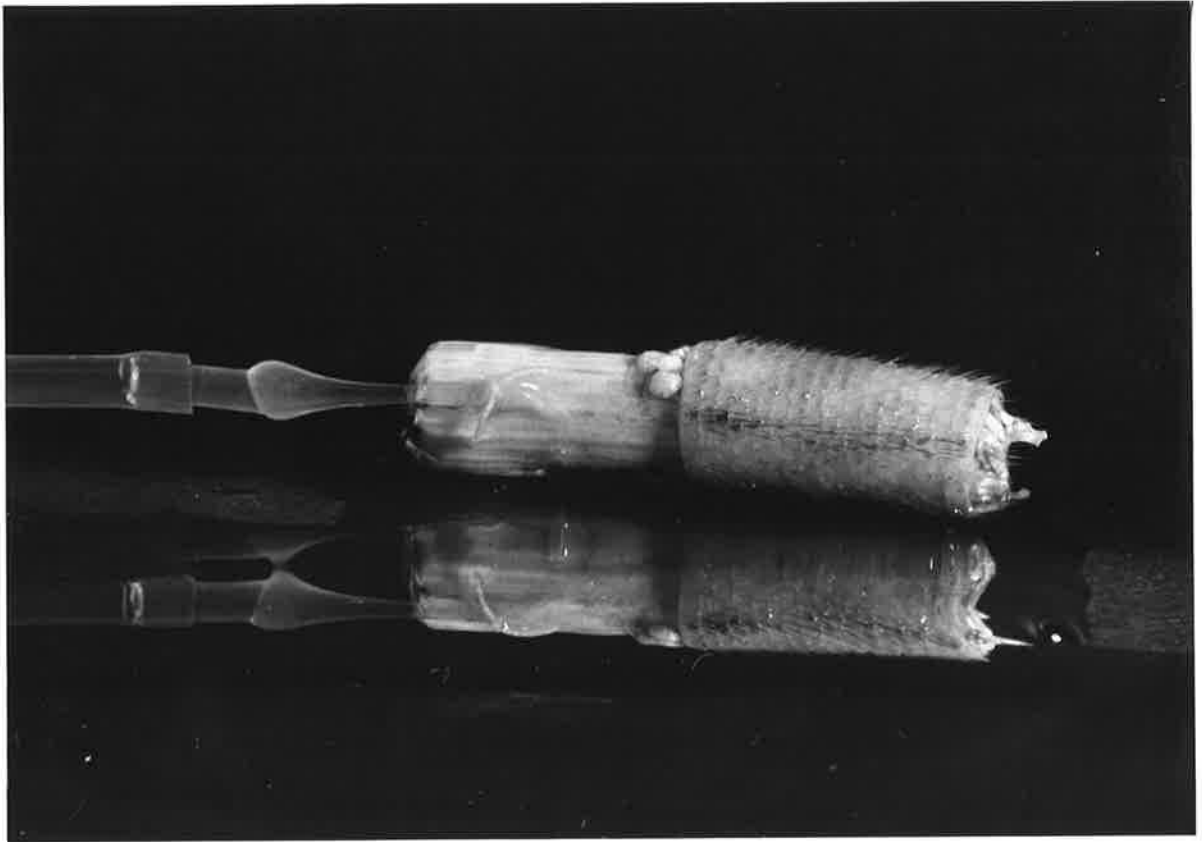The perfusion apparatus used in the study of rat tail segments.

Fig. 2.2

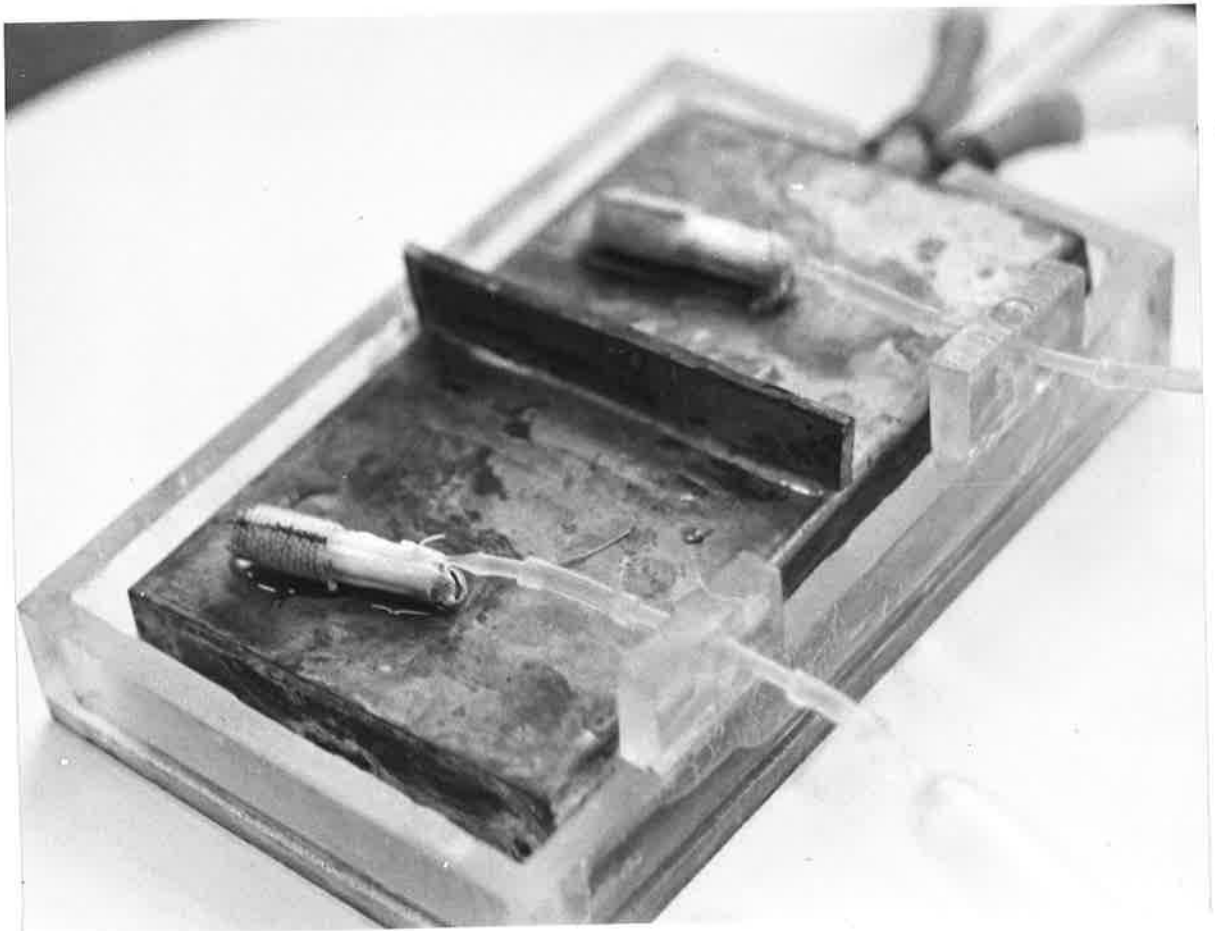The rat tail segment showing the cannula in the ventral artery.

Fig. 2.3

The warming chamber used in the study showing the rat tail
segment "in situ."

to change the perfusing drug solutions in some experiments and thus cumulative dose responses could be obtained. The tail segments were allowed to perfuse with Krebs for 30 minutes before any drugs were administered. This resulted in a resting perfusion pressure of approximately 30 mmHg. All drugs were added to the perfusion fluid and were thus applied intraluminally. Each test drug was maintained in contact with the intraluminal surface of the vessel until a maximum response was obtained.

2a. Perfusion of the isolated rat tail artery

Male albino Sprague-Dawley rats (weighing 250-350 g) were stunned and exsanguinated. The tail was then severed from the trunk and the ventral caudal artery isolated. A piece of this artery, approximately 3 cm in length, was removed from the proximal end. A Sterivac No. 3 heat-drawn polythene cannula was inserted into the proximal end with the aid of a dissecting microscope, and a finer cannula (Sterivac No. 2) was inserted and tied into the distal end of the artery. Approximately 1.5 to 2 cm of artery remained between the tips of the cannulae. The artery was then quickly set up in a vertical, double-jacketed organ bath (15 ml capacity), and bathed extraluminally and perfused intraluminally with Krebs-bicarbonate solution at 37°C. The upper distal cannula was held at a tension of 1 g. Figure 2.4 is an illustration of the perfusion apparatus, which was similar to that described by de la Lande et al. (1966). The perfusion fluid was gassed with 95% O_2, 5% CO_2 (Carbogen) and then pumped through a constant volume roller pump at a flow rate of 4-5 ml/min. Pressure changes were measured using a Statham P23GC or P23DC pressure
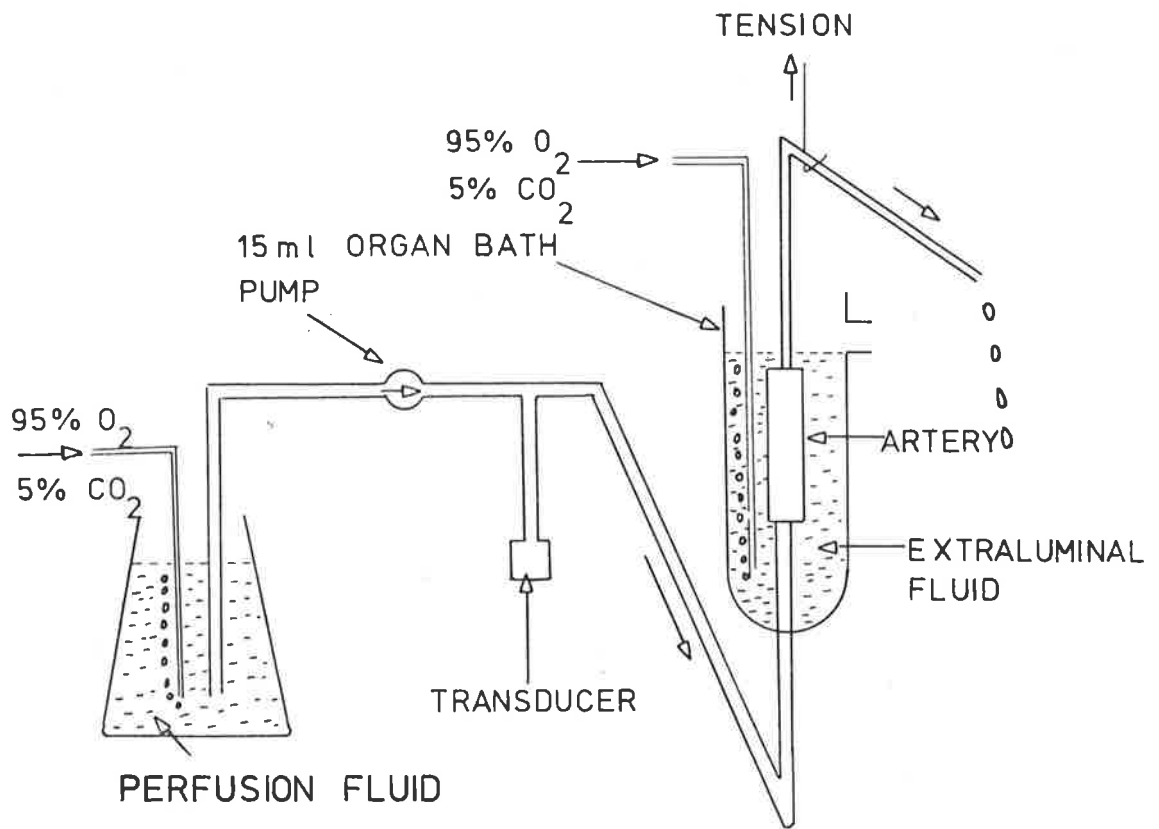
Fig. 2.4

The perfusion apparatus used in the studies involving isolated rat tail arteries and rabbit ear arteries.

transducer and recorded on a Rikadenki pen recorder. Two segments of artery from the same animal were always used and perfused simultaneously. The vessel was allowed to equilibrate for 30 minutes and the recorded resting perfusion pressure was approximately 30 mmHg. Absence of leakage between the extraluminal and intraluminal fluid compartments was routinely tested at the beginning of each experiment by observing the level of the extraluminal fluid in the organ bath. On some occasions, at the end of the experiment, leakage was also tested by perfusing the artery intraluminally with 1% Evans Blue dye and examining the extraluminal fluid for the appearance of the dye. Approximately one artery in six displayed evidence of leakage, usually through a side branch not detected during dissection; such arteries were rejected. Drugs were administered to the arteries either extraluminally or intraluminally and maintained in contact with the vessel until a maximal response was obtained.

2a. Perfusion of the rabbit ear artery

Semi-lop-eared rabbits, bred at the Central Animal House, Waite Agricultural Research Institute, University of Adelaide, were used in all perfusion experiments and generally weighed between 1.5 to 2.5 Kg. Rabbits were first stunned and exsanguinated. The overlying skin at the base of the ear was then removed. A 3-4 cm segment of artery up to the first major branch was dissected clear of adhering tissue (Fig. 2.5). Tissues were kept moist with Krebs-bicarbonate solution (see Appendix 1a), and a Sterivac No. 3 heat-drawn polythene cannula was inserted into the proximal end of the artery and tied into position. A finer cannula (Sterivac
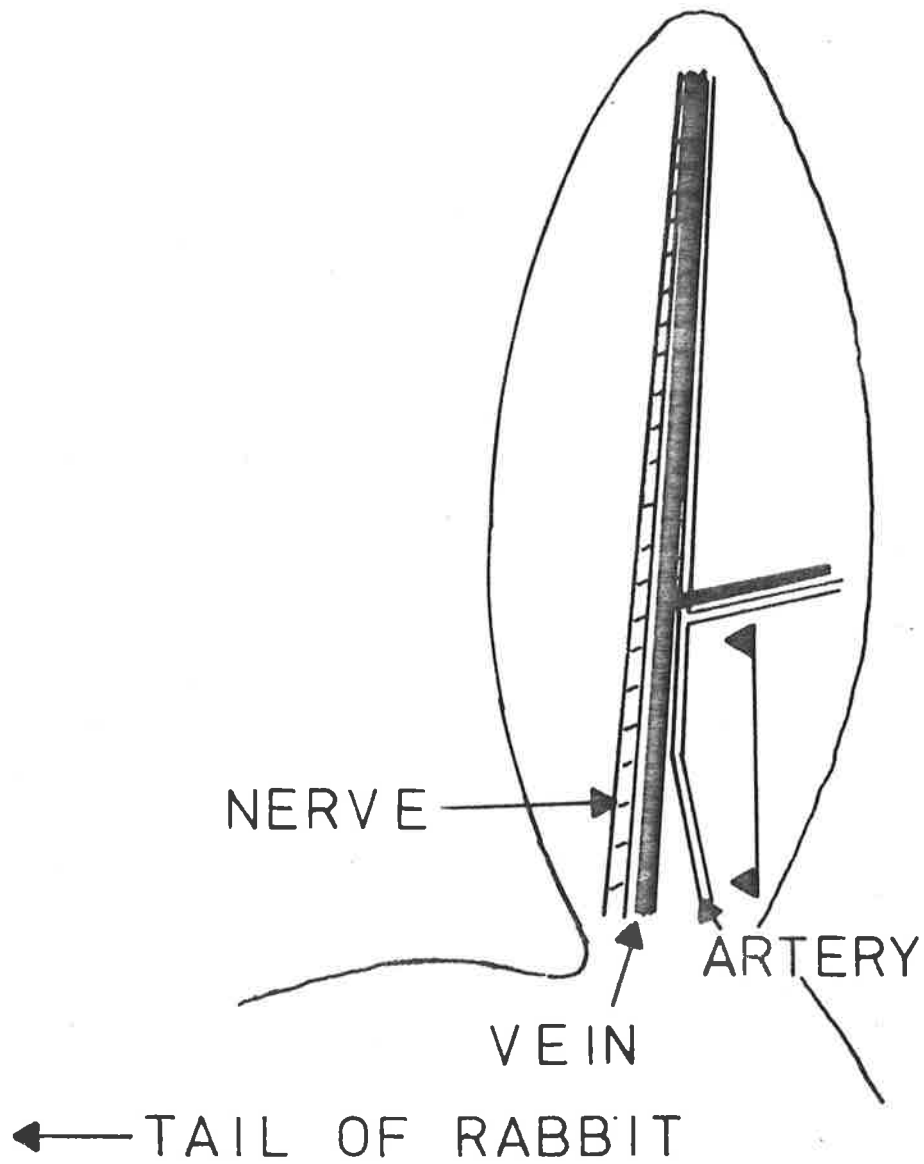
Fig. 2.5
The rabbit ear showing the anatomical location of the rabbit ear artery used in some of the studies.

No. 2) was inserted and tied into the distal end of the artery. Approximately 1.5 to 2 cm of artery remained between the tips of the cannulae. The artery was then quickly set up in a vertical, double-jacketed organ bath (15 ml capacity), bathed extraluminally and perfused intraluminally with Krebs-bicarbonate solution at 37° C. The upper distal cannula was held at a tension of 1 g. Figure 2.4 is an illustration of the perfusion apparatus, which was similar to that described by de la Lande et al. (1966). The perfusion fluid was gassed with 95% O_2, 5% CO_2 (Carbogen) and then pumped through a constant volume roller pump (see Appendix) at a flow rate of 4-5 ml/min. Pressure changes were measured using a Statham P23GC or P23DC pressure transducer and recorded on a Rikadenki pen recorder (see Appendix 1c). Two arteries from the same animal were always perfused and monitored simultaneously. The perfusate exiting from the distal cannula was discarded. Drugs could be added independently to either the Krebs solution bathing the adventitia (extraluminal application) or the internal perfusate (intraluminal application). Leaks from small side branches were rare and easily detected at the commencement of each experiment due to a spontaneous increase in the volume of the extraluminal bath. Leaking arteries were discarded.

3. Autoanalyser catecholamine assay

Estimations of tissue catecholamine content were made by the catecholamine assay technique described by Head et al. (1977). A Technicon Autoanalyser train was used, which consisted of a sampler, proportioning pump and fluorometer. The recorder was a Rikadenki Model B1G1 potentiometric recorder, which was matched

to the fluorometer by installation of a transistor emitter follower circuit. The fluorometer was set at maximum sensitivity, and height of responses (determined by catecholamine content in assay) was controlled by a millivolt attenuator on the recorder. The auto-analyser functioned by (1) sampling unknown and standard solutions of catecholamine automatically; (2) neutralising these solutions to pH 5.0-6.0; (3) oxidising catecholamines with potassium ferri-cyanide; (4) forming fluorescent tri-hydroxyindole (THI) derivatives by treating the oxidised catecholamine with sodium hydroxide in the presence of ascorbic acid (As/Acid); (5) estimating the fluorescence produced by THI derivatives.

The fluorescence was continuously measured at the following wavelengths: 410 nm for activation (interference filter) and emission at greater than 485 nm (Wratten No. 8 sharp cut filter). The flow diagram of the system is shown in Figure 2.6. The catecholamine content of the arteries was measured by removing the arteries from the tails, weighing them, and placing them into tubes containing 1 ml of EDTA (30 μM) in HCL (100 mM). The tissues were left 18-24 hours at 4 ^{o}C, and the catecholamine content was determined from this clear acid extract by using the autoanalyser system described above. Successful denervation of the tail artery was confirmed by detecting a substantial decrease in the catecholamine content of the surgically treated vessel by comparison with normal control arteries.

4. Surgical sympathetic denervation of the rabbit ear artery

It was found by de la Lande and Rand (1965) that removal of the homolateral superior cervical ganglion caused denervation
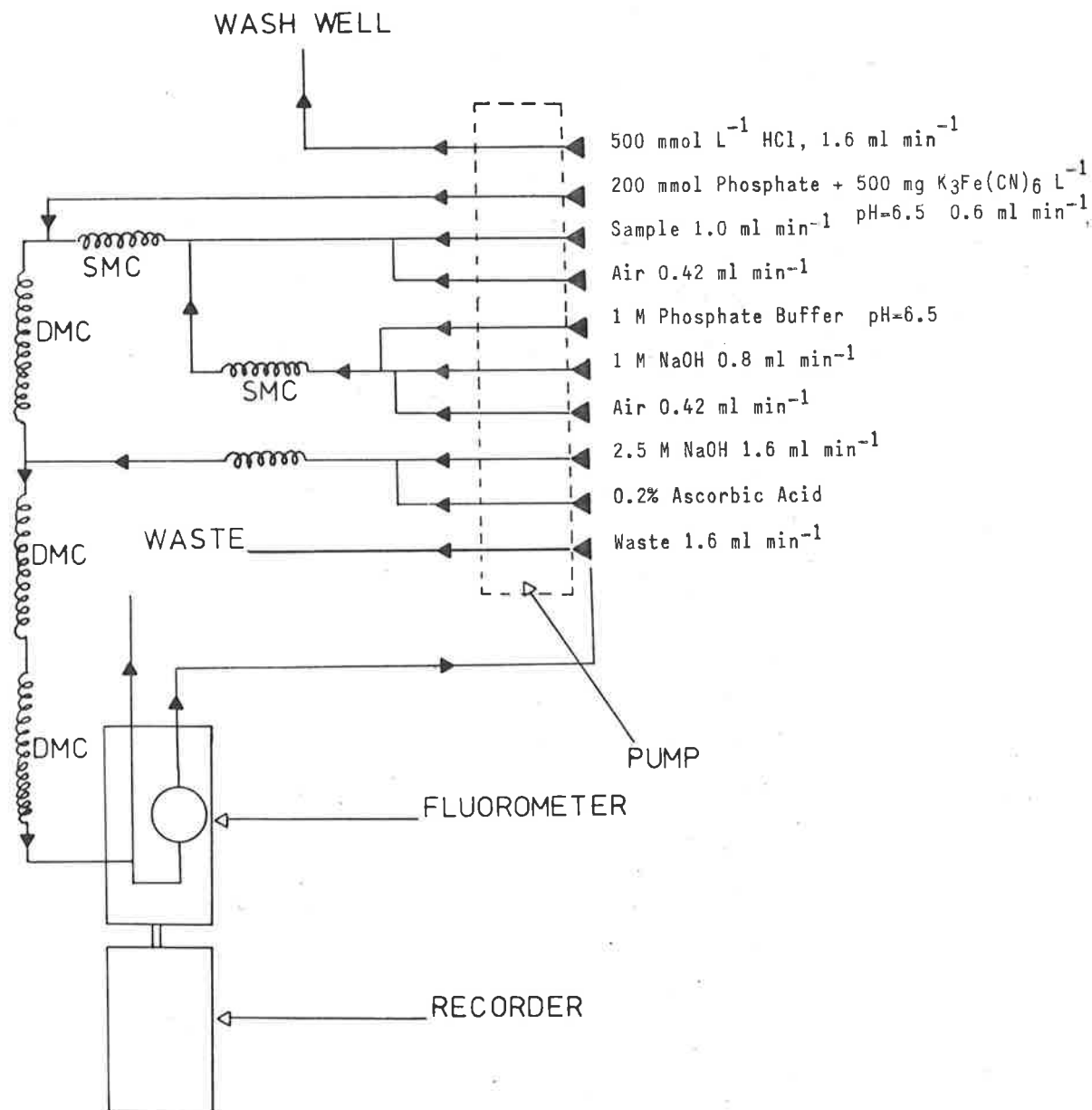
Fig. 2.6

Flow diagram for the autoanalyzer when used for the assay of catecholamines. The reagents, their concentrations and flow rates are shown. SMC and DMC refer to single mixing coil and double mixing coil, respectively.

of the rabbit ear artery. Rabbits were anaesthetised with pento-
barbitone (1:5 dilution with sterile saline) injected into the
marginal ear vein (Murdock, 1969). The dose of barbiturate varied
considerably but was usually within the range of 30-50 mg/Kg.
Injection at a slow rate continued until the toe reflex was weak
and respiration was decreased in rate but increased in depth. The
neck was extended, shaved and cleansed with Savlon 3% in 70%
ethanol. A midline incision through skin and muscle was made to
expose the trachea. The left carotid artery was located and the
superior cervical ganglion identified adjacent to the thyroid carti-
lage and under the angle of the mandible. The ganglion was
removed with approximately 1 cm of tissue attached on either side
to ensure that the pre- and post-ganglionic fibres had been taken.
The wound was closed with silk sutures. Antibiotic cover was not
routinely administered since the surgery was conducted using an
aseptic technique. Experiments using denervated arteries were
carried out as close to 21 days following ganglionectomy as pract-
icable.

5. ^{3}H-noradrenaline efflux protocol

Male Sprague-Dawley rats (250-350 g) were stunned and
exsanguinated. The tail was then severed from the trunk and the
ventral artery isolated. A piece of this artery, measuring approx-
imately 3 cm, was removed and placed in Krebs-bicarbonate solution.
The artery was blotted dry (with the intraluminal fluid being care-
fully expressed) and then weighed. The vessel was then pre-
incubated for 30 minutes in 1 ml Krebs-bicarbonate solution with
EDTA and ascorbic acid (see Appendix 1e) at 37^oC and bubbled

with 95% O_2, 5% CO_2 before being incubated in either a further 1 ml of ascorbic Krebs containing ($\pm$) ^{3}H-noradrenaline 1.18 μM or ^{14}C-sorbitol 1.1 μM for 30 minutes.

The equipment used in the efflux studies (designed and built by the author and illustrated in Figure 2.7) provided a reliable and efficient method for continuously washing the arteries with 1 ml aliquots of ascorbic Krebs.

Following incubation, the artery segment was transferred to the syringe containing 1 ml of ascorbic Krebs bubbled with 95% O_2, 5% CO_2 (Carbogen). After 1 minute, the wash cycle was initiated. Relay 1 was operated by timer 1 providing a sufficient period for the relay which clamped thin silicone tubing to remain open and allow the 1 ml wash to drain into a collection vial. Timer 1 then triggered timer 2, which caused a closing of relay 1 and an opening of relay 2, thus allowing a volume of 1 ml of ascorbic Krebs to be dispensed from reservoir 1. This Krebs solution was kept warmed to 37°C and gassed with Carbogen. Timer 2 then triggered timer 3, which closed relay 2 and opened relay 3, which in turn allowed a quantity of ascorbic Krebs to be released into reservoir 1 from reservoir 2 until overflow occurred. To ensure that a constant volume of 1 ml was always dispensed into the 1 ml syringe, a constant pressure head had to be maintained as well as accuracy of timing (provided by timer 2). Variation in volume rarely exceeded 1-2%, i.e., 0.01-0.02 ml. Timer 3 then triggered timer 4, which closed relay 3. Timer 4 provided a time buffer to obtain an interval of 1 minute between washes. Timer 4 then triggered timer 1, which opened relay 1 and repeated the cycle. This process continued for 20 minutes. Each 1 ml wash

RESERVOIR 2
(100 ml)

95% O$_2$
5% CO$_2$

R3

RESERVOIR 1
(20 ml)

OVERFLOW
TO WASTE

95% O$_2$
5% CO$_2$

R2

1 ml

SYRINGE

WARMING
CHAMBER

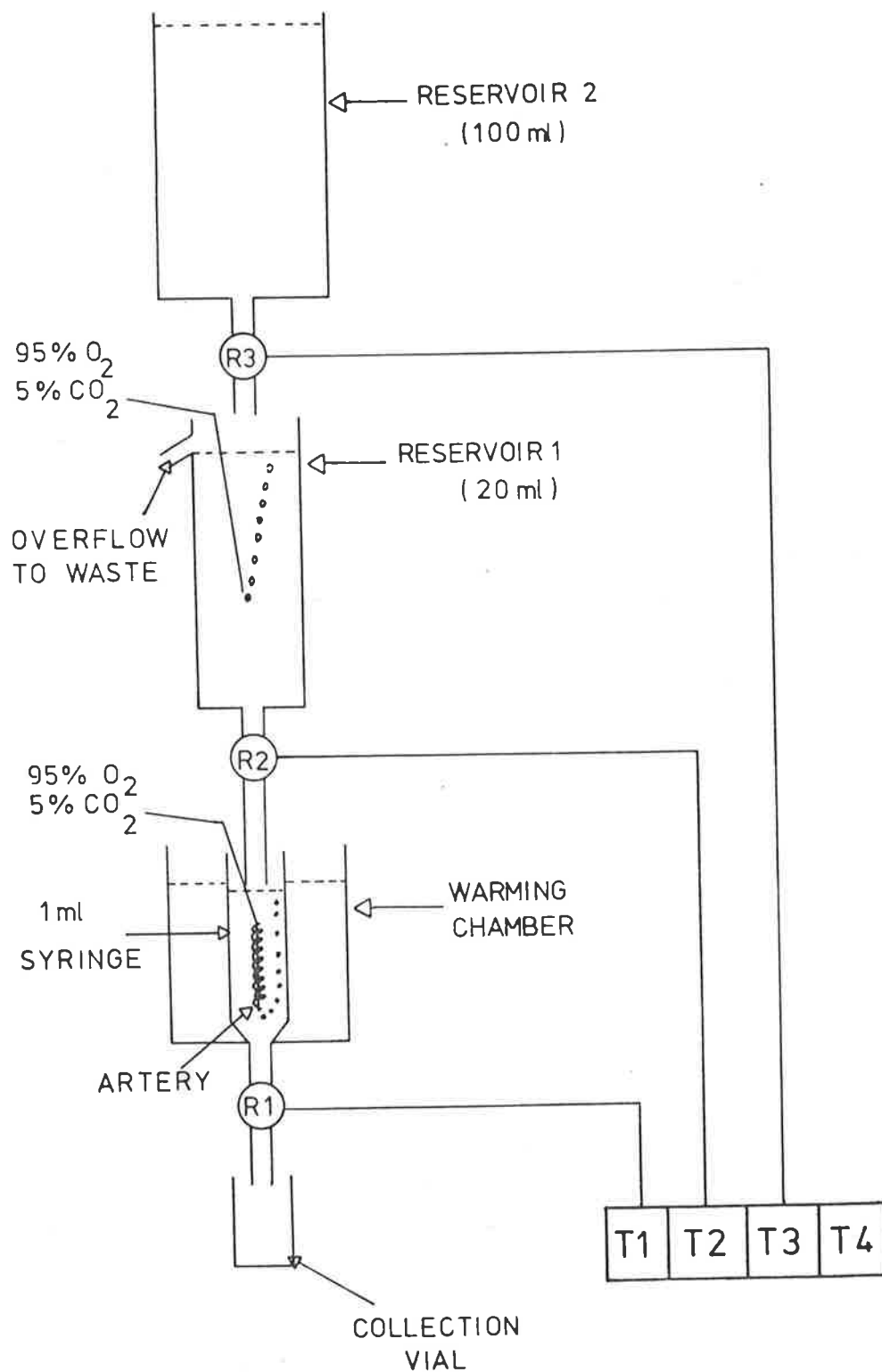ARTERY

R1

COLLECTION
VIAL

T1 | T2 | T3 | T4

Fig. 2.7

The system used to wash the tail arteries is shown in this diagram. Each timer T1–T3 was responsible for opening and closing relays R1–R3, thus allowing aliquots of Krebs to wash the artery and be subsequently collected.

collected during this period was retained and added to 10 ml of scintillation fluid (see Appendix 1f). The samples were counted using a Packard 2425 liquid scintillation spectrometer and corrected for efficiency using an automatic external standard. Results were expressed as pmol ^{3}H-NA/g/min.

6a. ^{3}H-noradrenaline and metabolite efflux protocol

Male Sprague-Dawley rats (250-350 g) were stunned and exsanguinated. The tail was then severed from the trunk and the ventral artery isolated. A piece of artery measuring approximately 7 cm was removed and placed in Krebs-bicarbonate solution. The artery was blotted dry (with the intraluminal fluid being carefully expressed) and then weighed. Weights varied from 14.0 to 21.0 mg. The vessel was then pre-incubated for 30 minutes in Krebs-bicarbonate solution with EDTA and ascorbic acid (ascorbic Krebs) (see Appendix 1e) at 37°C and bubbled with 95% O_2, 5% CO_2 before being incubated in (-)^{3}H-noradrenaline (^{3}H-NA) 1.18 μM for 30 minutes. The incubation fluid was sampled (0.1 ml) before and after each incubation. Following the incubation, the artery was removed and immersed in a 1 ml aliquot of ascorbic Krebs for 5 minutes. This procedure was repeated six times, with a fresh aliquot of ascorbic Krebs each time, using the equipment described in the previous methods section (Fig. 2.7). Drugs were introduced into the immersion (wash) fluid at the commencement of the third 5 minute period and maintained in contact with the segment for all subsequent washes up to the 35 minute maximum. The two 0.1 ml samples of incubation fluid and the seven 1 ml washes were then assessed for ^{3}H-NA and ^{3}H metabolite content using alumina and

Dowex columns (Graefe et al., 1973). A correction was made for crossover and recovery of NA metabolites (Morris and Irvine, personal communication; see Appendix 1g).

After the ^{3}H–NA and the various metabolites had been separated from each wash aliquot, a unit volume (1 ml) of each eluate or effluent was sampled and added to 10 ml scintillation fluid (see Appendix 1f). The samples were counted using either a Packard 2425 or 3310 liquid scintillation spectrometer and corrected for efficiency using an automatic external standard or by obtaining a channels ratio.

6b. Metabolite separation

The method used was similar to that described by Graefe et al. (1973). The 1 ml tritiated wash volume from the artery or the 0.1 ml sample from the incubation fluid (to which was added a further 0.9 ml Krebs–bicarbonate to keep the volumes consistent) was collected in a chilled vial containing 0.2 ml 0.1 N HCl and 0.1 ml 1% ascorbic acid. These samples were kept frozen until assay. Immediately prior to loading on to the alumina column, the following solutions were added to each sample – the Tris–buffer added last: (1) 0.1 ml 1% ascorbic acid; (2) 0.1 ml 12.5% Na_2SO_3; (3) 0.1 ml 10% EDTA; (4) 0.01 ml of a cold carrier solution containing a mixture of noradrenaline metabolites, each at a concentration of 1 mg/ml, i.e., VMA, MOPEG, DOMA, DOPEG, NMN and NA; (5) 0.5 ml 1 N Tris–buffer at pH 8.4. The total volume thus loaded on to the alumina column (see Appendix 1h) was 2.11 ml. The column was then washed with 4 ml of water. This effluent was allowed to run on to a Dowex 50Wx4 column (see Appendix 1i),

which was subsequently washed with 1 ml water. The final volume of the effluent was therefore 7.11 ml. This effluent contained 0-methylated deaminated noradrenaline metabolites (OMDA), i.e., methoxyhydroxyphenylglycol (MOPEG) and methoxyhydroxymandelic acid (VMA). No attempt was made to further separate these metabolites, and a 1 ml aliquot was added to 10 ml scintillation fluid and counted. The Dowex column was then washed with 2 ml of a 6 N HCl-ethanol mixture (ratio 1:1). This eluate contained normetanephrine (NMN) and 1 ml of this solution was also counted.

The alumina column was then washed with 2 ml 0.2 N acetic acid, and this wash allowed to run on to a second Dowex column and collected. This second Dowex column was washed with 2 ml water, making a final volume of 4 ml. This eluate contained dihydroxyphenylglycol (DOPEG). Again, 1 ml was sampled and counted as before. This second Dowex column was now washed with 2 ml 6 N HCl-ethanol (ratio 1:1). This eluate contained the noradrenaline fraction and 1 ml of this was sampled and counted. The alumina column was next washed with 4 ml 0.5 N acetic acid and this was discarded. As the final step, the alumina was washed with 2 ml 0.2 N HCl and this wash was collected. In this final wash, dihydroxymandelic acid (DOMA) had been eluted, and 1 ml was again sampled and counted as before. (Fig. 2.8).

7. Tests of significance

When comparing the effects of different drugs and these effects under different conditions, a Student's t-test was performed as defined by Runyon and Haber (1968). Paired or unpaired t-tests were performed, depending on which one of these was appropriate.
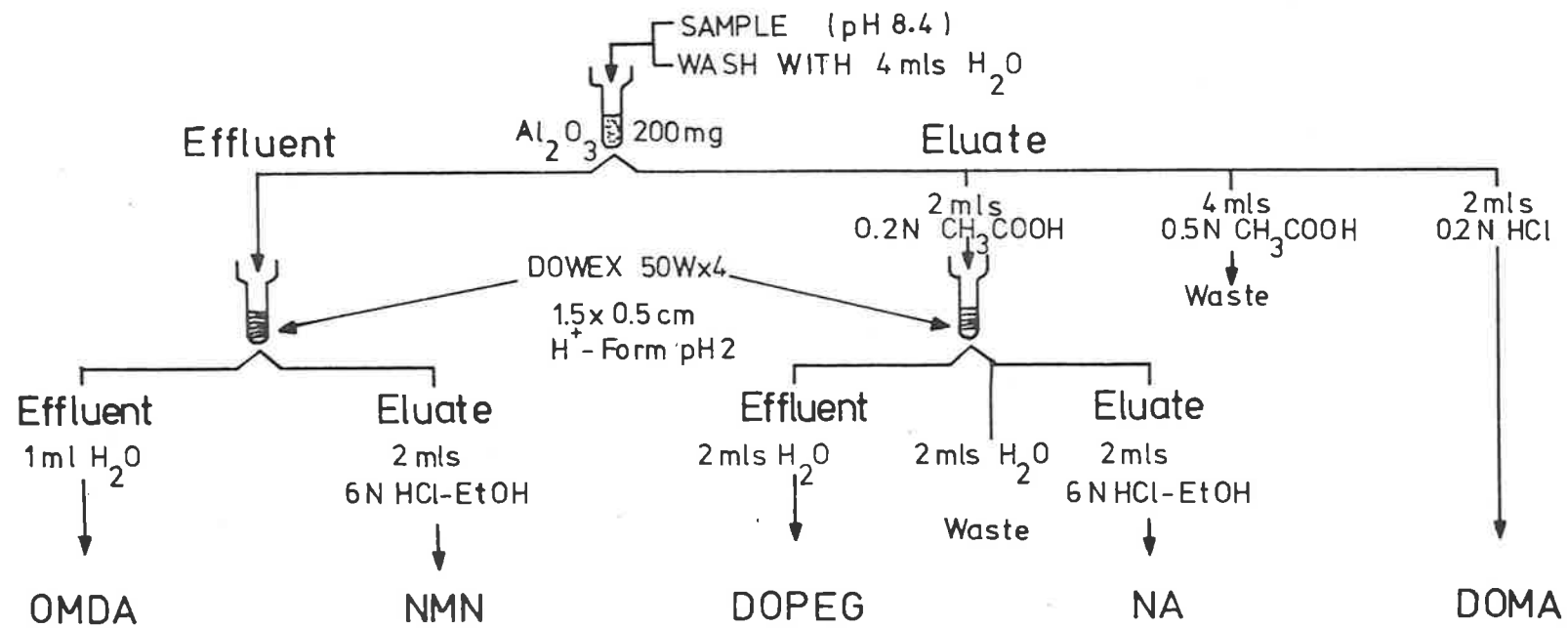
Fig. 2.8

The column separation technique used in this study to separate ^{3}H–NA and its various metabolites.

Where directional hypotheses were asserted (i.e., "the response was decreased" or "the response was enhanced") then one-tailed probability levels were applied to the t-values. Significance levels are generally indicated throughout the text; however, where convenient, differences between observations are described as "significant" ($p < 0.05$) or "not significant" ($p > 0.05$). Tests of significance for differences between dose-response curves were generally made at individual dose points and the overall significance indicated in the text.

*   *   *

CHAPTER 3

THE EFFECTS OF ETILEFRINE ON BLOOD
VESSELS IN THE RAT TAIL

Introduction

Etilefrine is a sympathomimetic amine which has been used in the treatment of patients with orthostatic hypotension (Miller et al., 1973). Offermeier and Dreyer (1970) demonstrated that etilefrine has both alpha- and beta-stimulant effects on the rat vas deferens and the rat atrium, respectively. Mellander (1966), using a cat calf muscle preparation, suggested that etilefrine had "a local constrictor effect which was mediated by alpha-receptors" and also a "distant" dilator effect which was activated via the sympathetic nervous system. He further showed that etilefrine caused a pattern of response which was quantitatively similar to that of noradrenaline. Coleman et al. (1975) showed in a clinical trial that etilefrine caused a positive chronotropic effect and also increased peripheral vascular resistance. They concluded that etilefrine has both alpha- and beta-adrenergic effects in man.

The experimental drug REN-293 (2-amino-3-[3-5-dihydroxyphenyl]-1-propanol) has also been developed as an agent for use in the treatment of orthostatic hypotension. This compound has been shown to have alpha-receptor activity in the rat vas deferens (personal communication – Boehringer Ingelheim). Their studies showed that reserpine pretreatment inhibited the contractile response to REN-293 and that repeated administration of this agent resulted in tachyphylaxis.

An important consideration in the selection of a sympatho-

mimetic agent to treat orthostatic hypotension is the ability the drug has to exert a direct constrictor effect on vascular smooth muscle. Parks et al. (1961), in reviewing the drug therapy of postural hypotension, showed that in patients with autonomic degeneration, the indirectly acting sympathomimetics, ephedrine and methylamphetamine, had no constrictor effect on denervated blood vessels. In this chapter, an attempt has been made to determine the mechanism of action of etilefrine and REN-293 using a sympathetically innervated vascular bed, i.e., a perfused rat tail segment, and to compare the effects of these drugs with other sympathomimetics on the same preparation.

Methods

The rat tail artery segment was single cannulated in this series of experiments and perfused with Krebs-bicarbonate solution as described in Chapter 2. Using the protocol outlined in that chapter, the following series of drug applications were carried out.

1. Increasing doses of adrenaline, noradrenaline, phenylephrine, metaraminol, etilefrine, REN-293, tyramine and ephedrine were applied to the artery and dose response curves constructed in a manner described by de la Lande, Glover and Head (1967). The relative constrictor activity of the drugs was determined by constructing a mean dose response curve for each agent and from it calculating the appropriate dose producing pressure responses of 50, 100, 150 and 200 mmHg. A mean was then obtained from the four available measurements and the shift to the right of the

adrenaline curve expressed as a ratio of 1:mean shift (i.e., a reciprocal was obtained).

2. Etilefrine and noradrenaline were administered to the tail artery segments from rats which had been pretreated with guanethidine (25 mg/Kg intraperitoneally on alternate days for a period of seven weeks).

3. The effects of etilefrine (5.5 μM), REN-293 (91 μM) and noradrenaline (148 nM) infusions were examined on tail segments from rats which had been pretreated with reserpine. The latter was injected intraperitoneally at a dose of 2.5 mg/Kg, 24 hours before the experiment.

4. The effect of the alpha-receptor blocking drug phentolamine on the vasoconstrictor response of etilefrine, phenylephrine, noradrenaline, adrenaline and REN-293 was examined. The doses of these agents were selected so as to give equivalent vasoconstriction on the rat tail segments, and the phentolamine (1.78 μM) was administered for ten minutes before and also during the testing of these drugs.

5. The response to an infusion of etilefrine (5.5 μM) was determined before and after a five minute infusion of each of the following drugs: (a) noradrenaline (148 nM), (b) tyramine (1.45 mM) and (c) cocaine (29.5 μM). In this series, etilefrine was also administered to a control artery segment with the same time sequence as that used in (a), (b) and (c) so as to monitor any spontaneous changes in vascular sensitivity which may have occurred during the experiment.

6. The response to an infusion of REN-293 (91 μM) was determined before and after a five minute infusion of each of the

following drugs: (a) tyramine (1.45 mM), and (b) cocaine (29.5 μM).   In this series, REN-293 was also administered to a control artery segment with the same time sequence as that used in (a) and (b) so as to monitor any spontaneous changes in vascular sensitivity which may have occurred during the experiment.

7. The response to 5.5 μM infusion of etilefrine was determined in artery segments from rats which had been pretreated with 6-hydroxydopamine.   The 6-hydroxydopamine was administered in a dose of 100 mg/Kg intraperitoneally on day 1 and on day 2, and the arteries then tested on day 3.   At least five rat tail artery preparations were used in each test series.   Mean values were obtained for all responses and the S.E.Ms calculated and included in the figures wherever appropriate.

Results

Figure 3.1 shows the dose response curves to adrenaline, noradrenaline, phenylephrine, metaraminol, etilefrine and REN-293 as obtained from the rat tail artery segment.   (Although tyramine and ephedrine were also tested on the preparation, the results for these agents are not shown in Figure 3.1.)   The responses for tyramine and ephedrine are shown in Table 3.1.   Adrenaline was found to be the most active agent on the preparation and ephedrine the least active.   The mean ratio of activity of the drugs compared to adrenaline was adrenaline : noradrenaline : phenylephrine : metaraminol : etilefrine, i.e., 1 : 0.38 : 0.06 : 0.02 : 0.01. With REN-293, tyramine and ephedrine it was not possible to obtain
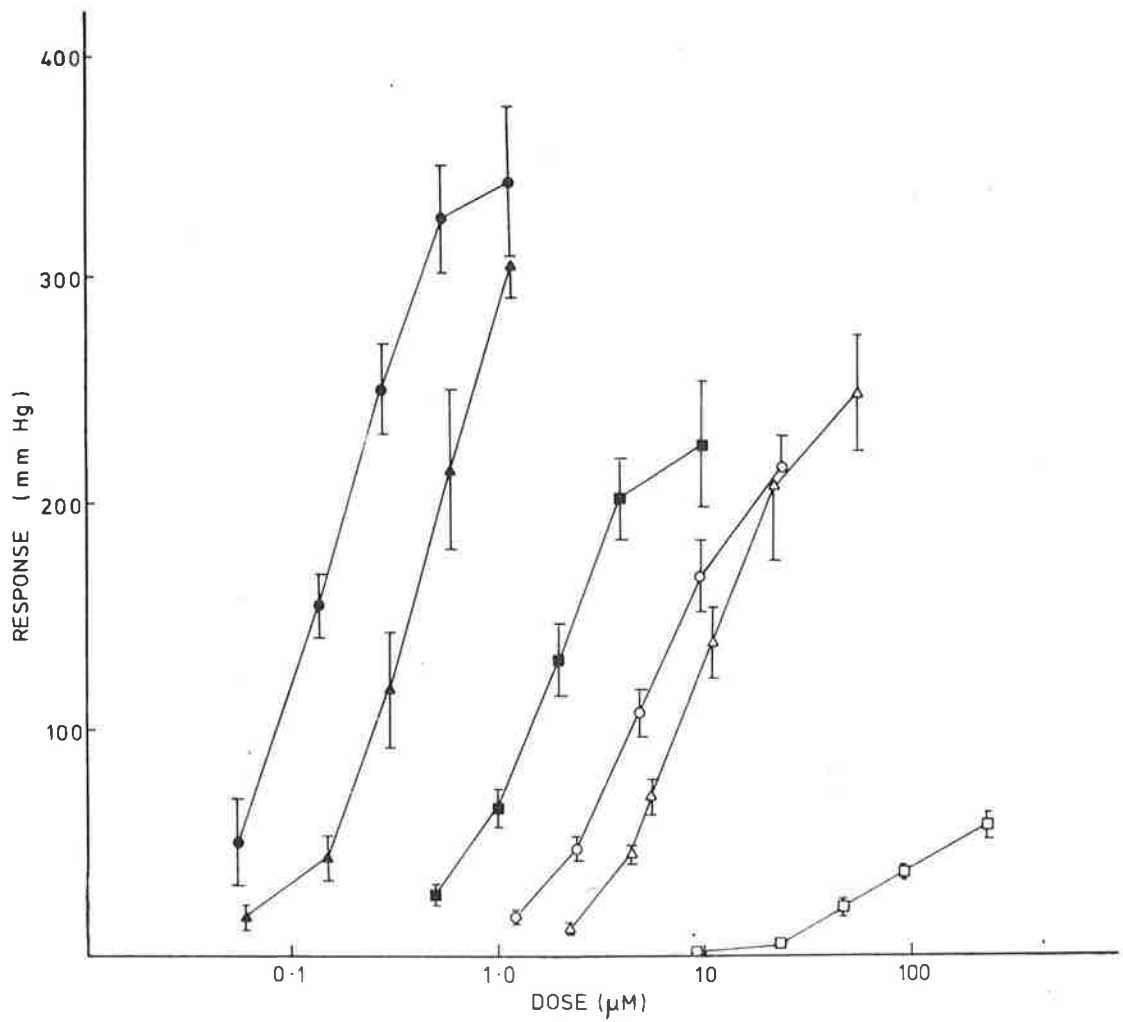
Fig. 3.1

Comparative dose response curves for adrenaline ($\bullet$), noradrenaline ($\blacktriangle$), phenylephrine ($\blacksquare$), metaraminol (O), etil-efrine ($\triangle$) and REN-293 ($\square$) on the rat tail artery. Each point represents the mean $\pm$ S.E.M. of the results of at least five experiments.

pressure responses above 100 mmHg. Tyramine exhibited tachy-phylaxis and ephedrine, too, showed very weak activity. Thus the calculation of shifts in dose response curve as described earlier could not be implemented.

The shape of the standard etilefrine response on the rat tail artery is shown in Figure 3.2a and compared with the response to the drug in a segment pretreated with 6-hydroxydopamine (Fig. 3.2b) and the vascular response to noradrenaline (Fig. 3.2c). The etilefrine response demonstrated a prompt initial phase which was followed by a slower rise in pressure, till a maximum was reached in approximately ten minutes. In contrast, the noradren-aline response was monophasic, with the maximum constriction being generated within a short time of the drug being applied to the preparation. 6-Hydroxydopamine pretreatment completely abolished the "slow-rise" phase of the etilefrine response. The response to noradrenaline in the 6-hydroxydopamine pretreated segment was within the normal range. REN-293, when administered as a 15 minute infusion at a dose of 91 μM, also exhibited a biphasic res-ponse similar to etilefrine. Figure 3.3 shows the response of tail artery segments from rats which had been chronically pretreated with guanethidine. The vasoconstriction produced by noradrenaline remained unaltered, while the magnitude of the response to etil-efrine was significantly reduced. Reserpine pretreatment signifi-cantly reduced the vasoconstriction produced by etilefrine on the rat tail artery segment ($p < 0.005$). The vascular response to nor-adrenaline was unaffected, while that of REN-293 was completely abolished.

Phentolamine, the alpha-receptor antagonist, blocked the
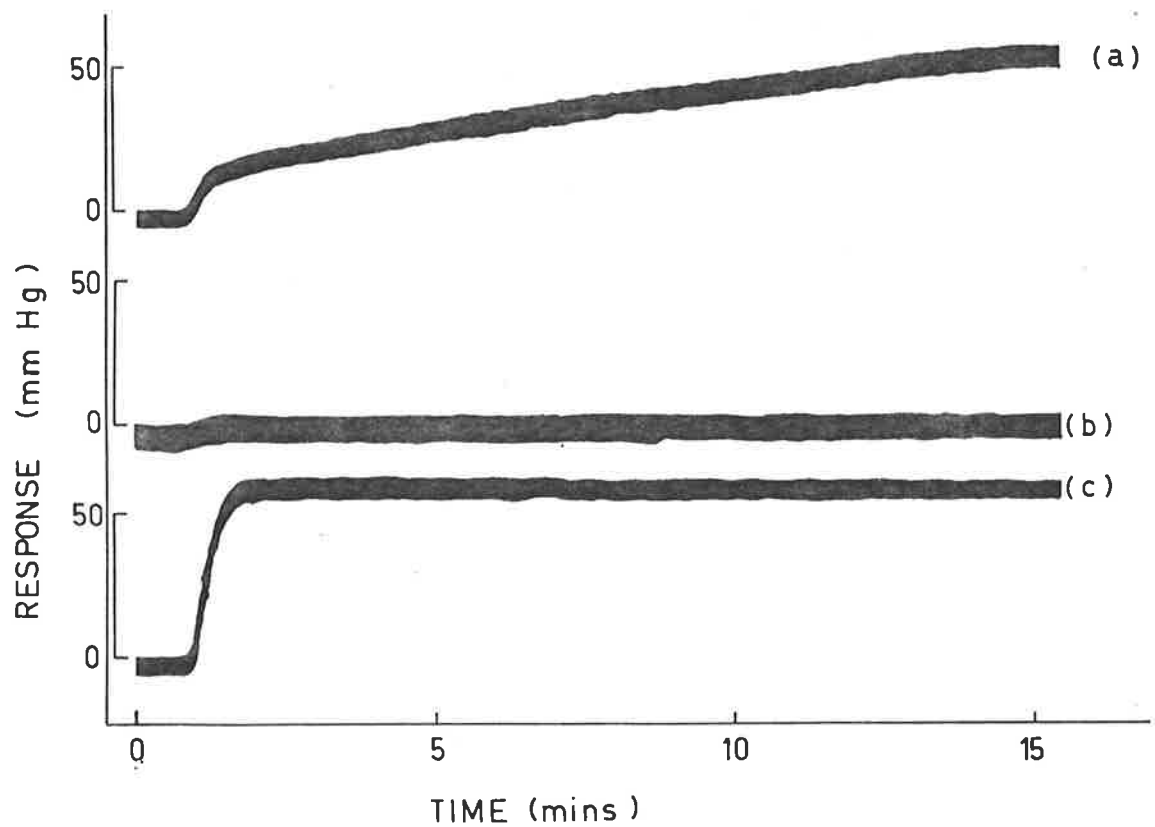
Fig. 3.2

The actual vascular response (mmHg) obtained to (a) a 5.5 μM infusion of etilefrine in an untreated artery; (b) a 5.5 μM infusion of etilefrine in an artery pretreated with 6-OH dopamine; and (c) a 148 nM infusion of noradrenaline in an untreated artery.

Fig. 3.3

Comparative dose-response curves to noradrenaline in tail artery segments of untreated rats (●) and in rats pretreated with guanethidine (▲). Comparative dose-response curves to etilefrine on the same blood vessels are also shown in untreated arteries (■) and pretreated arteries (o).

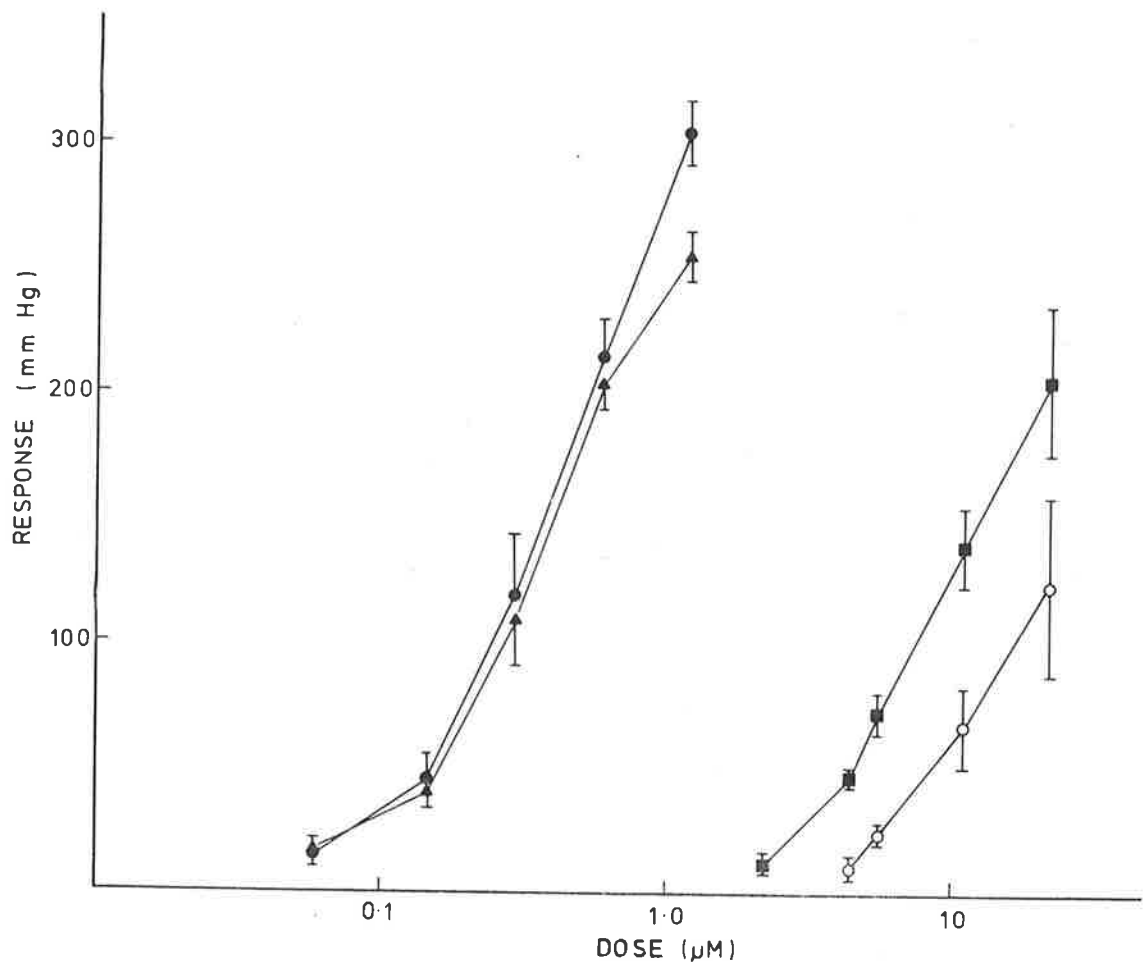constrictor response to etilefrine, adrenaline, noradrenaline, phenylephrine and REN-293 on the rat tail artery.

Following a 148 nM infusion of noradrenaline for five minutes, the response to etilefrine was significantly augmented (Fig. 3.4a). Following a 1.45 nM infusion of tyramine for the same time period, the etilefrine response was significantly decreased (Fig. 3.4b). Cocaine (29.5 μM) enhanced the response of the rat tail segment to etilefrine (Fig. 3.4c). The mean response to etilefrine in the time control remained unchanged from the initial value (Fig. 3.4d). An infusion of tyramine (1.45 mM) for five minutes significantly decreased the response to REN-293 (Fig. 3.5a). Cocaine (29.5 μM) also significantly depressed the response to REN-293 on the rat tail segment (Fig. 3.5b). In the time control, the response to REN-293 was significantly increased (Fig. 3.5c). The arteries from the rats pretreated with 6-hydroxydopamine showed a significant decrease in their responsiveness to a 5.5 μM infusion of etilefrine ($p < 0.005$).

Discussion

The results of this chapter indicate that etilefrine possesses a significant indirect sympathomimetic action on the rat tail artery. This statement is based on several findings.

Guanethidine has been shown (Lindmar and Muscholl, 1966) to be taken up into adrenergic neurones by the same uptake involved with noradrenaline accumulation and to displace intraneuronal noradrenaline from storage granules. Chronic guanethidine

Fig. 3.4

The open columns represent the mean ± S.E.M. response to a 5.5 μM infusion of etilefrine before treatment with noradren-. aline, tyramine and cocaine. The hatched columns show the response to a 5.5 μM infusion of etilefrine following an infusion of (a) 148 nM noradrenaline, (b) 1.45 mM tyramine, and (c) 29.5 μM cocaine. The cross hatched column (d) represents the response to etilefrine given to a control rat tail segment with the same time sequence as the second etilefrine dose in the treated arteries. This was to monitor any spontaneous change in vascular sensitivity with time
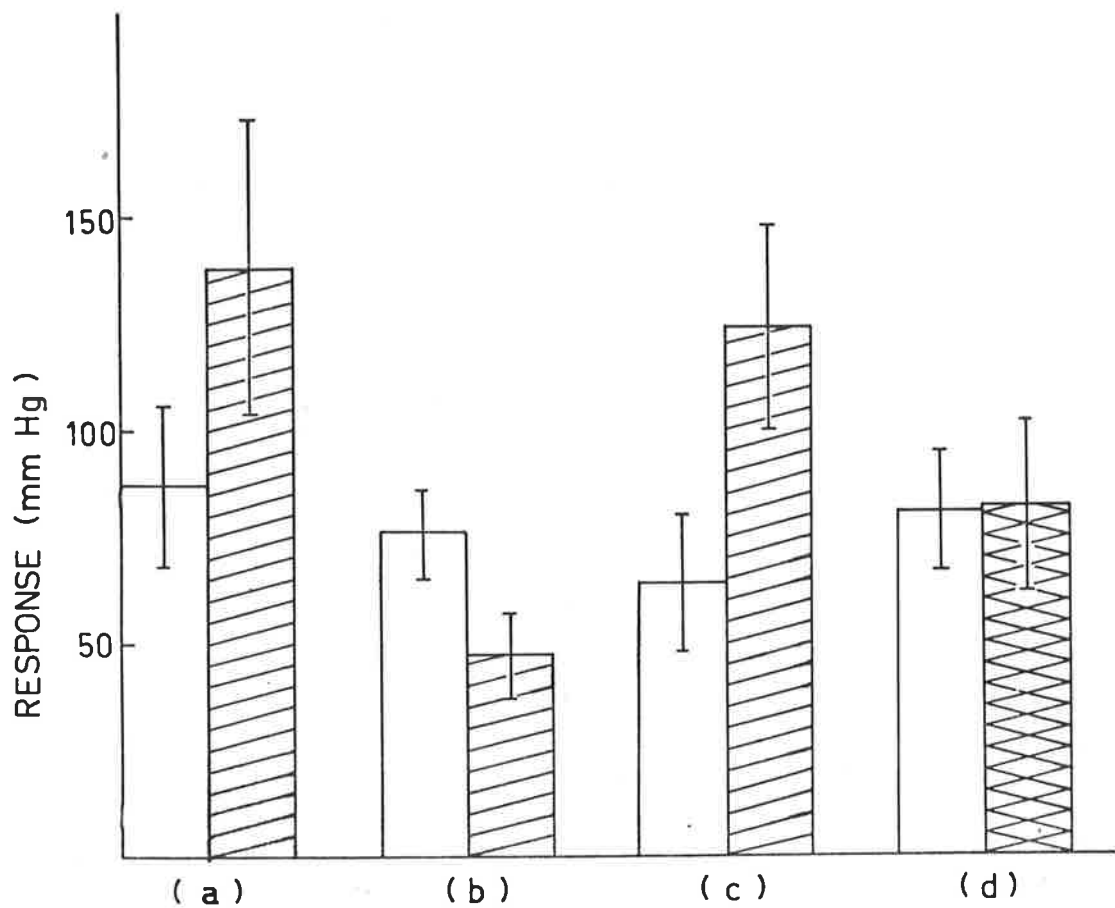
Fig. 3.5

The open columns represent the mean ± S.E.M. response to a 91 μM infusion of REN-293 before treatment with tyramine and cocaine. The hatched columns show the response to a 91 μM infusion of REN-293 following the infusion of (a) 1.45 mM tyramine and (b) 29.5 μM cocaine. The cross hatched column (c) represents the response to REN-293 given to a control rat tail segment with the same time sequence as the second REN-293 dose in the treated arteries. This was to monitor any spontaneous change in vascular sensitivity with time.
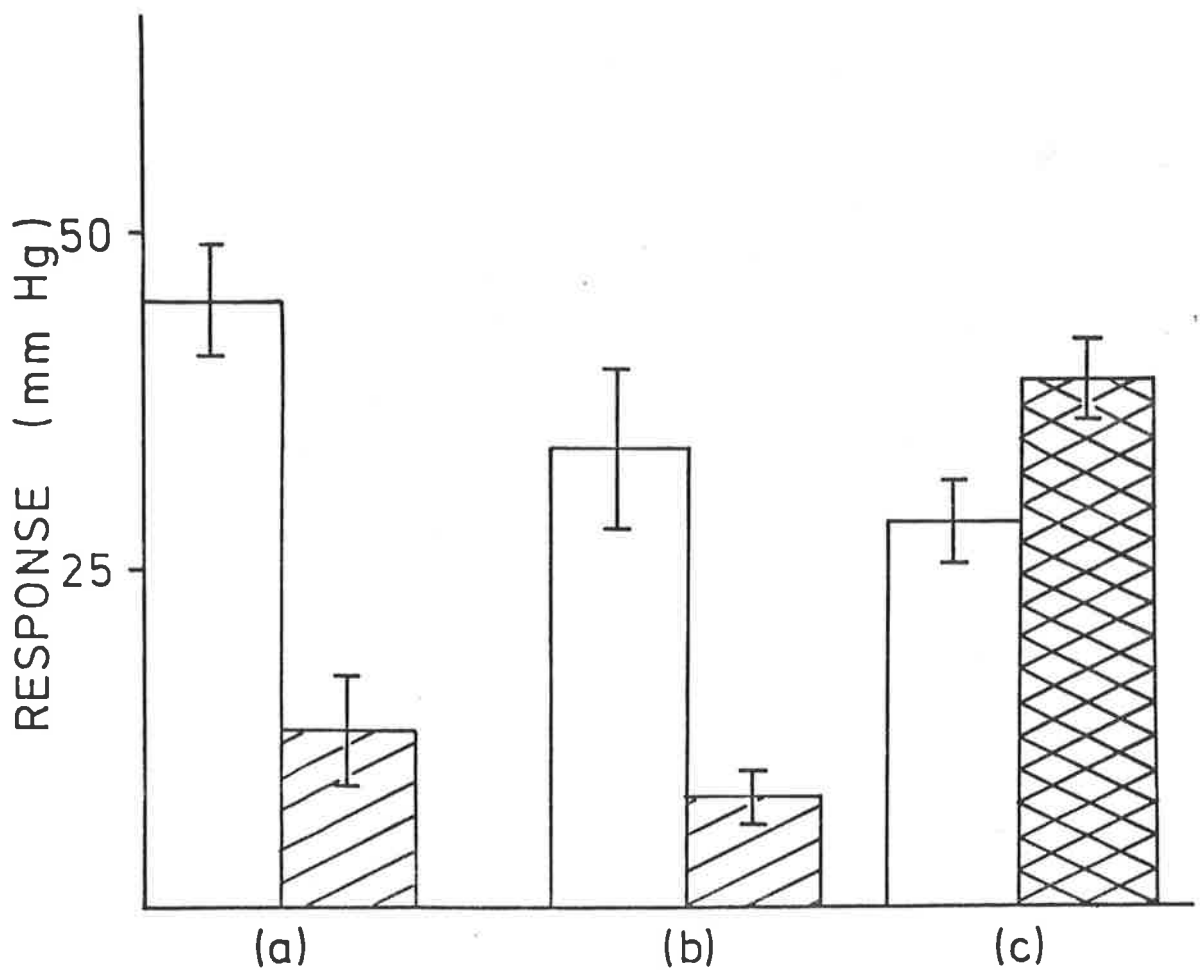
administration causes depletion of noradrenaline and an enhancement of the sensitivity of effector cells to catecholamines similar to that observed following sympathetic denervation. In the series of experiments using guanethidine, the results showed that the response to etilefrine was decreased, indicating that etilefrine is in part dependent on the integrity of the sympathetic nerves for its action. No supersensitivity to exogenous noradrenaline was observed with guanethidine pretreatment, which did not alter the noradrenaline response, except at the highest dose used, where a decrease in response was observed.

Reserpine has also been shown to deplete neuronal noradrenaline stores by inhibiting the uptake of transmitter into the intraneuronal storage vesicles (Roth and Stone, 1968). Reserpine differs from guanethidine in that it is not taken up by the neuronal uptake mechanism and has a slower rate of entry. The results show that the etilefrine response is significantly decreased following pretreatment with reserpine and that of REN-293 is completely abolished. The finding that reserpine completely eliminates the response to REN-293 is in agreement with preclinical data provided by the pharmaceutical company (Boehringer Ingelheim). Following pretreatment with 6-hydroxydopamine, which has been shown to selectively destroy sympathetic nerve endings (Thoenen and Tranzer, 1968), a reduction in the response to etilefrine was observed. 6-Hydroxydopamine inhibits the action of indirectly acting sympathomimetics due to its depletion of neuronal noradrenaline.

On the basis of the results using these three agents which compromise adrenergic neurone function, it seems likely that etilefrine has an indirect sympathomimetic component to its action.

The prior administration of tyramine (an indirectly acting sympathomimetic - Fleckenstein and Burn, 1953) significantly reduced the magnitude of the etilefrine response. Both these agents are presumably acting on a common pool of releasable noradrenaline and tend to modify the vascular effects caused by each other. When tyramine is administered before the addition of REN-293, the response to REN-293 is also depressed. This is similar to that observed for etilefrine, except the depression of the REN-293 response is greater, indicating the greater indirect action of the latter. The depression of the REN-293 response following cocaine was due to the inhibition of REN-293 uptake. Burgen and Iversen (1965) suggested certain criteria for a sympathomimetic amine to be a substrate of neuronal uptake. Based on these criteria, REN-293 would be a good substrate for uptake, and hence be blocked by cocaine, thus decreasing REN-293 uptake and hence depressing its noradrenaline releasing potential.

The vascular response to etilefrine, which showed a secondary "slow rise" phase and was similar to that observed with REN-293, strongly contrasted with the response to noradrenaline, suggesting an indirect sympathomimetic component. Additional supportive evidence for such a component came from the enhancement of the etilefrine response by both noradrenaline and cocaine. The former observation probably represents an augmentation of the releasable neuronal noradrenaline pool by infusion of this agent, while the latter finding suggests a block of re-uptake of noradrenaline released by etilefrine in the presence of cocaine (de la Lande et al., 1967). The complete abolition of the response to etilefrine, REN-293 and the other sympathomimetic amines produced by

phentolamine indicates that the vasoconstrictor effects of these drugs on the rat tail artery were totally mediated by alpha-adrenoreceptor stimulation.

An additional finding noted in the present study was when tyramine was administered to the vessel and a maximal response obtained, discontinuance of the tyramine infusion caused a further increase in perfusion pressure. This "wash out" response was only observed with tyramine and its origin is uncertain. The "wash out" response could be blocked by adding phentolamine at the commencement of the wash period. Farmer (1968) observed that tyramine had a biphasic response in the rabbit ear artery when injected into the perfusion fluid before it entered the artery. de la Lande and Waterson (1968) suggested that this was due to the fact that in Farmer's study intraluminal fluid was allowed to mix with extraluminal fluid and the biphasic response represented the direct action of tyramine intraluminally on smooth muscle and then the indirect action on sympathetic nerves reached more effectively from the extraluminal side. de la Lande and Waterson (1968) then suggested that the biphasic response was due to the difference in tyramine's potency when administered either extraluminally or intraluminally.

*   *   *

CHAPTER 4

THE EFFECTS OF AGENTS MODIFYING SYMPATHETIC NERVE FUNCTION ON THE RESPONSE OF THE ISOLATED RAT TAIL ARTERY TO ETILEFRINE AND TYRAMINE

Introduction

In the previous chapter, it was found that etilefrine possessed a significant indirect sympathomimetic component to its action. Since this previous series was carried out on the rat tail segment, it was decided to re-examine the effects of etilefrine on the isolated ventral caudal artery of the rat in the present chapter for two reasons. Firstly, the fact that the ventral artery has arterio-venous anastomoses with the tail vein seemed an important consideration in terms of drug distribution; for this reason, it was essential to ascertain the effects of the drug on one isolated artery as compared with those on the whole vascular bed. Secondly, it was decided to determine the relative response to etilefrine administered intra- and extraluminally to the vessel.

A special attribute of the ventral caudal artery is the rich sympathetic innervation it possesses (Hodge and Robinson, 1972) which is very similar in distribution to that seen in small human arteries (Frewin et al., 1971a, b); it thus serves as a good model for testing sympathomimetic agents. An additional part of the study involved the use of the indirectly acting sympathomimetic agent, tyramine, as a standard for comparison with etilefrine.

Methods

In this series of experiments, the rat tail artery was double cannulated and perfused according to the method described in Chapter 2. Drugs were administered to the arteries either extraluminally or intraluminally and maintained in contact with the vessel until a maximal response was obtained. The respective concentrations of these agents in either the external bathing medium or in the perfusion fluid are referred to in parentheses. Single administrations were used in this study, rather than complete dose response curves, due to the prominent indirect sympathomimetic action of the agents tested. The sensitivity of the rabbit ear artery to extraluminal tyramine has been shown to decrease with repeated administration of this agent (de la Lande and Waterson, 1968), and this phenomenon was also noted in preliminary experiments with etilefrine and tyramine on the ventral artery of the rat tail. The test concentrations of etilefrine and tyramine selected were those which lay about the middle of the dose response curve and which were very reproducible on repeated drug administration.

Etilefrine (5.52 µM) and tyramine (1.45 mM) were each administered internally and externally to arteries from untreated rats or rats pretreated with (a) iproniazid (200 mg/Kg i.p. each day for 2 days), (b) reserpine (2.5 mg/Kg i.p. each day for 2 days), (c) guanethidine (10 mg/Kg i.p. daily for 4 weeks), in the manner described by de la Lande and Waterson (1968). The extraluminal and intraluminal response to etilefrine (5.52 µM) was also determined in the presence of cocaine (29.5 µ M) and

and nialamide (335 μM).

The catecholamine content of segments of untreated and pre-treated arteries adjacent to those used in the perfusion experiments were determined using the fluorimetric assay as described in the general methods chapter. At least five rat tail artery preparations were used in each series. Mean responses were obtained and the S.E.Ms calculated and included in the figures wherever appropriate. Statistical analysis was performed on group data using a Student's t-test.

Results

The mean response to etilefrine (5.52 μ M) administered intraluminally (internally) and extraluminally (externally) in normal arteries and arteries treated with reserpine, guanethidine, iproniazid and cocaine is shown in Figure 4.1. Reserpine, guanethidine and iproniazid pretreatment all significantly decreased the response to both internally and externally applied etilefrine (p < 0.05). The external response was decreased to a greater extent in all cases. This is contrast to the normal arteries, where the external response was observed to be greater than the internal response (p < 0.05). The response to etilefrine in the presence of nialamide was similar to that seen in those arteries pretreated with iproniazid. Although the external response to etilefrine was augmented in the presence of cocaine in some preparations, for the group, the mean value was not significantly different from the control due to a large variance in the

Fig. 4.1
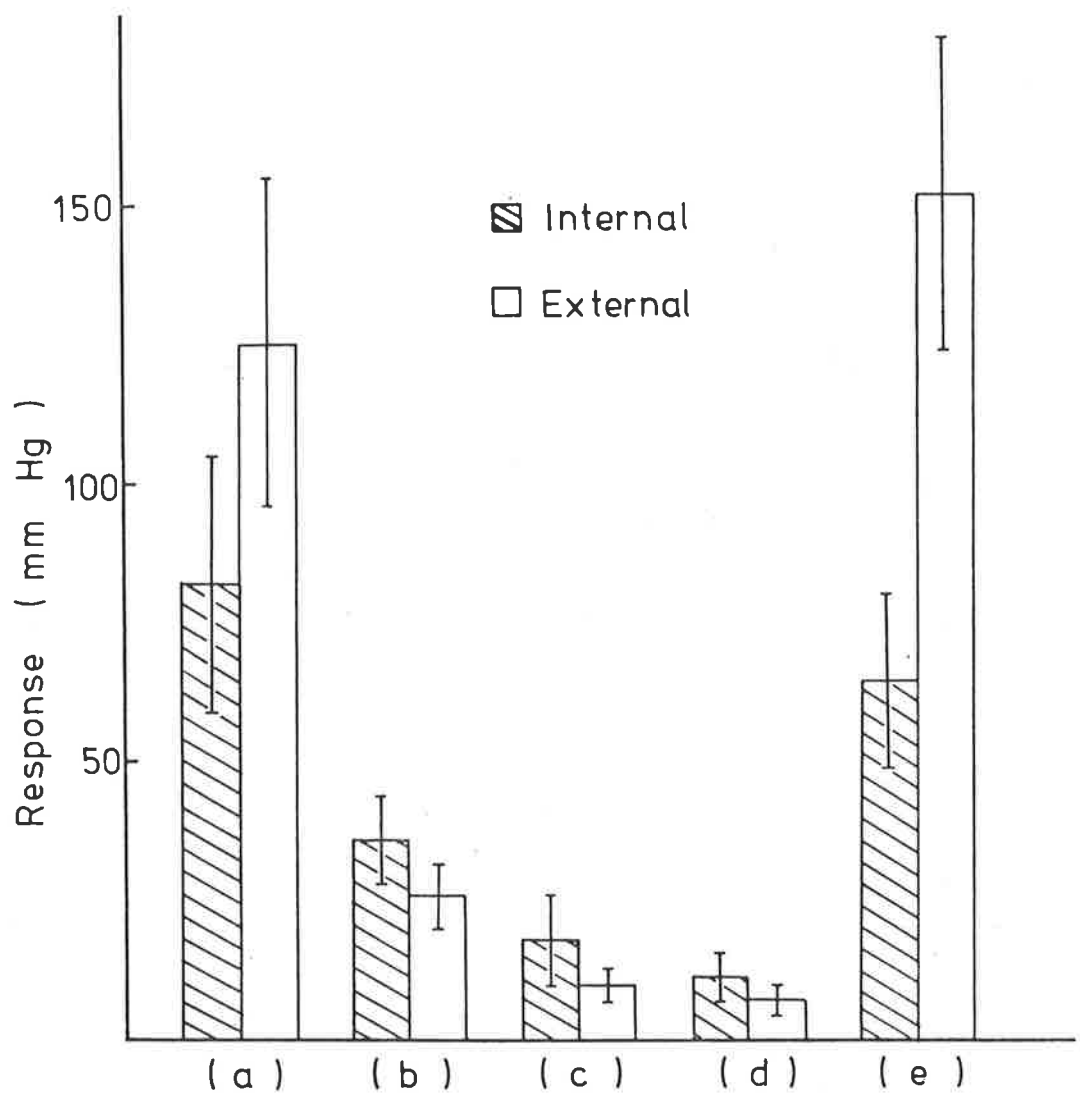
The response to etilefrine 5.52 μM in normal arteries (a) , and arteries pretreated with reserpine (b), guanethidine (c), iproniazid (d) and cocaine (e). The respective S.E.M's are also shown. ▨ Intraluminal (internal), □ Extraluminal (external).

results (Fig. 4.1).

Figure 4.2 shows the response to tyramine (1.45 mM) administered internally and externally in normal arteries and arteries pretreated with reserpine, guanethidine and iproniazid. Reserpine and guanethidine pretreatment completely abolished the response to tyramine both internally and externally. Iproniazid caused a depression in the response to tyramine, but the values obtained were not significantly different from the values in Figure 4.2a. The catecholamine content of the arteries was also determined and the results are represented in Figure 4.3. Reserpine and guanethidine both caused a significant decrease in catecholamine content when compared with the untreated vessels. The monoamine oxidase (MAO) inhibitor, iproniazid, caused a significant increase in the catecholamine content of the arteries.

Discussion

From the results obtained in this chapter it would seem that etilefrine does indeed possess a significant indirect sympathomimetic component to its action. In the present study, the response of the isolated artery to the drug was of a similar magnitude to that obtained when etilefrine was administered to the tail segment. In the presence of agents depleting the neuronal noradrenaline content in the arteries (e.g., reserpine and guanethidine), both tyramine and etilefrine exhibited an attenuated effect.

The response to tyramine was significantly greater ($p < 0.025$)

Fig. 4.2

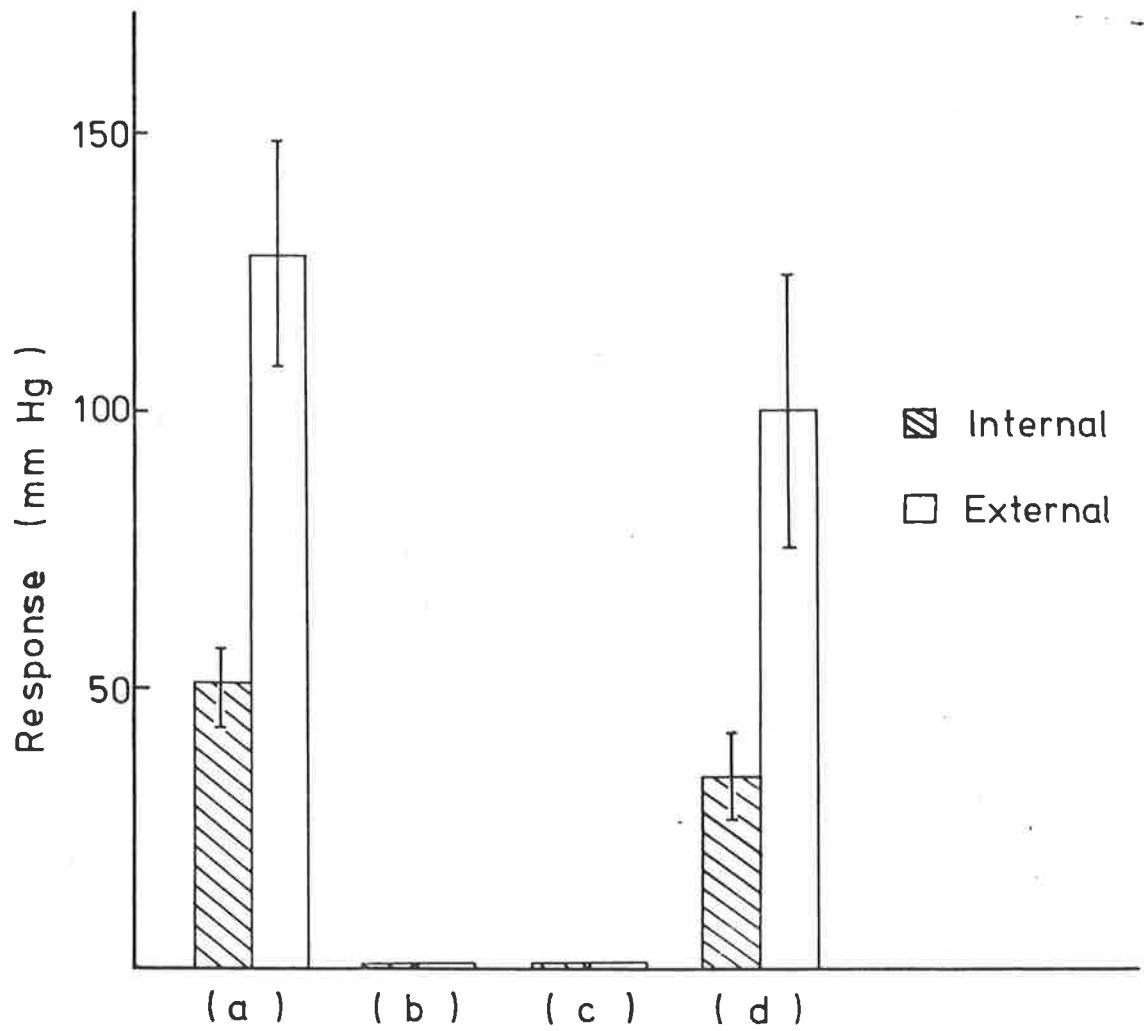The response to a 1.45 mM dose of tyramine in normal arteries (a), and arteries pretreated with reserpine (b), guanethidine (c), and iproniazid (d). The respective S.E.M's are also shown. ▨ Internal, ▢ External.
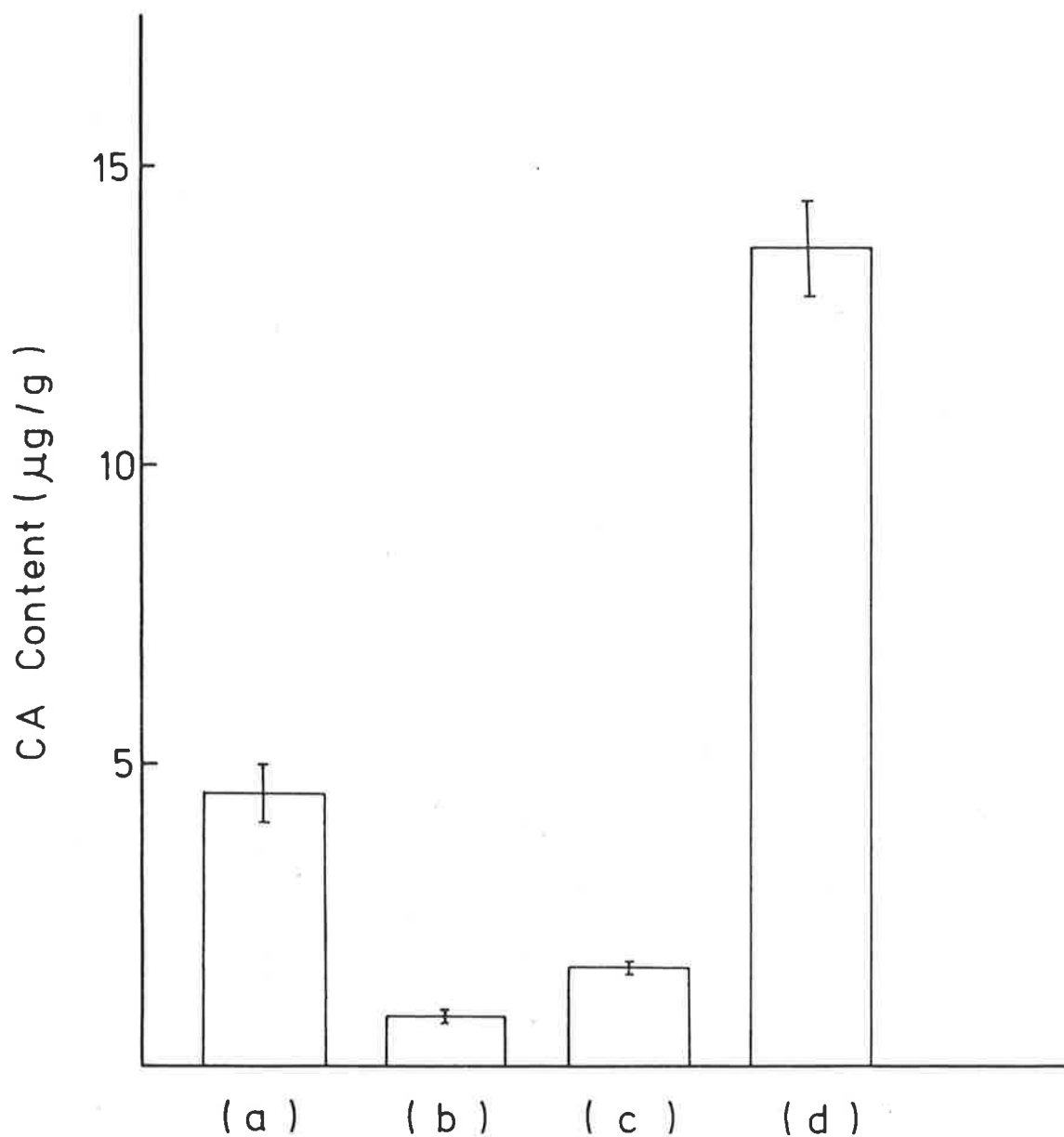
Fig. 4.3

    The mean catecholamine content in segments of normal arteries (a), and of those pretreated with reserpine (b), guanethidine (c), and iproniazid (d). The respective S.E.M's are also shown.

when applied extraluminally than when applied intraluminally (Fig. 4.2). This is in agreement with the findings of de la Lande and Waterson (1968) in the rabbit ear artery. Those authors also found that chronic denervation, which caused the noradrenergic storage structures to disappear, reduced the potency of extraluminal tyramine more than that of intraluminal tyramine. In the rat tail artery, it was found that the intra-luminal and extraluminal tyramine response was completely abol-ished by reserpine and guanethidine pretreatment. This indicated that the response to tyramine in this vessel is totally dependent on its noradrenaline-releasing potential. The fact that the etil-efrine response was significantly attenuated by both pretreatments (with the external response being affected to a greater degree than the internal response) is thus indicative of an indirect component to its action. Since the response to the drug was not completely abolished in the presence of the sympatholytic agents, it would seem that etilefrine also has a direct component to its vascular effect. Cocaine, in blocking neuronal noradrenaline re-uptake, appeared to increase the external (as compared with the internal) response to etilefrine, but the finding was not signifi-cant at the 5% level due to the large variance in the response.

The catecholamine content of the arteries correlated well with the perfusion results in the reserpine and guanethidine treated vessels, i.e., a decreased catecholamine content was associated with an attenuation of the vascular response to the sympathomimetic.

Iproniazid elevated the catecholamine level in the vessels as expected, but the decrease in response to etilefrine is in

contrast to what was anticipated. A similar trend was seen with tyramine, although the result was not significant at the 5% level. This depression of the response due to the MAO inhibitor, iproniazid, is difficult to interpret. MAO is believed to regulate the levels of transmitter monoamines which diffuse from their neuronal storage vesicles into the neuroplasm. Inhibition of MAO has been shown (Spector, Hirsch and Brodie, 1963) to elevate neuronal catecholamine levels. Weiner and Bjur (1972) suggested that intraneuronal MAO may regulate noradrenaline synthesis by preventing elevations in the unbound noradrenaline pool. These findings may in part explain why there were increased levels of catecholamines in the tissue but a decrease in indirect sympathomimetic agents due to the decrease in releasable vesicular noradrenaline. However, the fact remains that etilefrine, which has a larger direct component to its vasoconstrictor action than does tyramine, was depressed to a greater degree. This may be due to a depressant effect on the vascular smooth muscle caused by an interaction between etilefrine and the MAO inhibitor. A series of experiments using nialamide was also carried out to test the specificity of the depressant effect in relation to iproniazid. This produced results which were similar to those obtained with iproniazid. The precise mechanism for this finding still remains to be resolved.

*   *   *

CHAPTER   5

A STUDY OF THE VASCULAR EFFECTS OF ETILEFRINE
USING THE ISOLATED RABBIT EAR ARTERY

Introduction

The suggested use of etilefrine in the treatment of patients with orthostatic hypotension is based on the premise that it has an action similar to that of noradrenaline (Miller et al., 1973). In the two previous chapters it was demonstrated that etilefrine has a substantial indirect sympathomimetic action on the ventral artery of the rat tail. The present chapter examines the effects of etilefrine on a similar blood vessel, namely, the central artery of the rabbit ear. This vessel is known to have a rich sympathetic innervation (de la Lande, Frewin and Waterson, 1967) and has the added advantage that it can be surgically denervated by removal of the homolateral superior cervical ganglion (de la Lande and Rand, 1965). In addition, the effects of, and interactions with, other agents commonly employed in the therapy of orthostatic hypotension have been examined.

Methods

The central artery of the rabbit ear was double cannulated and perfused with Krebs-bicarbonate solution as described in Chapter 2. Using the general protocol described in that chapter, the following series of experiments were carried out. Drugs were administered to the arteries intraluminally and maintained in

contact with the vessel until a maximal response was obtained.

1. Increasing doses of noradrenaline, phenylephrine, etilefrine and ephedrine were applied to the artery and dose response curves constructed in a manner described by de la Lande, Glover and Head (1967). The relative constrictor activity of the drugs was determined by constructing a mean dose response curve for each agent and from this calculating the dose producing pressure responses of 50, 100, 150 and 200 mmHg. A mean was then obtained from the four available measurements and the shift to the right of the noradrenaline curve expressed as a ratio of 1:mean shift (i.e., a reciprocal was obtained).

2. Etilefrine was administered to the artery before and during an infusion of 9-alpha-fluorohydrocortisone at a dose of 22.8 nM or 22.8 μM. The 9-alpha-fluorohydrocortisone perfusion was commenced 10 minutes before the etilefrine was added. In concurrence with the foregoing procedure, etilefrine was administered with the same time sequence to a vessel untreated with the mineralocorticoid so as to monitor any spontaneous change in vascular sensitivity with time, i.e., a "time control" was employed. The response of the artery to noradrenaline was also examined before and during an infusion of 22.8 nM of 9-alpha-fluorohydrocortisone, which was commenced 10 minutes before the addition of the noradrenaline. "Time controls" were used as before to monitor any changes in vascular sensitivity with time.

3. Dose response curves to etilefrine and noradrenaline were obtained on arteries from rabbits in which the superior cervical ganglion had been surgically removed on one side three weeks previously. This procedure caused the sympathetic denervation of

the respective artery. The response to the indirectly acting amine ephedrine and to electrical stimulation was also examined to confirm denervation. Electrical stimulation was given at a level which caused release of neuronal noradrenaline, but did not stimulate the smooth muscle directly, i.e., at 70 volts, 38 pulses/sec for 5 seconds (de la Lande - personal communication).

4. The catecholamine content of the denervated arteries and normal arteries was also determined using the fluorometric catecholamine assay described in the general methods.

At least five arteries were used in each series. Mean responses were obtained and the S.E.Ms calculated and included in the figures whenever appropriate. Statistical analysis was performed on group data using a Student's t-test.

Results

Figure 5.1 shows the dose response curves to noradrenaline, phenylephrine, etilefrine and ephedrine in the rabbit ear artery. Noradrenaline was found to be the most active agent on the preparation and ephedrine the least active. The mean ratio of activity of the drugs compared to noradrenaline was noradrenaline : phenylephrine : etilefrine : ephedrine = 1.0 : 0.5 : 0.07 : 0.003.

When etilefrine was added to the rabbit ear artery before and during an infusion of 9-alpha-fluorohydrocortisone (22.8 nM or 22.8 μM) no significant increase in response was observed at all the doses used. The time controls also showed no significant difference over the duration of the experiment. The dose response

TABLE 5.1

Catecholamine levels (ng/g) in the
normal and denervated rabbit ear
artery segments (n = 5).

	$\bar{x}$	±	SEM
Denervated	0.16 ng/g*		0.01
Normal	0.33 ng/g		0.03

* significantly reduced (p<0.001).

curves to noradrenaline before and during an infusion of 9-alpha-fluorohydrocortisone (22.8 nM) showed no significant difference. The time controls from this series exhibited no change during the experiment over the entire dose range.

Figure 5.2 shows the response to etilefrine in sympathectomised and non-sympathectomised ear arteries obtained from the same rabbit. The denervated vessels showed a significant decrease in response for all the doses of etilefrine used. Figure 5.3 shows the response to noradrenaline in these arteries. There was no significant difference between the denervated and the normal arteries as a group; however, two of the five vessels examined showed an increase in the noradrenaline response. The denervated vessels also showed a greatly reduced response to the indirectly acting amine ephedrine ($p < 0.05$) and no response to electrical stimulation. The catecholamine levels in the denervated arteries were decreased (Table 5.1).

Discussion

The results of the present study on the central artery of the rabbit ear support the findings in the previous chapters of this thesis that etilefrine relies heavily on sympathetic nerves for its vasoconstrictor effect. The advantage of using the rabbit ear artery preparation is that it can be surgically sympathectomised, whereas the rat tail artery (used in the previous studies) cannot be successfully treated in this fashion. Denervation was substantiated by testing with electrical stimulation, assaying catecholamine
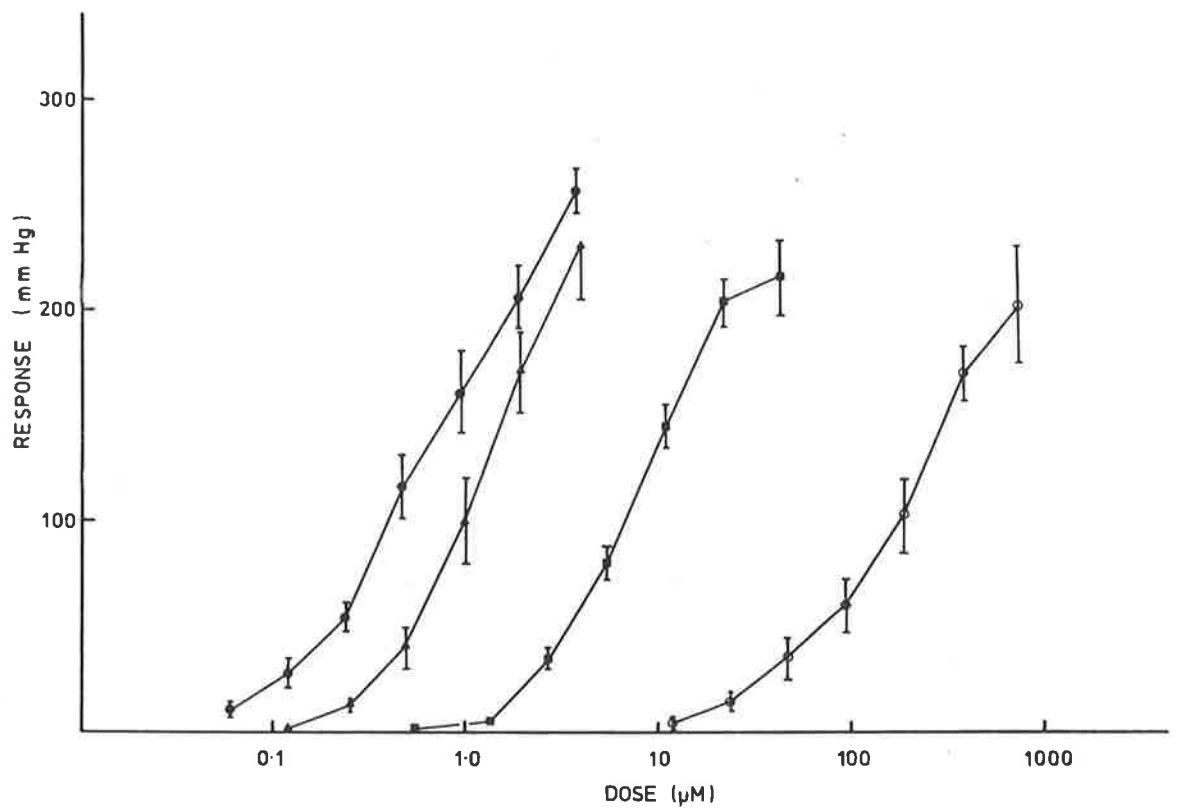
Fig. 5.1

Dose response curves to noradrenaline (●), phenylephrine (▲), etilefrine (■), and ephedrine (o). Noradrenaline was found to be the most active and ephedrine the least active on the isolated rabbit ear artery. The dose refers to the µM concentration of drug perfusing the artery.
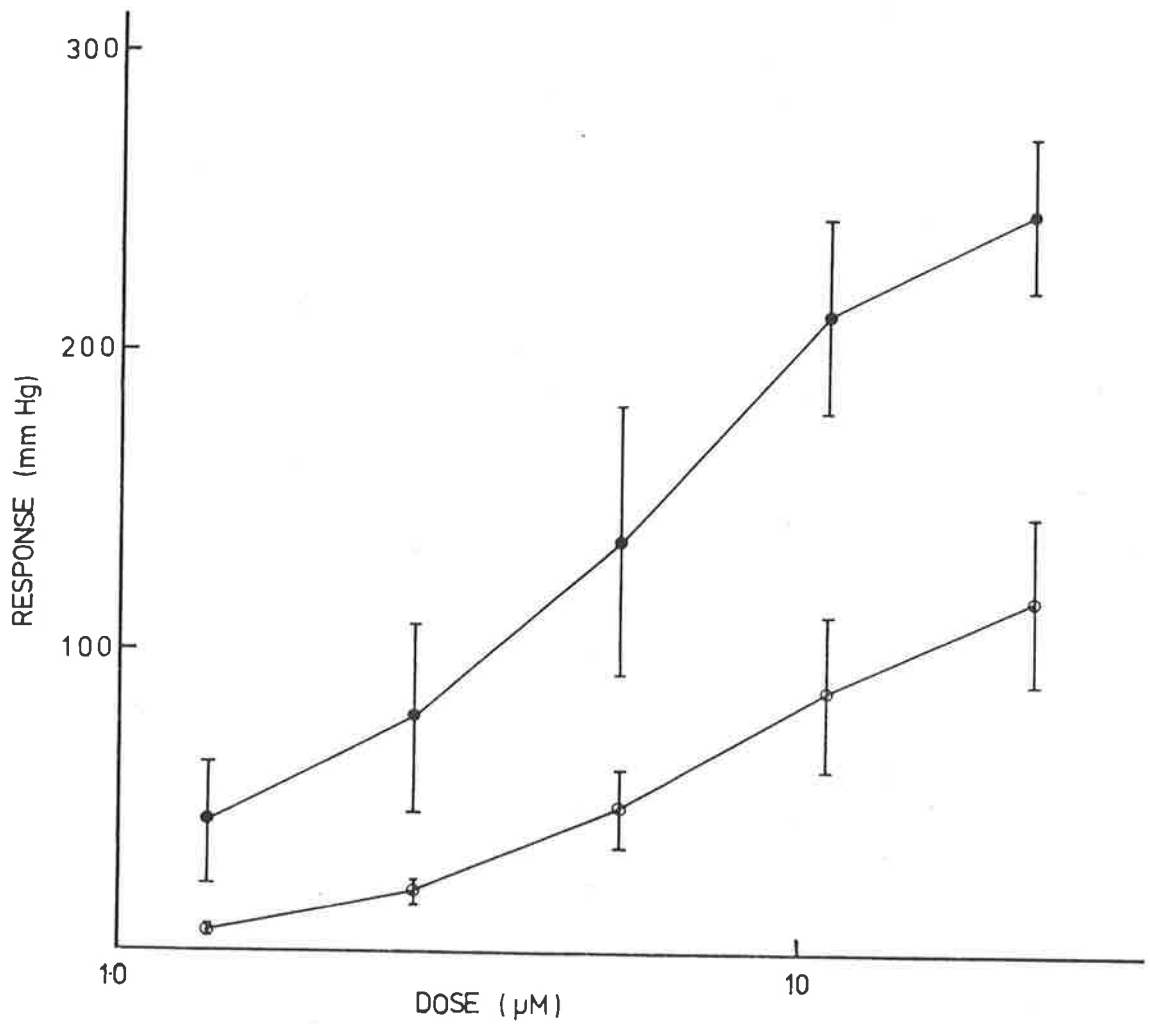
Fig. 5.2

Dose-response curves to etilefrine in sympathectomised (O) and non-sympathectomised (●) arteries. Sympathectomy decreased the response to etilefrine.

Fig. 5.3

Dose response curves to noradrenaline in sympathectomised
(●) and non-sympathectomised (■) arteries.

levels in the preparation and testing with the sympathomimetic amine ephedrine. In the face of the foregoing, it was shown that etilefrine's constrictor action was significantly attenuated in sympathectomised ear arteries, and the results demonstrated the strong dependence of the drug on these nerve endings to exert its pharmacological action.
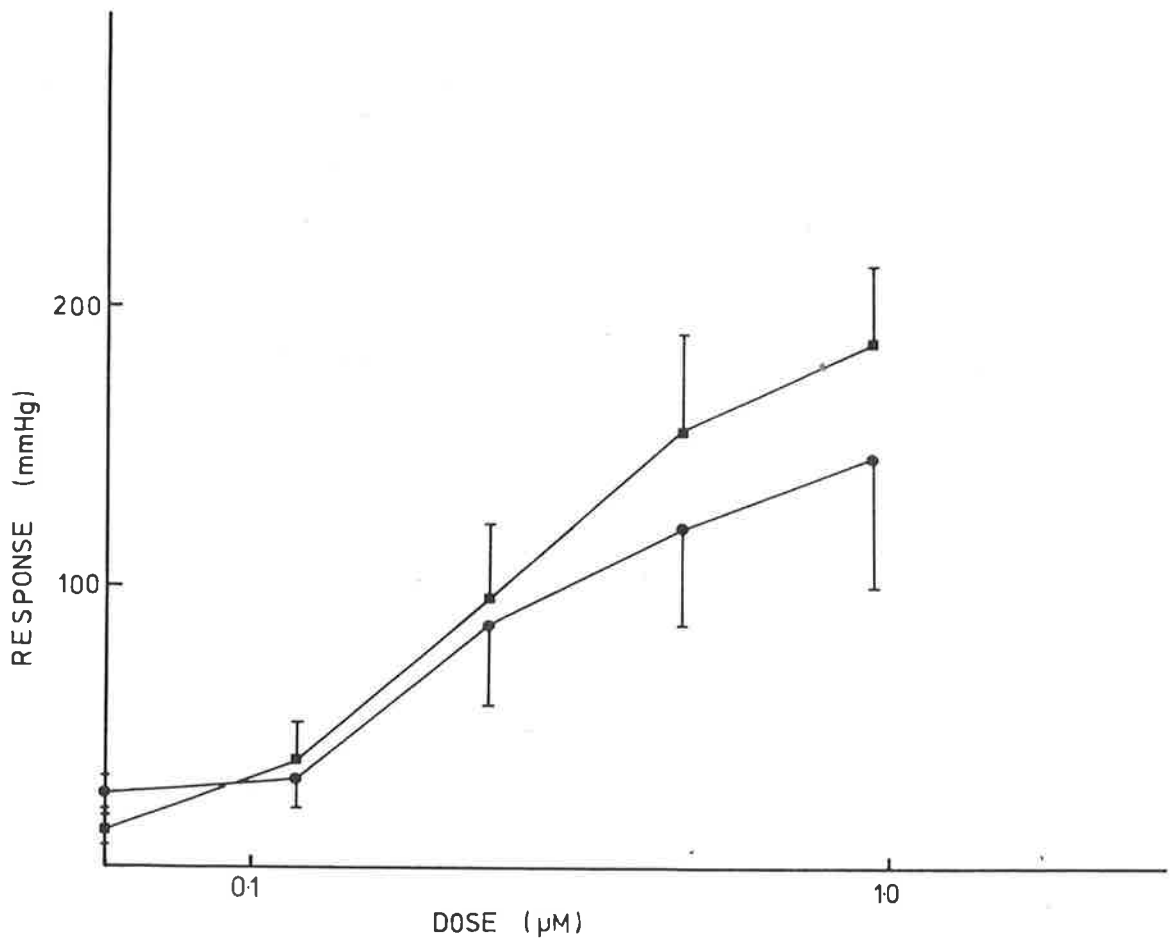
The mineralocorticoid 9-alpha-fluorohydrocortisone is frequently used in the therapy of patients with postural hypotension (Corbett, 1976). Since etilefrine's proposed therapeutic role is in the same clinical situation, it seemed appropriate to examine potential inter-actions between these two agents. The mineralocorticoid was used at two different dose levels, the low dose to approximate the circu-lating blood level of the drug during therapy and the high dose to approximate the dose of deoxycorticosterone acetate used to block extraneuronal noradrenaline uptake. Johnson and de la Lande (1978) showed that deoxycorticosterone acetate is a blocker of extraneuronal uptake in the rabbit ear artery. This is in agree-ment with the findings of Iversen and Salt (1970), who showed that corticosterone is an inhibitor of extraneuronal uptake. The potentiation of the noradrenaline response by 9-alpha-fluorohydro-cortisone has been reported by Schmidt et al. (1966). The reason for the failure of 9-alpha-fluorohydrocortisone to potentiate etil-efrine is difficult to determine from the present results. The potentiation was expected to occur by inhibition of extraneuronal uptake of released noradrenaline.

Phenylephrine and ephedrine were included in this study as two other sympathomimetic amines that have been used in treating postural hypotension (Corbett, 1976). A difference in potency of

noradrenaline, phenylephrine, etilefrine and ephedrine was observed between the rat tail artery used in Chapter 3 and the rabbit ear artery used in this chapter. It was found that noradrenaline was more potent in the tail artery and phenylephrine and ephedrine were more potent in the ear artery. Etilefrine was equipotent in the two vessels.

*   *   *

CHAPTER 6

THE INDIRECT SYMPATHOMIMETIC ACTIVITY

OF ETILEFRINE – A COMPARISON WITH TYRAMINE

AND EPHEDRINE USING ^{3}H–NORADRENALINE

Introduction

In view of the findings of the previous chapters, which indicated that etilefrine possesses considerable indirect sympathomimetic activity, it was decided to examine the noradrenaline releasing potential of this amine next, using ^{3}H–noradrenaline and the rat tail artery as the test vessel. Many studies have been carried out to examine various aspects of neuronal and extraneuronal ^{3}H–noradrenaline efflux. Trendelenburg (1963) reviewed the actions of several sympathomimetic amines on the cat nictitating membrane and other tissues with respect to their direct and indirect activity based on their pharmacological action. Paton (1973a, b) studied the effects of various sympathomimetics on the efflux of ^{3}H–noradrenaline from reserpine pretreated and MAO and COMT inhibited rabbit atria. His results indicated that tyramine, metaraminol, amphetamine and noradrenaline itself enhanced the neuronal efflux of ^{3}H–noradrenaline from this preparation. Eckert et al. (1976) and Henseling et al. (1976a, b) have examined the ^{3}H–noradrenaline distribution in rabbit aortic strips and its modification by inhibitors of noradrenaline metabolism and uptake. Those authors also studied the stereoselectivity of this distribution and subjected the resultant efflux curves to compartmental analysis.

The present chapter examines the action of etilefrine in

modifying the ^{3}H-noradrenaline efflux from rat tail arteries and compares its action with that of tyramine and ephedrine.   In addition, the effects of inhibitors of noradrenaline metabolism and uptake on the efflux are assessed.

Methods

This efflux study was carried out according to the protocol described in the general methods section (Chapter 2).   The initial series of experiments examined the efflux profile of ^{14}C-sorbitol over 20 minutes from the rat tail artery.   This efflux was also examined in the presence of (1) etilefrine 55.2 μM, (2) tyramine 1.45 mM, (3) cocaine 29.5 μM and (4) deoxycorticosterone acetate 26.9 μM with UO521 (55 μM) as an inhibitor of catechol-O-methyl transferase (Henseling et al., 1978).   The subsequent experiments examined the efflux of ^{3}H-noradrenaline in the presence and absence of the following agents: (1) etilefrine 55.2 μM, (2) tyramine 1.45 mM, (3) ephedrine (182 μM), (4) cocaine 29.5 μM, (5) etilefrine 55.2 μM + cocaine 29.5 μM, and (6) tyramine 1.45 mM + cocaine 29.5 μM.

On the basis of results obtained during the series with sorbitol, the experimental protocol was modified for the ^{3}H-noradrenaline study.   The modification consisted of an initial five minute wash in ascorbic/Krebs solution, followed by a 15 minute wash in ascorbic/Krebs containing the individual drug(s). A further series was carried out using arteries from rats that were variously treated.   These included: (1) pretreatment of

rats with the monoamine oxidase inhibitor iproniazid (200 mg/Kg i.p. each day for 2 days) and exposing the segments of the tail arteries during the washout period to ascorbic/Krebs containing cocaine 29.5 µM, deoxycorticosterone acetate 26.9 µM and U0521 55 µM; (2) pretreatment of rats with guanethidine (25 mg/Kg i.p.) on alternate days for 7 weeks before removing their caudal arteries.

The content of compounds with a dihydroxyphenol structure in the efflux was calculated by combining five one-minute (i.e., 1 ml) samples and subjecting each to a batch alumina procedure (de la Lande et al., 1967) to determine what percentage of the total radioactivity was adsorbed on to alumina.

Mean efflux curves were calculated from the results of at least five individual experiments.

Results

^{14}C-sorbitol efflux

The results of experiments in which the ^{14}C-sorbitol efflux was measured indicated that the counts obtained after five minutes of efflux had decreased to approximately 5% of the counts effluxed at the first minute. For this reason, the efflux of ^{3}H-noradrenaline from the artery during the first five minutes was discarded to avoid complicating the overall results with those due to this marked efflux from the extracellular space. The rate of efflux of ^{14}C-sorbitol was unchanged by the addition of any of the drugs used.

^{3}H-noradrenaline efflux

It was found that approximately 90% of the total radio-activity was adsorbed on to alumina, indicating that the collected efflux was predominantly dihydroxyphenol structured compounds – probably ^{3}H-noradrenaline or ^{3}H-3,4-dihydroxyphenylglycol (DOPEG), which are both of neuronal origin.

^{3}H-noradrenaline efflux in the presence of sympathomimetic amines

The comparative rates of efflux in control arteries and arteries treated with etilefrine, tyramine and ephedrine are shown in Figure 6.1. The rate of efflux was in the descending order tyramine > ephedrine ≡ etilefrine > control. In the presence of cocaine, no significant difference was observed between the rates of efflux for cocaine alone and cocaine and etilefrine (Fig. 6.2a). The efflux rate for tyramine in the presence of cocaine was significantly greater than the efflux curve obtained for cocaine alone, irrespective of whether tyramine was introduced at the start of minute six or at minute eleven (Fig. 6.2b).

^{3}H-noradrenaline efflux from pretreated arteries

The efflux curves from arteries which had been pretreated with guanethidine are shown in Figure 6.3. It can be seen that although the amount of ^{3}H-noradrenaline effluxed is lower than the values in the control efflux curves (Fig. 6.1), etilefrine has caused an enhanced efflux of ^{3}H-noradrenaline compared with the values obtained when the vessels were not treated with etilefrine. In Figure 6.4, the efflux curves from arteries treated with
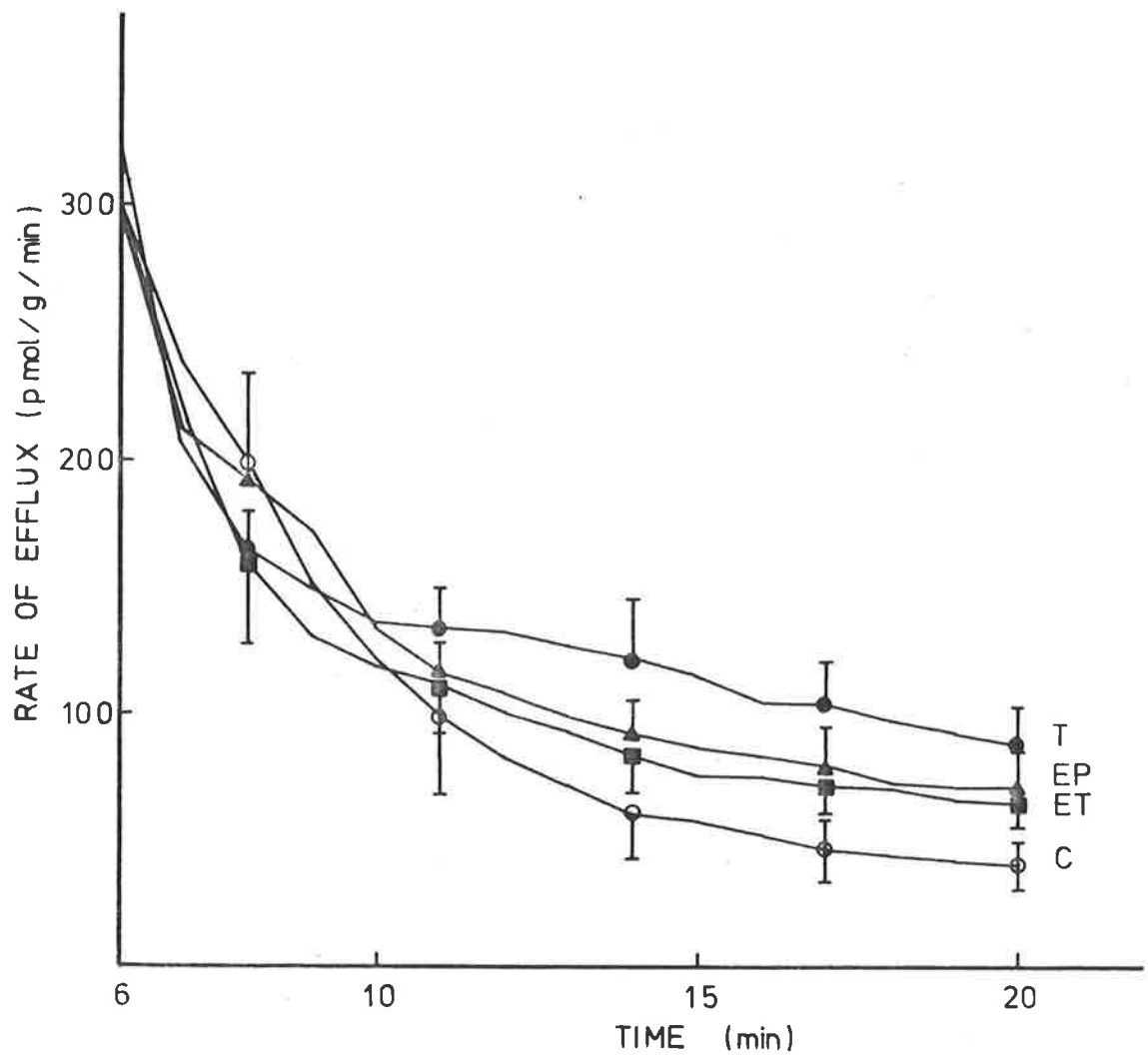
Fig. 6.1

Efflux curves of ^{3}H-NA from control (C) arteries and arteries treated with tyramine (T), ephedrine (EP) and etilefrine (ET). Each point represents the mean values ± S.E.M. of five experiments.
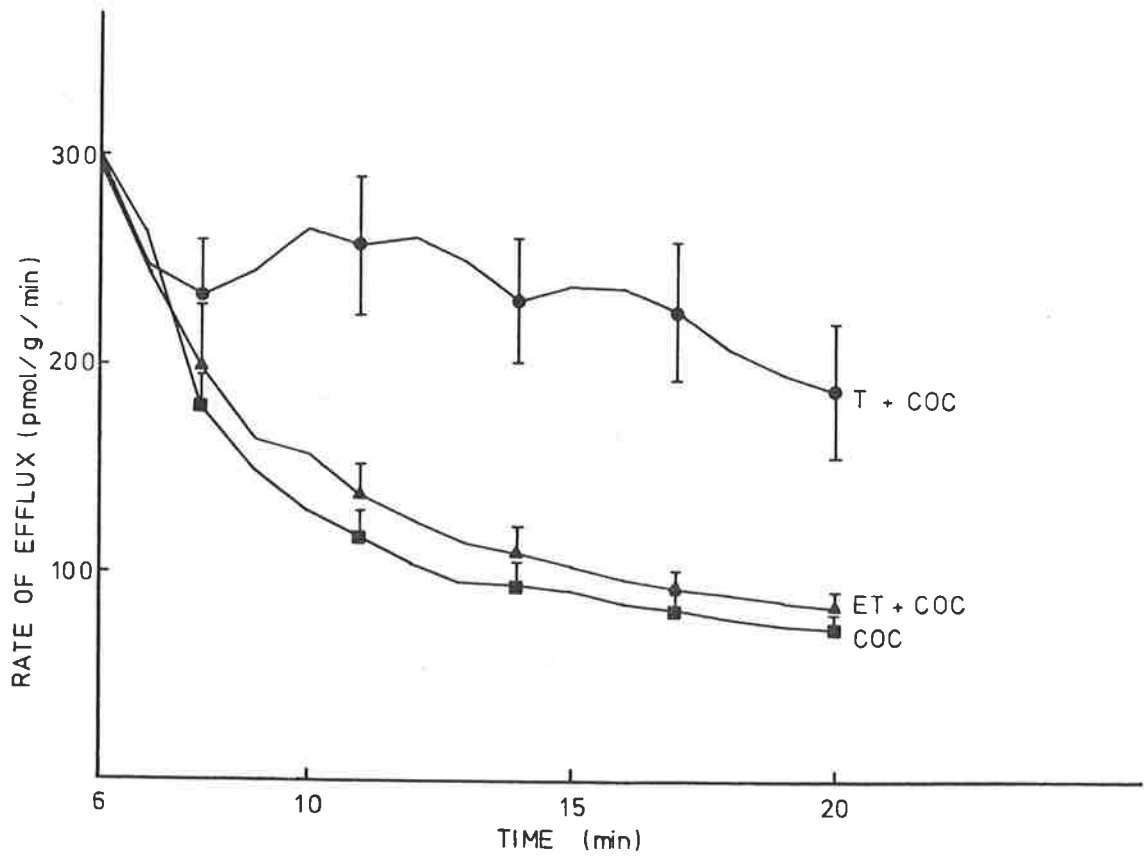
Fig. 6.2a

Efflux curves of ^{3}H-NA from arteries treated with cocaine (COC), etilefrine and cocaine (ET + COC), and tyramine and cocaine (T + COC). Each point represents the mean values ± S.E.M. of five experiments. The cocaine and tyramine were administered together at minute 5.
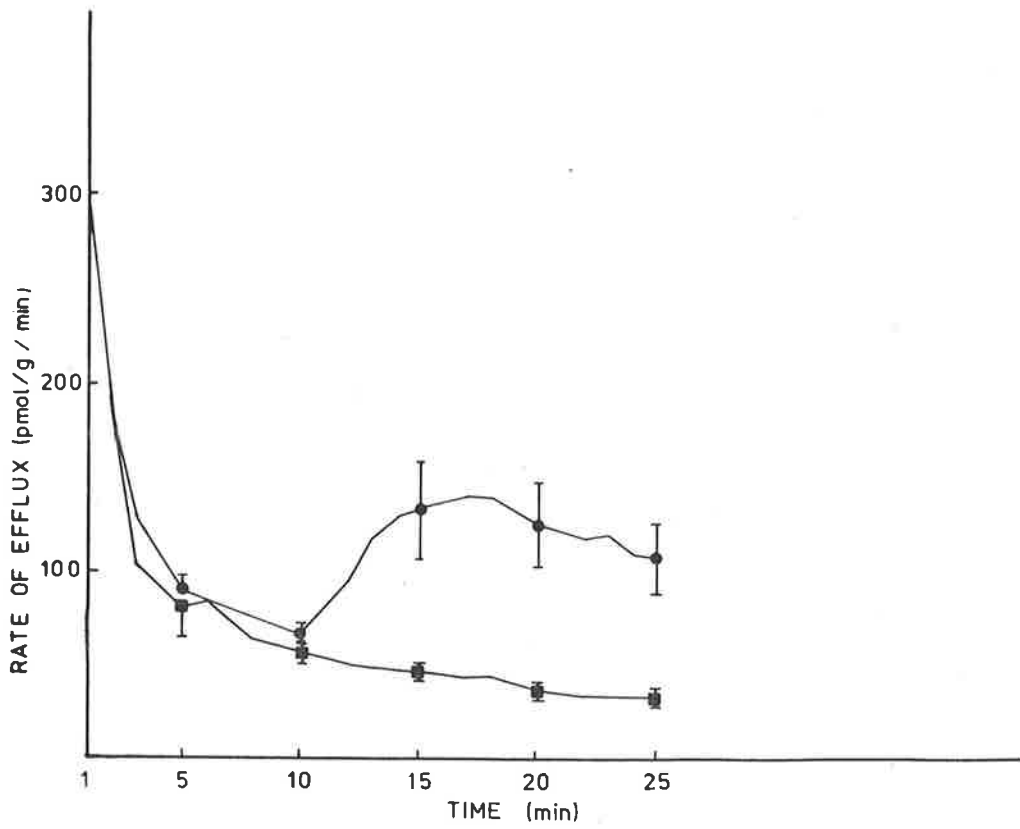
Fig. 6.2b

Figure 6.2b shows efflux curves where cocaine was added at minute 5 (■) and also where cocaine was added at minute 5 and tyramine at minute 10 (●).
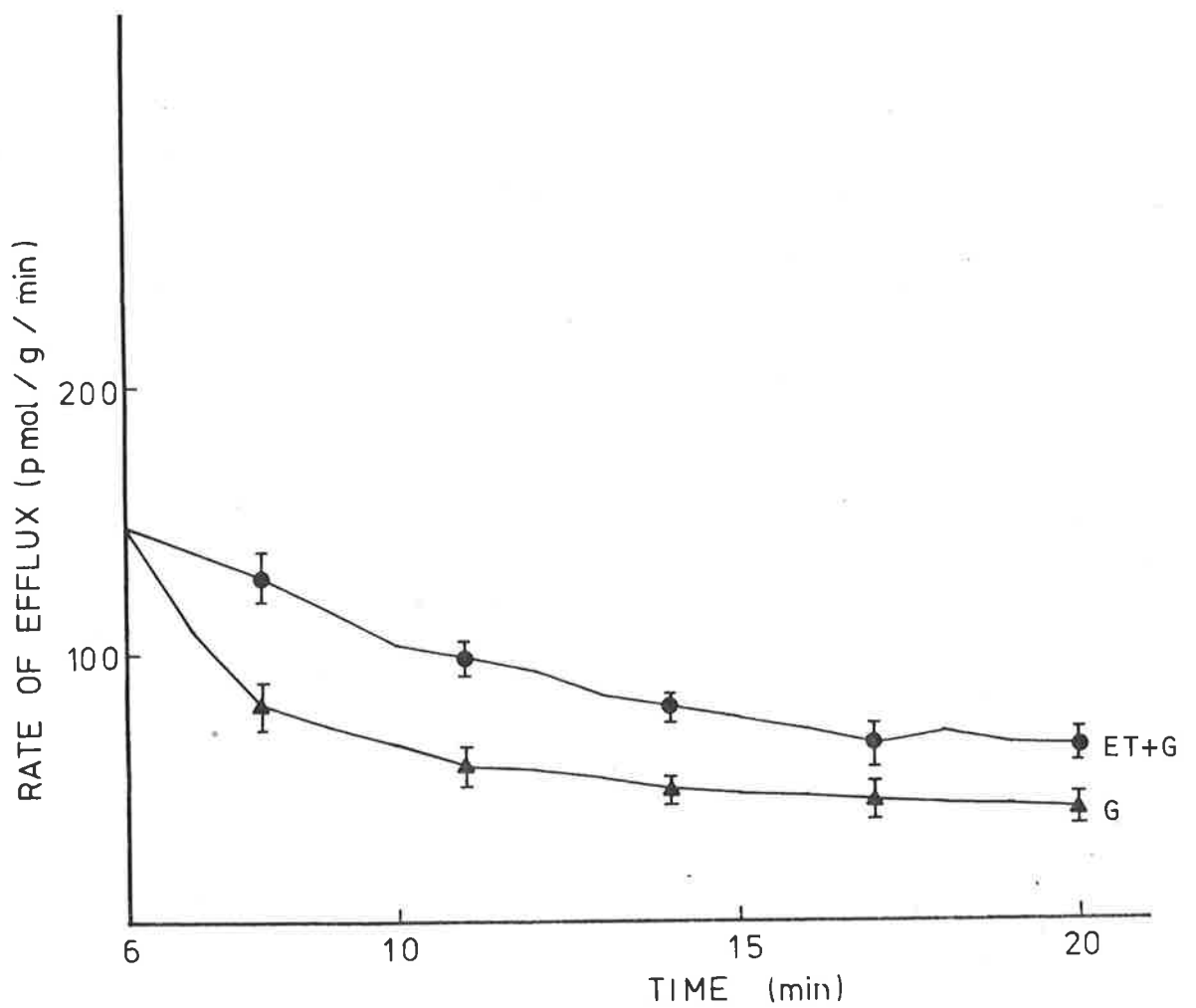
Fig. 6.3

Efflux curves of ^{3}H-NA from arteries obtained from rats chronically treated with guanethidine (G), or those pretreated with guanethidine to which etilefrine was added (ET + G). Each point represents the mean values ± S.E.M. of five experiments.
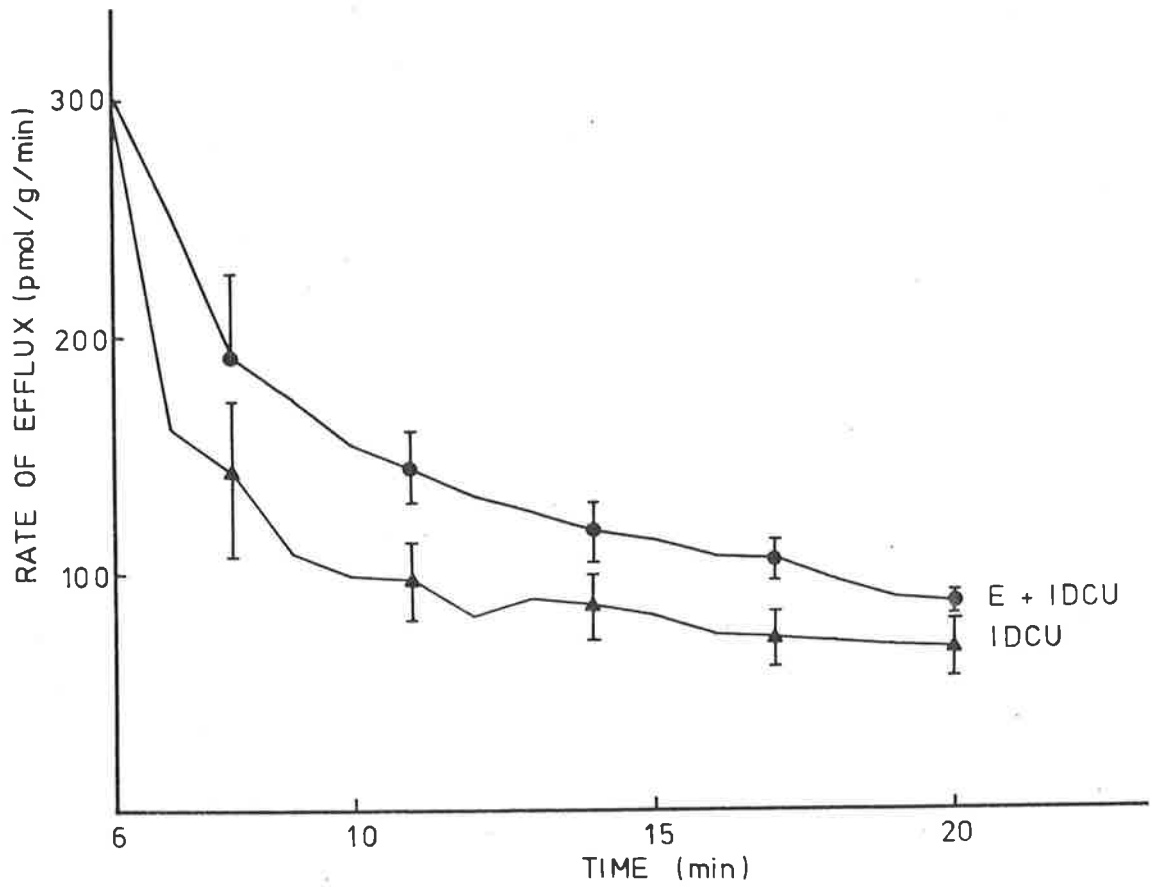
Fig. 6.4

Efflux curves of ^{3}H–NA from arteries treated with iproniazid to which doca, cocaine and UO521 were added (IDCU) or from those to which etilefrine was further added (E + IDCU). Each point represents the mean values ± S.E.M. of five experiments.

iproniazid, cocaine, deoxycorticosterone acetate and UO521 are depicted. The arteries treated with etilefrine show an enhanced rate of efflux in relation to the arteries which received no etilefrine. The efflux from the vessels treated with the MAO inhibitor, cocaine deoxycorticosterone acetate, and UO521 was higher than that from the control untreated arteries shown in Figure 6.1.

Discussion

The overall results of the present study support earlier findings that etilefrine possesses a significant indirect sympathomimetic component to its action. The results show that etilefrine enhanced the rate of efflux of ^{3}H-noradrenaline from the sympathetically innervated rat tail artery in comparison to control values. The rate of efflux was less than that induced by tyramine but comparable with that of ephedrine. These results are in agreement with those of Paton (1973a, b), who showed that tyramine enhanced the ^{3}H-noradrenaline efflux from the rabbit atria. Paton (1973a) suggested that the ability of some indirectly-acting sympathomimetics to enhance ^{3}H-noradrenaline efflux may be influenced by their affinities for carrier-dependent influx sites, their lipid solubilities and their ability to displace noradrenaline from reserpine-resistant intraneuronal binding sites. It would seem that many explanations are possible for the variation in the action of sympathomimetics. This present study differs from that of Paton (1973a) in that vesicular uptake and storage is intact and may thus represent a larger "pool" of

releasable noradrenaline. The predominant component of tyramine's sympathomimetic action relates to noradrenaline release from sympathetic nerve endings (Burn and Rand, 1958; Trendelenburg, 1963). Trendelenburg (1963) showed that tyramine is an indirectly acting sympathomimetic, whereas ephedrine can be classed as a "mixed" acting sympathomimetic with both direct and indirect actions. The results from the present study agree with these findings and suggest that etilefrine may also be a "mixed" acting amine.

Previous evidence (Tainter and Chang, 1927; Tainter, 1929; Ross and Renyi, 1966) showed that the actions of tyramine and ephedrine were antagonised by cocaine. In the present study, the efflux of ^{3}H-noradrenaline from tyramine treated arteries in the presence of cocaine was significantly augmented. This augmentation occurred when cocaine and tyramine were added together to the wash fluid and also when tyramine was added five minutes after the application of cocaine at the beginning of the eleventh minute. The increase in efflux of ^{3}H-noradrenaline in the presence of cocaine and tyramine may be due to the fact that the tyramine concentration is far higher than that of cocaine and so this dose of cocaine is ineffective in preventing uptake of tyramine into the neurone. If this is the case, then the combined neuronal uptake blocking action of cocaine and competition for uptake with tyramine would prevent re-uptake of tyramine released noradrenaline and thus account for the enhanced efflux from the tissue into the wash fluid. Another possibility is that tyramine is entering the neurone via an uptake system insensitive to cocaine and displacing intraneuronal vesicular noradrenaline.

With etilefrine, the increase of ^{3}H-noradrenaline efflux was not significantly different from the values obtained with cocaine alone. This may reflect a blocking of neuronal uptake of etilefrine by cocaine. Burgen and Iversen (1965), using the perfused rat heart, evaluated a number of sympathomimetics for their affinity as substrates for neuronal uptake, in relation to their structure. Based on structural criteria, etilefrine would be expected to have a low affinity for neuronal uptake, and cocaine would be able to inhibit the etilefrine uptake and thus its noradrenaline releasing potential. These findings are in agreement with the perfusion study carried out in Chapter 4. Another possibility for cocaine's action is advanced by Paton (1973b), who suggests that cocaine decreases the enhanced efflux induced by sympathomimetic amines by inhibiting ^{3}H-noradrenaline efflux rather than the uptake of the sympathomimetic agent. However, Henseling et al. (1976b) showed, using rabbit aortic strips, that cocaine reduced the neuronal accumulation of ^{3}H-noradrenaline and enhanced the efflux from the neuronal compartment, which indicates a difference in the action of cocaine on rabbit atria and rabbit aorta.

The depressed rate of efflux obtained following guanethidine pretreatment indicates that the storage potential for ^{3}H-noradrenaline has been decreased (presumably intraneuronally). Evidence for this phenomenon comes from the work of Bisson and Muscholl (1962) and Schanker and Morrison (1965), who showed that guanethidine is selectively accumulated in sympathetic nerves, thus affecting noradrenaline storage. Etilefrine's action in the guanethidine treated arteries which had been pre-incubated with ^{3}H-

noradrenaline appeared unimpaired. The difference in efflux rate when this drug was added was apparent over virtually the entire washout period.

Arteries treated with iproniazid, cocaine, deoxycorticosterone acetate and UO521 simultaneously had efflux rates higher than those of control vessels (Fig. 6.1). This was expected, since cocaine and deoxycorticosterone acetate block neuronal and extra-neuronal uptake, respectively, leading to increased efflux of ^{3}H-noradrenaline from the arteries. In addition, iproniazid and UO521 would tend to decrease the breakdown of ^{3}H-noradrenaline, thereby providing more ^{3}H-noradrenaline, rather than its metab-olites, to be available for release from the artery. Since Wyse (1976) and Venning and de la Lande (1979) showed that the extraneuronal uptake system in the rat tail artery is relatively unimportant, this increase above the control efflux values may indicate a predominant role for cocaine on the observed efflux.

Etilefrine also produced an enhanced efflux above the ipro-niazid, cocaine, DOCA and UO521 control values. This efflux was also greater than that observed when etilefrine was administered to normal untreated arteries.

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CHAPTER 7

THE EFFECT OF ETILEFRINE AND OTHER
SYMPATHOMIMETIC AGENTS ON NORADRENALINE AND
METABOLITE EFFLUX FROM THE RAT TAIL ARTERY

Introduction

In the previous chapter, it was found that etilefrine enhanced the efflux of radioactive material from rat tail arteries pre-incubated with tritiated noradrenaline. This effect was similar to that observed with tyramine and ephedrine. It was suggested that the efflux was probably of neuronal origin in the form of tritiated noradrenaline (^{3}H-NA) and tritiated dihydroxy-phenylglycol (^{3}H-DOPEG). It has been shown (Paton, 1973a, b) that various sympathomimetic amines can enhance the efflux of ^{3}H-NA from rabbit atria. In those studies, only axoplasmic efflux was examined, since vesicular storage of noradrenaline was impaired by pretreatment with reserpine and noradrenaline metabolism suppressed by treatment with pargyline and tropolone.

Brandao et al. (1978) found that electrical stimulation and tyramine enhanced the efflux of ^{3}H-NA and its metabolites from the canine saphenous vein. They also found that electrical stimulation primarily enhanced the efflux of noradrenaline, whereas tyramine enhanced the efflux of ^{3}H-DOPEG and noradrenaline.

This chapter examines the composition of the radioactive efflux from the rat tail artery and its modification by treatment with etilefrine and several other sympathomimetic agents. The effects of pretreatment with a monoamine oxidase inhibitor, ipro-niazid, and an inhibitor of neuronal uptake, cocaine, are also

examined.   In this study, only $(-)^3$H-noradrenaline was used in order to avoid any differences in the disposition of the isomers (Iversen et al., 1971).

Methods

This efflux study was carried out in accordance with the protocol described in the general methods.   The initial series of experiments examined the metabolite efflux profile over 35 minutes without any drugs present.   Subsequent experiments investigated the changes in metabolites when the following drugs were added individually at the commencement of the third five minute period:

    (1)   etilefrine   55.2   µM

    (2)   tyramine   1.45   mM

    (3)   ephedrine   182   µM

    (4)   REN-293   227   µM

    (5)   cocaine   29.5   µM

    (6)   cocaine   29.5   µM, followed by

        tyramine   1.45   mM at the start of the

                      fourth wash period.

A series was also carried out in which the arteries were pre-incubated with cocaine 29.5 µM before exposure to ^{3}H-noradrenaline to determine whether cocaine could inhibit neuronal uptake.

In a further series of experiments, rats were pretreated with iproniazid (200 mg/Kg i.p.) each day for two days.   The metabolite efflux profiles were examined, as before, in the presence and absence of etilefrine 55.2 µM.

TABLE 7.1

The mean value ± S.E.M. for the group of arteries studied is shown.

Metabolite	1	2	Efflux 3	Period 4	Number 5	6	7
Control Efflux							
NA	48.7±7.3	43.0±8.7	36.0±8.5	33.0±9.4	34.3±9.6	40.3±8.9	38.6±8.6
DOPEG	28.5±2.5	27.5±2.7	27.5±2.7	23.3±2.5	22.0±3.9	23.0±3.0	23.0±6.5
DOMA	4.0±0.4	3.3±0.8	3.7±0.4	4.0±0.4	4.0±0.6	4.0±0.5	5.0±0.1
OMDA	14.7±3.7	21.3±6.5	29.8±6.2	36.8±6.6	35.7±8.2	30.0±6.0	29.6±6.2
NMN	2.9±0.9	4.5±1.3	3.5±0.9	3.0±0.7	2.4±0.8	2.3±0.6	2.3±0.8
Etilefrine Efflux							
NA	31.4±4.3	18.4±2.9	18.6±3.3	31.2±2.4	11.8±2.6	12.2±2.5	12.2±2.2
DOPEG	36.0±2.4	30.0±2.8	44.0±3.6	50.0±2.8	52.7±4.4	51.8±3.6	51.2±3.8
DOMA	4.2±0.2	4.0±0.3	5.0±0.5	4.2±0.4	4.0±0.7	4.6±0.4	4.6±0.5
OMDA	24.8±2.7	44.4±2.7	29.0±5.8	29.8±2.0	29.3±4.0	29.4±2.7	30.4±3.4
NMN	2.7±0.5	3.0±0.5	3.0±0.4	1.4±0.2	1.4±0.3	1.4±0.2	1.1±0.09
Tyramine Efflux							
NA	49.3±2.4	32.3±4.4	41.7±4.3	40.3±6.1	46.7±5.3	41.0±6.5	37.6±6.1
DOPEG	30.0±1.2	27.5±4.6	43.7±4.1	48.3±5.4	41.3±5.2	45.6±5.7	49.0±5.2
DOMA	4.8±1.7	5.7±1.7	4.3±1.4	4.8±1.4	4.3±1.6	2.3±0.3	2.3±0.3
OMDA	14.8±2.1	33.5±5.0	9.7±1.6	6.8±1.4	6.0±1.1	7.6±1.6	8.7±1.4
NMN	0.5±0.07	1.7±0.3	1.3±0.2	1.3±0.4	1.1±0.2	2.0±0.4	2.0±0.3

The figures in each column represent the
percentage of the total efflux per period.

The amount of ^{3}H-NA, ^{3}H-DOPEG and ^{3}H-metabolites, i.e., normetanephrine (^{3}H-NMN), dihydroxymandelic acid (^{3}H-DOMA), the OMDA fraction (containing methoxyhydroxyphenylglycol - MOPEG, and methoxymandelic acid - VMA) effluxed per five minute period has been expressed as a percentage of the efflux over the first five minute period which is taken as 100%. At each efflux period, the percentage of the individual metabolites contributing to the total tritium effluxed was also determined. This was calculated for the control values and also for the effluxes following drug treatment. Appendix 4 shows the structures of the various metabolites and the enzymatic conversion pathways.

Results

Figure 7.1 shows the efflux profile of ^{3}H-NA, ^{3}H-DOPEG and the other noradrenaline metabolites from control arteries and arteries treated with etilefrine and tyramine. In the untreated arteries, there was a progressive decrease in the efflux of ^{3}H-NA, ^{3}H-DOPEG and the other metabolites over the 35 minute wash period. Both etilefrine and tyramine significantly increased the efflux of ^{3}H-DOPEG, commencing at the third efflux period. Tyramine caused a significant increase in ^{3}H-NA efflux. Although a similar trend in ^{3}H-NA efflux was observed with etilefrine, the values were not significant at the 5% level.

An increase in ^{3}H-DOMA efflux was also observed upon the addition of etilefrine or tyramine to the wash medium. However, as shown in Table 7.1, the ^{3}H-DOMA fraction represented only

TABLE 7.2

The mean value ± S.E.M. for the group of arteries studied is shown.

Metabolite			Efflux	Period	Number		
	1	2	3	4	5	6	7

REN-293 Efflux

Metabolite	1	2	3	4	5	6	7
NA	12.7±1.6	10.3±2.2	39.0±6.9	43.7±7.5	51.0±9.3	52.3±8.4	39.3±7.5
DOPEG	35.0±6.1	27.0±4.3	11.7±3.2	6.5±1.0	7.0±0.9	5.5±0.8	6.0±0.9
DOMA	5.0±1.1	4.8±2.3	3.0±0.8	4.0±0.7	2.5±0.7	5.2±0.7	5.3±0.6
OMDA	42.5±6.1	52.0±5.9	41.0±2.8	38.5±2.9	30.5±2.0	27.3±1.5	34.7±5.3
NMN	4.3±1.6	5.0±1.7	5.0±1.0	6.5±1.5	8.3±1.8	9.3±1.4	14.0±3.4

Ephedrine Efflux

Metabolite	1	2	3	4	5	6	7
NA	51.0±5.8	42.3±7.1	46.3±6.2	39.3±3.0	40.2±5.4	39.8±2.7	34.5±3.5
DOPEG	33.8±4.6	33.7±6.2	33.5±4.8	38.0±2.1	37.0±4.1	36.7±2.2	38.5±3.6
DOMA	4.0±0.4	4.0±0.7	4.3±0.6	4.3±0.3	4.8±0.3	3.8±0.3	4.0±0.4
OMDA	9.7±0.8	18.8±2.4	14.8±2.2	14.7±2.8	13.5±3.4	15.7±1.7	19.3±0.8
NMN	1.2±0.2	1.7±0.3	1.3±0.3	2.8±0.3	3.5±0.2	3.3±0.6	3.5±0.5

The figures in each column represent the percentage of the total efflux per period.

4.2% and 4.8% of the total tritium efflux, respectively. Tyramine also induced an increase in ^{3}H-NMN, but in this instance also this only represented 1.3% of the total tritium effluxed.

Figure 7.2 shows the efflux profile in the presence of REN-293 and ephedrine. REN-293 caused a large increase in ^{3}H-NA released compared with the untreated arteries and a decrease in the amount of ^{3}H-DOPEG effluxed. The ^{3}H-DOMA efflux varied over the seven periods, accounting for approximately 4% of the total tritium effluxed per period. REN-293 also caused an increase in ^{3}H-NMN over the latter five efflux periods, with ^{3}H-NMN comprising 14% of the total tritium effluxed in the final wash period. Ephedrine slowed the rate of decline of the ^{3}H-DOPEG efflux compared with the control values for all the time periods. The ^{3}H-NA efflux in the presence of ephedrine was significantly higher than the control only during periods three and four. Ephedrine also caused an increase in the efflux of the ^{3}H-NMN fraction, though this was not as marked as that initiated by tyramine and REN-293. From Table 7.2 it can be seen that in the case of ephedrine, the ^{3}H-NMN fraction contributed to approximately 3.5% of the total tritium effluxed during the latter periods of efflux.

Figure 7.3 shows the efflux from arteries treated with cocaine or cocaine and tyramine. Tyramine again caused a significant efflux of both ^{3}H-NA and ^{3}H-DOPEG. The ^{3}H-OMDA fraction was not changed significantly by the addition of tyramine. The enhancement of the ^{3}H-DOMA and ^{3}H-NMN efflux caused by the tyramine in the presence of cocaine accounted for approximately 2% and 1% of the total tritium effluxed, as observed

TABLE 7.3

The mean value ± S.E.M. for the group of arteries studied is shown.

Metabolite	Efflux Period Number						
	1	2	3	4	5	6	7
Cocaine Pre-incubation Efflux							
NA	21.0±5.0	6.0±1.0	4.5±1.1	3.0±0.4	3.5±0.9	4.0±0.5	2.2±0.3
DOPEG	23.0±3.0	1.0±0.1	1.0±0.2	0.8±0.05	0.8±0.2	0.6±0.1	0.5±0.1
DOMA	0.4±0.05	0 ±0	0.5±0.1	0.1±0.02	0.2±0.05	0.2±0.05	0.1±0.02
OMDA	41.5±5.2	92.0±2.0	94.0±2.0	95.5±0.5	94.5±2.2	95.0±2.0	97.0±3.4
NMN	0.7±0.2	1.0±0.2	0.5±0.2	0.2±0.05	0.3±0.05	0.1±0.02	0.1±0.02
Cocaine Control Efflux							
NA	17.0±4.9	10.0±3.2	10.5±3.1	13.2±2.1	10.3±3.1	10.8±0.8	10.5±4.9
DOPEG	26.3±5.9	28.3±6.1	23.5±3.8	25.5±3.2	18.0±4.0	24.0±5.1	22.3±6.4
DOMA	2.6±0.8	2.5±1.0	2.2±0.4	2.3±0.4	2.7±0.5	2.2±0.7	2.0±0.4
OMDA	52.5±9.8	58.8±8.2	63.3±6.2	58.0±7.2	67.5±8.2	61.8±8.1	64.0±6.3
NMN	0.5±0.06	0.4±0.05	0.5±0.1	0.5±0.06	0.5±0.09	0.6±0.1	0.5±0.1
Tyramine and Cocaine Efflux							
NA	33.0±4.2	12.8±2.0	8.9±1.3	17.5±1.5	18.0±3.0	22.0±4.1	20.0±3.0
DOPEG	23.0±3.0	23.4±2.7	14.4±2.3	27.5±3.5	37.5±2.5	32.0±3.0	30.5±3.1
DOMA	1.5±0.3	1.5±0.2	1.4±0.3	2.0±0.1	1.5±0.5	2.0±0.2	1.1±0.3
OMDA	41.5±8.1	61.0±6.2	74.5±8.4	52.5±2.5	41.0±6.0	43.0±4.5	47.5±2.5
NMN	0.6±0.2	0.6±0.1	0.8±0.2	0.9±0.05	0.9±0.07	1.0±0.3	0.7±0.1

The figures in each column represent the percentage of the total efflux per period.

in Table 7.3

Pre-incubation with cocaine decreased the uptake of ^{3}H-NA, so that ^{3}H-NA and ^{3}H-DOPEG efflux accounted for only 6% and 1% of the total tritium efflux, respectively (Table 7.3), whereas in the controls these values were 43% and 27% (Table 7.1), taken during the second five minute efflux period.

Figure 7.4 shows the efflux profiles in arteries pretreated with iproniazid. Table 7.4 shows that in the iproniazid control group the ^{3}H-DOPEG efflux contributes only 4.3% of the total efflux compared to 28.5% in the untreated group (Table 7.1). Etilefrine has now caused an increase in ^{3}H-NA efflux. An increase in ^{3}H-DOPEG efflux was also observed (as in arteries not treated with iproniazid) following etilefrine administration (Fig. 7.1). No change was observed in ^{3}H-NMN or ^{3}H-OMDA efflux following etilefrine administration; however, an unexplained fluctuation in ^{3}H-DOMA occurred in both iproniazid treated groups with and without etilefrine.

Discussion

The results of the present study indicate that the sympatho-mimetic amines examined have quite different effects on noradrenaline and metabolite efflux from a sympathetically innervated blood vessel. Leitz and Stefano (1971), using the rat heart, observed no increase in efflux of deaminated metabolites following exposure to tyramine or amphetamine. However, Brandao et al. (1978) found that tyramine induced an increase mainly in

TABLE 7.4

The mean value ± S.E.M. for the group of arteries studied is shown.

Metabolite	Efflux Period Number					
	1	2	3	4	5	6
Iproniazid Control Efflux						
NA	27.7±3.2	14.8±1.6	12.5±4.0	7.7±2.1	6.8±2.1	7.7±1.6
DOPEG	4.3±0.8	5.0±1.2	4.5±0.8	4.3±0.5	3.8±0.4	4.3±0.4
DOMA	0.5±0.1	0.5±0.1	0 ±0	0.3±0.1	0.2±0.05	0.6±0.1
OMDA	65.0±7.2	77.8±8.3	80.5±6.4	86.3±3.5	87.7±3.5	85.3±2.1
NMN	2.3±0.2	2.0±0.4	1.8±0.4	1.5±0.2	1.5±0.2	1.5±0.2
Iproniazid and Etilefrine Efflux						
NA	53.3±7.4	40.3±4.4	58.3±6.9	55.8±6.9	54.3±7.4	53.5±7.1
DOPEG	8.3±2.0	11.5±2.1	10.3±2.1	10.0±2.1	11.3±2.6	11.5±2.1
DOMA	4.3±0.8	5.3±0.9	2.7±0.4	4.3±0.7	3.0±0.2	4.3±0.8
OMDA	25.3±2.7	31.7±4.2	18.8±2.3	22.3±2.4	24.0±2.7	23.5±2.5
NMN	8.5±1.6	11.5±2.6	7.0±1.7	7.3±1.6	5.8±1.5	6.5±1.3

The figures in each column represent the
percentage of the total efflux per period.

^{3}H-DOPEG, whereas electrical stimulation predominantly enhanced the efflux of ^{3}H-NA followed by ^{3}H-DOPEG.

In the previous chapter, it was shown that etilefrine increased the efflux of neuronal tritium (NA and DOPEG) from the rat tail artery. The present study shows that etilefrine is in fact enhancing the release of ^{3}H-DOPEG to a larger extent than it is of noradrenaline. The increase in ^{3}H-DOPEG may be due to re-uptake of released noradrenaline and subsequent metabolism and diffusion into the wash fluid. This is in agreement with the findings of Vanhoutte and Webb (1979), who showed that neuronal uptake is an important mechanism of transmitter elimination from the synapse in the rat tail artery. Burgen and Iversen (1965) suggested certain structural requirements which need to be fulfilled for sympathomimetics to be substrates for neuronal uptake in the rat heart:

    (a) beta-hydroxylation caused a decreased affinity;

    (b) alpha-methylation resulted in an increased affinity;

    (c) phenolic hydroxyl groups, especially in the 3',4' position, enhanced affinity;

    (d) N-substitution with bulky groups decreased affinity;

    (e) O-methylation of phenolic groups caused a decrease in affinity for uptake.

Based on these criteria, etilefrine would be expected to be a poor substrate. Etilefrine is not a substrate for monoamine oxidase (MAO) and therefore would not compete with noradrenaline for this enzyme.

Tyramine has been shown to release neuronal noradrenaline (Burn and Rand, 1958; Paton, 1973a; Brandao et al., 1979).
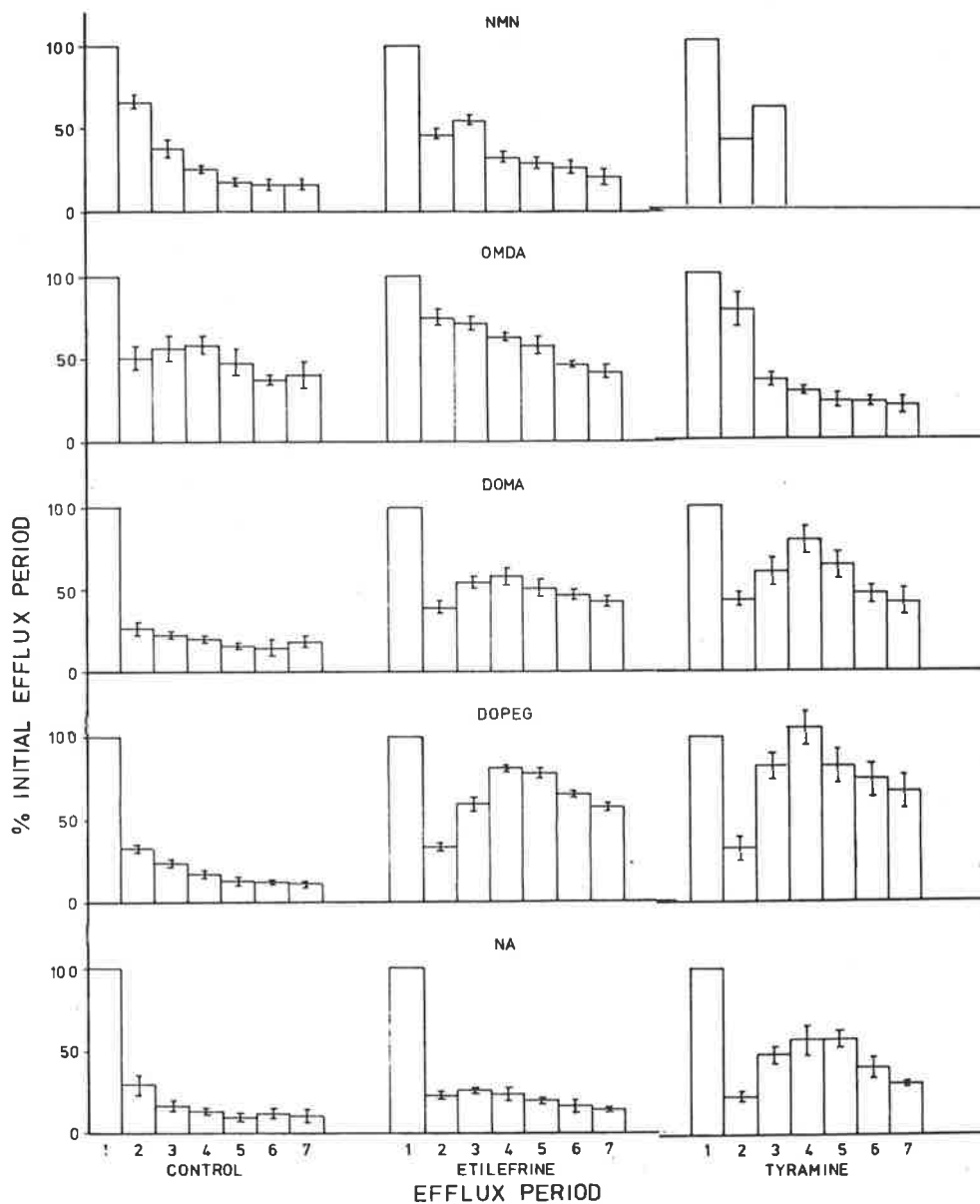
Fig. 7.1

The efflux of ^{3}H-NA, ^{3}H-DOPEG and the other metabolites (expressed as a percentage of the amount washed out in the first wash period) from the rat tail artery. The effect of etilefrine and tyramine (introduced at the beginning of the third wash period) is compared with control efflux.
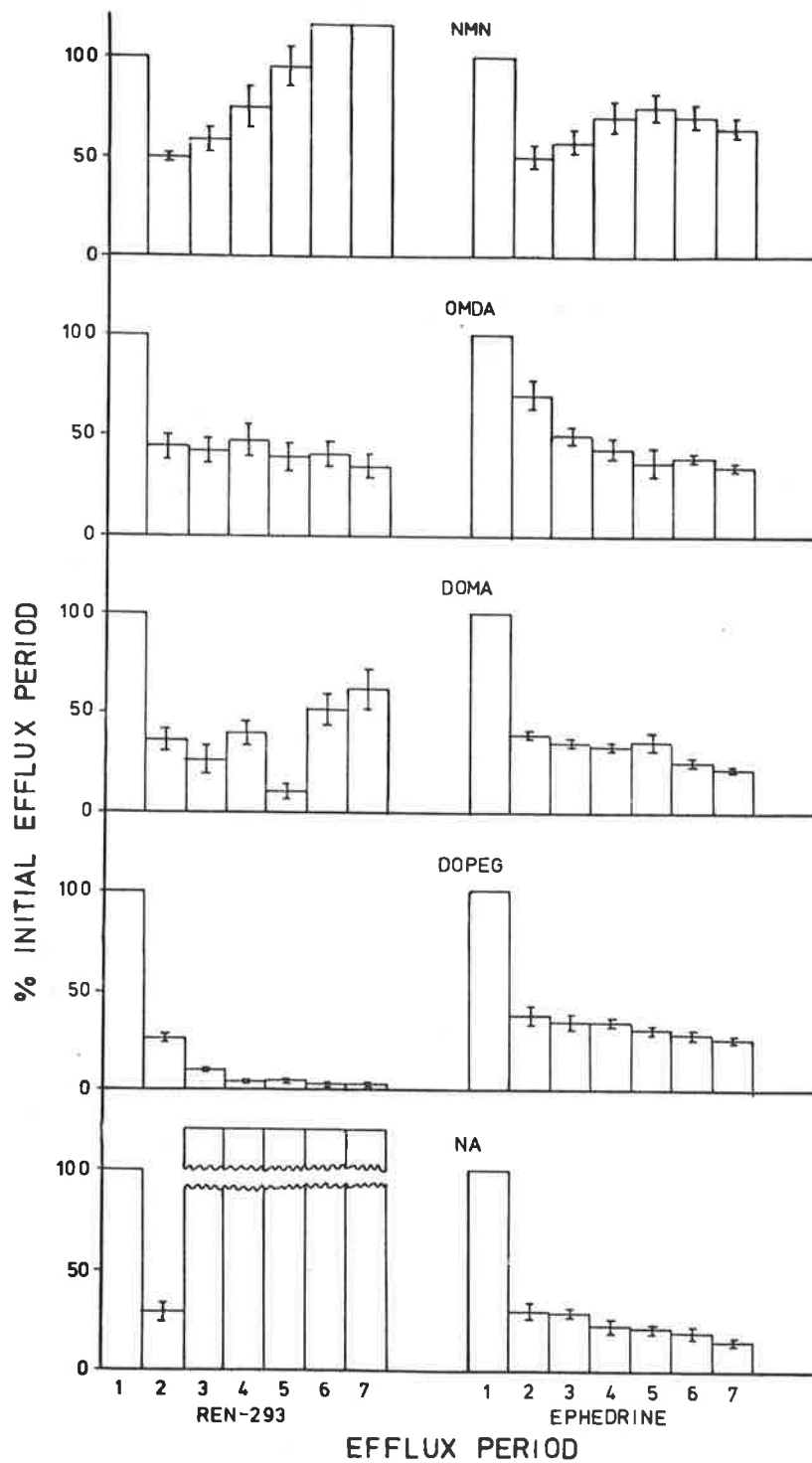
Fig. 7.2

The efflux of ^{3}H-NA, ^{3}H-DOPEG and the other metabolites (expressed as a percentage of the amount washed out in the first wash period) from the rat tail artery. The effect of REN-293 and ephedrine (introduced at the beginning of the third wash period) is shown.
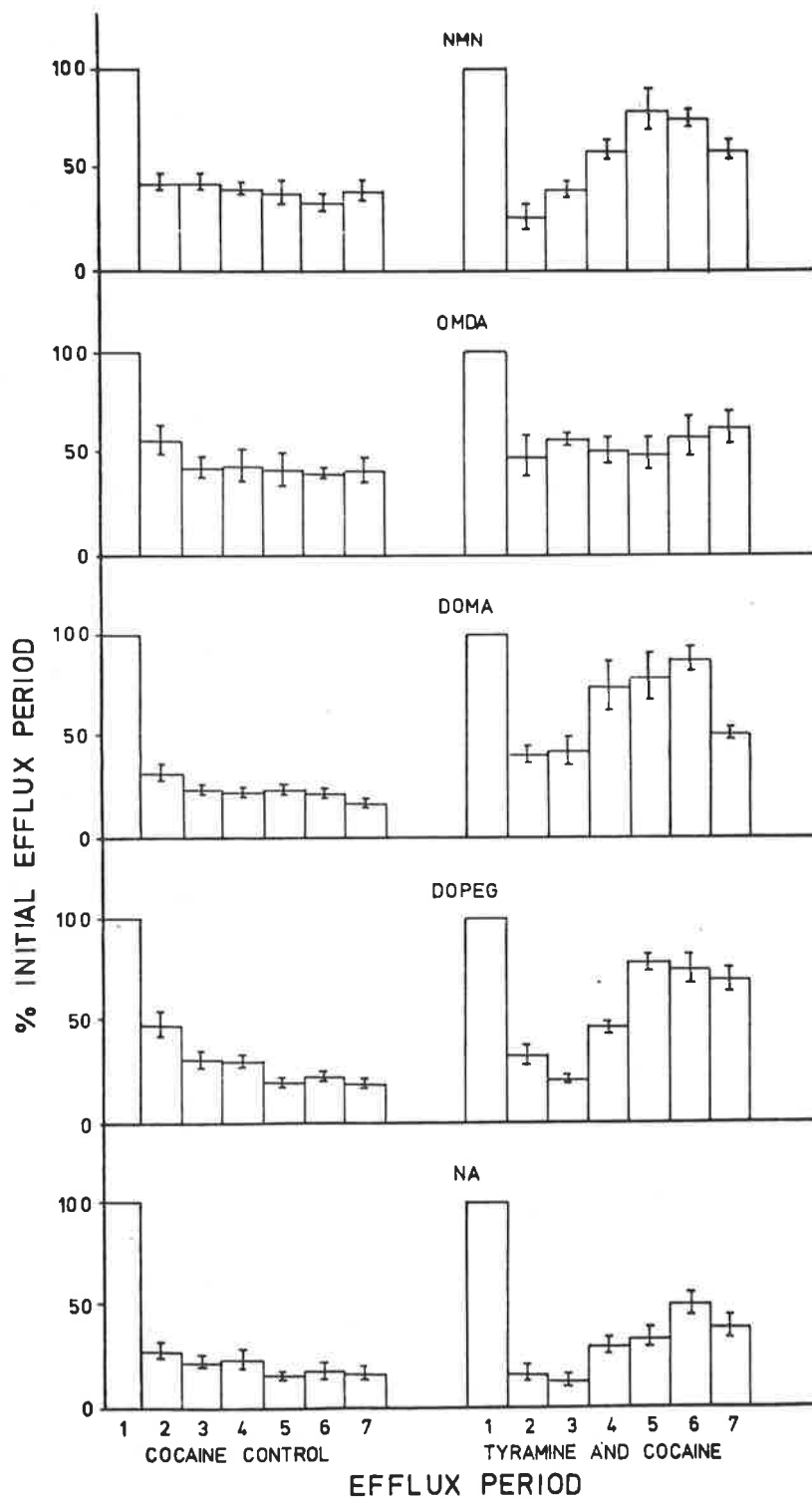
Fig. 7.3

The influence of cocaine and cocaine/tyramine on the efflux patterns from the tail artery. Cocaine was added at the commencement of the third five minute period and tyramine at the start of the fourth five minute wash period.
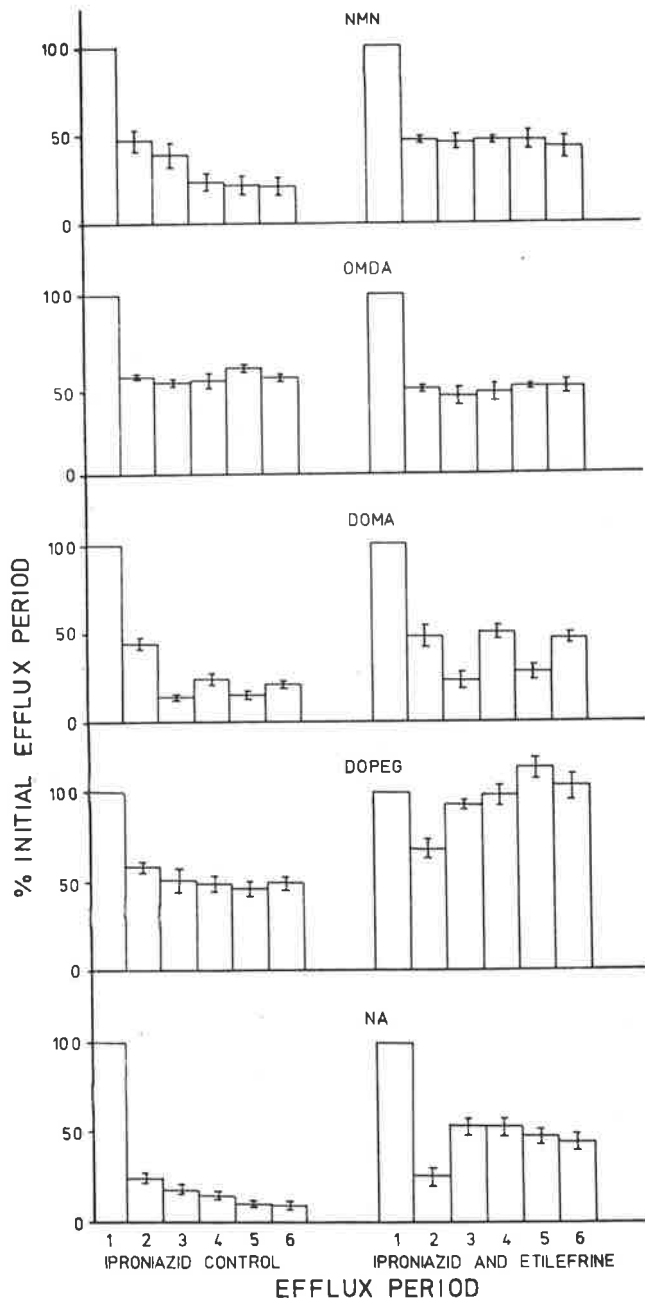
Fig. 7.4

Efflux patterns from rat tail arteries taken from animals pretreated with iproniazid for 48 hours. Etilefrine now causes a significant increase in ^{3}H–NA efflux.

Schümann and Philipu (1961) showed that tyramine is taken up into the nerve terminal, displacing noradrenaline from its storage vesicles into the cytoplasm and finally into the synapse. Our results show that there is an increase of both ^{3}H–NA and ^{3}H–DOPEG efflux in the presence of tyramine. Since tyramine has also been shown to be a good substrate for neuronal uptake (Burgen and Iversen, 1965), this would explain the ability of tyramine to enhance noradrenaline efflux by release of ^{3}H–NA and subsequent inhibition of re-uptake by competing with ^{3}H–NA for the Uptake 1 mechanism.

REN–293 induced a large increase in ^{3}H–NA efflux but a decrease in ^{3}H-DOPEG efflux. Previous observations (Chapter 3) have shown that REN–293 has a vasoconstrictor action in the rat tail artery which is blocked by phentolamine and abolished by pretreatment with reserpine.

Together, these results indicate that REN–293 has a strong indirect sympathomimetic action mediated via neuronal noradrenaline release. Applying the criteria of Burgen and Iversen (1965) once again, REN–293 would seem to be a good substrate for neuronal uptake and so would have a dual effect on noradrenaline efflux by enhancing release and inhibiting re-uptake. The decrease in ^{3}H-DOPEG is difficult to explain. Since REN–293 has been shown to be a substrate for MAO (personal communication, Boehringer Ingelheim), it is possible that ^{3}H-DOPEG formation has been inhibited by REN–293, firstly by inhibition of re-uptake and secondly by competition for MAO. However, on this basis one would not expect the increase in ^{3}H-DOPEG observed on the addition of tyramine. This difference may be explained if REN–

293 has a higher affinity than tyramine for either neuronal uptake or MAO. If REN-293 is a better substrate for MAO, then the resultant competition with noradrenaline for this enzyme may inhibit the formation of H-DOPEG. With tyramine this competition for MAO may not be as great and an increase in ^{3}H-DOPEG form-ation would occur.

Ephedrine tended to decrease the rate of decline of both ^{3}H-NA and ^{3}H-DOPEG efflux from the artery when compared with untreated controls. Ephedrine has been described as a "mixed acting amine" by Trendelenburg (1963) and these findings indi-cate a modest indirect action via neuronal noradrenaline release. Based on the criteria of Burgen and Iversen (1965), ephedrine would appear to have a relatively low affinity for neuronal uptake and so would be expected to be a poor releaser of noradrenaline and a poor inhibitor of re-uptake. The present findings are in agreement with these criteria.

Cocaine did not cause an enhancement of ^{3}H-NA or ^{3}H-DOPEG efflux, but when tyramine was added in the presence of cocaine, an increase in both ^{3}H-NA and ^{3}H-DOPEG was observed. This increase in the presence of cocaine may be due to an incomplete block of neuronal uptake by cocaine at the doses used in this study.

Iproniazid did not modify the trend in percentage ^{3}H-NA efflux, but the levels of ^{3}H-DOPEG expressed as a percentage of total tritium were reduced when compared to control values. The fact that etilefrine induced a significant increase in ^{3}H-NA efflux in the iproniazid treated vessels suggests that inhibition of MAO has prevented the metabolism of ^{3}H-NA and shifted the efflux

pattern in the direction of the latter. Cocaine pretreatment significantly inhibited the uptake of tritiated noradrenaline, so confirming the presence of a cocaine sensitive uptake process in the rat tail artery.

*   *   *

CHAPTER 8

GENERAL DISCUSSION

The project commenced as a study to examine the effects of the sympathomimetic amine etilefrine. Etilefrine had been used in the treatment of patients with orthostatic hypotension (Miller et al., 1973), and in a previous report Mellander (1966) had suggested that etilefrine had "a local constrictor effect mediated by alpha receptors" and a "distant" dilator effect activated via the sympathetic nervous system. With this in mind, it was decided to look at the mechanism of action of etilefrine on blood vessels, to determine whether or not it had any direct sympatho-mimetic effects. This was important, since it had been shown (Parks et al., 1961) that in the treatment of postural hypotension the indirectly acting sympathomimetics, such as ephedrine and methyl amphetamine, were ineffective in patients with autonomic degeneration.

In selecting a suitable vascular model to examine the actions of etilefrine, several factors were important. Firstly, it was considered essential that the model be similar to human arteries with respect to its sympathetic innervation and, secondly, that it was sensitive to sympathetic amines and exhibited repro-ducible dose response curves. Hodge and Robinson (1972) had shown that the rat tail artery had good sympathetic innervation and was similar in characteristics to the rabbit ear artery, which had been shown previously (Frewin et al., 1971b) to resemble the human digital artery in its histology and innerv-ation. Nicholas (1969) suggested that the rat tail artery was a sensitive preparation and that it would be suitable for the assay

of sympathomimetic amines. These facts, combined with the ease with which the preparation could be isolated, warranted the selection of the rat tail artery, and in some experiments the central artery of the rabbit ear, as vascular models for this study.

In Chapter 3, it was observed that the rat tail artery "in situ" did respond well to a number of directly and indirectly acting sympathomimetics. Etilefrine caused an increase in perfusion pressure which was comparable to that of metaraminol and this increase was mediated via alpha-adrenergic receptors as demonstrated by the inhibition of response caused by phentolamine. Of particular interest in this chapter was the response characteristics exhibited by etilefrine. The etilefrine response was composed of a short initial rise phase, followed by a prolonged slow rise phase. The shape of the response was qualitatively similar to that of ephedrine and contrasted markedly with the response of adrenaline and noradrenaline which typically had a monophasic response with a subsequent plateau. Trendelenburg (1963) categorised ephedrine as a "mixed-acting" amine, i.e., having both direct and indirect actions. This similarity of etilefrine and ephedrine, combined with the supportive evidence that the slow rise phase of etilefrine was abolished by pretreatment with 6-hydroxydopamine, indicated that etilefrine possessed some indirect sympathomimetic activity. The sympatholytic agents reserpine and guanethidine also caused a decrease in the etilefrine response.

Further supportive evidence for a noradrenaline releasing role came from the fact that prior administration of a noradrenaline depleting agent (namely, tyramine) depressed the response

to etilefrine. Tyramine has been shown to be the classic example of an indirectly acting sympathomimetic (Carlsson et al., 1957; Burn and Rand, 1958) and, as such, by depressing the etilefrine response, a common mechanism of action between these two amines was speculated. The augmentation of the etilefrine response by cocaine indicated that etilefrine may not be dependent on the cocaine sensitive uptake and that the enhanced response was due to the inhibition of re-uptake of noradrenaline released by etilefrine. In Chapter 4, it was decided to examine the effects of etilefrine on the isolated rat tail artery. This was instigated in an attempt to elucidate further the drug's mechanism of action by comparing the responses of externally and internally administered etilefrine. de la Lande and Waterson (1968), who investigated the action of intraluminally and extraluminally administered tyramine, postulated that the difference in response to the drug via the two routes was related to tyramine's indirect action and hence its access to intraneuronal storage sites. Etilefrine showed a larger response when administered externally to the rat tail artery, and when the same sympatholytic agents (reserpine and guanethidine) were used as in the previous chapter, the external response was depressed to a greater extent than the internal response. The internal and the external responses to tyramine were similar to etilefrine in normal arteries, with the external response being larger than the internal response. The greater indirect sympathomimetic activity of tyramine was demonstrated by the complete inhibition of its response by reserpine and guanethidine compared to a partial depression in the response observed with etilefrine.

Chapter 5 examined the actions of some of the sympatho-
mimetic amines used in previous chapters on the rabbit ear
artery. The selection of the ear artery as a vascular model was
for two reasons: firstly, it seemed advantageous to compare the
responses obtained in the rat tail artery with those on another
vessel and, secondly, the rabbit ear artery could be surgically
denervated with relative ease. In relation to the use of etil-
efrine in orthostatic hypotension, the interaction of this drug
with 9-alpha-fluorohydrocortisone (one of the main therapeutic
agents used in this condition) was also evaluated. Since 9-alpha-
fluorohydrocortisone is used therapeutically at a low dose level,
a dose was selected which it was thought would approximate
physiological levels. In addition, a higher dose was also
selected which was comparable to that used with other mineralo-
corticoids for the purpose of inhibiting extraneuronal uptake.
However, no change in the etilefrine response was observed in the
presence of either dose of the mineralocorticoid.

Further evidence of the postulated partial indirect action
of etilefrine was suggested when it was found that the etilefrine
response was depressed in surgically sympathectomised rabbit ear
arteries. To further substantiate the pharmacological findings,
the next step was to determine whether or not there was a modif-
ication of the tritiated noradrenaline efflux from the tail artery
in the presence of etilefrine. It has been shown (Paton, 1973a,
b) that various sympathomimetic amines can enhance the efflux
of tritiated noradrenaline from rabbit atria. The results of the
study in Chapter 6 showed that etilefrine did enhance the trit-
iated efflux from arteries pre-incubated with tritiated

noradrenaline. The enhancement observed was equivalent to that of ephedrine but substantially less than that induced by tyramine. The findings seen with tyramine and ephedrine were in agreement with the actions of those amines as suggested by Trendelenburg (1963). Tyramine was found to cause an increased efflux in the presence of cocaine, and it was suggested that tyramine may enter the neurone via an alternative uptake to that inhibited by cocaine or may just be present in quantities large enough to render the cocaine block ineffective. Chronic guanethidine administration reduced the capacity of the neurones to store noradrenaline, but an increase in tritium efflux was still evident upon administration of etilefrine.

Inhibition of the various noradrenaline disposition mechanisms by drugs inhibiting transmitter uptake and metabolism increased the rate of efflux with respect to control values. Etilefrine also caused an enhanced efflux in those arteries which had been treated with iproniazid, UO521, cocaine and deoxycorticosterone acetate. This efflux was greater than that released from control arteries and also those vessels treated with the inhibitors of transmitter uptake and metabolism alone. This increased tritium efflux was thought to be due to the release of more unmetabolised noradrenaline from the arteries in the presence of etilefrine.

To further verify the composition of the tritiated efflux from the rat tail artery, it was decided to separate the various metabolites within the radioactive efflux. This procedure enabled direct determination of whether the enhanced efflux induced by the various sympathomimetics was due to increased noradrenaline

or metabolite displacement. In this investigation, it was found that tyramine again caused an increase in tritium efflux and this could be attributed to enhanced ^{3}H-NA and ^{3}H-DOPEG efflux. Etilefrine, on the other hand, caused an increase in ^{3}H-DOPEG efflux and only a very slight, non-significant increase in ^{3}H-NA. However, following pretreatment with iproniazid, a larger increase in ^{3}H-NA efflux was observed following etilefrine administration. Together, these findings suggested that, although etilefrine caused an increase in ^{3}H-NA release, re-uptake and subsequent metabolism probably occurred during the five minute efflux periods monitored. Thus, the increase in effluxed ^{3}H-NA was only unmasked when MAO was inhibited by iproniazid. Tyramine was again responsible for an increase in ^{3}H-NA and ^{3}H-DOPEG efflux in the presence of cocaine. The possible reasons for cocaine being unable to inhibit tyramine's action has been discussed previously.

The sympathomimetic REN-293 is a drug which is under investigation by the pharmaceutical agency Boehringer Ingelheim as a possible successor to etilefrine. Samples of the drug were received late in this investigation and, as such, data on this agent are only included in Chapter 3 and Chapter 7. REN-293, on the basis of the work carried out in Chapter 3, appears to be an indirectly acting sympathomimetic in the rat tail artery, i.e., via the action of noradrenaline on alpha-receptors. Its indirect effect seems equivalent to that of tyramine as suggested by the total suppression of the response of both agents in reserpine pretreated arteries. These findings are in agreement with company investigational literature (Boehringer Ingelheim). From

the dose response curves generated in the present study, REN-293 would appear to be more potent than tyramine in this vessel.

The response to REN-293 following tyramine administration was significantly depressed. This suggested that tachyphylaxis to the REN-293 response occurred following the addition of tyramine. This would be expected if the two agents were acting on a common pool of releasable noradrenaline. The inhibition of the REN-293 response by cocaine suggested that its action is dependent to a large extent on the cocaine sensitive uptake. Based on the criteria of Burgen and Iversen (1965), REN-293 would be expected to be a good substrate for neuronal uptake, which provides supportive evidence for the cocaine inhibition. The results in Chapter 7 regarding REN-293 show that this agent does enhance ^{3}H-NA release from the tail artery. The depression in ^{3}H-DOPEG efflux, however, was postulated to be due to an inhibition of re-uptake by REN-293 and competition for the enzyme MAO.

In conclusion, it would seem that etilefrine does have an indirect sympathomimetic component to its action which is comparable with ephedrine but not as great as that of tyramine. This is of relevance in the clinical situation where the use of this agent in treating postural hypotension has been proposed. REN-293 would appear to have an indirect action similar to that of tyramine and, as such, is of interest as a pharmacological tool for examination of sympathetic nerve function. REN-293 as a drug of clinical importance is doubtful, since it is far less potent than etilefrine and due to its indirect action would be subject to tachyphylaxis and loss of effect in autonomic neuropathy,

and so be limited in its therapeutic usefulness.

It is obvious that the sympathomimetic agents examined in this thesis have varying modes of action on blood vessels. The interplay between the various neuronal and extraneuronal disposition mechanisms with respect to the actions of these agents is yet to be fully elucidated. As Trendelenburg (1963) suggests, "sympathomimetic amines possess a continuous spectrum of actions ranging from purely direct to completely indirect in a variety of species and tissues within each species."

*   *   *

APPENDIX

1a. Krebs bicarbonate solution

Composition of the Kreb's bicarbonate solution used in this study.

Constituent	Grams/Litre	Millimolar Equivalent
NaCl	6.9	120.0
KCl	0.29	3.9
$CaCl_2$	0.28	2.5
$MgCl_2$	0.10	1.1
$NaHCO_3$	2.10	25.0
KH_2PO_4	0.16	1.0
glucose	1.00	5.5
EDTA	0.0045	0.01

All compounds, except $CaCl_2$, $MgCl_2$ and ethylenediamine-tetracetic acid (EDTA), were dissolved in the required volume of distilled water. $CaCl_2$ and $MgCl_2$ were added from standardised 10% stock solutions. The EDTA was added from a stock solution of 30 μM. The fully constituted Krebs solution was filtered before use and gassed with 5% carbon dioxide and 95% oxygen (Carbogen). The pH of the gassed solution was 7.4.

1b. Perfusion pump

The pump used was a peristaltic roller pump model 131900 (Desaga Germany).

1c. Pressure recorder

Pressure changes in the arteries were measured using a pressure transducer (Statham, P23GC) and recorded on a twin channel potentiometric recorder (Rikadenki model B-261). The 5 and 10 mV attenuator settings were used.

1d. Sequential timer

A five channel sequential timer, designed and constructed by the author, was used to change drug solutions or wash volumes automatically. The timer consisted of five LM2905 integrated circuit (IC) timers (National Semiconductor Corp.) in parallel. The output from one IC was used to trigger the next. Each individual timer had a continuously variable range from 0-20 minutes. The output from each timer was used to trigger a relay, which in turn triggered a further relay modified to clamp thin silicone rubber tubing and thus prevent the flow of perfusion or wash fluid.

1e.

Ascorbic Krebs was Krebs-bicarbonate to which ascorbic acid had been added to give a concentration of 113 μM to inhibit noradrenaline oxidation (Hughes and Smith, 1978).

1f. Liquid scintillation spectrometry

Radioactivity was counted using a Packard 2425 or 3310 Tri-Carb liquid scintillation spectrometer.

The scintillation fluid had the following composition (g/litre): PPO (2,5-diphenyloxazole) 8.25, POPOP (1,4-di(2[5-phenyloxazolyl]benzene)) 0.25. The PPO and POPOP were dissolved in one litre of toluene to which was added 500 ml of Triton X-100.

1g. Separation of unlabelled noradrenaline metabolites

To verify that the Dowex and alumina column technique used in this thesis was separating the various ^{3}H-noradrenaline metabolites, the recoveries and crossovers of unlabelled metabolites were determined.

The technique was that used by Morris and Irvine (personal

communication). Each metabolite (100 μg in 0.0167 ml), i.e., MOPEG, VMA, NMN, DOPEG, NA and DOMA or a blank containing no metabolites, was individually added to the following: 0.2 ml 1% ascorbic acid, 0.2 ml 0.1 N HCl, 0.1 ml 10% EDTA, 0.1 ml 12.5% Na_2SO_3, 1 ml Krebs, 0.5 ml 1 M Tris (pH 8.4). This mixture was loaded on to an alumina column and the procedure followed as described in the "metabolite separation" section in the general methods.

The fractions eluted from the alumina and Dowex columns, as well as the two washes, were then assayed for native fluorescence on a Perkin-Elmer 202 fluorometer (excitation = 288 nm, emission = 315 nm).

	FRACTION (Expected Metabolite)	ACTUAL % RECOVERY					
		MOPEG	VMA	NMN	DOPEG	NA	DOMA
1.	MOPEG+VMA	96.8	92.3	0	0		0
2.	NMN	2.0	0.7	100	0	0.5	0
3.	DOPEG	0	1.2	0	95.8	0.2	0
4.	NA	1.1	1.1	0	0	95.1	2.3
5.	DOMA	0	0	0	1.9	1.7	56.3
6.	Wash 1	0	0	0	0	0	0
7.	Wash 2	0	4.8	0	2.3	2.5	41.4

On the basis of these results crossover corrections were made.

1h. Preparation of alumina for columns

The procedure used was similar to that used by Crout (1961). Alumina (200-300 g) was boiled in one litre of 2 N HCl for 30 minutes and the acid decanted. The alumina was then washed twelve times with distilled water, allowing five minutes

each time for the alumina to settle. When the water was clear, the pH of the mixture was approximately 4–5. The alumina was then dried at 100°C for two hours and stored for use.

1i. Preparation of Dowex for columns

The procedure used was similar to that described by Graefe et al. (1973). Dowex 50 Wx4 (200–400 mesh) was washed twice with 2 N sodium hydroxide (containing 1% di-sodium EDTA) at 50°C until the supernatant was colourless. The Dowex was then washed with distilled water and subsequently with 2 N HCl. Following a further wash with distilled water and equilibration with 0.01 N HCl to pH 2, the Dowex was finally washed with 6 N HCl-ethanol (ratio 1:1, v/v) and again equilibrated with 0.01 N HCl to pH 2. The Dowex was then stored ready for use in 0.01 N HCl.

1j. pH measurements

The pH of solutions was measured with a digital pH meter Radiometer (Copenhagen), model PHM62, using a Radiometer glass electrode.

1k. Electrical stimulation

This was performed using an electrical stimulator (Grass model S4E). The following settings were used: voltage 20 V, pulse delay 0.01 ms, pulse duration 5 ms, frequency varied from 1–20 pulses/sec.

2. Purification of ^{3}H-noradrenaline

The ^{3}H-noradrenaline purification procedure was similar to that described by Head et al. (1977). The ^{3}H-noradrenaline (0.5 ml), as received from Amersham or New England Nuclear,

was added to 700 mg alumina, 100 mg ascorbic acid, 100 mg EDTA, 0.1 ml 0.25 M sodium acetate and 2 ml 0.001 M HCl. This mixture was bubbled with nitrogen and the pH adjusted to 8.4 using 0.1 M sodium carbonate and maintained at this pH for four minutes. The alumina was allowed to settle and the effluent was removed. The alumina was then washed with two aliquots of 10 ml of distilled water. The ^{3}H-noradrenaline was first eluted with 5 ml 0.3 M acetic acid, then with a further 2 ml 0.3 M acetic acid. The 7 ml of eluate was then stored at 4°C.

A 0.1 ml sample from the foregoing was added to 2 ml 0.5 M HCl and assayed for noradrenaline content using the fluorometric assay technique described in the general methods. A 0.1 ml sample of the eluate was also counted for radioactivity and the counts corrected for efficiency using an automatic external standard. This enabled the specific activity expressed in dpm/ng of the purified ^{3}H-noradrenaline to be determined. The purity varied from 80% to 95%. Unlabelled noradrenaline was added to the eluate and the specific activity ranged from 25,000 to 40,000 dpm/ng.

Prior to an experiment the required volume of the ^{3}H-noradrenaline stock solution was frozen using a mixture of solidified CO_2 and ethanol. The sample was then freeze-dried using a Dynavac FDA/24 hour freeze dryer and a volume of ascorbic-Krebs added to give the required concentration of ^{3}H-noradrenaline for the experiment.

3. Drugs

The following drugs were used:

etilefrine hydrochloride (Effortil)	Boehringer Ingelheim
cocaine hydrochloride	MacFarlan-Smith
tyramine hydrochloride	Koch-Light
reserpine (Serpasil)	Ciba
guanethidine sulphate (Ismelin)	Ciba
iproniazid phosphate (Marsilid)	Roche
nialamide	Pfizer
(-)-noradrenaline bitartrate	Koch-Light
(-)-adrenaline bitartrate	Koch-Light
phenylephrine hydrochloride (Neo-synephrine)	Winthrop
metaraminol bitartrate (Aramine)	Merck, Sharp & Dohme
phentolamine mesylate (Regitine)	Ciba
6-hydroxydopamine hydrobromide	Sigma
^{14}C-sorbitol (10 mCi/mmol)	Amersham
$(\pm)^{3}$H-noradrenaline hydro-chloride (10.8-12.0 Ci/mmol)	Amersham
$(-)^{3}$H-noradrenaline hydro-chloride (10.8-12.0 Ci/mmol)	Amersham
(1-5 Ci/mmol)	New England Nuclear
ephedrine hydrochloride	Knoll
U0521 (3'4'-dihydroxy-2-methylpropiophenone)	Upjohn
deoxycorticosterone acetate	Steraloids
REN-293 (2-amino-3-(3,5-di-hydroxyphenyl)-1-propanol hydrochloride	Boehringer Ingelheim

Preparation of drugs

Most drugs were prepared in 0.9% sodium chloride containing ascorbic acid (0.57 mM), i.e., ascorbic saline.

Ascorbic/saline. This was a 9 gram/litre solution of NaCl in distilled water to which had been added 1 ml of a 1 gram/ 10 ml solution of ascorbic acid. The ascorbic saline solution was adjusted to give a final pH of 5.4–5.6.

Deoxycorticosterone acetate was prepared in a stock solution of 67 mM in ethanol.

9-alpha-fluorohydrocortisone was prepared in a stock solution of 22.8 mM in ethanol.

Nialamide solution was prepared by dissolving the required amount of nialamide in 20 ml of water with the aid of gentle heat. This solution was then made up to the appropriate volume with Krebs solution immediately prior to use.

Concentrations of adrenaline, noradrenaline, phentolamine, nialamide and reserpine refer to the bases. Other drug concentrations refer to the salts.

* * *

Appendix 4.
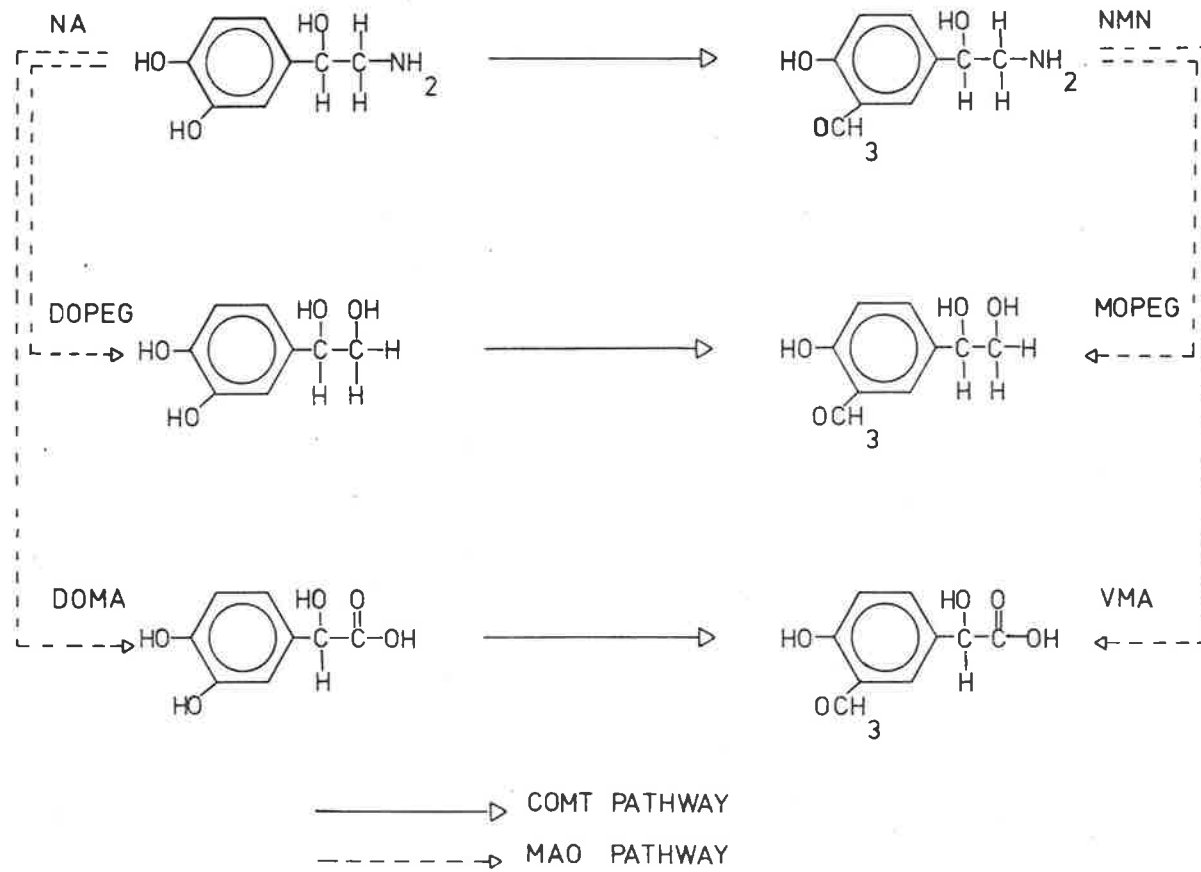
Products formed from noradrenaline (NA) by the combined action of
COMT (catechol-O-methyl transferase) and MAO (monoamine oxidase).

BIBLIOGRAPHY

ABEL, G. (In Hartung, W. H., 1931). Epinephrine and related compounds: influence of structure on physiologic activity. Chem. Rev., 9, 389-465.

AHLQUIST, R. P. (1948). A study of adrenotropic receptors. Am. J. Physiol., 153, 586-600.

AVAKIAN, O. V. and GILLESPIE, J. S. (1968). Uptake of nor-adrenaline by adrenergic nerves, smooth muscle and connective tissue in isolated perfused arteries and its correlation with the vasoconstrictor response. Br. J. Pharmacol., 32, 168-184.

AXELROD, J., WEIL-MALHERBE, H. and TOMCHICK, R. (1959). The physiological disposition of ^{3}H-epinephrine and its metabolite metanephrine. J. Pharmacol. exp. Ther., 127, 251-256.

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

BARNETT, A., STAUB, M. and SYMCHOWICZ, S. (1969). The effect of cocaine and imipramine on tyramine induced release of nor-adrenaline-^{3}H from the rat vas deferens in vitro. Br. J. Pharmacol., 36, 79-84.

BARQ, Z. M. (1934). La pharmacologie du système nerveux auto-nome, et particulierement due sympathique d'après la theorie neurohumorale. Ann. Physiol. Physiochim. Biol., 10, 467.

BERTLER, A., CARLSSON, A. and ROSENGREN, E. (1956). Release by reserpine of catecholamines from rabbit hearts. Naturwissen-schaften, 43, 521.

BEVAN, J. A. and SU, C. (1973). Sympathetic mechanisms in blood vessels: Nerve and muscle relationships. Ann. Rev. Pharmacol., 13, 269-285.

BISSON, G. M. and MUSCHOLL, E. (1962). The relation between the guanethidine concentration in the rat heart and its noradrena-line content. Naunyn Schmiedeberg's Arch. exp. Path., 244, 185-195.

BLASCHKO, H., RICHTER, D. and SCHLOSSMANN, H. (1937). Oxid-ation of adrenaline and other amines. Biochem. J., 31, 2187-2196.

BONISCH, H., UHLIG, W. and TRENDELENBURG, U. (1974). Analy-sis of the compartments involved in the extraneuronal storage and metabolism of isoprenaline in the perfused heart. Naunyn Schmiedeberg's Arch. Pharmacol., 283, 223-244.

BRANDAO, F., MONTEIRO, J. G. and OSSWALD, W. (1978). Differ-ences in the metabolic fate of noradrenaline released by electrical stimulation or by tyramine. Naunyn Schmiedeberg's Arch. Phar-macol., 305, 37-40.

BURGEN, A. S. V. and IVERSEN, L. L. (1965). The inhibition of

noradrenaline uptake by sympathomimetic amines in the rat isolated heart. Br. J. Pharmacol. Chemother., 25, 34-49.

BURN, J. H. (1932). The action of tyramine and ephedrine. J. Pharmacol. exp. Ther., 46, 75-95.

BURN, J. H. and RAND, M. J. (1958). The action of sympathomimetic amines in animals treated with reserpine. J. Physiol., Lond., 144, 314-336.

BURN, J. H. and RAND, M. J. (1960). The effect of precursors of noradrenaline on the response to tyramine and sympathetic stimulation. Br. J. Pharmacol. Chemother., 15, 47-55.

BURN, J. H. and TAINTER, M. L. (1931). An analysis of the effect of cocaine on the actions of adrenaline and tyramine. J. Physiol., Lond., 71, 169-193.

CANNON, W. B. and URIDIL, J. E. (1921). Studies of the conditions of activity in endocrine glands. VIII. Some effects on the denervated heart of stimulating the nerves of the liver. Am. J. Physiol., 58, 353-354.

CARLSSON, A., HILLARP, N. A. and WALDECK, B. (1963). Analysis of the Mg^{++}-ATP dependent storage mechanism in the amine granules of the adrenal medulla. Acta physiol. scand., 59, Suppl. 215, 38pp.

CARLSSON, A., ROSENGREN, E., BERTLER, A. and NILSSON, J. (1957). Effect of reserpine on the metabolism of catecholamines. In "Psychotropic Drugs" (Eds. Garattini, S. and Ghetti, V.).

COLEMAN, A. J., LEARY, W. P. and ASMAL, A. C. (1975). The cardiovascular effects of etilefrine. Eur. J. Clin. Pharmacol., 8, 41-45.

COQUIL, J. F., GORIDIS, C., MACK, G. and NEFF, N. H. (1973). Monoamine oxidase in rat arteries: evidence for different forms and selective localisation. Br. J. Pharmacol., 48, 590-599.

CORBETT, J. L. (1976). Postural hypotension: causes and management. Current Therapeutics, 17, 53-64.

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

DE LA LANDE, I. S. (1975). Adrenergic mechanisms in the rabbit ear artery. A review. Blood Vessels, 12, 137-160.

DE LA LANDE, I. S., CANNELL, V. A. and WATERSON, J. G. (1966). The interaction of serotonin and noradrenaline on the perfused artery. Br. J. Pharmacol., 28, 255-272.

DE LA LANDE, I. S., FREWIN, D. and WATERSON, J. G. (1967a).

The influence of sympathetic innervation on vascular sensitivity to noradrenaline. Br. J. Pharmacol., 31, 82-93.

DE LA LANDE, I. S., FREWIN, D., WATERSON, J. G. and CANNELL, V. (1967b). Factors influencing supersensitivity to adrenaline in the isolated perfused artery: Comparative effects of cocaine, denervation and serotonin. Circulat. Res., 20-21, Suppl. 111, 177-181.

DE LA LANDE, I. S., GLOVER, W. E. and HEAD, R. (1967d). The use of the rabbit ear artery in the bioassay of catecholamines in urine. J. Pharm. Pharmacol., 19, 674-681.

DE LA LANDE, I. S., HILL, B. D., JELLETT, L. B. and McNEIL, J. M. (1970). The role of monoamine oxidase in the response of the isolated central artery of the rabbit ear to tyramine. Br. J. Pharmacol., 40, 249-256.

DE LA LANDE, I. S. and JELLETT, L. B. (1972). Relationship between the roles of monoamine oxidase and sympathetic nerves in the vasoconstrictor response of the rabbit ear artery to nor-epinephrine. J. Pharmacol. exp. Ther., 180, 47-55.

DE LA LANDE, I. S., JELLETT, L. B., LAZNER, M. A., PARKER, D. A. S. and WATERSON, J. G. (1974). Histochemical analysis of the diffusion of noradrenaline across the artery wall. Aust. J. exp. Biol. med. Sci., 52, 193-200.

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

DE LA LANDE, I. S. and RAND, M. J. (1965). A simple isolated nerve-blood vessel preparation. Aust. J. exp. Biol. med. Sci., 43, 639-656.

DE LA LANDE, I. S. and WATERSON, J. G. (1968). The action of tyramine on the rabbit ear artery. Br. J. Pharmacol., 34, 8-18.

DENGLER, H. J., MICHAELSON, I. A., SPIEGEL, H. E. and TITUS, E. (1962). The uptake of labelled norepinephrine in brain and other tissues of the cat. Int. J. Neuropharmacol., 1, 23-38.

DRASKOCZY, P. R. and TRENDELENBURG, U. (1970). Intraneuronal and extraneuronal accumulation of sympathomimetic amines in the isolated nictitating membrane of the cat. J. Pharmacol. exp. Ther., 174, 290-306.

ECKERT, E., HENSELING, M., GESCHER, A. and TRENDELENBURG, U. (1976a). Stereoselectivity of the distribution of labelled nor-adrenaline in rabbit aortic strips after inhibition of the nor-adrenaline-metabolizing enzymes. Naunym Schmiedeberg's Arch. Pharmacol., 292, 219-229.

ECKERT, E., HENSELING, M. and TRENDELENBURG, U. (1976b). The effect of inhibitors of extraneuronal uptake on the distribution of $^3H(\pm)$ noradrenaline in nerve free rabbit aortic strips. Naunyn Schmiedeberg's Arch. Pharmacol., 293, 115-127.

EISENFELD, A. J., AXELROD, J. and KRAKOFF, L. (1967). Inhibition of the extraneuronal accumulation and metabolism of norepinephrine by adrenergic blocking agents. J. Pharmacol. exp. Ther., 156, 107-113.

ELLIOTT, T. R. (1905). The action of adrenaline. J. Physiol., Lond., 32, 401-467.

EULER, U. S. von (In Euler, U. S. von, 1972). Synthesis, uptake and storage of catecholamines in adrenergic nerves. The effects of drugs. In "Catecholamines" (Eds. Blaschko, H. and Muscholl, E.). Handb. exp. Pharmakol., Vol. 33, Springer-Verlag, Berlin, p.p. 186-230.

EULER, U. S. von and LISHAJKO, F. (1964). Uptake of l- and d- isomers of catecholamines in adrenergic nerve granules. Acta physiol. scand., 60, 217-222.

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

FALCK, B. and TORP, A. (1962). New evidence for the localization of noradrenaline in the adrenergic nerve terminals. Med. exp., 6, 169-172.

FARMER, J. B. (1966). The biphasic response of an isolated artery to tyramine. Br. J. Pharmacol. Chemother., 28, 340-347.

FLECKENSTEIN, A. and BURN, J. H. (1953). The effect of denervation on the action of sympathomimetic amines on the nictitating membrane. Br. J. Pharmacol., 8, 69-78.

FLEMING, W. W. and TRENDELENBURG, U. (1961). Development of supersensitivity to norepinephrine after pretreatment with reserpine. J. Pharmacol. exp. Ther., 133, 41-51.

FREWIN, D. B., HUME, W. R., WATERSON, J. G. and WHELAN, R. F. (1971a). The histochemical localization of sympathetic nerve endings in human gingival blood vessels. Aust. J. exp. Biol. med. Sci., 49, 573-580.

FREWIN, D. B., WATERSON, J. G. and WHELAN, R. F. (1971b). Catecholamine fluorescence in primate digital arteries. Aust. J. exp. Biol. med. Sci., 49, 421-424.

FROST, B. R., GERKE, D. C. and FREWIN, D. B. (1976). The effects of 2-phenylalanine-8-lysine vasopressin (Octapressin) on blood vessels in the rat tail. Aust. J. exp. Biol. med. Sci., 54, 403-411.

FURCHGOTT, R. F. (1955). The pharmacology of vascular smooth muscle. Pharmacol. Rev., 7, 183.

FURCHGOTT, R. F. and GARCIA, P. S. (1968). Effects of inhibition of monoamine oxidase on the actions and interactions of norepinephrine, tyramine and other drugs on guinea-pig left atrium. J. Pharmacol. exp. Ther., 163, 98–122.

GIACHETTI, A. and SHORE, P. A. (1966). Optical specificity of monoamine oxidase. Life Sci., 4, 1373–1378.

GILLESPIE, J. S. (1973). Uptake of noradrenaline by smooth muscle. Br. Med. Bull., 29, 136–141.

GILLESPIE, J. S. and MUIR, T. C. (1970). Species and tissue variation in extraneuronal and neuronal accumulation of noradrenaline. J. Physiol., 206, 591–604.

GILLESPIE, J. S. and TOWART, R. (1973). Uptake kinetics and ion requirements for extraneuronal uptake of noradrenaline by arterial smooth muscle and collagen. Br. J. Pharmacol., 47, 556–557.

GRAEFE, K. H. and ECKERT, E. (1972). On the stereoselectivity of the neuronal uptake in the cat's nictitating membrane. Naunyn Schmiedeberg's Arch. Pharmacol., 275, 45–68.

GRAEFE, K. H., STEFANO, F. J. E. and LANGER, S. Z. (1973). Preferential metabolism of (-)-^{3}H-norepinephrine through the deaminated glycol in the rat vas deferens. Biochem. Pharmacol., 22, 1147–1160.

GRAEFE, K. H. and TRENDELENBURG, U. (1974). The effect of hydrocortisone on the sensitivity of the isolated nictitating membrane to catecholamines: Relationships to extraneuronal uptake and metabolism. Naunyn Schmiedeberg's Arch. Pharmacol., 286, 1–48.

HAMBERGER, B., MALMFORS, T., NORBERG, K. A. and SACHS, C. (1964). Uptake and accumulation of catecholamines in peripheral adrenergic neurones of reserpinized animals, studied with a histochemical method. Biochem. Pharmacol., 13, 841–844.

HEAD, R. J., CRABB, G. A., DE LA LANDE, I. S. and FREWIN, D. B. (1977a). Semi-automated catecholamine assay. Aust. J. exp. Biol. med. Sci., 55, 213–223.

HEAD, R. J., JOHNSON, S. M., BERRY, D. M. and DE LA LANDE, I. S. (1975). Denervation and O-methylation of noradrenaline in the rabbit ear artery. Clin. exp. Pharmacol. Physiol., Suppl. 2, 39–42.

HEAD, R. J., STITZEL, R. H. and DE LA LANDE, I. S. (1974). Changes in adenosine triphosphate, monoamine oxidase and catechol-O-methyl transferase accompanying chronic denervation of the

rabbit ear artery. Proc. Aust. Physiol. Pharmacol. Soc., 5, 80–81.

HEAD, R. J., STITZEL, R. H., DE LA LANDE, I. S. and JOHNSON, S. M. (1977b). Effect of chronic denervation on the activities of monoamine oxidase and catechol-O-methyl transferase and on the contents of noradrenaline and adenosine tri-phosphate in the rabbit ear artery. Blood Vessels, 14, 229–239.

HENSELING, M., ECKERT, E. and TRENDELENBURG, U. (1976a). The distribution of ^{3}H-($\pm$) noradrenaline in rabbit aortic strips after inhibition of the noradrenaline-metabolising enzymes. Naunyn Schmiedeberg's Arch. Pharmacol., 292, 205–217.

HENSELING, M., ECKERT, E. and TRENDELENBURG, U. (1976b). The effect of cocaine on the distribution of labelled noradrenaline in rabbit aortic strips and on efflux of radioactivity from the strips. Naunyn Schmiedeberg's Arch. Pharmacol., 293, 231–241.

HENSELING, M. and TRENDELENBURG, U. (1978). Stereoselectivity of the accumulation and metabolism of noradrenaline in rabbit aortic strips. Naunyn Schmiedeberg's Arch. Pharmacol., 302, 195–206.

HERTTING, G. and AXELROD, J. (1961). Fate of tritiated noradrenaline at the sympathetic nerve endings. Nature, London, 192, 172–173.

HERTTING, G., AXELROD, J., KOPIN, I. J. and WHITBY, L. G. (1961). Lack of uptake of catecholamines after chronic denervation of sympathetic nerves. Nature, London, 189, 66.

HILLARP, N. A. and MALMFORS, T. (1964). Reserpine and cocaine blocking of the uptake and storage mechanisms in adrenergic nerves. Life Sci., 3, 703–708.

HODGE, R. L. and ROBINSON, S. M. (1972). The action of clonidine on isolated arterial preparations. Aust. J. exp. Biol. med. Sci., 50, 517–526.

HORITA, A. and LOWE, M. C. (1972). On the extraneuronal nature of cardiac monoamine oxidase in the rat. Advanc. biochem. Psychopharmacol., 5, 227–242.

HUGHES, I. E. and SMITH, J. A. (1978). The stability of noradrenaline in physiological saline solutions. J. Pharm. Pharmacol., 30, 124–125.

INNES, I. R. and KOHLI, J. D. (1969). Excitatory action of sympathomimetic amines on 5-hydroxytryptamine receptors of gut. Br. J. Pharmacol., 35, 383–393.

IVERSEN, L. L. (1963). The uptake of noradrenaline by the isolated perfused rat heart. Br. J. Pharmacol. Chemother., 21, 523–527.

IVERSEN, L. L. (1965a). The uptake of catecholamines at high perfusion concentrations in the rat isolated heart: A novel catecholamine uptake process. Br. J. Pharmacol., 25, 18-33.

IVERSEN, L. L. (1965b). The uptake of adrenaline by the rat isolated heart. Br. J. Pharmacol., 24, 387-394.

IVERSEN, L. L., JARROTT, B. and SIMMONDS, M. A. (1971). Differences in the uptake and metabolism of (+) and (-)-noradrenaline. Br. J. Pharmacol., 43, 845-855.

IVERSEN, L. L. and SALT, P. J. (1970). Inhibition of catecholamine uptake 2 by steroids in the isolated rat heart. Br. J. Pharmacol., 40, 528-530.

IVERSEN, L. L., SALT, P. J. and WILSON, H. A. (1972). Inhibition of catecholamine uptake in the isolated rat heart by haloalkylamine related to phenoxybenzamine. Br. J. Pharmacol., 46, 647-657.

JARROTT, B. (1971a). Occurrence and properties of monoamine oxidase in adrenergic neurons. J. Neurochem., 18, 7-16.

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

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

JARROTT, B. and LANGER, S. Z. (1971). Changes in monoamine oxidase and catechol-O-methyl transferase activities after denervation of the nictitating membrane of the cat. J. Physiol., 212, 549-559.

JOHNSON, S. M. and DE LA LANDE, I. S. (1978). The action of deoxycorticosterone acetate on the central artery of the rabbit ear. Blood Vessels, 15, 231-246.

KALSNER, S. (1969). Steroid potentiation of responses to sympathomimetic amines in aortic strips. Br. J. Pharmacol., 36, 582-593.

KAUMANN, A. J. (1972). Potentiation of the effects of isoprenaline and noradrenaline by hydrocortisone in cat heart muscle. Naunyn Schmiedeberg's Arch. Pharmacol., 273, 134-153.

KOPIN, I. J., FISCHER, J. E., MUSACCHIO, J. M., HORST, W. D. and WEISE, V. K. (1965). "False neurochemical transmitters" and the mechanism of sympathetic blockade by monoamine oxidase inhibitors. J. Pharmacol. exp. Ther., 147, 186-193.

KOPIN, I. J. and GORDON, E. K. (1962). Metabolism of norepinephrine-^{3}H released by tyramine and reserpine. J. Pharmacol. exp. Ther., 138, 351-359.

LANGLEY, J. N. (1901). Observations on the physiological action of extracts of the supra-renal bodies. J. Physiol., Lond., 27, 237-256.

LEITZ, F.. H. and STEFANO, F. J. E. (1971). The effect of tyramine, amphetamine and metaraminol on the metabolic disposition of ^{3}H-norepinephrine released from the adrenergic neurone. J. Pharmacol. Exp. Ther., 178, 464-473.

LEWANDOWSKY, M. (1898). Ueber einer Wirkung des Nebennieren-extractes auf das Auge. Zentbl. Physiol., 12, 599-600.

LIGHTMAN, S. L. and IVERSEN, L. L. (1969). The role of uptake$_2$ in the extraneuronal metabolism of catecholamines in the isolated rat heart. Br. J. Pharmacol., 37, 638-649.

LINDMAR, R. and MUSCHOLL, E. (1964). Die wirkung von Pharmaka auf die Elimination von Noradrenalin aus der Perfusions-flüssigkeit und die Noradrenalin-aufnahme in das isolierte Herz. Arch. exp. Path. Pharmakol., 247, 469-492.

LINDMAR, R. and MUSCHOLL, E. (1966). Der Einfluss von Pharmaka auf die Aufnahme von Guanethidin in das Herz. Naunyn Schmiedeberg's Arch. exp. Path. Pharmakol., 253, 67.

LISSAK, K. (1939). Liberation of acetylcholine and adrenaline by stimulating isolated nerves. Am. J. Physiol., 127, 263.

LOEWI, O. (1921). Über humorale Übertragbarkeit der Herznerven-wirkung. Pflügers Arch. ges. Physiol., 189, 239-242.

MACMILLAN, W. H. (1959). A hypothesis concerning the effect of cocaine on the action of sympathomimetic amines. Br. J. Pharmacol. Chemother., 14, 385-391.

MAENGWYN-DAVIES, G. D. and KOPPANYI, T. (1966). Cocaine tachyphylaxis and effects on indirectly acting sympathomimetic drugs in the rabbit aortic strip and in splenic tissue. J. Pharmacol. exp. Ther., 154, 481-492.

MARLEY, E. (1962). Action of some sympathomimetic amines on the cat's iris, in situ or isolated. J. Physiol., Lond., 162, 193-211.

MELLANDER, S. (1966). Comparative effects of acetylcholine, butyl-nor-Synephrine (Vasculat), noradrenaline, and ethyl adrianol (Effontil) on resistance, capacitance, and precapillary sphincter vessels and capillary filtration in cat skeletal muscle. Angiologica, 3, 77-99.

MILLER, E., WIENER, L. and BLOOMFIELD, D. (1973). Etilefrine in the treatment of levodopa-induced orthostatic hypotension. Arch. Neurol., 29, 99-103.

MIREYLEES, S. E. and FOSTER, R. W. (1973). 3-methoxy-isopren-

aline: A potent selective uptake$_2$ inhibitor. J. Pharm. Pharmacol., 25, 833–835.

MURDOCK, H. R. (1969). Anaesthesia in the rabbit. Fed. Proc., 28, 1510–1516.

MUSACCHIO, J. M., KOPIN, I. J. and WEISE, V. K. (1965). Subcellular distribution of some sympathomimetic amines and their beta–hydroxylated derivatives in the rat heart. J. Pharmacol. exp. Ther., 148, 22–28.

MUSCHOLL, E. (1960). Die Hemmung der Noradrenalin–Aufnahme des Herzens durch Reserpin und die Wirkung von Tyramin. Arch. exp. Path. Pharmakol., 240, 234–241.

MUSCHOLL, E. and WEBER, E. (1965). Die Hemmung der Aufnahme von a–methyl–noradrenalin in das Herz durch sympathomimetische amine. Naunyn Schmiedeberg's Arch. exp. Path. Pharmakol., 252, 134–143.

NANDA, R. N., BOYLE, F. C., GILLESPIE, J. S., JOHNSON, R. H. and KEOGH, H. J. (1977). Idiopathic orthostatic hypotension from failure of noradrenaline release in a patient with vasomotor innervation. J. Neurol. Neurosurg. Psychiat., 40, 11–19.

NASH, C. W., WOLFF, S. A. and FERGUSON, B. A. (1968). Release of tritiated noradrenaline from perfused rat hearts by sympathomimetic amines. Canad. J. Physiol. Pharmacol., 46, 35–42.

NICHOLAS, T. E. (1969). A perfused tail artery preparation from the rat. J. Pharm. Pharmacol., 21, 826–832.

OFFERMEIER, J. and DREYER, A. C. (1971). A comparison of the effects of noradrenaline, adrenaline and some phenylephrine derivatives on alpha, beta $_1$ and beta $_2$ adrenergic receptors. S.A. Med. J., 45, 265–267.

ORDEN, L. S. van III, BENOCH, K. G., LANGER, S. Z. and TRENDELENBURG, U. (1967). Histochemical and fine structural aspects of the onset of denervation supersensitivity in the nictitating membrane of the spinal cat. J. Pharmacol. exp. Ther., 157, 274–283.

PARKS, V. J., SANDISON, A. G., SKINNER, S. L. and WHELAN, R. F. (1961). Sympathomimetic drugs in orthostatic hypotension. Lancet, 1, 1133–1136.

PATON, D. M. (1973a). Mechanism of efflux of noradrenaline from adrenergic nerves in rabbit atria. Br. J. Pharmacol., 49, 614–627.

PATON, D. M. (1973b). Effect of sympathomimetic amines on the efflux of noradrenaline from adrenergic nerves in rabbit atria. Br. J. Pharmacol., 49, 143P.

POWIS, G. (1973). Binding of catecholamines to connective tissue

and the effect upon the responses of blood vessels to noradrenaline and to nerve stimulation. J. Physiol., 234, 145-162.

ROSS, S. B. and RENYI, A. L. (1966). Uptake of tritiated tyramine and (+)-amphetamine by mouse heart slices. J. Pharm. Pharmacol., 18, 756-757.

ROTH, R. H. and STONE, E. A. (1968). The action of reserpine on noradrenaline biosynthesis in sympathetic nerve tissue. Biochem. Pharmacol., 17, 1581-1590.

RUNYON, R. P. and HABER, A. (1968). Fundamentals of Behavioural Statistics. Addison-Wesley Publishing Co., Reading, Massachusetts; Menlo Park, California; London;, Don Mills, Ontario.

SCHANKER, L. S. and MORRISON, A. S. (1965). Physiological disposition of guanethidine in the rat and its uptake by heart slices. Int. J. Neuropharmacol., 4, 27-39.

SCHMID, P. G., ECKSTEIN, J. W. and ABBOUD, F. M. (1966). Effect of 9-α-fluorohydrocortisone on forearm vascular responses to norepinephrine. Circulation, 34, 620-626.

SCHMIDT, J. L. and FLEMING, W. W. (1963). The structure of sympathomimetics as related to reserpine induced sensitivity changes in the rabbit ileum. J. Pharmacol. exp. Ther., 139, 230-237.

SCHÜMANN, H. J. and PHILIPU, A. (1961). Untersuchungen zum Mechinismus der Freiselzung von Brenzcatechinaminen durch Tyramin. Naunyn Schmiedeberg's Arch. exp. Path. Pharmakol., 241, 273-280.

SHELLEY, J. H. (1978). REN-293 CI-investigational brochure. Boehringer Ingelheim Pty Ltd.

SMITH, C. B. (1966). The role of monoamine oxidase in the intraneuronal metabolism of norepinephrine released by indirectly-acting sympathomimetic amines or by adrenergic nerve stimulation. J. Pharmacol. exp. Ther., 151, 207-220.

SPECTOR, S., HIRSCH, C. W. and BRODIE, B. B. (1963). Association of behavioural effects of pargyline, a non hydrazide MAO inhibitor with increase in brain norepinephrine. Internat. J. Neuropharmacol., 2, 81.

STJÄRNE, L. and von EULER, U. S. (1966). Stereospecificity of amine uptake mechanisms in nerve granules. J. Pharmacol. exp. Ther., 150, 335-340.

TAINTER, M. L. (1929). Comparative effects of ephedrine and epinephrine on blood pressure, pulse and respiration with reference to their alteration by cocaine. J. Pharmacol. exp. Ther., 36, 569-594.

TAINTER, M. L. and CHANG, D. K. (1927). The antagonism of the pressor action of tyramine by cocaine. J. Pharmacol. exp. Ther., 30, 193-207.

THOENEN, H. and TRANZER, J. P. (1973). The pharmacology of 6-hydroxydopamine. A. Rev. Pharmacol., 13, 169-180.

TRENDELENBURG, U. (1959). The supersensitivity caused by cocaine. J. Pharmacol. exp. Ther., 125, 55-65.

TRENDELENBURG, U. (1963). Supersensitivity and subsensitivity to sympathomimetic amines. Pharmacol. Rev., 15, 225-276.

TRENDELENBURG, U. (1972). Classification of sympathomimetic amines. In: "Catecholamines" (Eds. Blaschko, H. and Muscholl, E.). Handb. exp. Pharmakol., Vol. 33, Springer-Verlag, Berlin, p.p. 336-362.

TRENDELENBURG, U., HÔHN, D., GRAEFE, K. H. and PLUCHINO, S. (1971). The influence of block of catechol-O-methyl transferase on the sensitivity of isolated organs to catecholamines. Naunyn Schmiedeberg's Arch. Pharmakol., 271, 59-92.

TRENDELENBURG, U., MAXWELL, R. A. and PLUCHINO, S. (1970). The importance of the intraneuronal uptake in the cat's nictitating membrane as assessed with methoxamine, a sympathomimetic amine which is not taken up by adrenergic nerves. J. Pharmacol. exp. Ther., 172, 91-99.

TRENDELENBURG, U., MUSKUS, A., FLEMING, W. W. and GOMEZ ALONSO DE LA SIERRA, B. (1962). Modification by reserpine of the action of sympathomimetic amines in spinal cats; a classification of sympathomimetic amines. J. Pharmacol. exp. Ther., 138, 170-180.

TSAI, T. H., LANGER, S. Z. and TRENDELENBURG, U. (1967). Effects of dopamine and α methyl-dopamine on smooth muscle and on the cardiac pacemaker. J. Pharmacol. exp. Ther., 156, 310-324.

VANHOUTTE, P. M. and WEBB, C. (1979). Inactivation of released norepinephrine in rat tail artery by neuronal uptake. Br. J. Pharmacol., 66, 116P.

VENNING, M. and DE LA LANDE, I. S. (1979). Relative influences of neuronal and extraneuronal uptake on the response of the rat tail artery to adrenaline. Proc. Australas. Soc. Clin. Exp. Pharmacol., Dunedin, N.Z., No. 15.

WADE, D. N. and BEILIN, L. J. (1970). Vascular resistance in the perfused isolated rat tail. Br. J. Pharmacol., 38, 20-36.

WATERSON, J. G. and DE LA LANDE, I. S. (1967). Function of sympathetic nerves in blood vessels. Ann. Aust. Coll. dent. Surg., 1, 60-69.

WATERSON, J. G. and DE LA LANDE, I. S. (1974). The influence of replacing sodium by potassium ions on uptake of noradrenaline in the rabbit ear artery. Aust. J. exp. Biol. med. Sci., 52, 201–205.

WATERSON, J. G. and SMALE, D. E. (1967). Location of noradrenergic structures in the central artery of the rabbit ear. Aust. J. exp. Biol. med. Sci., 45, 301–308.

WEINER, N. and BJUR, R. (1972). The role of intraneuronal monoamine oxidase in the regulation of norepinephrine synthesis. In: "Advances in Biochemical Pharmacology," Raven Press (New York), Vol. 5, p. 409.

WHITBY, L. G., AXELROD, J. and WEIL-MALHERBE, H. (1961). The fate of ^{3}H-norepinephrine in animals. J. Pharmacol. exp. Ther., 132, 193–201.

WYSE, D. G. (1976). Inactivation of neural and exogenous norepinephrine in rat tail artery studied by the oil immersion technique. J. Pharmacol. exp. Ther., 198, 102–111.

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