PRE-SYNAPTIC ALPHA ADRENOCEPTORS AND NORADRENALINE IN NEUROTRANSMISSION IN THE MOUSE ISOLATED VAS DEFERENS

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ABSTRACT

The mouse vas deferens is a sympathetically innervated tissue which contains large concentrations of noradrenaline. Field stimulation of the isolated tissue preparation causes it to release noradrenaline and to contract. The contraction, the form of which depends upon the frequency of stimulation, can be blocked by tetrodotoxin and by guanethidine. Unlike other sympathetically innervated tissues the response of the mouse vas deferens to stimulation is inhibited by exogenous noradrenaline, tyramine, and by agents which block the uptake of noradrenaline into the sympathic neurone or effector tissue. Furthermore it is potentiated by alpha-adrenoceptor blocking agents.

The tissue is shown to contain presynaptic alpha-adrenoceptors. Their stimulation produces a frequency and ion dependent inhibition of noradrenaline release from the sympathetic neurone, and inhibition of the contraction. The inhibition produced by noradrenaline, tyramine and uptake blockade has characteristics similar to that produced by the selective presynaptic alpha-adrenoceptor agonist clonidine. The potentiation produced by high concentrations of the less selective alpha-adrenoceptor blocking agents is similar to that produced by low concentrations of the selective presynaptic alpha-adrenoceptor blocking agent yohimbine. It is suggested that the inhibition produced by noradrenaline, tyramine and uptake blockade is mediated via presynaptic alpha-adrenoceptors. Possible reasons for the preferential action of exogenous and endogenous noradrenaline upon these receptors are discussed. It is proposed that the presynaptic

receptors are stimulated by endogenous noradrenaline, forming a negative feedback loop which is interrupted by alpha-adrenoceptor blocking agents producing a potentiation of the tissue response. The inability of these drugs to block the tissue response, and implications of this finding for the interpretation of the role noradrenaline in this tissue are discussed.

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GENERAL INTRODUCTION

1. Adrenergic Neuroeffector Transmission

The first suggestion that nerves exerted their excitatory action on muscle by liberating a chemical substance was probably made by Du Bois-Reymond in 1877. This was followed by the observations of Lewandowsky (1899) and Langley (1901) noting the similarity between the response to injected extracts of adrenal gland and stimulation of sympathetic nerves. It was Elliott (1904) however who suggested that sympathetic nerves might release adrenaline on stimulation. After this early predominance of the sympathetic system in the investigation of chemical transmission the most significant advances were now made with the parasympathetic system. Dixon (1907) noticed the relationship between vagal stimulation and the effects of muscarine and suggested that the vagus released a muscarine like substance on stimulation. In 1914 Dale thoroughly investigated the pharmacological actions of acetyl choline and coined the term parasympathetic. In 1921 Loewi began his classical experiments with frog hearts which provided the first real evidence of the chemical mediation of nerve impulses. Loewi discovered the release of a substance which accelerated the heart when the accelerans nerve was stimulated. He christened this 'Acceleransstoff' and suggested that it could be adrenaline (which was correct in this case as adrenaline as opposed to noradrenaline is released from adrenergic nerves in most anuran amphibians). Cannon and co-workers firmly established the release of a chemical substance from mammalian sympathetic nerves which they called 'sympathin'. It was Von Euler (1946) who proposed that 'sympathin' was in fact noradrenaline and not adrenaline as originally proposed. Peart (1949) conclusively

demonstrated the release of noradrenaline from sympathetic nerves in the cat spleen.

While the true nature of 'sympathin' was being investigated the site of its action on the effector organ was also being explored. The concept of a 'receptor' as the site of a drug's action was discussed as early as 1878 by Langley, and the first reference to a 'receptor substance' for adrenaline was made by Lewandowsky (1899). Elliott (1905) noted that in certain tissues the effect of adrenaline was inhibitory while in others it was excitatory, and suggested that it did not react with the muscle fibre directly, but reacted with a substance from the muscle. He called this an irritant substance and suggested that the combination of adrenaline with it determined whether the action of adrenaline was motor or inhibitory. It was not in fact until 1948 that Ahlquist postulated that adrenaline reacted with two types of receptor which he termed alpha - which were responsible for the excitatory effects except in the heart but including the inhibitory effects in the intestine, or beta - which produced inhibitory effects and also the excitatory effects in the heart. Though agents which block the alpha-adrenoceptor had been known for many years (such as Dale's classical work with ergot alkaloids) it was not until 1958 that antagonists selective for beta-adrenoceptors were discovered. The beta receptor was later subclassified into $beta_1$ and $beta_2$ (Lands et al 1967) with antagonists selective for each subclass. The examples given above now form the basis for the modern concept of sympathetic neurotransmission as expounded by Von Euler (1959), that noradrenaline is the only transmitter

released by sympathetic neurones(with the exception of certain post-ganglionic sympathetic cholinergic nerves) which then acts upon either alpha or beta receptors on the effector organ.

In 1932 Burn suggested that peripheral tissues may take up and store catecholamines. However it was not until 1959 that Axelrod, Weil-Malherbe and Tomchick were able, with the use of radio-labelled compounds, to demonstrate this'uptake'. Strömblad and Nickerson (1961) first suggested that uptake might represent an important mechanism for the physiological inactivation of catecholamines. In a series of papers Iversen investigated the uptake phenomenon and found it to be two mechanisms, one in the neurone ($Uptake_1$) and the other in the effector tissue ($Uptake_2$). These uptake mechanisms are selectively blocked by different compounds. Brown (1965) showed the blockade of $Uptake_1$ increased the overflow of noradrenaline and potentiated the response of the innervated organ to nerve stimulation. From the above discussion it is apparent that the response of the innervated tissue to sympathetic nerve stimulation can be altered by certain drugs. Alpha or beta adrenoceptor blocking agents will inhibit the response of the tissue to sympathetic nerve stimulation, while uptake blocking agents will potentiate it. Several other drugs have been instrumental in developing our understanding of the sympathetic system. One of the oldest of these is reserpine, a constituent of Rauwolfia which is a plant that has been used in Eastern medicine for centuries. It is only comparatively recently that its ability to produce a long lasting depletion of catecholamines from adrenergic neurones was discovered.

(Holzbauer and Vogt 1956). Depletion of adrenergic terminals leads to a decreased release of noradrenaline on stimulation and therefore a decreased effector response. A second class of drugs produces an immediate and comparatively short lasting release of noradrenaline, these drugs are known as the indirectly acting amines. Whereas reserpine releases noradrenaline from, and disrupts the noradrenaline storage vesicles, the indirectly acting amines, such as tyramine, release noradrenaline from its storage sites without disrupting the vesicles (Burn and Rand 1958). The release of noradrenaline by tyramine potentiates the effects of neuronal stimulation and can be blocked by depleting the catecholamine content of the tissue (Axelrod et al 1962; Carlsson, Rosengren, Bertler and Nilsson 1957). Drugs which block the release of noradrenaline from adrenergic neurones, such as Guanethidine (Abercrombie and Davies (1963) have been a useful tool for identifying adrenergically innervated tissues.

In conclusion, present knowledge about the sympathetic nervous system allows us to describe certain features of the system, and its response to drugs, which will help in the identification of other sympathetically innervated tissues:-

(1) The nerve supply to the tissue contains noradrenaline. This is released on stimulation and its effects will be mimicked by exogenous noradrenaline.

(2) The innervated tissue contains alpha and/or beta receptors for noradrenaline. These receptors can be selectively blocked by drugs, leading to an inhibition of the tissue's response to neuronal stimulation and

to exogenous noradrenaline.

(3) The response of the tissue to neuronal stimulation can be increased by blocking the neuronal and tissue uptake systems for noradrenaline. Uptake blockade potentiates the effect of exogenous noradrenaline.

(4) Adrenergic neurone blocking drugs decrease the release of noradrenaline upon stimulation and thus decrease the response of the innervated tissue to neuronal stimulation. The effect of exogenous noradrenaline is not blocked and may be potentiated as adrenergic blocking drugs have been reported to block the uptake of noradrenaline (Hertting, Axelrod and Patrick 1962).

(5) The neurone can be depleted of its noradrenaline content by reserpine, this depletion leads to a decrease in the response of the tissue to electrical stimulation. (Muscholl and Vogt 1958).

(6) Noradrenaline is released by the indirectly acting amines such as tyramine which mimic the response to nerve stimulation.

2. The Role of The Adrenergic Innervation in the Vas Deferens

Langley and Anderson (1895) demonstrated that stimulation of the upper lumbar nerves produced contraction of the ipsilateral vas deferens in several species. Subsequently it was found that the ganglia in this anatomically sympathetic pathway lay close to the effector tissue making the post-ganglionic fibres unconventionally short (Söjstrand 1962, Kuriyama 1963, Ferry 1967). The vas deferens exhibits many of the properties expected of an adrenergically innervated tissue which have been outlined above. In most species the vas deferens receives

an adrenergic innervation which forms a dense plexus (Sojstrand 1962), with nerve fibres containing catecholamines present in the musculature (Falck 1962). Exogenous noradrenaline contracts the tissue, and endogenous noradrenaline is released upon stimulation (Hughes 1972). Adrenergic neurone blockers inhibit the response to stimulation of the hypogastric nerve (Bentley and Sabine 1963). Reserpine depletes the noradrenaline content of the tissue (Gillespie and McGrath 1974). Large doses of reserpine (0.5 mg/kg followed by 1 mg/kg) cause a progressive decrease in the twitch response to electrical stimulation which can be reversed by addition of noradrenaline (Hukovic 1961). Tyramine releases noradrenaline, which mimics nerve stimulation in rats but not guinea-pigs (Ambache, Dunk, Verney and Zar 1971).

The vas deferens is however atypical in its respone to adrenoceptor blocking agents. Boyd, Chang and Rand (1960) used the Hukovic preparation of the isolated guinea-pig hypogastric nerve - vas deferens, which contains ganglia. They reported a potentiation of the response to electrical stimulation by the alpha-adrenoceptor blocking drugs, Tolazoline 0.1-100 mg/ml, Phenoxybenzamine 1-30 µg/ml, Yohimbine 0.2-1 µg/ml, Ergotamine 3-30 µg/ml and Piperoxan 1-3 µg/ml. It was not until concentrations of 500, 130, 30, 70 and 100 µg/ml respectively, were reached that an inhibition of the response was seen. Finding that all these drugs inhibited cholinesterase, and that the anti-cholinesterase, eserine, potentiated the response of the tissue to electrical stimulation, these authors concluded that it was the inhibition

of this enzyme that produced the potentiation. Only with higher concentrations of these drugs was the alpha-adrenoceptor blockade apparent, producing the inhibition of the twitch response. The authors concluded that the enzyme blockade either increased the direct action of acetylcholine on the effector cells, or its intermediate action in liberating noradrenaline. Using the same preparation of the rat and guinea-pig vas deferens Ohlin and Strömblad (1963), reported a similar potentiation with Phenoxybenzamine 3.3×10^{-5}M, Dihydroergotamine 3.4×10^{-4}M, Tolazoline 2.8×10^{-7}M and Yohimbine 3.1×10^{-3}M. The contraction produced by acetyl choline was increased in the presence of all these drugs. The contraction produced by exogenous noradrenaline however was reduced by 3.3×10^{-8}M Phenoxybenzamine. These authors found no inhibition of cholinesterase by these drugs at concentrations used in their experiment and therefore concluded that Boyd et al were incorrect in their explanation of the potentiation, though they did not offer an alternative explanation for the results. They did point out that as the stimulus was applied to the preganglionic fibre drugs may produce changes in transmission at the ganglia and have no direct effect on transmission at the nerve ending. This makes the study of motor transmission difficult.

To avoid this problem Birmingham and Wilson (1963) used the field stimulated vas deferens preparation which did not contain ganglia and directly stimulated the postganglionic neurones. They showed that concentrations of alpha adrenoceptor blocking agents required to reduce the electrically induced response of the tissue also blocked the response of

the tissue to noradrenaline, acetyl choline and potassium. These results suggest that these concentrations of alpha adrenoceptor blocking agents directly depressed the muscle, rather than specifically blocking the receptors.

Electrophysiological studies of the mouse vas deferens by Holman (1970) showed that noradrenaline decreased the excitatory junction potentials induced by nerve stimulation. Exogenous noradrenaline produced excitatory junction potentials which were easily blocked by alpha adrenoceptor blocking agents whereas those due nerve stimulation were very resistant to these drugs. Blockade of noradrenaline uptake into the neurone by cocaine (1 μg/ml) or desmethyl imipramine (1 μg/ml) had little or no effect on nerve induced excitatory junction potentials.

The inability of adrenoceptor antagonists to block the electrically induced response of the vas deferens led Ambache and Zar (1971) and Ambache, Dunk, Verney and Zar (1972) to carry out a comprehensive study of the role of noradrenaline in the guinea-pig, rat and rabbit vasa deferentia. They found that the guinea-pig vas deferens varied in its sensitivity to noradrenaline between animals and in general high concentrations were required to produce a contraction (10-50 μg/ml). Preparations which were relatively insensitive to noradrenaline produced normal responses to electrical stimulation. When noradrenaline was added during stimulation it inhibited the response via alpha-adrenoceptors, and did not affect the sensitivity of the muscle to muscarine. Phenoxybenzamine (10^{-6} gms/ml) produced a thousand fold decrease in the sensitivity of the tissue to noradrenaline without decreasing the response to electrical stimulation.

Neither was the latter affected by beta-adrenoceptor, or a combination of both alpha and beta adrenoceptor blocking agents. The twitch response, and the noradrenaline inhibition of i were seen in vasa deferentia from reserpinised animals, though the twitch response was reduced as compared to that in untreated animals. Tyramine inhibited the electrically induced twitch response but potentiated the noradrenaline contractions, and caused contraction of the rat but not guinea-pig vasa deferentia. The tyramine inhibition could be abolished by alpha adrenoceptor blocking agents or by pretreating the animals with reserpine. Both these procedures abolished the contraction of the rat vas deferens caused by tyramine. In both species neuronal uptake blockade by cocaine inhibited the response to electrical stimulation in normal but not in reserpinised animals. These authors concluded that in these species noradrenaline was not the motor transmitter. Von Euler and Hedqvist (1975) supported this view but in addition found that a $beta_2$ receptor was partly responsible for the noradrenaline inhibition. Ambache et al (1971) also excluded the possibility of ATP, GABA, histamine or 5-hydroxytryptamine being motor transmitters. Using the mouse vas deferens Jenkins, Marshall and Nasmyth (1977) showed that the response to electrical stimulation of this tissue was not antagonised by alpha or beta adrenoceptor blocking agents, but was inhibited by noradrenaline. They concluded that the motor transmitter was either a substance, so far not identified, released from sympathetic nerves, or noradrenaline acting upon an adrenoceptor not blocked by conventional antagonists. They

also suggested that noradrenaline's inhibition of the twitch may be due to stimulation of presynaptic alpha adrenoceptors.

The first observation of the effect of presynaptic alpha adrenoceptors is often credited to Brown and Gillespie (1957), though at the time the significance of their results was not recognised. These authors reported that Phenoxybenzamine increased the overflow of noradrenaline from the cat spleen in response to sympathetic nerve stimulation. The reason for this has since been variously ascribed as being due to blockade of noradrenaline uptake, or of post-junctional receptors, supposedly involved in noradrenaline inactivation. That Phenoxybenzamine may facilitate the release of noradrenaline was first considered by Kirpekar and Cervoni (1963), though they rejected this concept. Häggendal (1970) excluded the inhibition of enzymic degradation, uptake, or of vasoconstriction as explanations for the effect of phenoxybenzamine, and proposed that the drug may increase the release per impulse either by a direct action on the nerve terminal or by some feedback mechanism connected to the effector cell response. The trans-synaptic feedback was later ruled out when it was found that alpha adrenoceptor antagonists facilitate transmission in the heart where the post-synaptic receptors are of the beta type (Enero and Langer 1973). The importance of alpha adrenoceptors in this regulation was emphasised by the finding that two alpha adrenoceptor agonists xylazine and clonidine reduced the stimulated overflow of noradrenaline from the cat spleen and rabbit heart (Heise and Kronebert 1970,

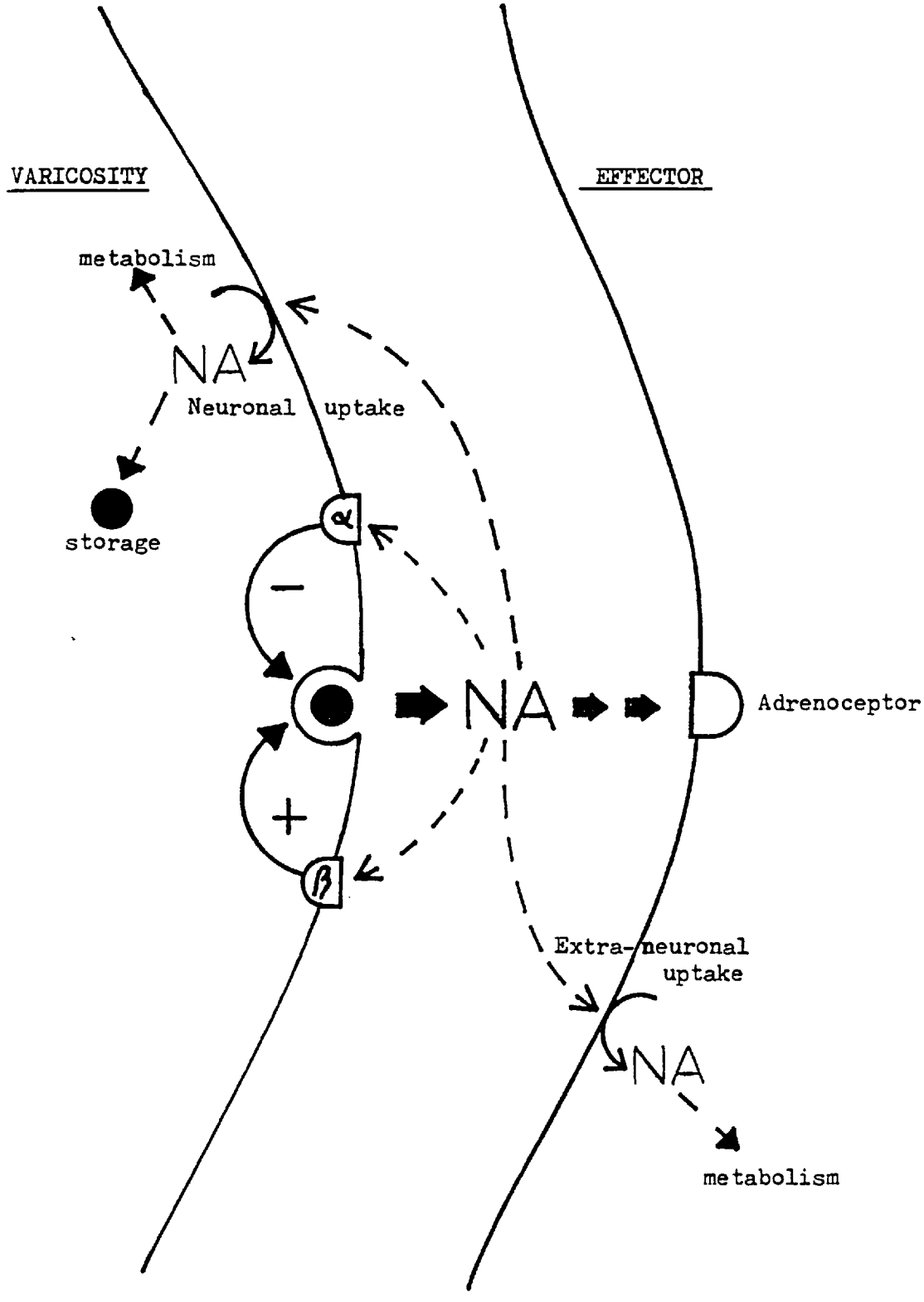
Werner et al 1970, original texts reviewed by Starke 1977). Farnebo and Hamberger (1971) showed that phenoxybenzamine increased the overflow of noradrenaline from the rat iris and suggested that this could be due to blockade of alpha adrenoceptors on the nerve terminals. However it was Kirpekar and Puig (1971) who from their study of the cat spleen using a stop flow technique finally concluded that - 'Noradrenaline released by nerve stimulation acts on a presynaptic alpha site to inhibit its own release by a negative feedback mechanism. Adrenoceptor blocking agents enhance the noradrenaline overflow from the spleen because they remove this autoinhibition by blocking presynaptic alpha sites'. Enero, Langer, Rothin, and Stefano (1972) proposed the same regulatory mechanism to explain the increased noradrenaline overflow from the isolated cat nictitating membrane in the presence of phenoxybenzamine. These authors also suggested that this mechanism could explain the lack of an increase in noradrenaline overflow when the catecholamine uptake systems are blocked, as observed in their experiments and by Blakeley, Brown and Ferry (1963). They proposed that any increase in the concentration of noradrenaline in the synaptic cleft caused by uptake blockade would activate the negative feedback mechanism causing a decrease in transmitter release. This proposal would explain for example, why Holman (1970) found that cocaine did not increase the excitatory junction potentials caused by nerve stimulation in the mouse vas deferens. And why Jenkins et al (1977) found that uptake blockade inhibited the twitch response of the same tissue.

Vizi, Somogyi, Hadhāzy and Knoll (1973) provided direct evidence for the effect of presynaptic alpha adrenoceptors by measuring both noradrenaline overflow and the response of the effector organ in the guinea-pig atrium and rat vas deferens.

Adler-Grashinsky and Langer (1975) have suggested that there is also a positive presynaptic feedback mechanism mediated via beta adrenoceptors. Stimulation of these receptors would increase the overflow of noradrenaline, in situations where there is a low release of noradrenaline. Thus to the basic description of the adrenergic neurone-effector junction given in the first part of this introduction, consisting of uptake systems and post synaptic receptors, we must now add this presynaptic control system of transmitter release. The junction is represented diagramatically in figure 1.

At the present time the role of noradrenaline in the vas deferens is still uncertain. Acceptance of noradrenaline as a motor transmitter is impeded by the fact that exogenous and endogenous noradrenaline produce inhibition of the twitch which is itself resistant to adrenoceptor blockade. Explanations for this resistance have been put forward by Burnstock and Holman (1964). These authors suggest that it may be due to the poor penetration of these drugs to the post junctional receptors, and conclude in later work that alpha adrenoceptor blocking drugs are reliable pharmacological tools only for those smooth muscles with large neuromuscular distances (Burnstock and Costa,1975), the minimum neuromuscular distance in the vas deferens being only 10-30 nm (Watanabe 1969). However this explanation has a number of

Fig. 1 Schematic representation of an adrenergic neuro-effector junction.



drawbacks. For example uptake blockade is thought to be more effective in tissues with narrow junctions, producing a greater potentiation of the response to stimulation. In the mouse vas deferens uptake blockade inhibits the response to stimulation (Jenkins et al 1977), thus the importance of the 'close junction' in this tissue is difficult to assess. As yet no explanation is available for the resistance of this tissue to adrenoceptor blockade. However the inhibition of the twitch response by exogenous and endogenous noradrenaline and its potentiation by alpha adrenoceptor blocking agents could be explained in terms of presynaptic alpha adrenoceptors. This work examines the role of noradrenaline and the importance of presynaptic alpha adrenoceptors in this tissue.

PART 1

The twitch response of the mouse vas deferens to electrical stimulation and the effect of drugs upon it.

INTRODUCTION

The isolated preparation of the mouse vas deferens used in this work was first described by Birmingham and Wilson (1963). It contains post ganglionic motor nerves, electrical stimulation of which liberates neurotransmitter which produces a contraction of the musculature. The vas deferens contains both circular and longitudinal muscle and the part each plays in the response to stimulation has been extensively investigated by Anton, Duncan and McGrath (1977). The contractions recorded in this work were probably the result of contractions of both these types of muscle. The response to electrical stimulation and to exogenous acetyl choline is described in this first section of the thesis. Hughes (1972) demonstrated that the twitch response of the isolated mouse vas deferens was increased if magnesium was omitted from the Krebs solution. This procedure has been followed in these experiments, though the effect of adding magnesium and of lowering the calcium content of the Krebs upon the response to stimulation is shown in this section.

As described in the general introduction an adrenergically innervated tissue has various properties. These properties are investigated in the mouse vas deferens in this section in the following order:

A. The tissue content of noradrenaline and the reduction of its concentration by inhibiting its synthesis, or by the use of specific depleting agents.

B. The effect of depletion of the tissue noradrenaline content on the twitch response to electrical stimulation.

C. The release of tissue noradrenaline by electrical stimulation. The noradrenaline overflowing from the tissue as a result of stimulation and its increase by the use of uptake blocking agents (Iversen 1963) or alpha adrenoceptor blocking agents. (Gillespie and Muir 1967).

D. The uptake of noradrenaline by the neuronal and extraneuronal mechanisms.

E. The effect of noradrenaline uptake blockade on the response of the tissue to electrical stimulation.

F. The effect of the adrenergic neurone blocking agent guanethidine on the response to electrical stimulation.

G. The effect of the release of endogenous noradrenaline by tyramine, an indirectly acting amine, on the response to electrical stimulation. The effects of the neuronal uptake blocker cocaine, or depletion of the tissue noradrenaline content on the action of tyramine.

H. The effect of adrenoceptor blocking agents on the response of the tissue to electrical stimulation and to exogenous noradrenaline.

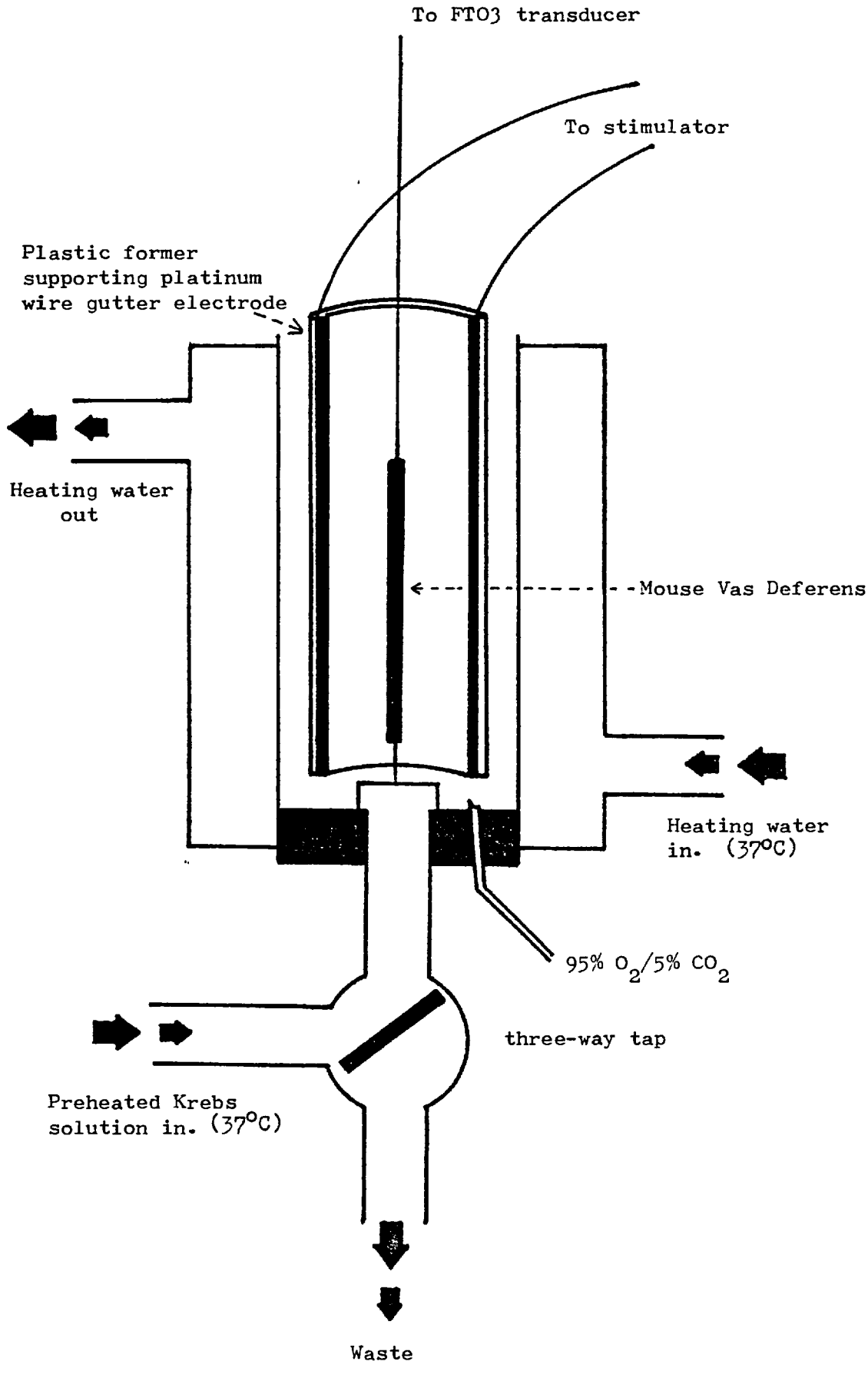
METHODS

Stimulation of and recording of contractions from the isolated mouse vas deferens

The following method has been used in the experiments described in parts I and II of this thesis unless otherwise stated. The animals used in all experiments were male T.O. strain mice weighing 25-40g. Krebs solution of the following composition (mM) was used; NaCl, 119.0; KCl, 4.7; $CaCl_2$, 2.5; $Na\ H_2\ PO_4.2H_2O$, 1.2; $Na\ HCO_3$, 25.0; and glucose, 11.0; and gassed with 95% O_2/5% CO_2.

The mouse was stunned and killed by cervical dislocation. The vasa deferentia were removed and dissected free of surrounding blood vessels and mesentery under Krebs solution. A single vas deferens was suspended in a 2 ml organ bath supplied with Krebs at 37°C and oxygenated with 95% O_2/5% CO_2 (Fig. 2). A tension of 500 mg was applied and changes in tension were recorded by a Grass FT03 isometric transducer. The transducer was mounted on an S.R.I. myograph stand allowing the tensions to be maintained by raising or lowering the platform. The recorded tension was displayed on a Grass TC polygraph. Before stimulation the tissue was allowed to equilibriate for 20 minutes. The preparation was stimulated via platinum wire gutter electrodes, square wave pulses being generated by a Grass S48 stimulator. The stimulation voltage remained constant in all experiments at 64v, the pulse width was varied as stated in results between 0.1 and 2 ms, frequency was also varied between 0.2 and 16Hz. At 0.2, 1 or 5Hz the preparation was stimulated every five minutes, at 10 and 16Hz the preparation was stimulated every ten minutes,

Fig. 2 Diagram showing tissue bath with mouse vas deferens in situ



Drugs were added either to the Krebs solution, in which case a twenty minute equilibration period in the presence of the drug was allowed, or they were introduced directly into the bath by an Agla micrometer syringe. In the latter case antagonists were added two minutes before stimulation and agonists either thirty seconds before stimulation or in some experiments using 0.2 Hz stimulation, after the sixth pulse.

Pre-dosing of animals

In sections B and G animals were predosed with the tyrosine hydroxylase inhibitor alpha methyl-p-tyrosine (∝MepT) or the noradrenaline depleting agent reserpine. The dosing schedules used were as follows:

∝MepT, the methyl ester was given intraperitoneally 4, 2 and 1 hours before killing the animals, 200 mg/kg at each dose. Reserpine, 5 mg/kg sub-cutaneously, was given 48 hours before killing, and 24 hours later 2.5 mg/kg was given intra-peritoneally. Dosing with reserpine caused the mice to loose weight, details of which appear in appendix 1.
In some cases both treatments were combined using the same schedules as above. Both drugs were made up in saline.

Measurement of the uptake of tritiated noradrenaline by the mouse vas deferens.

Materials

1. Tritiated noradrenaline (^{3}HNA) 5.90 µM, diluted to this concentration with 1% sodium metabisulphite.
2. Krebs containing ascorbic acid (114 µM) and EDTA (27 µM), gassed with 95% oxygen, 5% carbon dioxide.
3. 0.4 M Perchloric acid, kept ice cold.
4. Alumina, prepared by the method of Anton and Sayre (1962), containing sodium metabisulphite as one part to forty parts of alumina.
5. 2.0 M Tris buffer pH 9.2, containing 5% EDTA.
6. EDTA di-sodium salt.
7. Packard 'Instagel'.

Method

Mice were stunned and killed by cervical dislocation. Their vasa deferentia were removed, cleaned of surrounding blood vessels and mesentery and placed in oxygenated Krebs. Nine hundred µl of the Krebs solution, or the Krebs solution including drugs were placed in a 10 ml flask in a shaking incubator at 37°C. After warming the Krebs for 10 minutes one vas deferens was placed in each flask and allowed to equilibriate for 15 minutes. The incubation was begun by adding 100 µl of the ^{3}HNA solutions to the flask. The incubation was ended by pouring off the Krebs solution and washing rapidly with three 2.0 ml aliquots of the appropriate Krebs solution not containing ^{3}HNA. Then a further 2.0 ml was added to the flask and the tissue was incubated for a further 5 minutes in the absence of ^{3}HNA. The vas deferens

was then washed once more and placed on ice in 2.0 mls of the appropriate Krebs. Each vas deferens was blotted dry, weighed on aluminium foil, and then homogenised in 1.0 ml of the chilled perchloric acid. The homogenate was poured into a centrifuge tube and placed on ice. The mortar and pestle were washed with 1.0 ml perchloric acid and this was added to the centrifuge tube. After centrifuging at 30,000 g for 15 minutes at 4°C the supernatant was added to a tube containing 100 mg of alumina and 50 mg of EDTA. The absorption of catechols onto the alumina was begun by adding 2.0 mls of Tris pH 9.2 bringing the final pH of the solution to approximately 8.6. The alumina mixture was shaken for 15 minutes, and then centrifuged for 5 minutes at 2,500 r. min^{-1}. The supernatant was then aspirated off and the alumina was washed three times with 2.0 ml aliquots of distilled water. The wash was discarded and the alumina was then washed into a liquid scintillation counting vial with three 0.5 ml aliquots of distilled water. An equal volume of 'Instagel' was added to each vial which was then shaken. Samples were counted using a liquid scintillation counter (see appendix 2). Samples containing known amounts of ^{3}HNA were run in parallel with the assay samples during the alumina extraction procedure to estimate the efficiency of the extraction.

Estimation of released and endogenous tissue noradrenaline by a radioenzymic assay method.

Materials

1. Various standard concentrations of noradrenaline made up in 0.2 M hydrochloric acid containing 2.63 mM sodium metabisulphite.
2. (^{3}H)-s-adenosyl methionine (^{3}HSAME) solution made up in dithiothreitol (freshly made before each assay) to give a final radioactive concentration of 1μCi/μl (see appendix 1).
3. Phenylethanolamine-n-methyl transferase (PNMT) prepared from bovine adrenals.
4. Hydrochloric acid, 0.2M. (HCl).
5. Tris buffer (2.0M) containing 5% EDTA di sodium salt, pH 9.2.
6. Tris buffer (2.0M) containing 5% EDTA di sodium salt and 0.5M sodium phosphate, pH 8.6.
7. dodeca-Tungstophosphoric acid 0.5 gm/ml, made up freshly before each assay.
8. Unlabelled SAME (2.3mM) made up in a solution of 0.2M HCl, containing 1.57 mM noradrenaline.
9. Potassium phosphate 1.0M, pH 7.15.
10. Toluene containing 1% diethylhexylphosphoric acid (DHP).
11. Chilled perchloric acid 0.1M.
12. Sodium metabisulphite solution, 2.63mM, made up freshly before each assay.
13. Alumina, prepared by the method of Anton and Sayre (1962)
14. Liquiflor; 50g 2.5-diphenyloxazole (PPO), and 0.625 g

1, 4-di (^{2}-(5-phenyloxazolyl))benzene (POPOP) in 500 mls of toluene.

Sample Preparations

a) Tissue samples. Vasa deferentia were homogenised in 0,2M HCl containing 2.63mM sodium metabisulphite. The homogenate was diluted ten fold with the same 0.2M HCl and a 200µl sample taken for assay. Hearts were homogenised in 10.0 mls of the 0.2M HCl and a 200 µl sample was taken for assay.

b) Samples of Krebs solution bathing the vasa deferentia. The vasa deferentia were removed from the mice and cleaned of surrounding mesentery and blood vessels. The vasa deferentia were then tied in groups of eight and suspended in a 1.0 ml organ bath under 1.0 gm tension. The tissues were bathed in Krebs containing 114 µM ascorbic acid and gassed with 95% O_2/5% CO_2. After a 20 minute equilibriation period the preparation was stimulated, immediately after which the Krebs solution bathing the preparation was collected in a tube containing 10 µl of the sodium metabisulphite solution (see materials). The sample was then stored at 4^{o}C until 200 µl was taken for assay, which was always carried out on the same day.

Assay procedure

The assay was based upon the method of Henry, Starman, Johnson and Williams (1975), which is dependent upon the conversion of noradrenaline to adrenaline by PNMT using (^{3}H) SAME as the methyl donor.

A reaction mixture was made freshly before each assay containing: 37.5µl Tris buffer pH 9.2; 2.5 µl (^{3}H) SAME,

and 10 µl of the enzyme solution; per sample to be assayed. A 200 µl aliquot of the sample and 50 µl of the reaction mixture were placed in a reaction tube and incubated in a shaking water bath at 37°C for 40 minutes. The reaction was stopped by adding 2.0 mls of Tris buffer pH 8.6. Alumina (100 mg) was added to each tube, which was shaken for 15 minutes and then centrifuged for 5 minutes at 2,000 r. min^{-1}. The supernatant was removed and discarded. The alumina was washed three times with 2.0 ml aliquots of distilled water, which were discarded after each wash. Perchloric acid, 1.0 ml, was added to the alumina and mixed well, followed by 100 µl of the unlabelled SAME solution and 200 µl d-tungstophosphoric acid. This mixture was then placed on ice for 15 minutes. This procedure precipitated out any remaining (^{3}H) SAME, and removed catechols from the alumina. The mixture was then centrifuged at 2,000 r. min^{-1} for 10 minutes. The supernatant was removed and added to tubes containing 10.0 ml of the toluene/DHP mixture and 1.0 ml potassium phosphate. This mixture was shaken for 15 minutes to extract the tritiated adrenaline into the toluene and then centrifuged at 2,000 r. min^{-1} for 10 minutes. Nine mls of the supernatant was then removed and added to scintillation vials containing 400 µl of liquiflor. The samples were counted by liquid scintillation spectrometry. (see appendix 2). Noradrenaline standards were assayed in parallel with the samples to produce a standard curve for each assay.

Presentation of Results

The results obtained from all experiments are presented as mean plus or minus the standard error. The number of tissues examined, not the number of observations, is given as 'n'. The significance of the results was determined using Students t-test. When the value for 'P' was less than 0.05 the difference between two results was deemed significant.

Response of the tissue to field stimulation

In general the tension developed in response to stimulation increased with increasing pulse width from 0.25 to 2.0 ms, though there is no significant increase between 0.5 and 1 ms, or 1.5 and 2.0 ms. (table 1). The tension also increased with increasing frequency from 1.0 - 16 Hz at the 2 ms pulse width. (table 2). Addition of magnesium sulphate (1.1 mM) to the Krebs solution decreased the tension developed at frequencies up to 10 Hz, but not the maximum (16 Hz). (table 2). Halving the calcium concentration of the Krebs solution from 2.5 mM to 1.25 mM decreased the tension developed up to 16 Hz(table 2).

At 0.2 and 1 Hz discreet twitch responses were seen, but above this frequency the twitches fused to form a single contraction. (fig 3). This pattern was not changed when the calcium and magnesium content of the Krebs solution was altered as described above. Twitch tension progressively decreased with constant stimulation of the tissues at low frequencies (up to 1 Hz). This 'fall off' in response is shown in figure 4 as a percentage decrease from the initial tension in tissues stimulated at 0.1 Hz, 2.0 ms. Stimulation at higher frequencies (5-16 Hz) produced a rapid rise in tension which then decayed. The rate of this decay being somewhat variable between tissues (fig 3). No second rise in tension was seen, however long the period of stimulation. The responses produced by all frequencies and pulse widths in these experiments were completely blocked by 0.63 μM tetrodotoxin.

Table 1

Response of the mouse vas deferens to field stimulation (0.2 Hz) at varying pulse widths

PULSE WIDTHS (ms)	TENSION (mg)	n
0.25	147.0 ± 13.1	31
0.5	252.2 ± 11.9	30
1.0	244.9 ± 14.7	29
1.5	328.0 ± 29.2	11
2.0	346.0 ± 20.5	29

Table 2

The response of the mouse vas deferens to field stimulation (2.0 ms) at varying frequencies. The effect of varying the ionic composition of the Krebs solution

	TENSION (mg)		
FREQUENCY (Hz)	CONTROL (no Mg SO_4) n = 20	PLUS $MgSO_4$ n = 6	HALF $CaCl_2$ (1.25 mM) n = 14
0.2	284.6 ± 48.0	45.3 ± 12.6	68.6 ± 22.2
1	301.3 ± 32.8	76.2 ± 23.0	87.3 ± 24.7
5	1164.2 ± 270.2	406.7 ± 81.6	451.8 ± 100.2
10	1800.0 ± 144.8	1168.0 ± 147.4	1155.1 ± 161.9
16	2129.0 ± 119.4	1914.7 ± 107.0	1544.2 ± 143.9

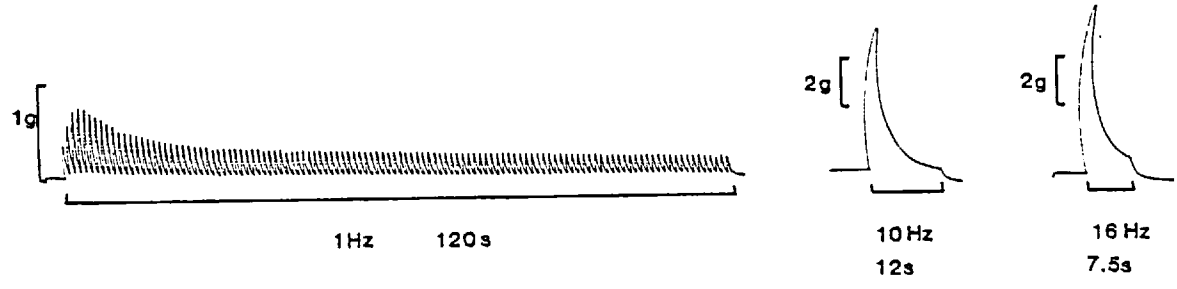


Fig. 3 Response of the mouse vas deferens to different frequencies of stimulation as recorded on a Grass Polygraph.

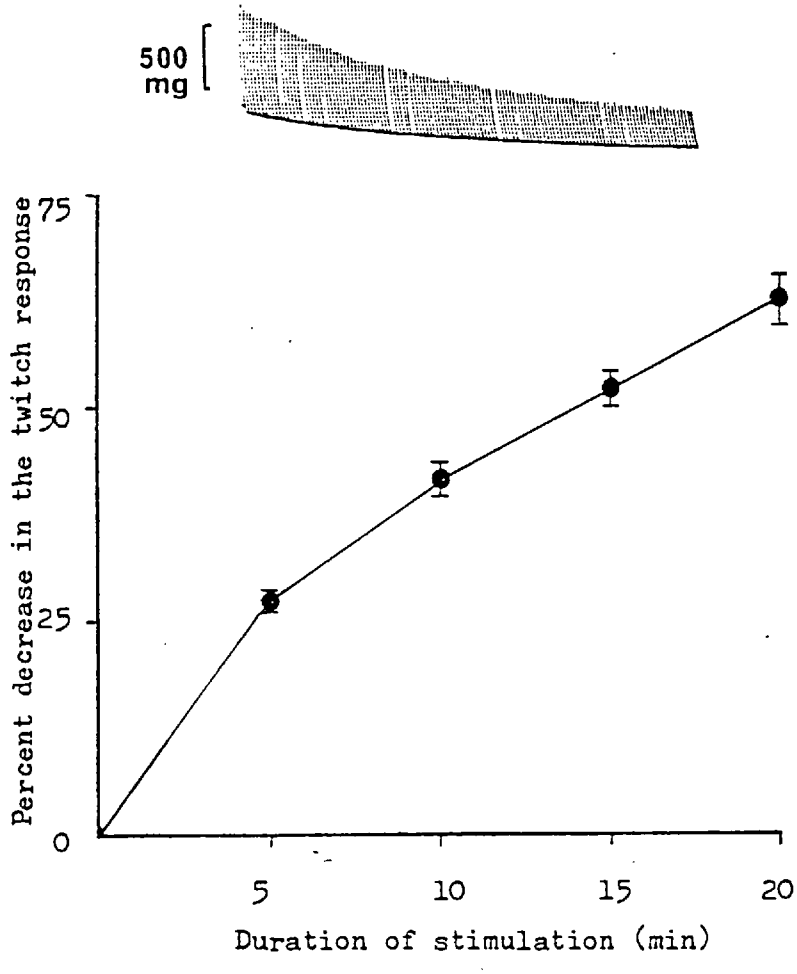


Fig. 4 The 'fall off' of the twitch response of the mouse vas deferens on continuous field stimulation. (0.1Hz, 2ms).

A. The noradrenaline content of the mouse vas deferens and the effect of depleting agents

Tissue samples were assayed for noradrenaline by the radioenzymic method already described. Predosing mice with reserpine, alpha methyl-p-tyrosine (∝Mept), or both compounds (dosing schedules are described in the methods section), reduced the noradrenaline content of the vas deferens. The combined treatment was most effective, producing a 99.4% depletion (table 3). The noradrenaline content of the hearts of the mice from which these vasa deferentia were taken were also assayed. This provides a comparison of the effects of these drugs in a tissue where noradrenaline is known to play an important transmitter role and the vas deferens. The volumes of perchloric acid required to homogenise the hearts diluted the concentration of noradrenaline in them after pretreatment with reserpine or reserpine and ∝Mept below that which could be assayed by this method.

Table 3

The noradrenaline content of the vas deferens and heart from naive animals and animals pretreated with ∝Mept, reserpine or both compounds

TISSUE	TREATMENT	NORADRENALINE CONTENT OF TISSUE	PERCENT REDUCTION FROM CONTROLS	n
vas deferens	control	9.95 ± 0.4 µg/gm		18
vas deferens	∝Mept	6.10 ± 0.5 "	39.7	20
vas deferens	reserpine	0.35 ± 0.03 "	96.5	14
vas deferens	reserpine + ∝Mept	0.06 ± 0.008 "	99.4	14
heart	control	408.4 ± 30.8 ng/gm		6
heart	∝Mept	272.1 ± 29.1 "	33.4	9
heart	reserpine	<20 ng "	95.1	4
heart	reserpine + ∝Mept	<20 ng "	95.1	4

B) The effect of depletion of tissue noradrenaline on the twitch response

Vasa deferentia from animals pretreated with reserpine, α-methyl-p-tyrosine (αMept), or reserpine and αMept were suspended in an organ bath and stimulated at 0.2 Hz with pulse widths of 0.25-2.0 ms. The tension developed in response to stimulation is given in table 4. Pretreatment with αMept had no significant affect on the tension developed. Both reserpine and reserpine plus αMept pretreatment decreased the tension developed at all pulse widths ($P<0.01$). The two pretreatments produce the same twitch tension at pulse widths from 0.25-1.0 ms, but the vasa deferentia from reserpine pretreated mice produced significantly less tension than those from reserpine plus αMept pretreated mice at 1.5 ms ($P<0.05$) and 2.0 ms ($P< 0.001$).

The possible involvement of released noradrenaline in the decrease in twitch response on constant stimulation at 0.1 Hz, 2.0 ms, was investigated by examining the decrease in vasa deferentia from pretreated animals. The decrease seen at various times during stimulation is given in fig. 5. Pretreatment with reserpine or reserpine plus αMept increased the rate and magnitude of the decline in the twitch response at all times. ($P< 0.01$). A curve for reserpine plus αMept is not given in fig. 5 as there was no significant difference between the rate of decline of the twitch in these vasa deferentia and those from reserpine pretreated animals ($P > 0.05$) except at 20 minutes, when the reduction produced in the former group was 83.7± 3.0% ($P < 0.05$). Vasa deferentia

Table 4

Tension developed (mg) by vasa deferentia from mice pretreated with catecholamine depleting agents in response to stimulation at various pulse widths

		PULSE WIDTHS (ms)				
TREATMENT	n	0.25	0.5	1.0	1.5	2.0
CONTROL	10	147.4 ± 13.1	252.0 ± 11.9	244.9 ± 14.7	328.0 ± 29.2	346.0 ± 20.5
αMept	12	178.9 ± 10.7	245.0 ± 9.3	267.3 ± 11.3	266.3 ± 22.8	345.3 ± 22.7
RESERPINE	10	48.3 ± 3.2	59.4 ± 4.0	81.9 ± 8.6	106.7 ± 17.9	121.9 ± 7.2
αMept + RESERPINE	12	47.0 ± 6.0	60.0 ± 4.0	92.0 ± 13.0	156.0 ± 15.0	158.0 ± 7.0

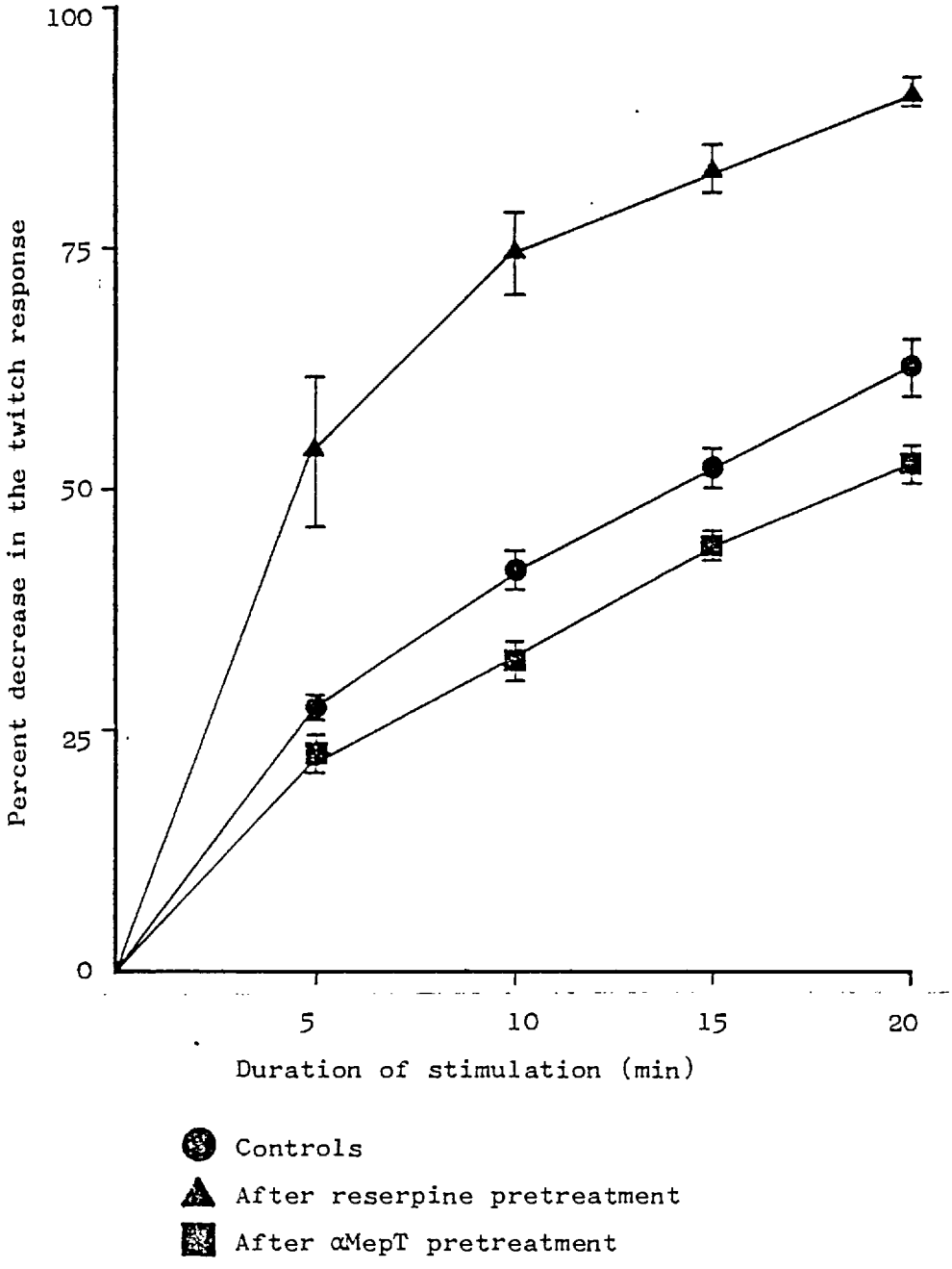


Fig. 5 The influence of depletion of the noradrenaline content of the mouse vas deferens on the twitch response to continuous field stimulation (0.1Hz, 2ms) n=4

from mice pretreated with ∝Mept alone were inhibited less than controls at each time ($P < 0.05$).

C) Release of endogenous noradrenaline by electrical stimulation

The vas deferens preparation was given 120 pulses at 1, 10 or 16 Hz and the resulting overflow of noradrenaline into the Krebs solution assayed as described previously. The maximum twitch tension developed at each frequency was recorded and is given along with the corresponding noradrenaline overflow in table 5. Twitch tension increased significantly ($P < 0.05$) between 1 and 10 Hz but not between 10 and 16 Hz, there was no increase in noradrenaline output at either increase in frequency of stimulation.

Inhibition of noradrenaline uptake into neurones by cocaine (10 μM) and into extraneuronal tissue by oestradiol (3.7 μM) would be expected to increase the noradrenaline overflow. The effects of this uptake blockade are shown in table 6. Results are expressed as percent change from control in each individual tissue because inter-tissue variation in actual overflows is large. The effect of the addition of 10 μM phentolamine in the presence of uptake blocking agents is also shown in table 6. The addition of oestradiol and cocaine produced an average 2.5 to 3 fold increase in overflow at all frequencies. The increase in overflow on the further addition of phentolamine, however, was frequency dependent, giving a further 4.7 fold increase at 0.2 Hz but only a 2.2 fold increase at 16 Hz.

From the estimates of noradrenaline content given in section B, it is possible to calculate the release of noradrenaline as a fraction of that present in the tissue, these values are given in table 7.

Table 5

Tension developed by and noradrenaline overflow from eight vasa deferentia in response to stimulation at varying frequencies

FREQUENCY Hz	n	TWITCH TENSION gm	NORADRENALINE OVERFLOW ng/gm OF TISSUE	OVERFLOW pg gm^{-1} $pulse^{-1}$
1	10	5.3 ± 0.3	3.13 ± 0.50	26.05 ± 4.20
10	4	8.4 ± 0.8	2.42 ± 0.70	19.98 ± 6.07
16	4	93 ± 0.6	2.98 ± 0.76	24.80 ± 6.32

Table 6

Percentage increase in the overflow of noradrenaline upon the addition of uptake blocking agents and an alpha adrenoceptor blocking agent

FREQUENCY Hz	PERCENTAGE INCREASE IN NORADRENALINE OVERFLOW			
	n	OESTRADIOL AND COCAINE	n	OESTRADIOL AND COCAINE AND PHENTOLAMINE
1	10	263.4 ± 58.7	5	1,235.5 ± 261.7
10	3	268.8 ± 79.5	4	659.7 ± 131.7
16	3	303.5 ± 34.3	4	664.2 ± 115.9

Table 7

Fractional release of noradrenaline ($\times 10^3$) in controls and in the presence of uptake blocking agents and an alpha-adrenoceptor blocking agent

FREQUENCY Hz	CONTROL	OESTRADIOL (3.7 μM) AND COCAINE (10 μM)	OESTRADIOL (3.7 μM) AND COCAINE (10 μM) AND PHENTOLAMINE (10 μM)
1	0.371 ± 0.072	1.05 ± 0.13	4.17 ± 1.18
10	0.244 ± 0.070	0.701 ± 0.283	3.23 ± 1.40
16	0.417 ± 0.132	1.03 ± 0.42	2.86 ± 0.78

D) Uptake of ^{3}H-noradrenaline by the mouse vas deferens

Uptake of ^{3}H-noradrenaline was estimated by the assay described in Methods. Uptake was found to increase with time (fig. 6) and with concentration of ^{3}H-noradrenaline (fig. 6). As the isolated tissue work has been carried out in magnesium free Krebs, uptake studies were conducted likewise. Addition of magnesium to the Krebs had no effect upon uptake ($P > 0.05$). However, decreasing the sodium chloride content of the Krebs from 119.0 mM to 32.8 mM significantly reduced the ^{3}H-noradrenaline uptake ($P < 0.05$, fig 7).

The effects of cocaine, a neuronal uptake blocking agent, and oestradiol, an extraneuronal uptake blocking agent, on uptake were examined using a 10.0 minute incubation period with ^{3}H-noradrenaline. Cocaine caused a dose related inhibition of uptake but oestradiol (3.7 μM) potentiated uptake (table 8). The composition of the tritium present in the vas deferens after ^{3}H-noradrenaline uptake was assayed by a column chromatographic method (described in Part III), which separated the tritium into ^{3}H-noradrenaline and its tritiated metabolites (Table 9). The majority of the tritium in the vas deferens was found to be noradrenaline and this proportion was not altered by oestradiol (table 9) indicating that the increase caused by oestradiol is due to a real increase in ^{3}H-noradrenaline uptake and not prevention of a metabolic process taking place after uptake.

Several other drugs whose effect on the twitch response will be investigated, have been examined for their effect

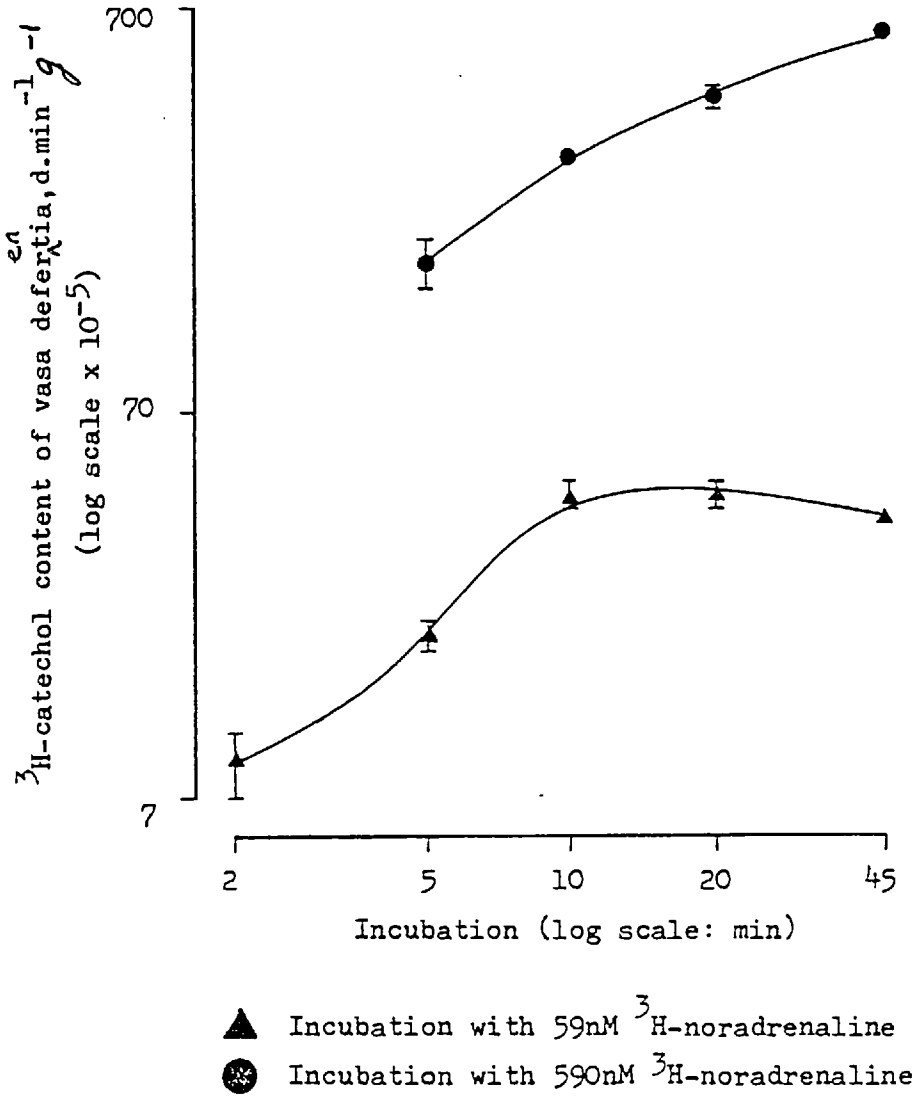


Fig. 6. The effect of increasing the concentration of ^{3}H-noradrenaline in the incubation media on the uptake of ^{3}H-catechols. n=6

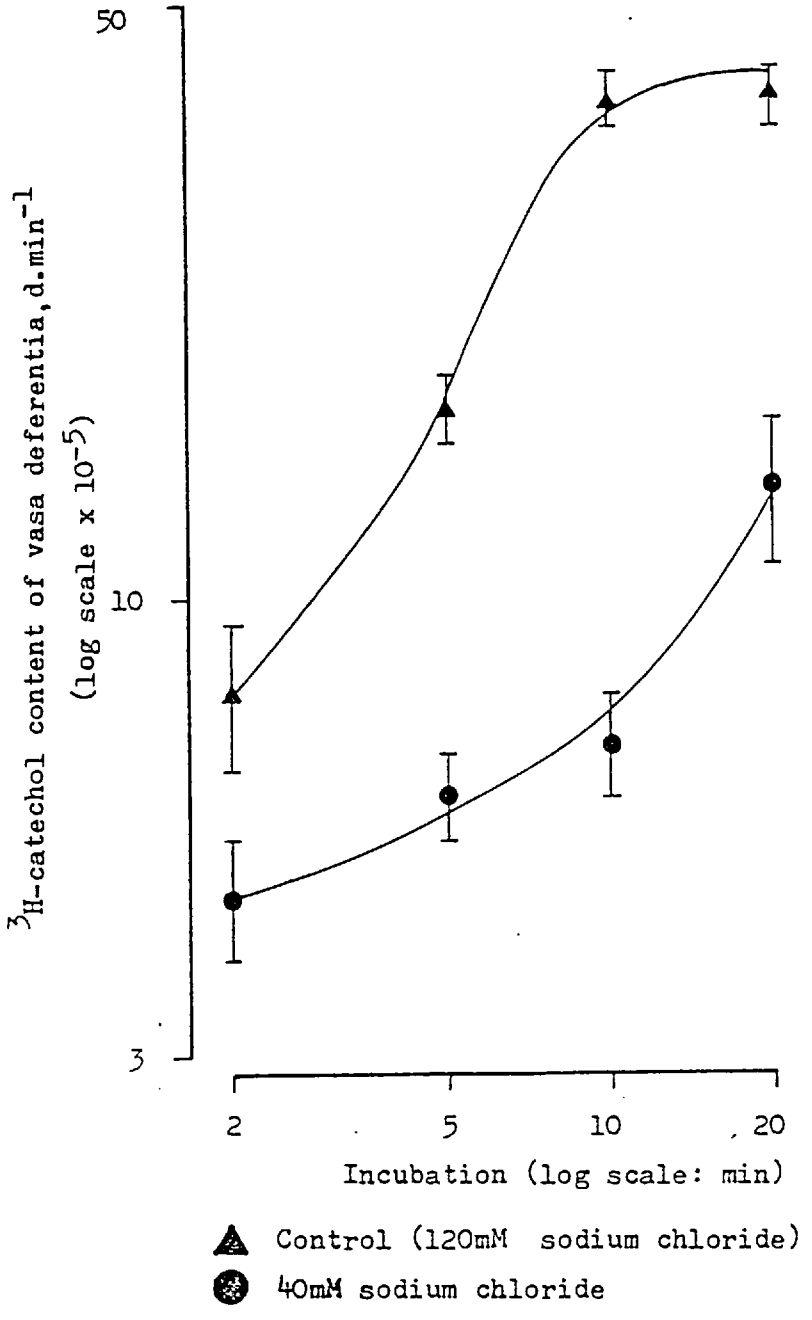


Fig. 7 The effect of reducing the sodium chloride content of the incubation medium on the uptake of ^{3}H-catechols during incubation with 59nM ^{3}H-noradrenaline. n=6

Table 8

The influence of cocaine and oestradiol on the uptake of (^{3}H) noradrenaline by the mouse vas deferens during a 10 minute incubation period

DRUG	CONCENTRATION μM	UPTAKE $d.min^{-1}g^{-1}$	PERCENT CHANGE
CONTROL		$36.3 \pm 5.5 \times 10^4$	
COCAINE	10	$2.56 \pm 0.12 \times 10^4$	-92.9
	40	NOT DETECTABLE	-100
OESTRADIOL	3.7	$53.7 \pm 5.0 \times 10^4$	+47.8
COCAINE AND OESTRADIOL	10 / 3.7	$4.73 \pm 0.64 \times 10^4$	-87

- = inhibition + = potentiation n = 4 for all experiments

Table 9

The effect of oestradiol (3.7 μM) on the percentage of (^{3}H)-noradrenaline and (^{3}H) metabolites in the total tritium taken up by the vas deferens during a 10 minute incubation

METABOLITE	CONTROL	PLUS OESTRADIOL
Non catechols	3.3 ± 0.2	2.8 ± 0.3
DOPEG	1.0 ± 0.2	1.4 ± 0.4
Noradrenaline	87.8 ± 0.4	88.9 ± 2.1
DOMA	7.5 ± 0.3	6.0 ± 1.1

n = 4 for all experiments

on uptake (Table 10). None of these drugs increased uptake, the majority decreased uptake. Yohimbine and Clonidine had no effect, this may be due to the low concentrations of these two drugs.

Table 10

Effect of various drugs in the Krebs solution on the uptake of (^{3}H)-noradrenaline by the mouse vas deferens after incubation for 10 minutes with 59 nM (^{3}H)-noradrenaline

DRUG	CONCENTRATION	% INHIBITION OF UPTAKE	n
Tyramine	3 µM	50.9 ± 3.7	4
	30 µM	83.2 ± 0.7	4
Phenoxybenzamine	33 nM	18.4 ± 2.8	4
	100 nM	25.3 ± 3.2	4
	1 µM	32.8 ± 6.7	4
	5 µM	68.1 ± 1.9	4
	15 µM	88.9 ± 1.41	5
Yohimbine	10 nM	0	4
	128 nM	0	4
Guanethidine	200 nM	15.9 ± 11.2	3
Propranolol	1 µM	8.8 ± 1.5	3
	10 µM	52.8 ± 3.3	4
	30 µM	88.3 ± 1.3	4
Phentolamine	1 µM	0	4
	10 µM	36.8 ± 3.9	4
	30 µM	58.9 ± 1.1	4
Clonidine	11.2 nM	0	4

E) Effect of uptake blockade on the twitch response of the mouse vas deferens

Cocaine produced a dose related inhibition of the twitch response to stimulation at 0.2 Hz, 0.25-2.0 ms ($P < 0.05$, fig 8). Cocaine (10 μM) and oestradiol (3.7 μM) also inhibited the twitch response at all these pulse widths ($P < 0.05$) producing approximately the same degree of inhibition as 10 μM cocaine alone (fig 8).

Uptake blockade inhibits the peak tension developed at all frequencies of stimulation (see part II), however it also produces a change in the form of the response at 5, 10 and 16 Hz. At these frequencies uptake blockade decreases the rate of decay of the peak tension, producing in some tissues an apparent second phase to the contraction (fig 9).

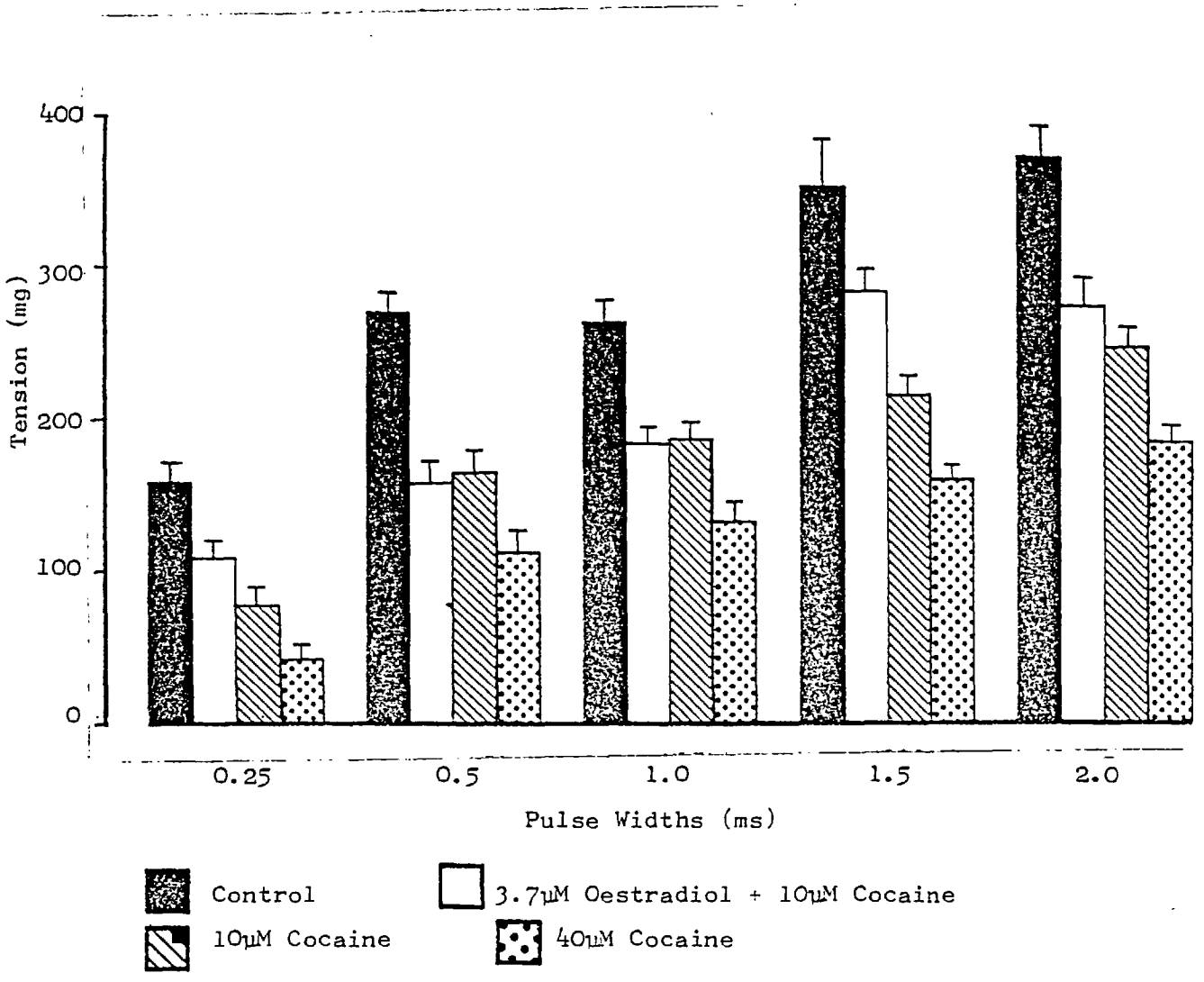


Fig. 8 The effect of uptake inhibitors upon the response of the mouse vas deferens to field stimulation. n=11

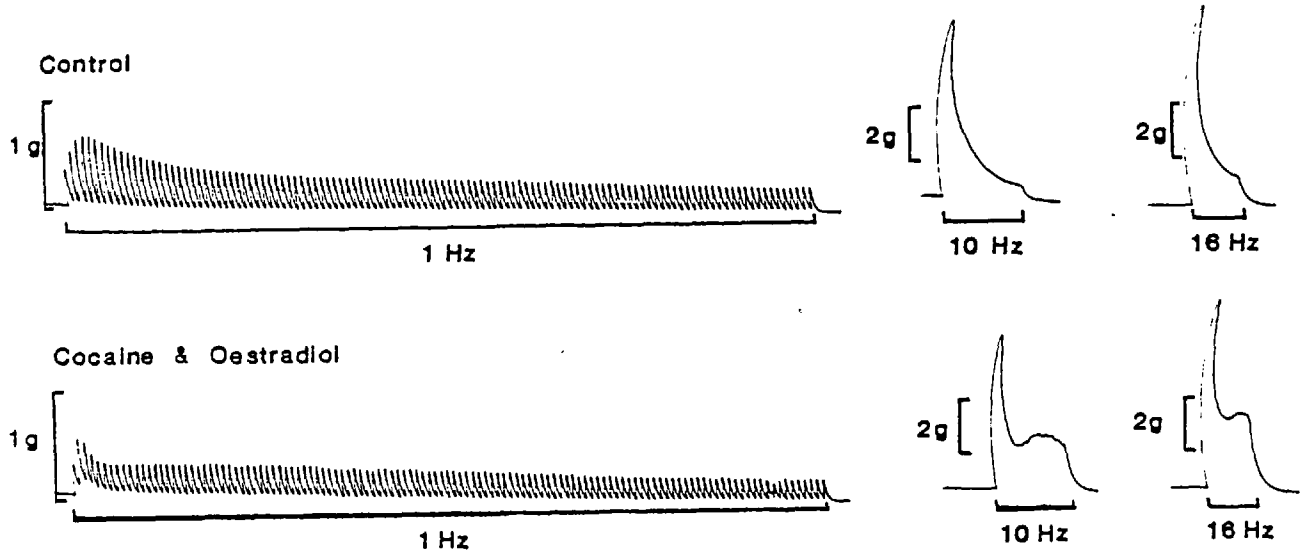


Fig. 9 The qualitative effect of cocaine (10μM) and oestradiol (3.7μM) on the tissue response at various frequencies of stimulation.

F) Effect of adrenergic neurone blockade on the response of the mouse vas deferens to electrical stimulation

Adrenergic neurone blockade was produced by guanethidine, which caused a dose related inhibition of the twitch response to stimulation at 0.2 Hz, 2.0 ms, when added to the Krebs solution twenty minutes before stimulation (Table 11). The inhibition took the form of an increased rate of fall off in twitch heights compared with that which occurs in the absence of guanethidine. The fall off in contrast to that occurring with presynaptic alpha-adrenoceptor agonists (see part II) is progressive. The response to stimulation of presynaptic alpha-adrenoceptors reaches equilibrium after five or six stimuli. Consequently, the figures given in table 11 represent the degree of inhibition produced after 6 stimuli.

Table 11

The effect of guanethidine on the twitch response of the mouse vas deferens to electrical stimulation

CONCENTRATION OF GUANETHIDINE (M)	% INHIBITION OF THE TWITCH	N
3.4×10^{-11}	0	2
3.4×10^{-10}	20.6 ± 6.2	4
3.4×10^{-8}	44.8 ± 7.9	4
3.4×10^{-7}	68.8 ± 6.7	4
6.8×10^{-3}	84.0 ± 6.8	4

G) Effect of the indirectly acting amine tyramine on the twitch response of the mouse vas deferens

Tyramine produces a dose related inhibition of the twitch response of the mouse vas deferens (fig 10), which is decreased in a dose related manner by cocaine (fig 10), or by producing a depletion of the noradrenaline content of the vas deferens (fig 11). Tyramine (3-300 μM) never produced a contraction of the vas deferens in normal Krebs solutions. However, if the calcium content of the Krebs is reduced from 2.5 mM to 1.25 mM eratic contractions occur during stimulation in the presence of tyramine. This occurs at all frequencies, but particularly after the initial peak tension seen at 5-16 Hz (fig 12). The tyramine induced contractions can be abolished by 33 nM phenoxybenzamine (fig 12), shown to block contractions due to exogenous noradrenaline (see section H).

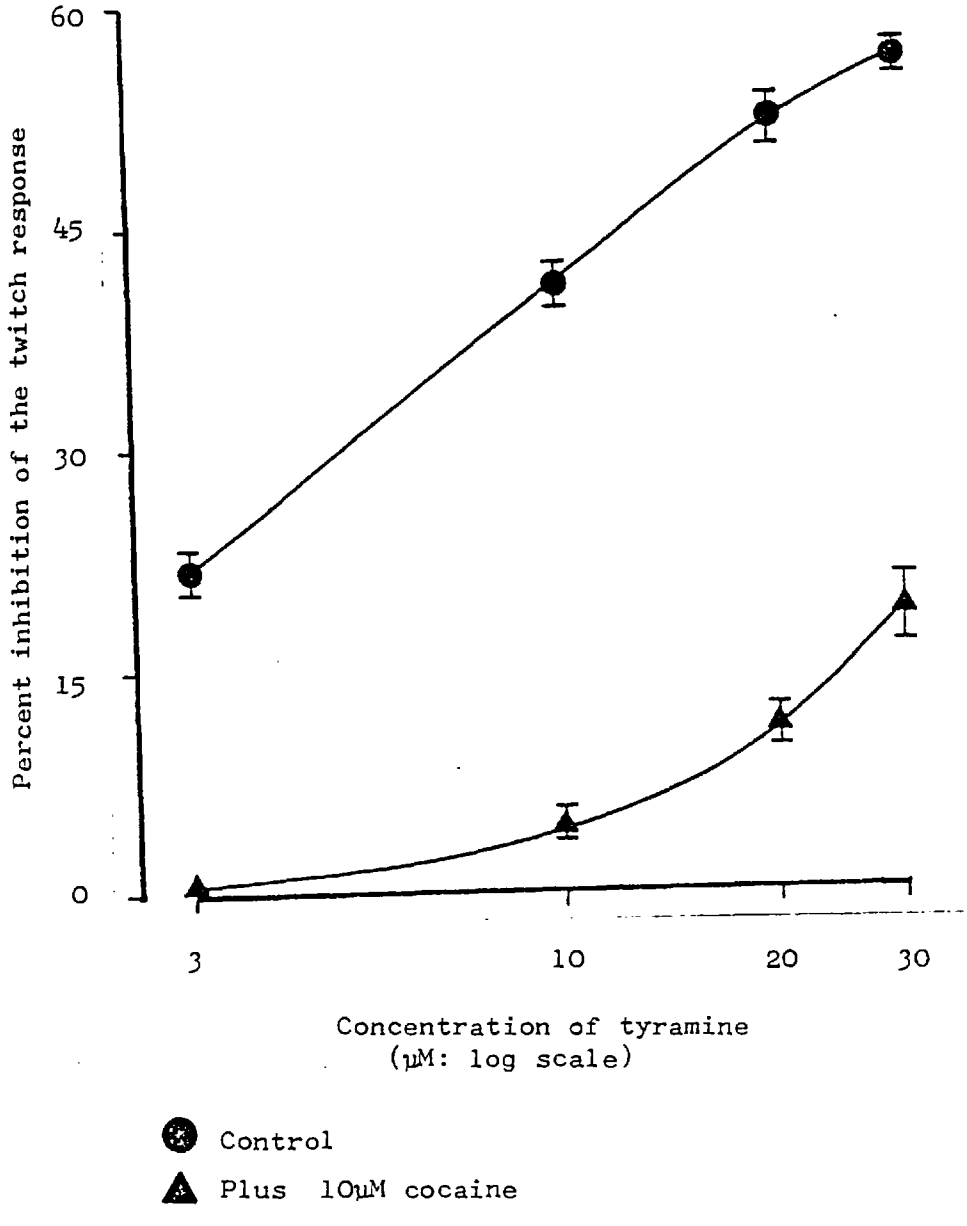


Fig. 10 Antagonism by cocaine of the inhibitory effect of tyramine on the twitch response of the mouse vas deferens field stimulation (2.0ms, 0.2Hz, n=4).

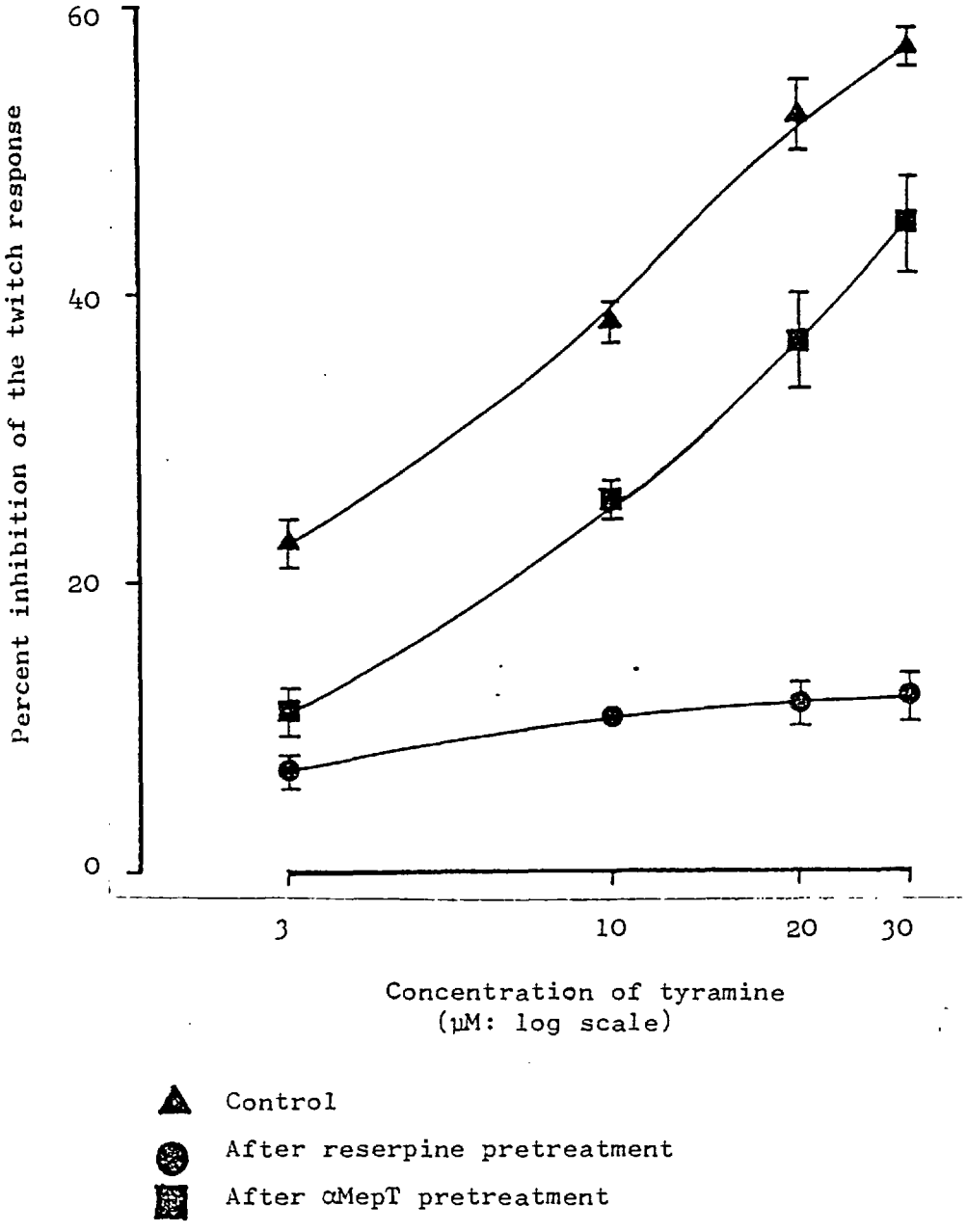
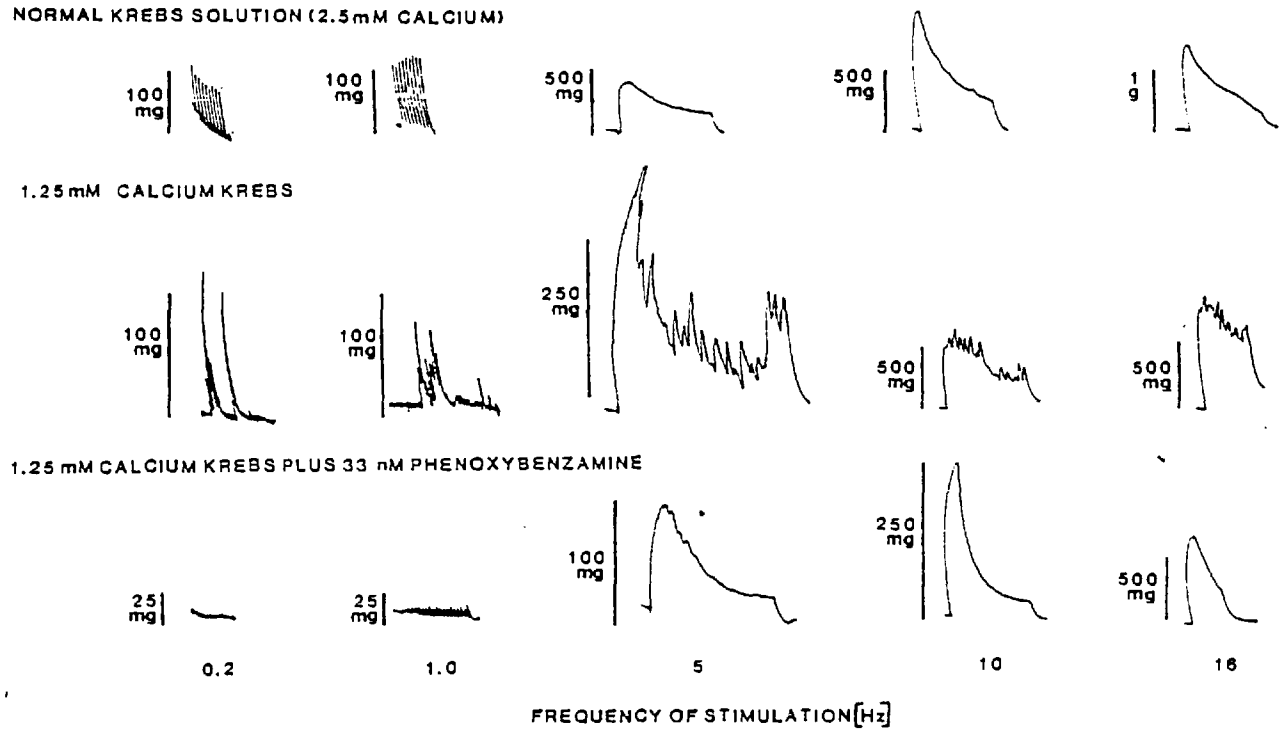


Fig. 11 The influence of depletion of the noradrenaline content of the mouse vas deferens on the inhibition by tyramine of the twitch response to field stimulation (2.0ms, 0.2Hz) n=4



Tyramine (20μM) present during each of these recordings.

Fig. 12 The qualitative effect of halving the calcium ion concentration (1.25mM) on the tissue response in the presence of tyramine (20μM) at various frequencies of stimulation.

H) The effect of exogenous noradrenaline and adrenoceptor blocking agents upon the response of the mouse vas deferens to electrical stimulation

In the absence of stimulation noradrenaline causes a dose related contraction of the tissue. These contractions are completely abolished by the addition of 33 nM phenoxybenzamine to the Krebs, and the dose response curve is moved to the right by 500 nM phentolamine. (fig 13). In view of the contractions produced by tyramine in Krebs containing only 1.25 mM calcium chloride but not in Krebs containing 2.5 mM calcium chloride (see previous section), it was interesting to see if the sensitivity of the tissue to noradrenaline was increased by halving the calcium content of the Krebs. Dose response curves for noradrenaline were repeated in Krebs of varying calcium concentration, but there was no increase in sensitivity (table 12).

If noradrenaline is added before or during stimulation it inhibits the twitch response in a dose dependent manner (fig 14). This inhibition is not blocked by 33 nM phenoxybenzamine as is the contraction. In fact this concentration of phenoxybenzamine potentiates the noradrenaline inhibition. 1.2 μM noradrenaline inhibits the twitch response by 45.0 ± 3.0 % and this is increased by 33 nM phenoxybenzamine to 66.3 ± 2.8 %, 5 μM phenoxybenzamine further potentiates the effect to 82.7 ± 10.4 % and it is not until 15 μM phenoxybenzamine is reached that the inhibition is blocked (9.05 ± 0.25 %). Furthermore, 33 nM phenoxybenzamine has no significant effect on the twitch response to electrical stimulation except at 0.25 ms

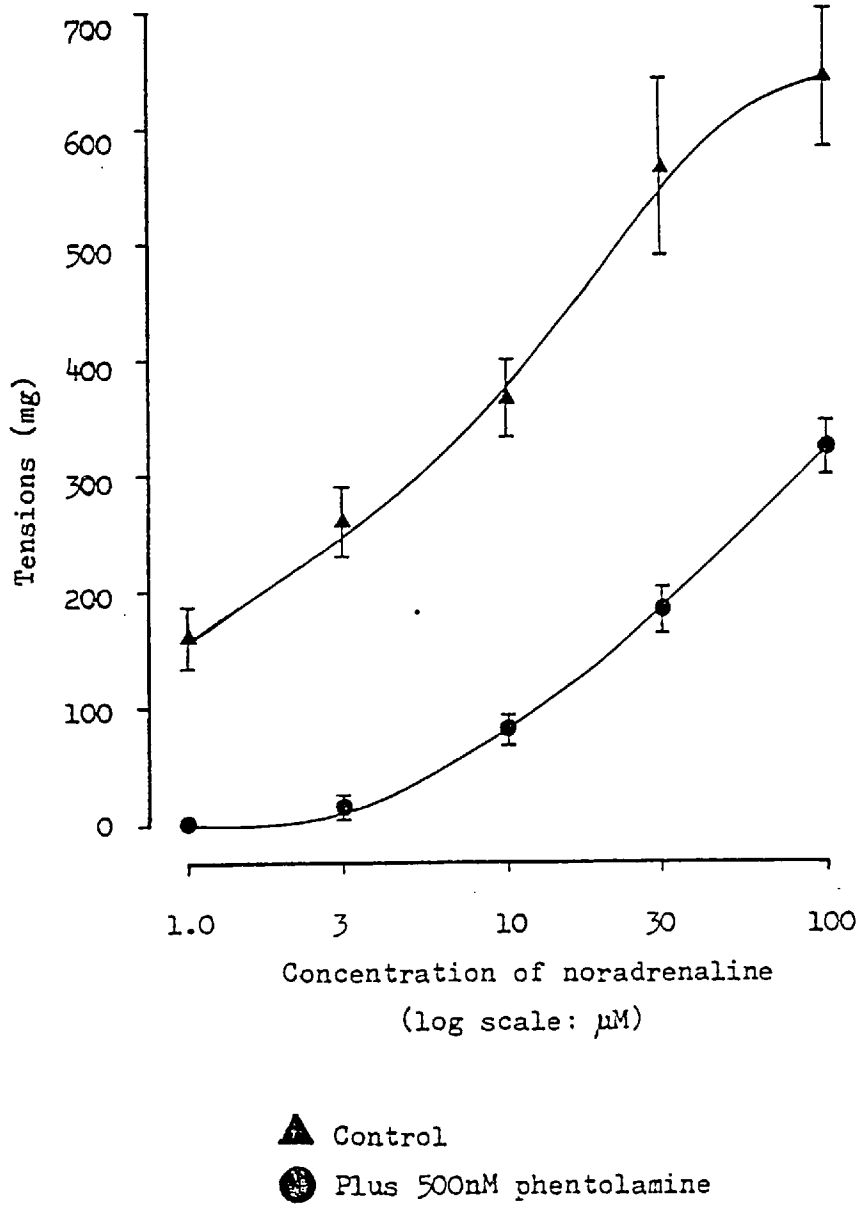


Fig. 13 The effect of phentolamine (500nM) on the tension developed by the mouse vas deferens in response to exogenous noradrenaline n=4.

Table 12

Effect of halving the calcium content of the Krebs solution (from 2.5 mM to 1.25 mM) on the tension developed by the mouse vas deferens in response to noradrenaline in the absence of field stimulation

CONCENTRATION OF NORADRENALINE (μM)	TENSION (mg)	
	CONTROL (2.5 mM $CaCl_2$)	1.25 mM $CaCl_2$
1.0	120.1 ± 17.7	159.3 ± 27.5
3.0	233.1 ± 46.1	260.2 ± 29.5
10.0	380.5 ± 53.6	369.0 ± 34.0
30.0	593.8 ± 111.2	566.1 ± 77.7
100.0	663.2 ± 94.3	639.0 ± 61.3

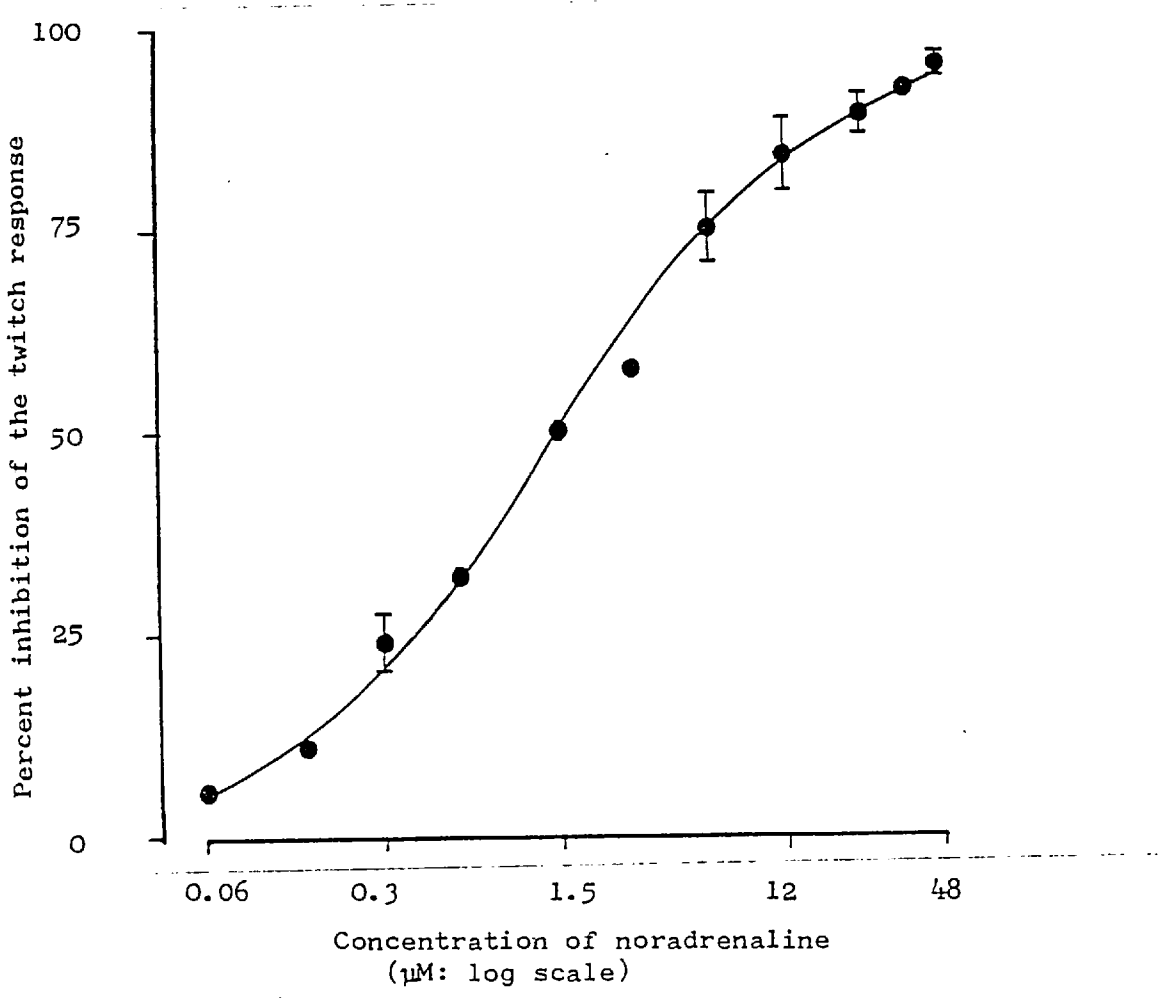


Fig. 14 Inhibition of the twitch response of the mouse vas deferens to field stimulation (2ms, 0.2Hz) by noradrenaline. n=4

(table 13), but if the concentration is raised to 1 μM there is a significant inhibition of the twitch response except at 0.25 ms (fig 15). Increasing the concentration of phenoxybenzamine to 15 μM significantly potentiates the twitch response ($P < 0.05$, fig 15). Phentolamine (10 μM) also potentiates the twitch response (fig 15). In fig 15 and table 13, the results are presented as a percentage of the 2.0 ms control response to reduce variation between vasa deferentia.

While the potentiation by alpha adrenoceptor blocking agents of the peak tension developed in response to stimulation is seen at all frequencies, these drugs also increase the rate of decay of the response at 5-16 Hz, and abolish the apparent second phase response seen in the presence of noradrenaline uptake blockers (fig 16).

Table 13

The effect of various concentrations of phenoxybenzamine on the twitch response of the mouse vas deferens to electrical stimulation

PERCENT OF 2 ms CONTROL RESPONSE.

CONCENTRATION OF PHENOXYBENZAMINE

PULSE WIDTHS (ms)	CONTROL	33 nM	100 nM	1 μM	5 μM	15 μM
	n = 12	n = 7	n = 3	n = 4	n = 6	n = 8
0.25	11.0 ± 3.2	32.5 ± 9.6	45.9 ± 8.9	17.2 ± 4.0	13.5 ± 3.0	74.4 ± 4.7
0.5	54.2 ± 2.9	54.1 ± 8.7	61.6 ± 14.2	34.5 ± 7.4	41.4 ± 9.0	112.6 ± 3.8
1.0	68.1 ± 2.9	65.1 ± 8.8	70.9 ± 11.5	50.8 ± 11.2	51.6 ± 9.6	134.3 ± 5.4
1.5	81.7 ± 1.6	75.0 ± 1 .8	88.4 ± 9.3	65.4 ± 10.6	62.7 ± 8.9	145.8 ± 5.6
2.0	100	95.5 ± 12.9	102.7 ± 12.6	70.4 ± 5.2	75.0 ± 10.0	175.7 ± 7.7

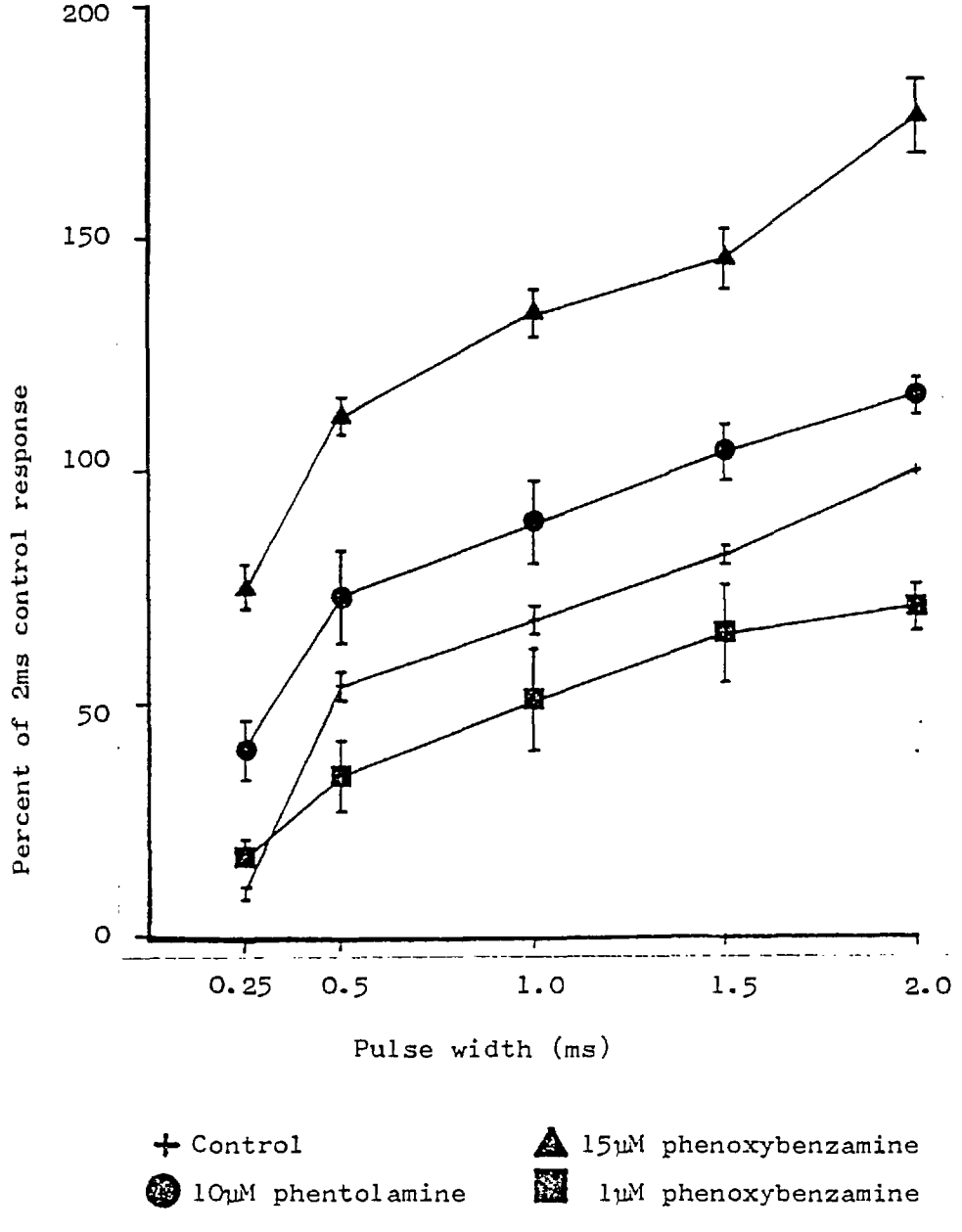


Fig. 15 Effect of various concentrations of α-adrenoceptor blocking agents on the response of the mouse vas deferens to field stimulation (0.2Hz, n=6).

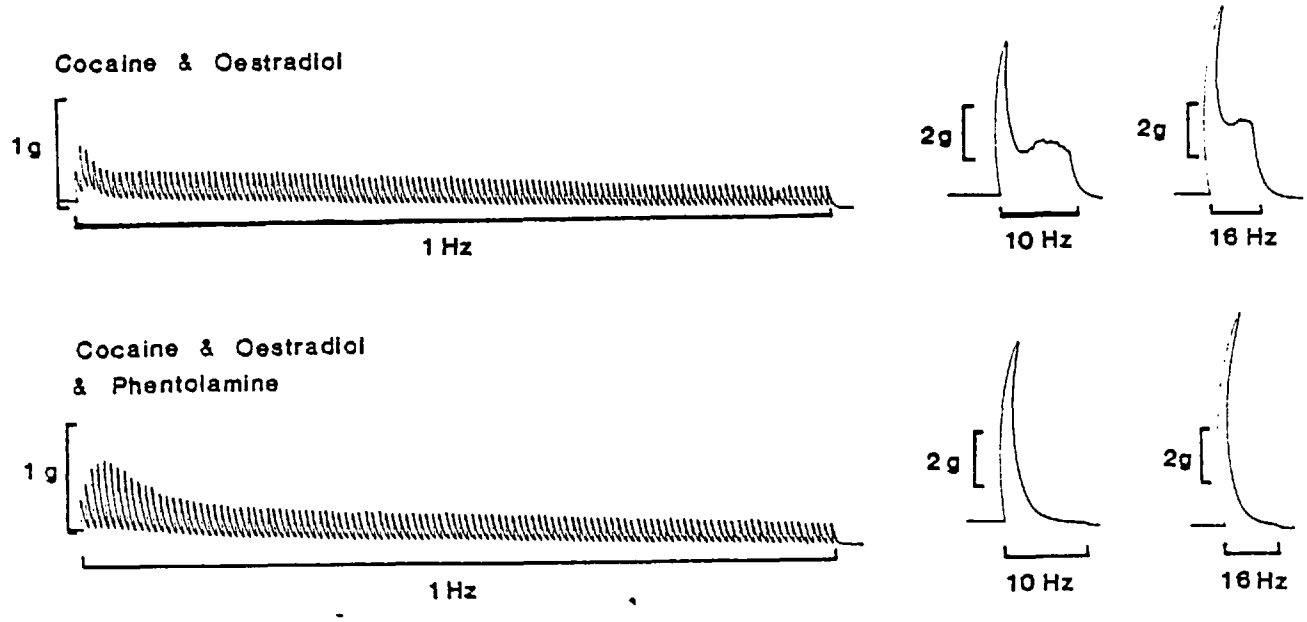


Fig. 16 The qualitative effect of phentolamine (10μM) on the tissue response in the presence of cocaine (10μM) and oestradiol (3.7μM) at various frequencies of stimulation.

DISCUSSION

The tension developed in response to electrical stimulation of the mouse vas deferens is proportional to both the frequency of stimulation and the duration of the stimulating pulse. At 0.2 and 1 Hz a discrete twitch response is seen. At 5-16 Hz the twitches fuse to form a single prolonged increase in tension. This latter response can be considered in two parts, the first initial rise to the peak tension, and the decay phase from this peak, known as the first and second phase respectively. The decay may be prolonged and occasionally a second slight increase in tension may occur. However the tension always falls back to resting levels as soon as stimulation ceases. No distinct second phase contraction occurs, such as was reported by Swedin (1971) in the rat and guinea pig vasa deferentia. Decreasing the calcium content of the Krebs or adding magnesium decreases the magnitude of these responses but not their form. Both these alterations in ionic composition of the Krebs produce an apparently frequency related inhibition of the twitch. This may be due to factors similar to those described by Younkin (1974) to explain facilitation. This author proposed that at high frequencies of stimulation calcium influx into neurones was in excess of that required for transmitter release, whereas at low frequencies the influx was sufficient only for the appropriate release of transmitter. If this were so then restriction of calcium availability (or competition for its binding sites by magnesium) will have a greater effect at low frequencies than at high.

All these electrically induced twitches were completely

abolished by tetrodotoxin indicating that they are all nerve mediated. The twitch responses are also blocked by guanethidine, an adrenergic neurone blocking agent. This suggests that the neurotransmitter, even if it is not noradrenaline is released from the adrenergic neurone. The concentration of guanethidine required to block these responses is of the same order of magnitude as that used by other authors to block nerve mediated contractions of the vas deferens (Jones and Spriggs, 1975. Westwood and Whaler, 1968). The noradrenaline content of the mouse vas deferens is comparable with that found by Sojstrand (1972) in the mouse (NIMR strain, 5.4 $\mu g.g^{-1}$), rat (8 $\mu g.g^{-1}$) and guinea pig (10 $\mu g.g^{-1}$). Compared with the mouse hearts assayed by the same method the noradrenaline content of the mouse vas deferens is high, being approximately 24 fold greater. This high concentration reflects the dense adrenergic innervation of the tissue reported by Sojstrand (1972). Inhibition of noradrenaline synthesis by pre-treating the animal with alpha methyl-p-tyrosine reduced the noradrenaline content of the vas deferens by 38.7% and that of the heart by 33.4%. This depletion did not affect the twitch tension developed by the vas deferens in response to electrical stimulation. However there was a significant ($P < 0.05$) decrease in the 'fall off' seen during constant stimulation. Pretreatment with reserpine or reserpine and alpha methyl-p-tyrosine produced a large depletion of the noradrenaline content of the vas deferens (96.5% and 99.4% respectively) and the heart.(Too little noradrenaline remaining to be measured by this assay). Large doses of reserpine were given to the animals as Sojstrand and Swedin (1968), and

Gillespie and McGrath (1974), have both reported that it is difficult to deplete the noradrenaline content of the vas deferens. The former authors thought this to be due to the poor blood supply to this tissue while the latter concluded that it was due to the less frequent stimulation of the vas deferens compared to other tissues and showed that stimulation of the sympathetic nerves to the vas deferens increased the depletion. However as one can deplete both the vas deferens and the heart to the same degree using alpha methyl-p-tyrosine both these explanations are doubtful. The blood supply would affect both alpha methyl-p-tyrosine and reserpine in the same way and if the supply to the vas deferens is poor there would be a lower depletion than in the heart. During stimulation it is the newly synthesised noradrenaline that is preferentially released (Kopin, Breese, Krauss, and Weise, 1968). Preventing synthesis would mean that stored noradrenaline has to be released on stimulation and thus depletion will be proportional to the rate of stimulation and other losses such as metabolism and 'leaking' through the neuronal membrane. Therefore either the vas deferens has an unusually high rate of loss by other routes or, one must conclude, that as the depletion of the vas deferens and the heart are the same so is the frequency of stimulation. Both reserpine and reserpine plus alpha methyl-p-tyrosine pretreatment decreased the response of the vas deferens to stimulation and increased the 'fall off' on constant stimulation. The reduction in twitch response is consistent with the observation of Lee (1967) who could detect no decrease in the response of other sympathetically innervated tissues

unless their noradrenaline content was reduced by more than 50%. These results are also in accord with those of Wakade and Krusz (1972) who investigated the effect of reserpinisation upon the guinea-pig vas deferens.

The overflow of noradrenaline per pulse from the stimulated vas deferens was found to be constant from 1 - 16 Hz agreeing with the results obtained by Henderson and Hughes (1976). The variability in the overflow between the groups of vasa deferentia was very large but when examining the effects of drugs this variability was reduced by expressing changes relative to individual controls. Oestradiol and cocaine produce an increase in the overflow at all rates of stimulation as one would expect when the sites of noradrenaline uptake are blocked. The increase in overflow was statistically the same at each frequency. The further addition of phentolamine produces a frequency related increase in the overflow, being greatest at 1 Hz and least at 16 Hz.

An increase in noradrenaline overflow caused by alpha-adrenoceptor blocking agents has been reported by several authors including Farnebo and Malmfors (1973). Though phentolamine blocks noradrenaline uptake in this tissue the increase in overflow is not related to this, as oestradiol and cocaine are already present, and also the increase in overflow produced by them is not frequency related. This would suggest that the increase in overflow produced by phentolamine is related to its alpha-adrenoceptor blocking properties.

The vas deferens takes up (^{3}H)-noradrenaline by a time and

concentration dependent system which is depressed by decreasing the sodium chloride content of the bathing solution. Eighty-seven per cent of the tritium taken up is recovered as (^{3}H)-noradrenaline indicating that little metabolism of the (^{3}H)-noradrenaline which is taken up occurs, this is not altered by adding oestradiol to the incubation medium. The uptake is decreased in a dose dependent manner by cocaine, but increased by oestradiol. This increase can be explained by proposing that as oestradiol blocks extra-neuronal uptake a larger concentration of (^{3}H)-noradrenaline is present in the synaptic cleft and available for neuronal uptake. These properties are consistent with the uptake of (^{3}H)-noradrenaline examined in this assay being primarily due to a neuronal uptake mechanism (Iversen 1963). Analysis of the effects of other drugs used in this work, upon uptake, showed that all but clonidine and yohimbine decreased uptake. However only low concentrations of these drugs were examined and it is possible that higher concentrations may block uptake. It must be noted that when examining the effects of drugs known to release noradrenaline such as tyramine and guanethidine, some of the apparent inhibition of uptake may be due to release of (^{3}H)-noradrenaline recently taken up. Uptake blockade inhibited the twitch response of the vas deferens to electrical stimulation. As uptake blockade increases the concentration of noradrenaline in the synaptic cleft and hence overflow of transmitter, one would expect there to be a potentiation of the response to stimulation such as is found in other sympathetically innervated tissues

(Iversen 1963). At 5 - 16 Hz while the peak tension developed is decreased by uptake blockade, the second phase response is increased.

The effects of endogenous noradrenaline in this tissue were examined by the use of tyramine, a drug known to act by releasing noradrenaline (Burn and Rand 1958 and 1960). In agreement with Ambache, Dunk, Verney and Zar's (1972) findings in the guinea-pig and rabbit vasa deferentia, tyramine produced no contraction of this tissue in concentrations up to 1 mM in normal Krebs solutions. However when the calcium content of the Krebs solution was halved 20 μM tyramine caused contractions. This could be explained if lowering the calcium increased the sensitivity of the tissue to noradrenaline. Dose response curves to exogenous noradrenaline were however the same in normal and low calcium Krebs. As the release of noradrenaline by tyramine is independent of calcium it is unlikely that there would be any change in the amount of noradrenaline released by tyramine (Chubb, De Potter, &De Schaepdryver, 1972). Thus the reasons for these contractions are not clear, but as they were blocked by 33 nM phenoxybenzamine it appears that they were due to noradrenaline. In concentrations lower than those required to produce contractions tyramine inhibited the response of the tissue to electrical stimulation, in a dose dependent manner. This is due to noradrenaline as it is prevented by neuronal uptake blockade and by depletion of the noradrenaline content of the vas deferens. Axelrod et al (1962) showed that tyramine produced the same effect as nerve stimulation in the isolated rat heart. This would suggest that the role of noradrenaline in the vas deferens

is in fact inhibitory.

Exogenous noradrenaline produces contractions of the tissue in the absence of electrical stimulation. These contractions are abolished by 33 nM phenoxybenzamine. If noradrenaline is added before or during stimulation it inhibits the twitch response. This inhibition is not blocked by 33 nM phenoxybenzamine, but is potentiated, probably due to blockade of uptake as this concentration of phenoxybenzamine inhibits the uptake of (^{3}H)-noradrenaline by 18%. This blockade probably explains the inhibition of the response to electrical stimulation by this and concentrations up to 5μM phenoxybenzamine. It has already been shown that uptake blocking agents inhibit the twitch response. A concentration of 15 μM phenoxybenzamine is required to block the inhibition produced by exogenous noradrenaline. This concentration of phenoxybenzamine also potentiates the tissue response to electrical stimulation. Phentolamine (10 μM) will also potentiate the twitch response, and the peak tension developed at 5 - 16 Hz. However phentolamine increases the rate of decay of the peak tension and abolishes the second phase response at these higher frequencies. Clearly the receptors responsible for the noradrenaline contraction and inhibition of the twitch response are not the same. And apparently both these receptors are different from the receptor mediating the twitch response, which is not blocked by 15 μM phenoxybenzamine as the other two are.

It is clear from these results that the twitch response at 0.2 - 1 Hz is somewhat different from the response at 5 - 16 Hz. While the low frequency responses resemble in their behaviour

the peak or first phase of the contraction produced by high frequency stimulation, the second phase of this contraction must be considered separately. The latter is potentiated by uptake blocking agents and inhibited by alpha-adrenoceptor blocking agents, this is consistent with this response being mediated via noradrenaline acting upon a conventional alpha-adrenoceptor. The low frequency response and the first phase of the contraction to high frequency stimulation are affected in the opposite way by these drugs, and in addition, are inhibited by tyramine and exogenous noradrenaline. While these results provide no direct information about the nature of the transmitter mediating these responses, two points are apparent. Firstly, the transmitter is released from neurones susceptible to blockade by guanethidine, which are therefore probably adrenergic. Secondly, if the transmitter is noradrenaline it is acting upon a receptor which is not blocked by the conventional alpha-adrenoceptor blocking agents phenoxybenzamine and phentolamine. To suggest that in the adrenergic innervation of this tissue there is a system for the synthesis, storage, release and destruction and/or uptake of a neurotransmitter other than noradrenaline would be making the neuroeffector junction unnecessarily complex. It is known that noradrenaline is present in, and released from this tissue. It is more economical to suggest that noradrenaline is the motor transmitter, but acts upon a receptor which is sufficiently different from the conventional adrenoceptor so as not to be blocked by conventional blocking agents. While it is true that there is no precedent for this theory (as there

is none for a new transmitter theory) it has been suggested that receptor classification merely represents extremes of a constantly variable population. (Van Rossum 1965). Therefore it is only necessary to propose that this receptor is one extreme of this possible variable.

The usual responses of this tissue to exogenous noradrenaline, tyramine and noradrenaline uptake blockade, could be construed as the interaction of noradrenaline with a twitch control system. This control is probably mediated via an alpha adrenoceptor as it is susceptible to blockade by alpha adrenoceptor antagonists. If stimulation of this receptor inhibited the twitch response it would explain the inhibition caused by exogenous and endogenous noradrenaline; and if it was normally affected by concentrations of noradrenaline released on stimulation, then its blockade by alpha adrenoceptor blocking agents would explain the potentiation of the twitch response produced by these compounds. Such a system could act as a negative feedback control of noradrenaline release if it were the transmitter, or may control the release of another transmitter.

Such a system has been proposed by several authors for a presynaptic alpha adrenoceptor. The existence of this receptor in the mouse vas deferens is investigated in the second part of this thesis.

PART II

Presynaptic alpha-adrenoceptors and the twitch response of the mouse vas deferens to electrical stimulation.

INTRODUCTION

It has already been suggested that inhibition of the twitch response of the mouse vas deferens by noradrenaline, tyramine, oestradiol and cocaine, and the potentiation of the twitch by phentolamine or phenoxybenzamine, is due to their effect on a presynaptic alpha-adrenoceptor. In this section the effect of the selective presynaptic alpha-adrenoceptor agonist clonidine, (Starke, Montel, Gayk and Merker, 1974) and the selective antagonist yohimbine, (Starke, Borowski and Endo, 1975), have been examined on the twitch response. The dependence of the effects of presynaptic alpha-adrenoceptors upon the frequency of stimulation and the calcium concentration have also been examined. Vizi, Somogyi, Hadházy and Knoll (1973), reported that the degree of inhibition of the electrically induced twitch response of the rat vas deferens produced by clonidine or noradrenaline was inversely proportional to the frequency of stimulation. These authors explained this by proposing that as the frequency of stimulation increased, the concentration of noradrenaline in the synaptic cleft also increased, producing a greater stimulation of presynaptic alpha-adrenoceptors. Thus, at high frequencies there is already considerable stimulation of presynaptic alpha-adrenoceptors, and the degree by which it can be increased by exogenous agonists is small. Whereas at low frequencies, where the amount of endogenous noradrenaline released on stimulation is lower, the effect of exogenous agonists is more apparent. A second hypothesis for the mechanism of this frequency dependence is based upon the proposed mode of action of presynaptic alpha-adrenoceptors,

namely the reduction of calcium availability at a site required for transmitter release. Electrical stimulation of nerves is known to cause an influx of calcium which is required for transmitter release. Repeated stimulation of nerves at low frequencies often produces facilitation of the response. Younkin (1974) developed the residue hypothesis to explain this. He suggested that each nerve impulse left some calcium bound to the active site involved in transmitter release. At high frequencies more than enough calcium enters the neurone to saturate the active site and the decrease in calcium influx caused by presynaptic alpha-adrenoceptor stimulation has no effect. At low frequencies however the interval between stimuli is longer and a diffusion of calcium from the active sites is assumed to occur preventing accumulation. Any decrease in calcium influx due to stimulation of presynaptic alpha-adrenoceptors then leads to a decrease in the number of active sites occupied by calcium and hence a decrease in transmitter release. If the latter explanation for the frequency dependency is correct one would expect the effect of presynaptic alpha-adrenoceptors to be calcium dependent. Increasing calcium influx into the neurone would antagonise the effect of presynaptic alpha-adrenoceptor stimulation, while decreasing influx would act synergistically with it.
In this section the effect of clonidine and yohimbine on the twitch response of the mouse vas deferens are established at various frequencies. Attempts to vary the calcium influx have been made by altering the calcium and magnesium content of the Krebs, and by replacing two-thirds of the sodium

chloride content of the Krebs with lithium chloride. This latter substitution has been reported by Baker, Blaustein, Hodgkin and Steinhardt (1969), to increase the calcium influx into squid axon. The effects of noradrenaline, tyramine, oestradiol and cocaine, phenoxybenzamine and phentolamine are then examined under varying conditions and compared with clonidine and yohimbine. The selectivity of clonidine and yohimbine for presynaptic alpha-adrenoceptors in this tissue has been described previously by Marshall, Nasmyth, Nicholl and Shepperson (1978).

The effect of changing the ionic composition of the Krebs solution on the response of the mouse vas deferens to electrical stimulation and to acetyl choline

The preparation was stimulated at 0.2 - 16 Hz in Krebs varying in magnesium and calcium content and in solutions where two-thirds of the sodium chloride was replaced with lithium chloride (table 14). Dose response curves to acetyl choline were obtained in each of these solutions (table 15). Addition of magnesium to the Krebs decreased the tension developed at 0.2 - 10 Hz, but had no significant effect at 16 Hz. The response to acetyl choline was not significantly affected except that to 11 μM which was potentiated ($P < 0.05$) in this Krebs solution. Decreasing the calcium content of the Krebs from 2.5 mM to 1.25 mM significantly decreased the tension developed in response to stimulation at all frequencies ($P < 0.05$), and depressed the response to higher concentrations of acetyl choline ($P < 0.05$ at 5.5 and 11 μM). Increasing the calcium content of the Krebs to 12.5 mM increased the twitch tension developed at 0.2 Hz, but had no significant effect at any other frequency. No significant effect on the response of the tissue to acetyl choline was seen. Replacing two-thirds of the sodium chloride with lithium chloride had no significant effect on the twitch response except at 5 Hz, which was potentiated. The responses to all concentrations of acetyl choline were depressed.

Table 14

Response of the vas deferens to electrical stimulation in Krebs of varying ionic composition

FREQUENCY Hz	TWITCH TENSION (mg)				
	CONTROL 2.5 mM $CaCl_2$	1.25 mM $CaCl_2$	12.5 mM $CaCl_2$	2.5 mM $CaCl_2$ 1.20 mM $MgCl_2$	2.5 mM $CaCl_2$ 80 mM LiCl 40 mM NaCl
0.2	284.6 ± 48.0	68.6 ± 22.2	431.1 ± 42.7	45.3 ±12.6	426.7 ± 77.5
1	301.3 ± 32.8	87.3 ± 24.7	348.3 ± 44.8	76.2 ± 23.0	247.8 ± 53.7
5	1164.2 ± 270.2	451.8 ± 100.2	1561.7 ± 188.9	406.7 ± 81.6	1722.2 ± 149.5
10	1800.0 ± 144.8	1155.1 ± 161.9	2071.7 ± 233.8	1168.0 ± 147.4	1811.1 ± 60.7
16	2119.0 ± 119.4	1544.2 ± 143.9	2338.9 ± 163.4	1914.7 ± 107.0	2111.1 ± 60.7
n	20	14	6	6	6

Table 15

Response of the vas deferens to acetyl choline in Krebs of varying ionic composition

CONCENTRATION OF ACETYL CHOLINE μM	TWITCH TENSION (mg)				
	CONTROL 2.5 mM $CaCl_2$	1.25 mM $CaCl_2$	12.5 mM $CaCl_2$	2.5 mM $CaCl_2$ 1.2 mM $MgCl_2$	2.5 mM $CaCl_2$ 80 mM LiCl 40 mM NaCl
0.11	3.3 ± 3.3	2.6 ± 1.1	0 ± 0	0 ± 0	0 ± 0
0.55	50.4 ± 6.6	67.7 ± 11.0	45.8 ± 18.3	34.2 ± 7.4	13.4 ± 3.0
1.1	110.7 ± 13.2	105.8 ± 13.2	95.6 ± 23.9	109.3 ± 5.6	49.6 ± 3.5
5.5	322.4 ± 28.9	173.4 ± 34.5	250.0 ± 44.2	419.9 ± 46.3	200.9 ± 23.2
11	359.3 ± 36.4	233.3 ± 7.4	242.6 ± 52.7	498.3 ± 60.0	280.8 ± 10.4
n	20	14	6	6	6

The effect of clonidine on the twitch response of the mouse vas deferens

Clonidine inhibited the twitch response to electrical stimulation at 0.2 Hz, in a dose dependent manner (fig 17). This inhibition was inversely proportional to the frequency of stimulation, (fig 18 and 19) being greatest at 0.2 Hz and least at 16 Hz. Halving the calcium content of the Krebs solution or the addition of magnesium to it increased the inhibition at each frequency ($P < 0.05$, fig 18 and 19).

Increasing the calcium composition of the Krebs five fold significantly decreased the clonidine inhibition at 0.2 Hz and 1 Hz, had no effect at 5 Hz and 10 Hz, and potentiated it at 16 Hz ($P < 0.05$, fig 18). Replacing two-thirds of the sodium chloride in the Krebs with lithium chloride decreased the inhibition at all frequencies, producing a significant potentiation at 1 Hz ($P < 0.025$ fig 19).

The effect of clonidine on contractions produced by exogenous noradrenaline in the absence of stimulation

Clonidine is known to be a presynaptic alpha-adrenoceptor agonist, however it is possible that part of its inhibition of the twitch response could be due to an interaction with post-junctional receptors, or a direct depression of smooth muscle. To investigate this the effect of 56 nM clonidine on contractions of the vas deferens produced by noradrenaline in the absence of stimulation was investigated. Clonidine was added 30 seconds before the noradrenaline and had no effect on the development of, or on the peak tension obtained (table 16).

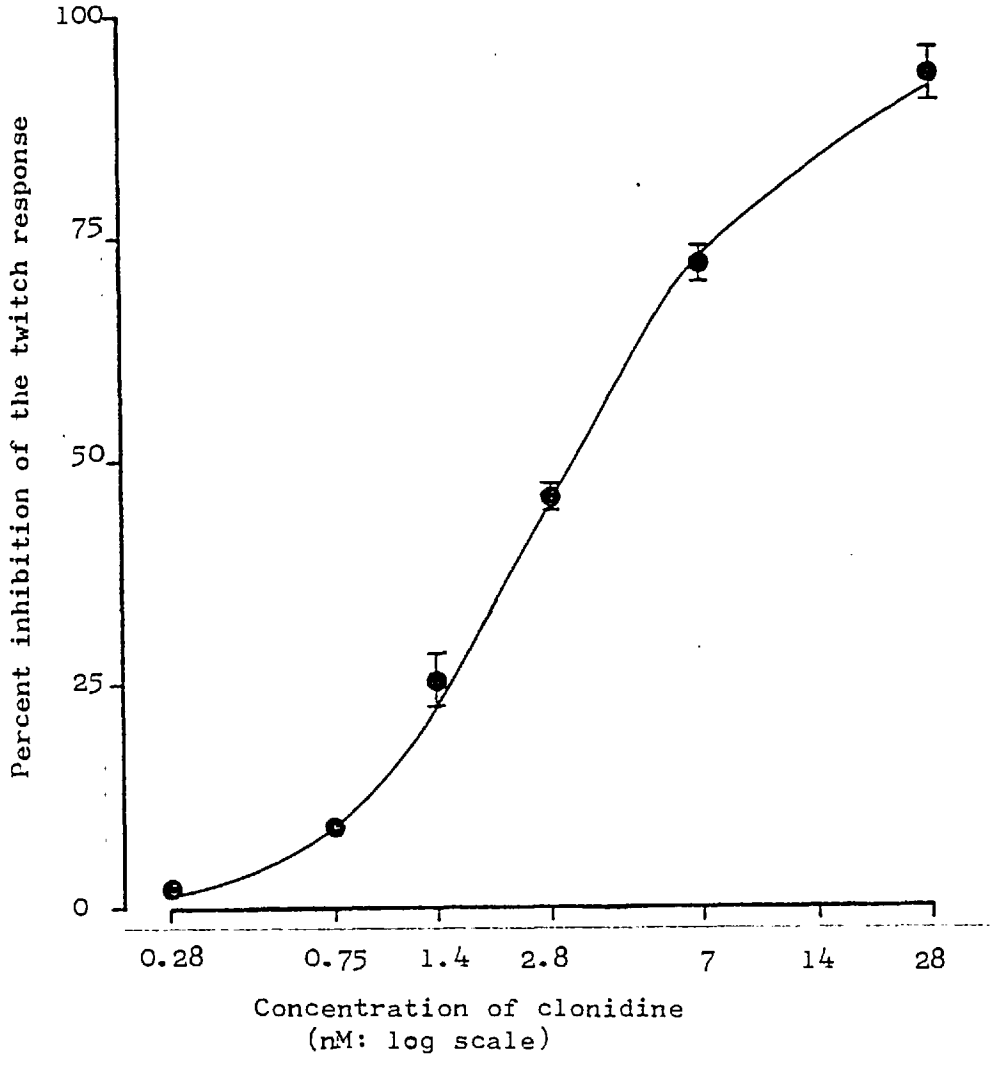


Fig.17 Inhibition of the twitch response of the mouse vas deferens to field stimulation (2ms, 0.2Hz) by clonidine. n=6

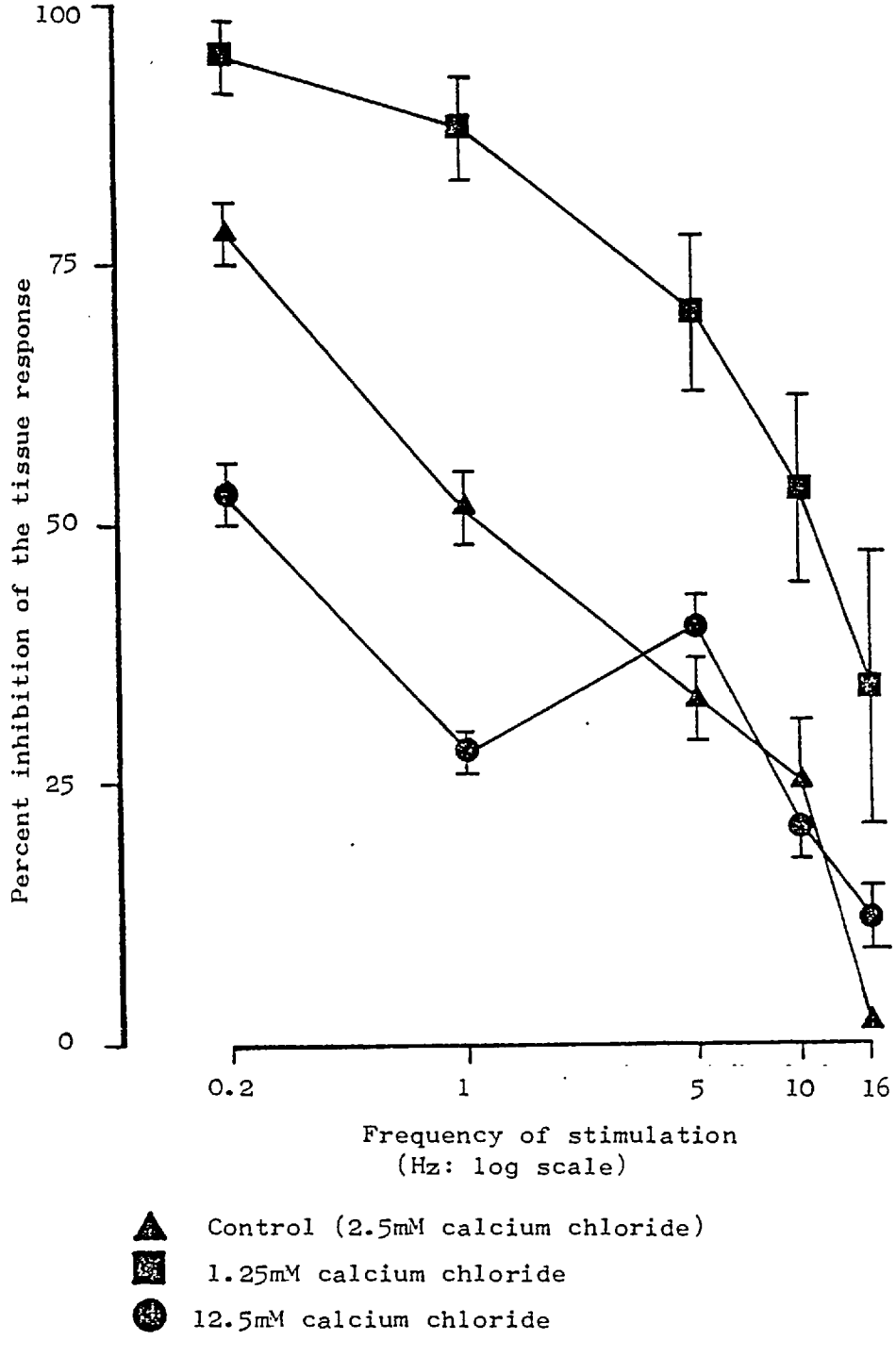


Fig. 18 The effect of changing the ionic composition of the Krebs solution upon the response of the mouse vas deferens to Clonidine (5.6nM) n=4

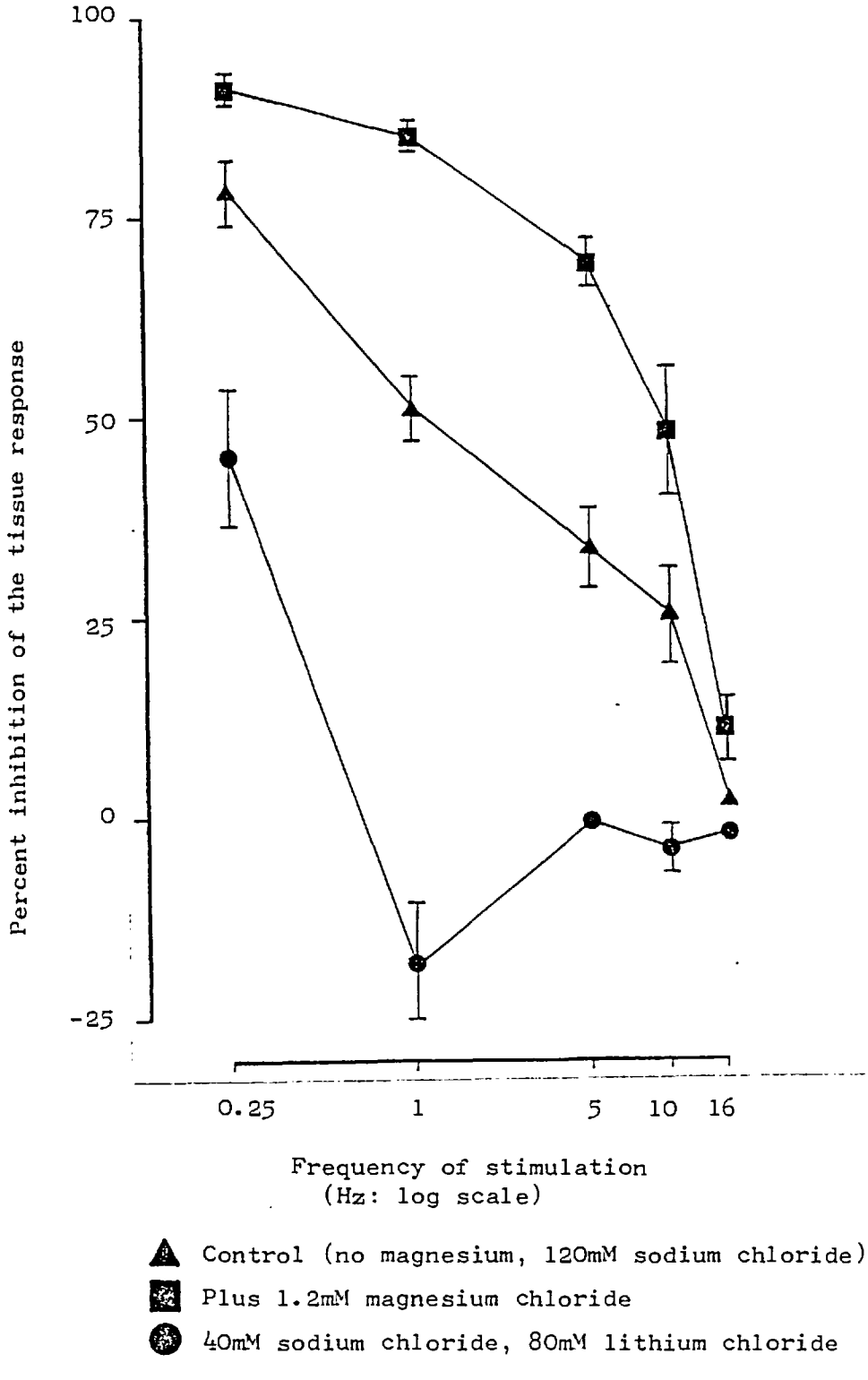


Fig. 19 The effect of changing the ionic composition of the Krebs solution upon the response of the mouse vas deferens to clonidine (5.6nM) n=4

Table 16

Effect of 56 nM clonidine on the tension developed by the mouse vas deferens in response to noradrenaline

	TENSION (mg)				
CONCENTRATION OF NA (μM)	1	3	10	30	100
CONTROL	165.0 ± 30.0	297.0 ± 17.5	503.5 ± 83.5	844.0 ± 97.6	747.0 ± 64.0
PLUS CLONIDINE	162.5 ± 20.0	273.8 ± 19.2	444.0 ± 66.9	712.5 ± 96.5	731.3 ± 55.3

figures are mean ± S.E. n = 4

The effect of yohimbine on the twitch response of the mouse vas deferens

Yohimbine potentiated the twitch response to electrical stimulation at 0.2 Hz in a dose dependent manner (fig 20). Unlike other alpha-adrenoceptor blocking agents (Part I, Section H) which blocked the second phase developed after the peak tension at 10 Hz and 16 Hz, yohimbine did not affect it (fig 21). Yohimbine (10 nM) decreased the inhibition produced by clonidine (fig 22).

The yohimbine potentiation of the twitch response was frequency dependent, increasing from 0.2 Hz to 1.0 Hz and decreasing as the frequency was further increased (fig 23 and 24). Decreasing the calcium content of the Krebs by fifty percent shifted the frequency response curve to the right, moving the peak to 5 Hz, the potentiation at 0.2 Hz and 1 Hz being less than in normal Krebs, and being greater at 5 Hz, 10 Hz and 16 Hz. (fig 23). Adding magnesium to the Krebs solution also shifted the curve to the right, moving the peak to 5 Hz. However the potentiation was greater than in normal Krebs at all frequencies except 0.2 Hz. The increase in potentiation of the twitch response produced by magnesium was far greater than the increase produced by decreasing the calcium. (fig 24).

Increasing the calcium content of the Krebs five fold had no significant effect on the frequency response curve. Replacing two-thirds of the sodium chloride with lithium chloride significantly reduced the potentiation from that in normal Krebs at 1 Hz, 5 Hz and 10 Hz though the peak potentiation remained at 1 Hz (fig 24).

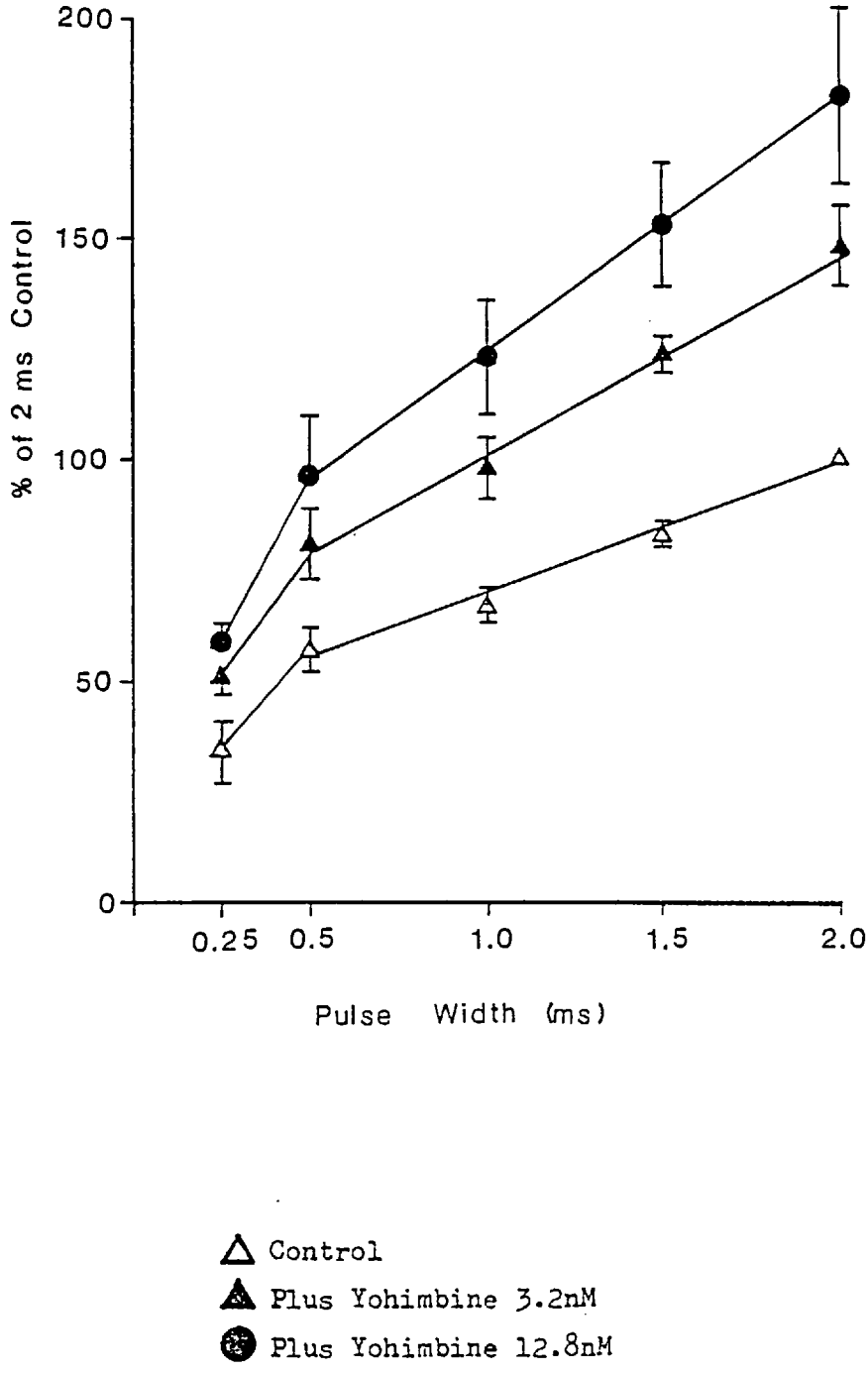


Fig. 20 Response of the mouse vas deferens to field stimulation (64v., 0.2Hz) at various pulse widths and its potentiation by yohimbine. Results are expressed as a percentage of the 2ms control response of each tissue to minimise variation between experiments. n=4

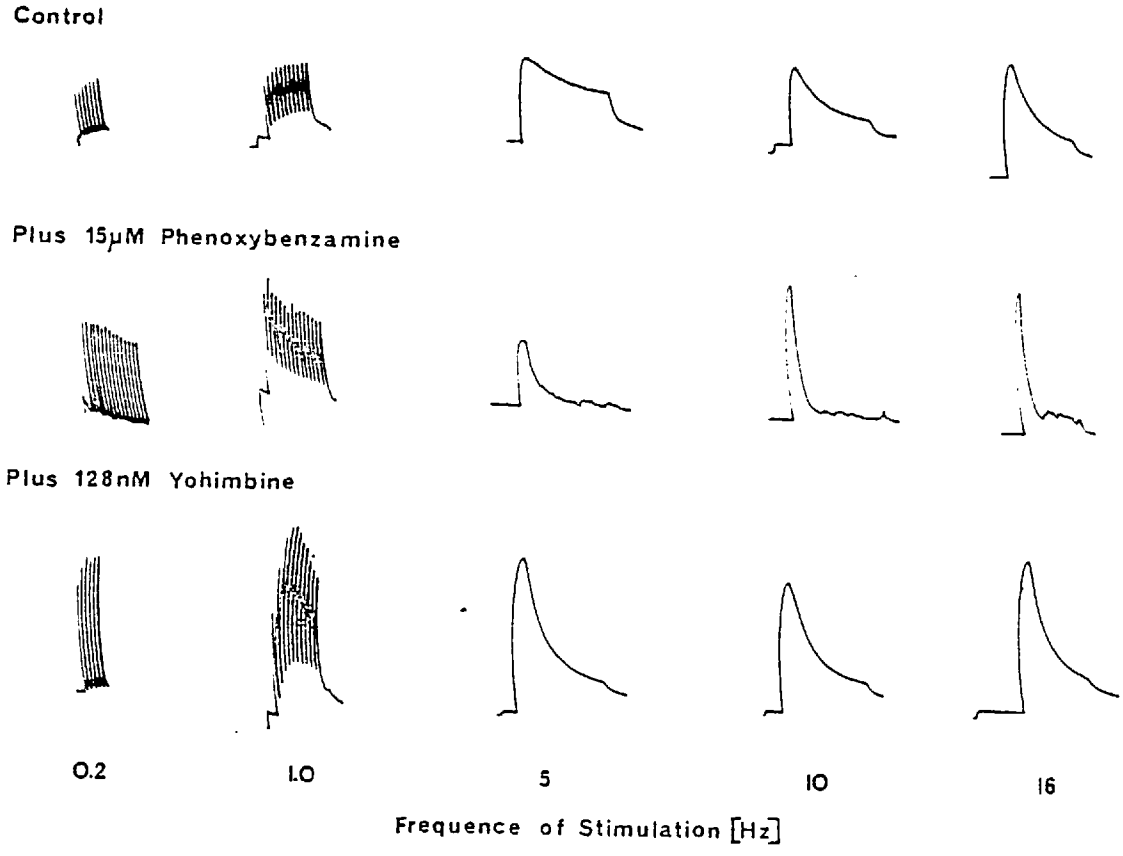


Fig. 21 The qualitative effect of pre- and post-synaptic alpha adrenoceptor blocking agents on the tissue response at various frequencies of stimulation.

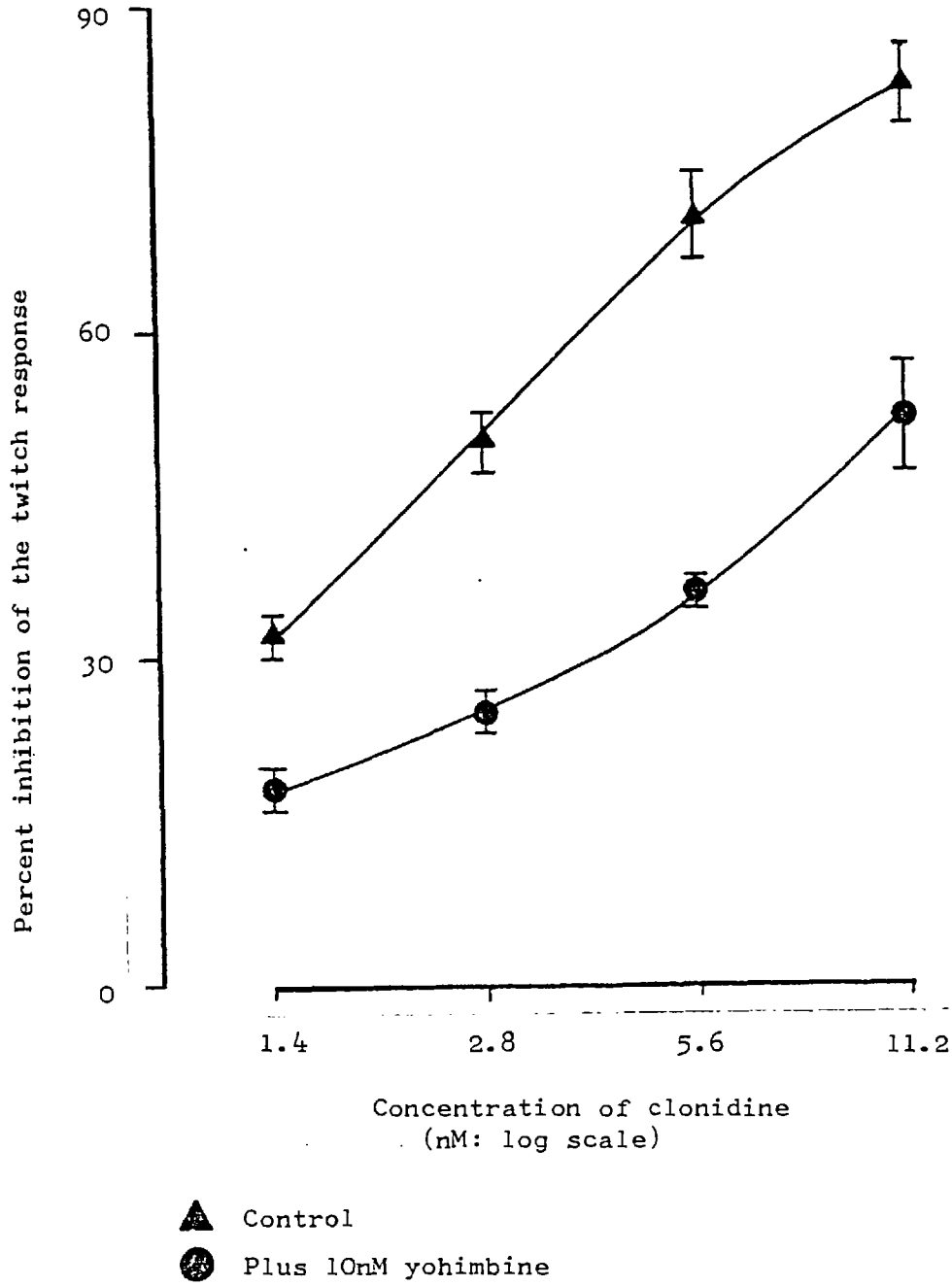


Fig. 22 The effect of yohimbine (10nM) on the inhibition of the twitch response of the mouse vas deferens to field stimulation (2.0ms, 0.2Hz) by clonidine. n=4

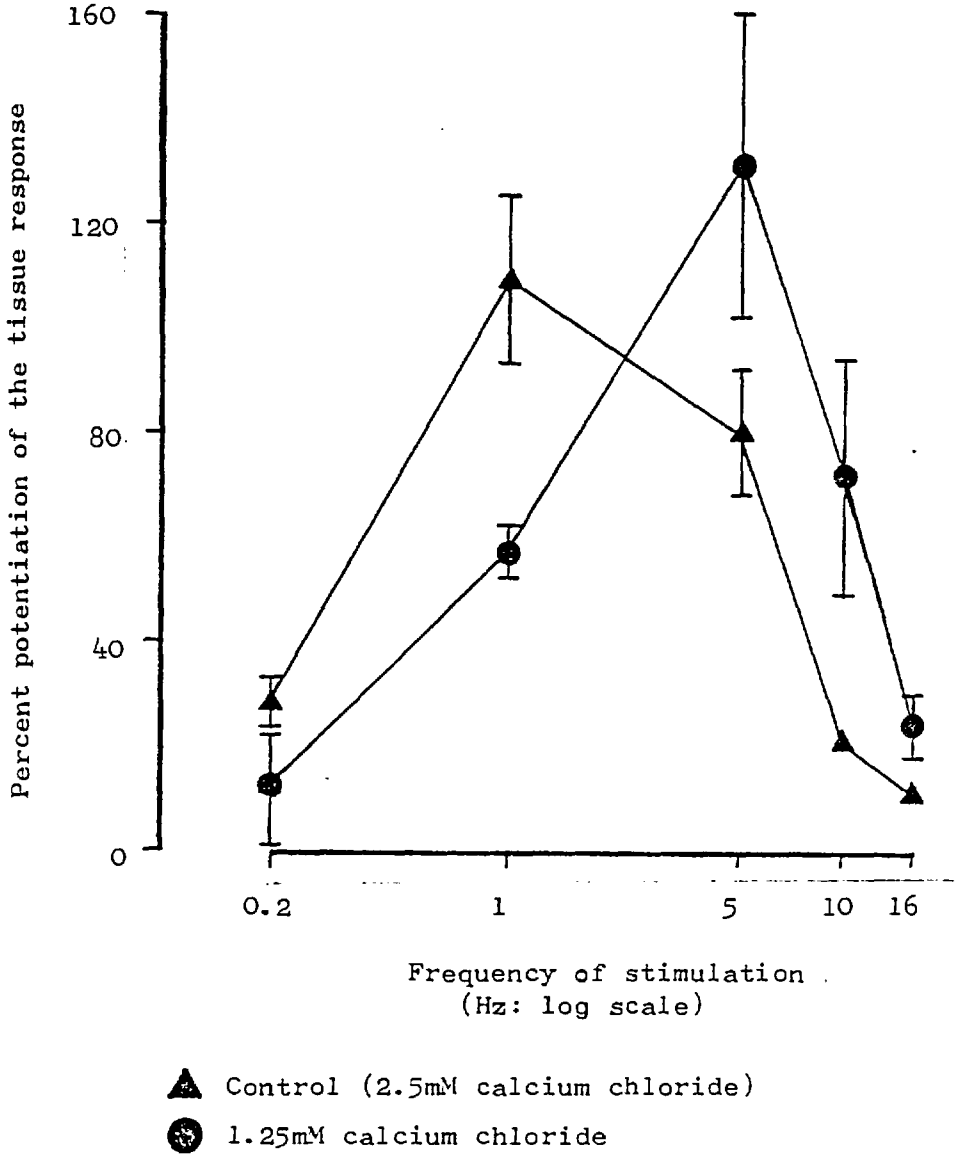


Fig. 23 The effect of halving the calcium ion concentration of the Krebs solution upon the potentiation of the tissue response by yohimbine (128nM) n=4

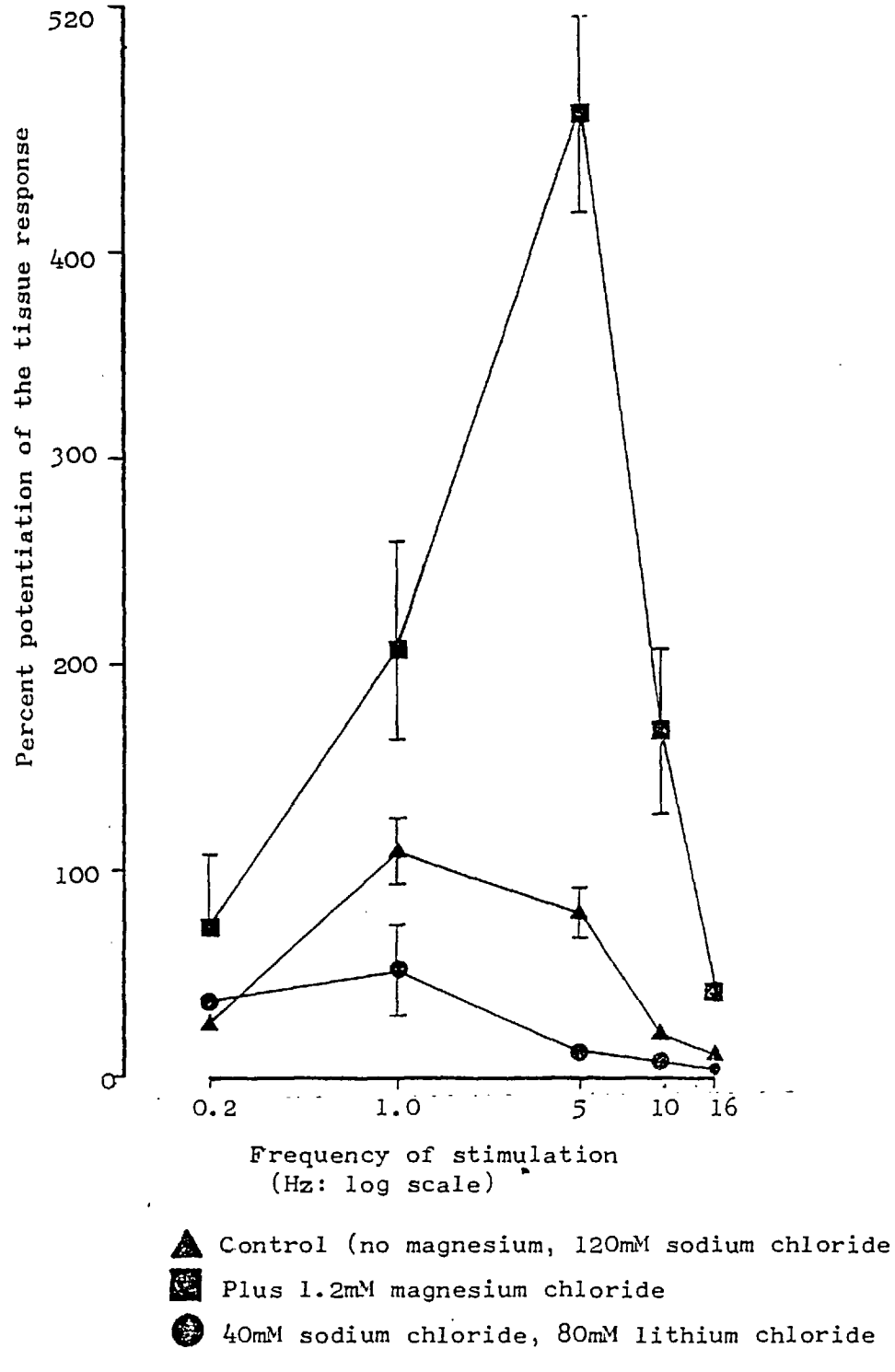


Fig. 24 The effect of changing the ionic composition of the Krebs solution upon the response of the mouse vas deferens to Yohimbine (128nM) n=4

Characterisation of the noradrenaline inhibition of the twitch response of the mouse vas deferens

The noradrenaline inhibition of the twitch response at 0.2 Hz was decreased by yohimbine. If the yohimbine (10 nM) was added to the organ bath 2 minutes before stimulation of the tissue the noradrenaline dose response curve was moved to the right ($P < 0.05$, fig 25). However if the yohimbine (10 nM) was added to the Krebs 20 minutes before stimulation the noradrenaline inhibition became a potentiation (fig 26). Furthermore this potentiation by yohimbine could be converted back to an inhibition, which was smaller than that produced by noradrenaline alone, by adding phentolamine (10 μM) to the Krebs in the presence of yohimbine (fig 26). The noradrenaline inhibition of the twitch was frequency dependent (fig 27) with a frequency response curve resembling that of clonidine. In Krebs containing only half the normal calcium concentration noradrenaline added to the organ bath 30 seconds before stimulation (see methods, Part 1) produced contractions of the tissue which interfered with the assessment of its effect on the twitch response. To eliminate these contractions 33 nM phenoxybenzamine was added to the Krebs solution. This procedure did not effect the frequency response curve in normal Krebs. Halving the calcium concentration of the Krebs solution increased the noradrenaline inhibition of the twitch response at all frequencies of stimulation (fig 27).

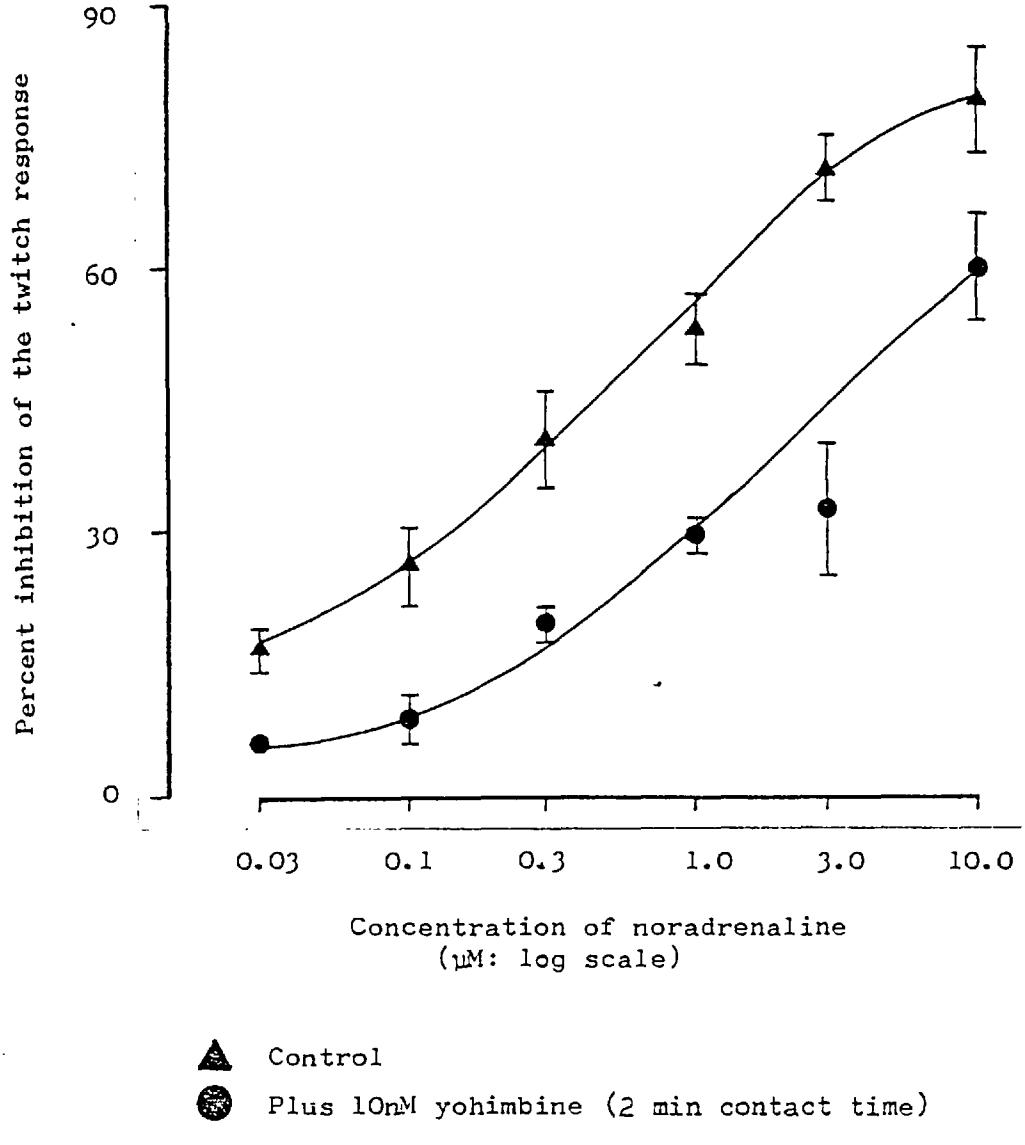


Fig.25 Effect of yohimbine (10nM) on the inhibition of the twitch response of the mouse vas deferens to field stimulation (2.0ms, 0.2Hz) by noradrenaline n=4

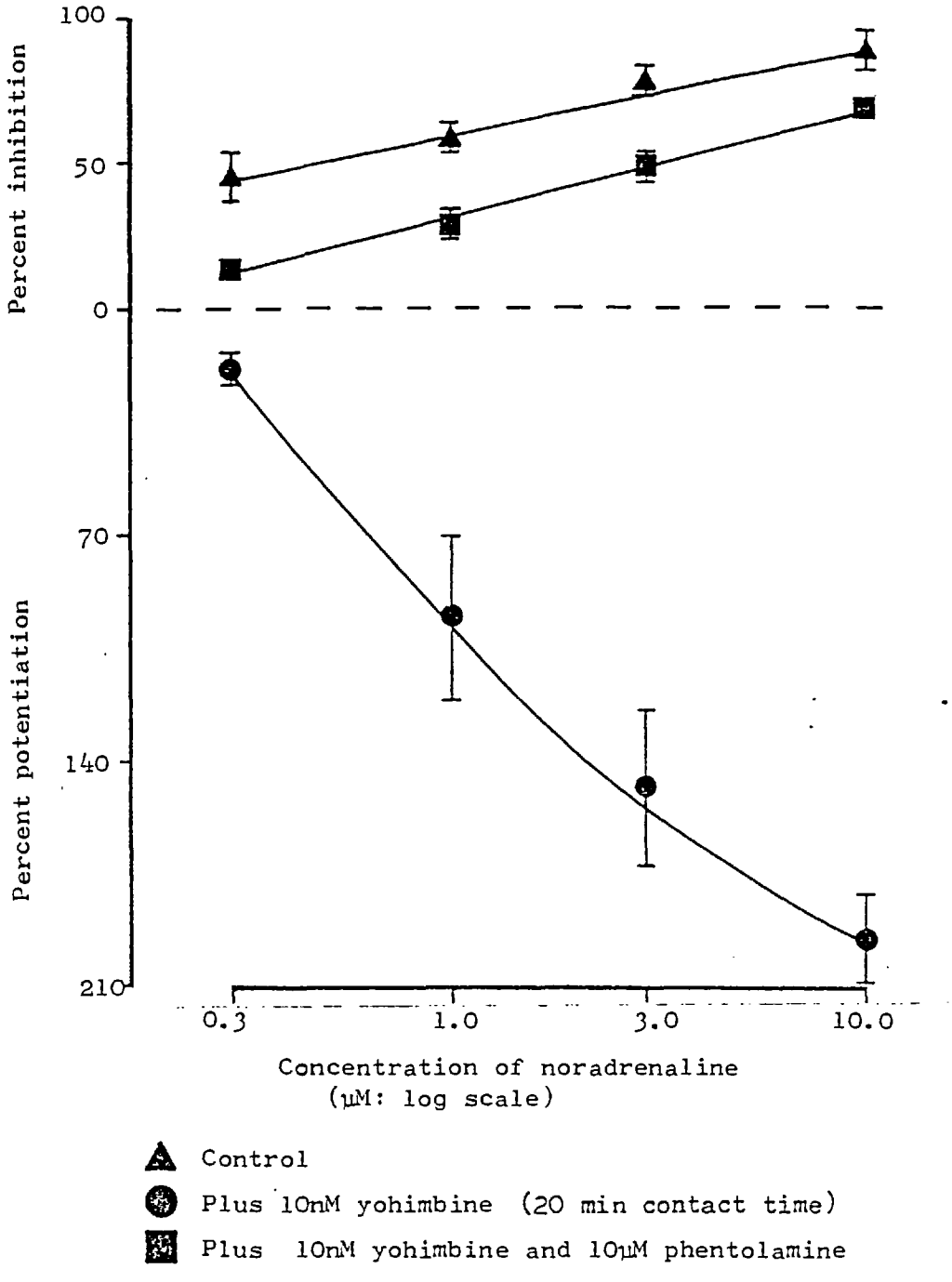


Fig. 26 The effect of α-adrenoceptor blocking agents on the noradrenaline inhibition of the twitch response of the mouse vas deferens to field stimulation (2Hz, 2ms, n=4)

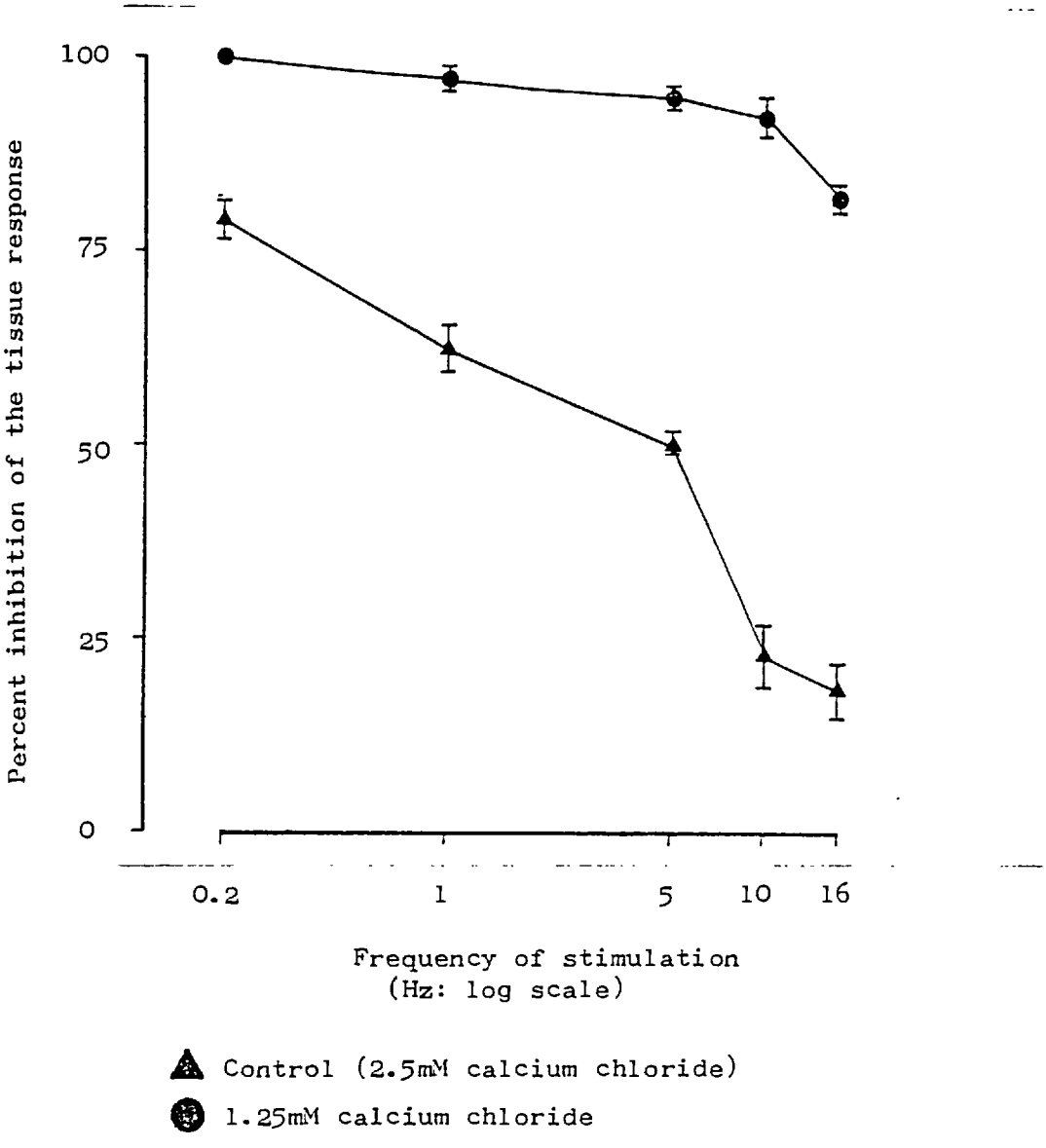


Fig.27 The effect of halving the calcium ion conentration of the Krebs solution upon the inhibition of the tissue response by noradrenaline (3μM) n=4

Characteristics of the inhibition of the twitch response of the mouse vas deferens to electrical stimulation, due to uptake blockade

The inhibition of the twitch response to electrical stimulation at 0.2 Hz due to oestradiol (3.7 μM) and cocaine (10 μM) was reversed to a potentiation by yohimbine (fig 28). The inhibition due to uptake blockade with these drugs was frequency dependent, as was the inhibition produced by cocaine alone (fig 29). In both cases the inhibition peaked at 1 Hz, resembling the yohimbine frequency response curve (fig 23 and 24) more than the clonidine curve (fig 18 and 19). The cocaine inhibition was significantly greater than that due to oestradiol and cocaine at all frequencies ($P < 0.05$), the latter combination only producing a significant inhibition at 0.2 Hz and 1 Hz (fig 29). Halving the calcium content of the Krebs solution increased the inhibition produced by oestradiol and cocaine at all frequencies of stimulation (fig 30, $P < 0.05$). This curve resembles that of clonidine, the greatest inhibition being at 0.2 Hz and not 1 Hz.

The characteristics of the tyramine inhibition of the twitch response of the mouse vas deferens to electrical stimulation

The tyramine inhibition of the twitch response was antagonised by yohimbine (10 nM) added 2 minutes before stimulation. (fig 31). When the yohimbine (10 nM) was added to the Krebs solution 20 minutes before stimulation the inhibition was further reduced. Addition of 100 nM yohimbine to the Krebs solution 20 minutes before stimulation turned the tyramine inhibition into a potentiation of the twitch response.

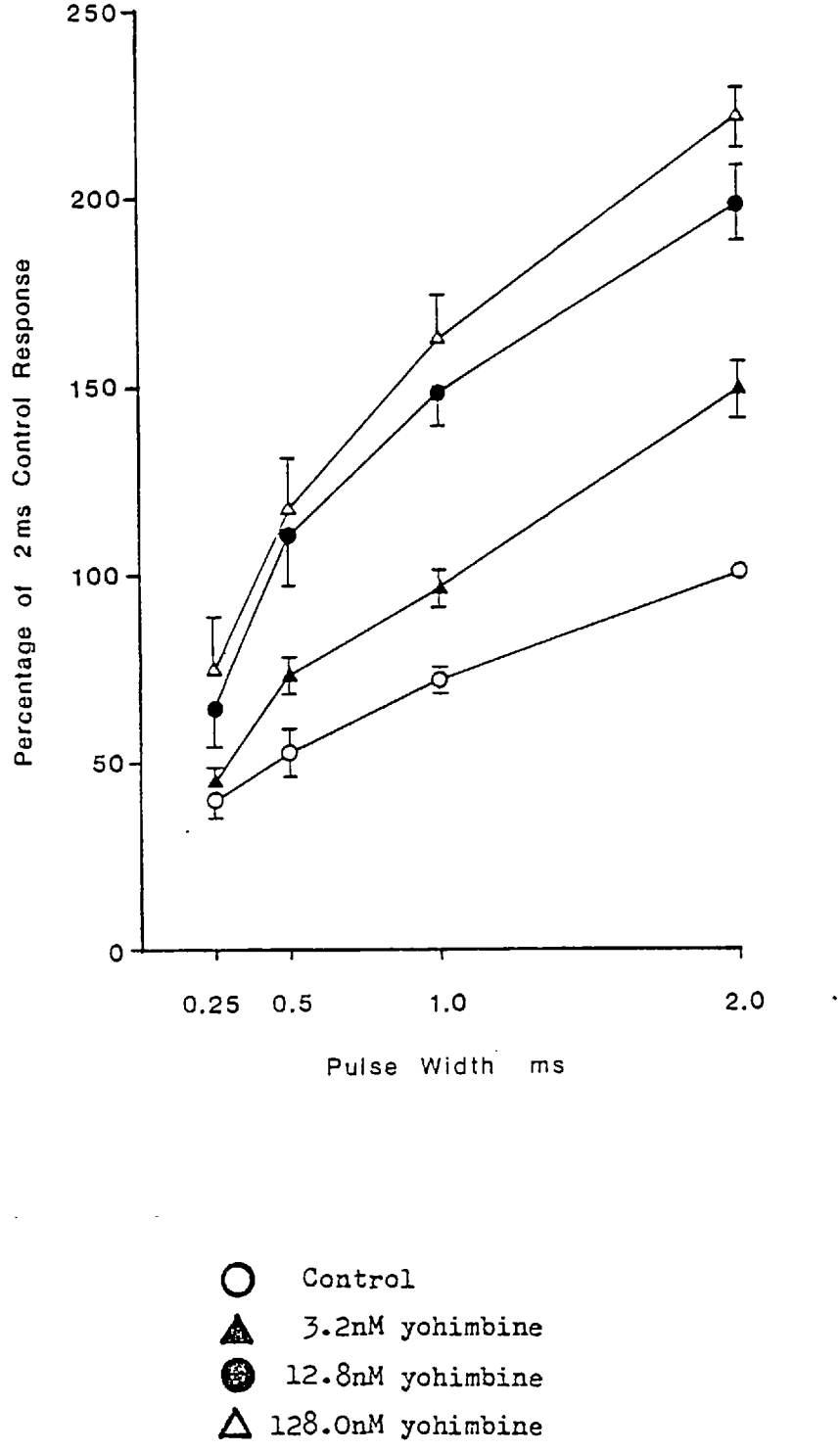


Fig. 28 The effect of yohimbine upon the response of the mouse vas deferens to field stimulation in the presence of oestradiol (3.7μM) and cocaine (10μM). n=4

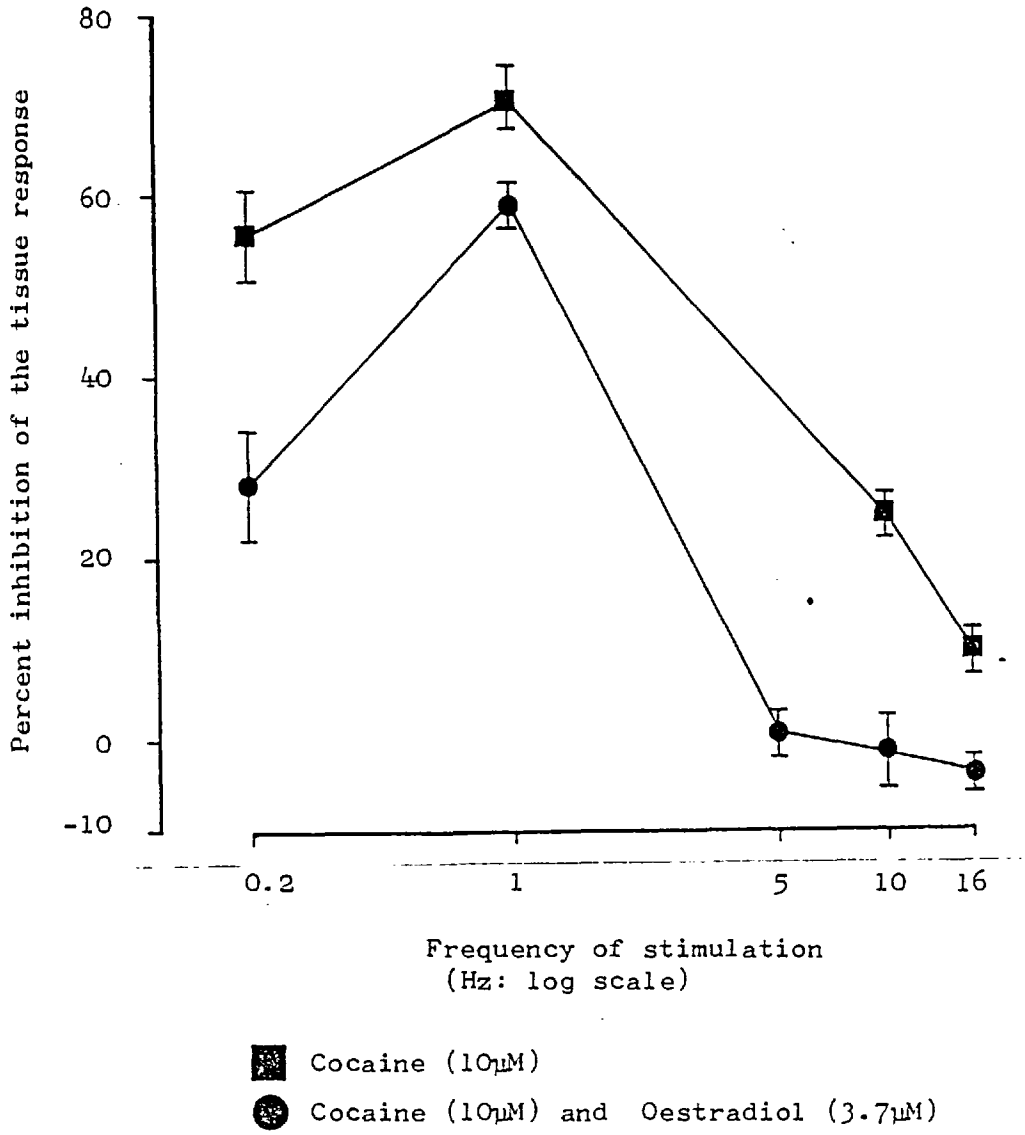


Fig. 29 Inhibition by cocaine and cocaine plus oestradiol of the response of the mouse vas deferens to field stimulation at various frequencies. n=4

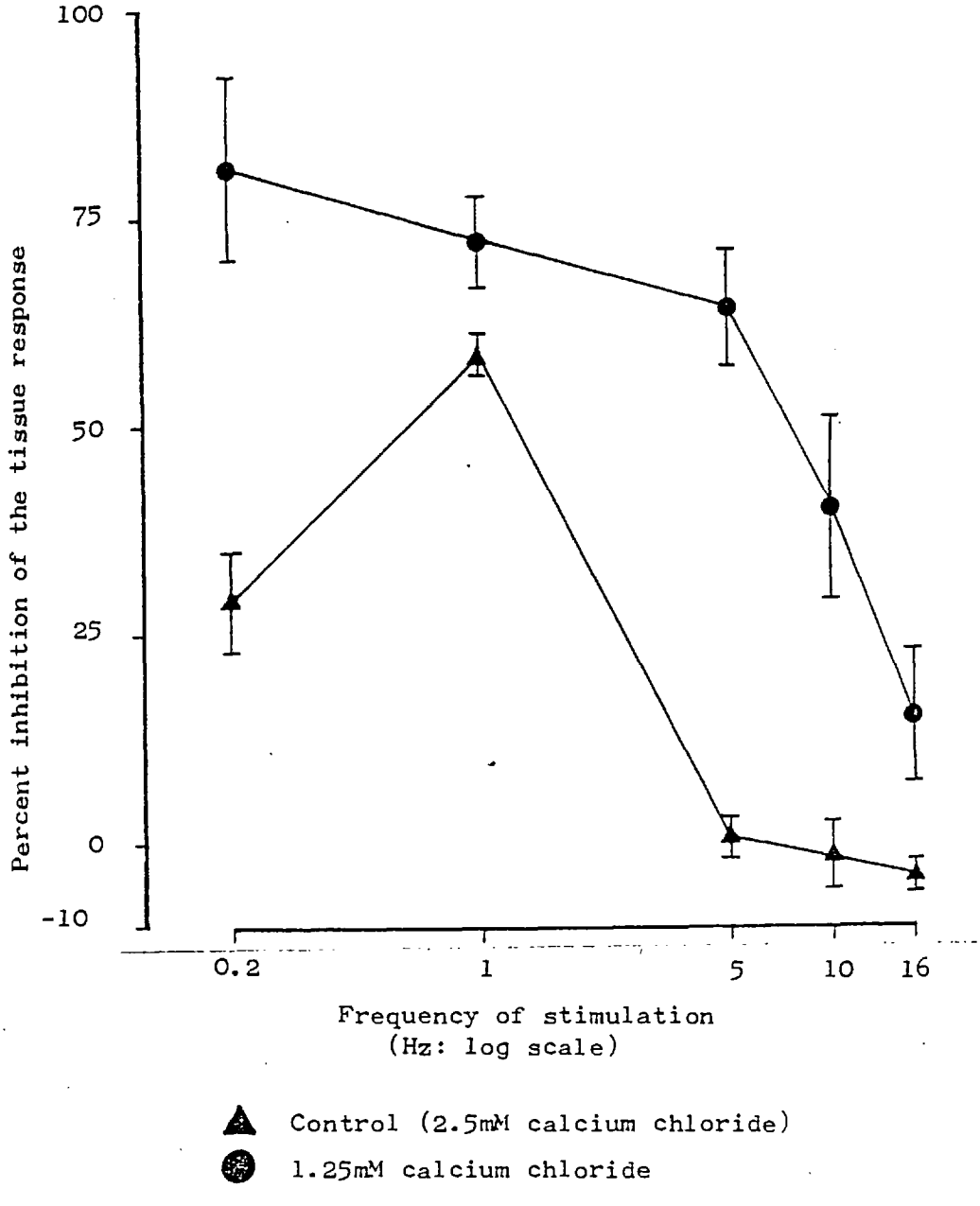


Fig. 30 The effect of halving the calcium ion concentration of the Krebs solution on the inhibition of tissue response by oestradiol (3.7μM) + cocaine (10μM). n=4

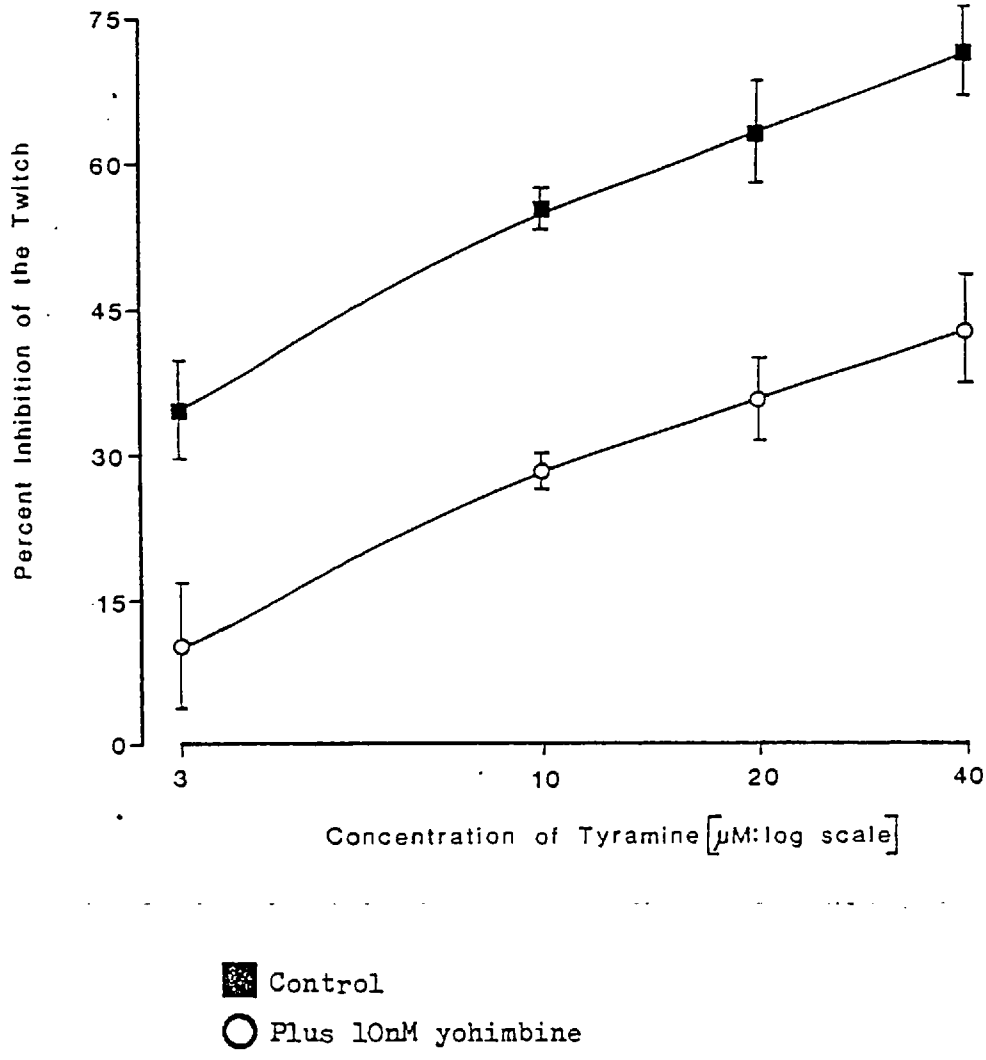


Fig. 31 The effect of yohimbine (10nM) on the inhibition of the twitch response of the mouse vas deferens by tyramine n=4.

(fig 32). This potentiation can be reversed once more to an inhibition by addition of 33 nM phenoxybenzamine to the Krebs in the presence of 100 nM yohimbine (fig 32).
As discussed in Part 1, when the calcium content of the Krebs solution is halved, tyramine produces erratic contraction of the tissue during stimulation. To prevent these erratic contractions 33 nM phenoxybenzamine was added to the Krebs solution. In normal Krebs solution the inhibition produced by tyramine was frequency dependent being greatest at 0.2 Hz and least at 16 Hz (fig 33). In Krebs solution containing only half the normal calcium concentration this inhibition was potentiated at all frequencies of stimulation (fig 33).

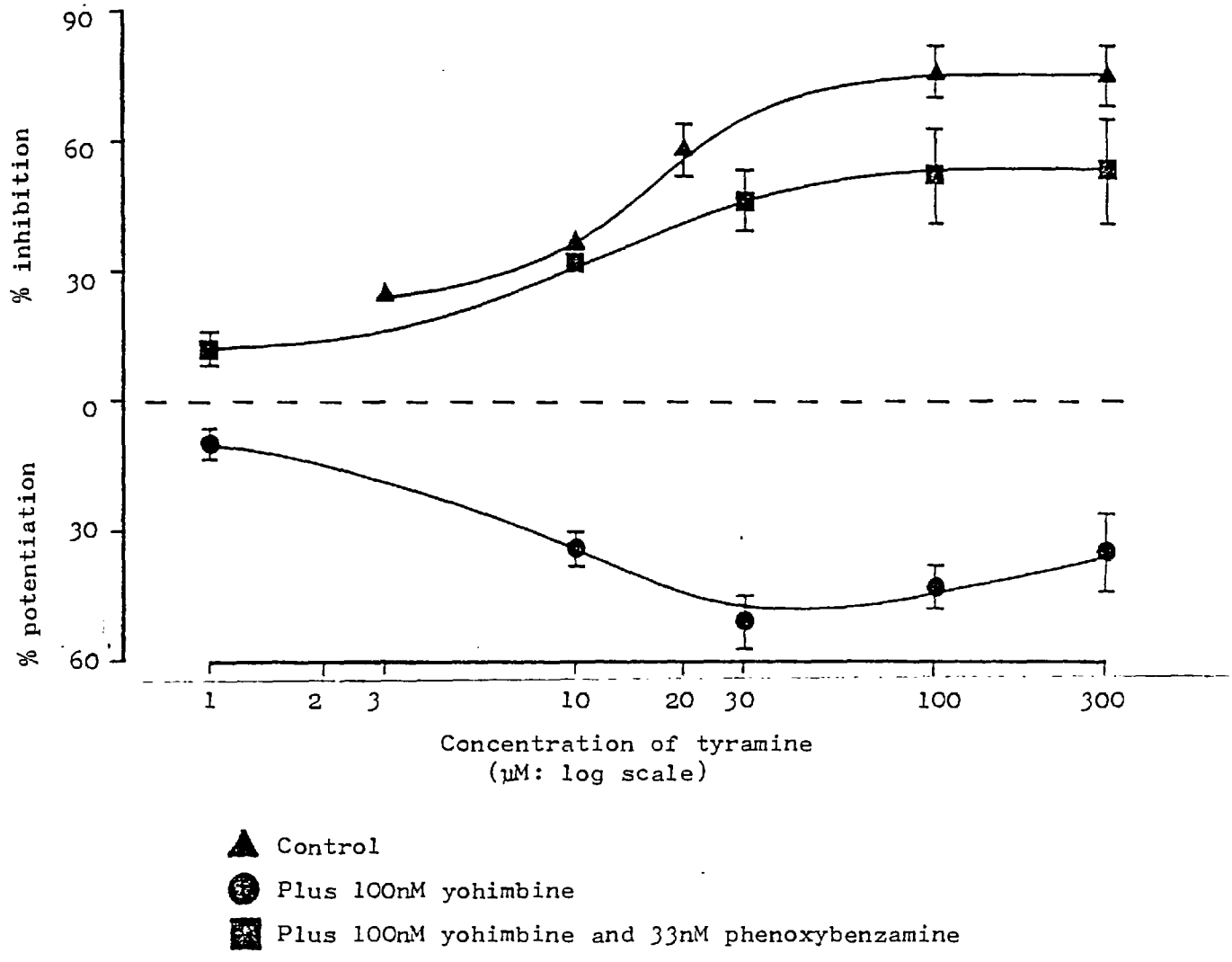


Fig 32 The effect of α-adrenoceptor blocking agents on the tyramine inhibition of the twitch response of the mouse vas deferens to field stimulation (2Hz, 2ms) n=4

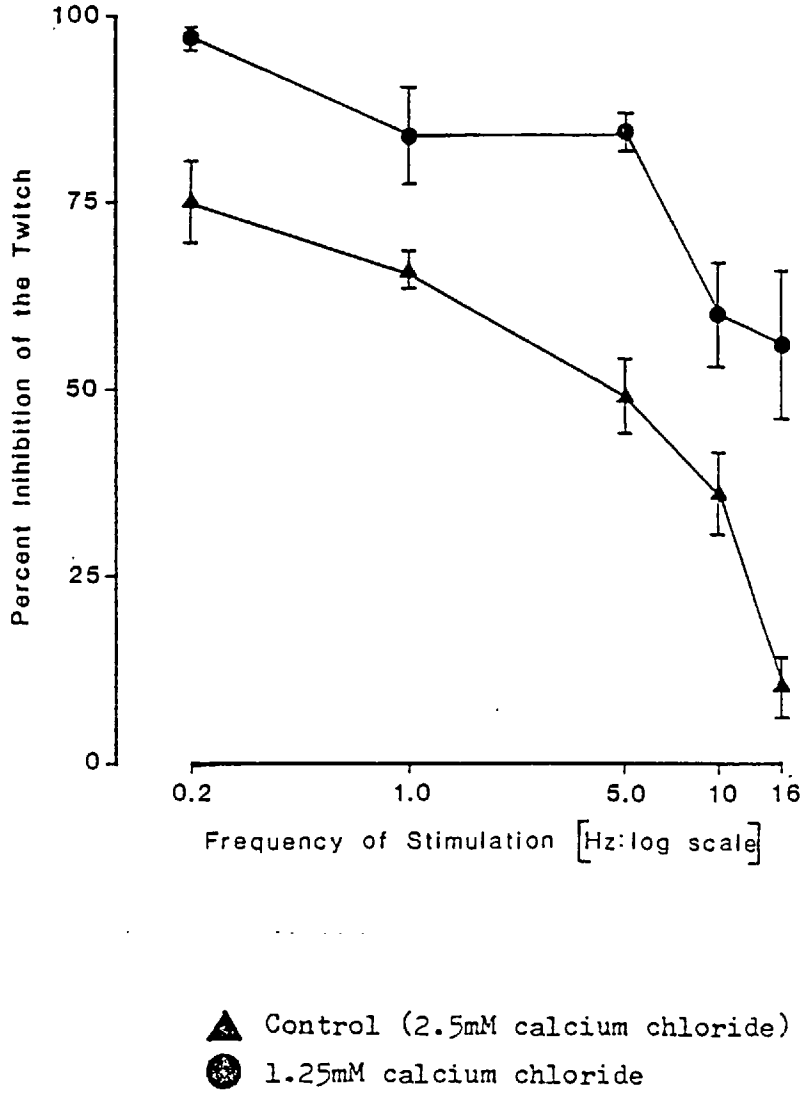


Fig. 33 The effect of halving the calcium ion concentration of the Krebs solution upon the inhibition of the tissue response by tyramine (20μM) in Krebs containing 33nM phenoxybenzamine. n=4.

Characteristics of the potentiation of the twitch response of the mouse vas deferens by alpha-adrenoceptor blocking agents

The potentiation of the twitch response produced by 15 μM phenoxybenzamine was frequency dependent, with a peak at 1 Hz (fig 34) resembling the curve for yohimbine. Halving the calcium content of the Krebs solution increased the potentiation at 0.2 Hz and 1 Hz ($P < 0.05$) but not at 5 - 16 Hz ($P > 0.05$) (fig 34). This produced a peak potentiation at 0.2 Hz and not at 5 Hz as was seen with yohimbine in half calcium Krebs.

Phenoxybenzamine (33 nM) does not potentiate the twitch or alter the inhibition produced by clonidine (1.5 - 128 nM), however 15 μM phenoxybenzamine completely abolishes the clonidine inhibition up to and including a concentration of 5.6 μM.

The potentiation of the twitch response produced by 10 μM phentolamine was frequency dependent with a maximum at 1 Hz (fig 35). This potentiation was less at 0.2 Hz and 1 Hz and greater at 10 Hz and 16 Hz than that produced by phenoxybenzamine (15 μM). To investigate the role of uptake blockade in this potentiation 10 μM cocaine was added with the 10 μM phentolamine. This caused a small but non-significant rise in the potentiation at 0.2 Hz and 1 Hz and a fall at 10 Hz and 16 Hz, producing a curve resembling more closely that of phenoxybenzamine. Halving the calcium content of the Krebs solution (in the absence of cocaine) increased the potentiation produced by phentolamine at 1 - 5 Hz producing a peak at 5 Hz ($P < 0.05$, fig 35).

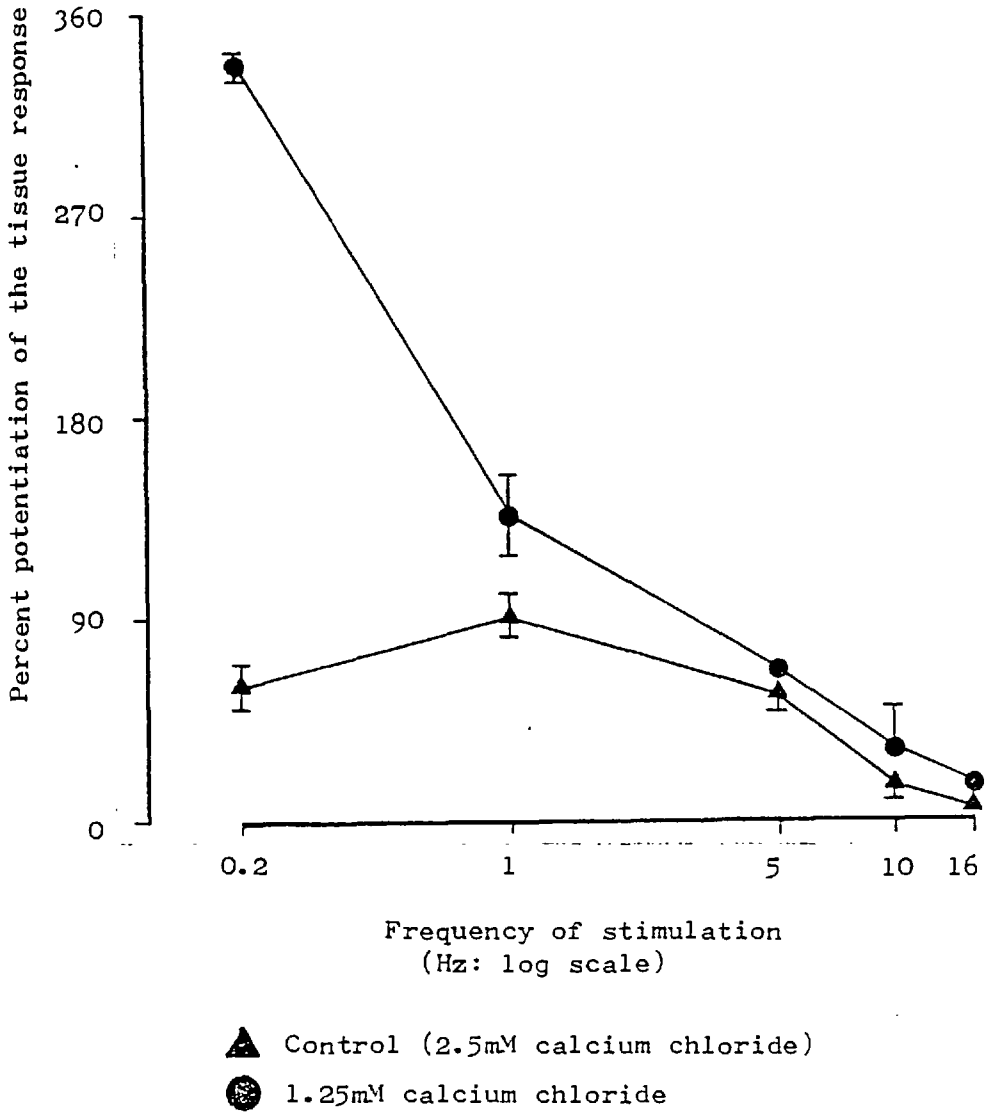


Fig. 34 The effect of halving the calcium ion concentration of the Krebs solution upon the potentiation of the tissue response by phenoxybenzamine (15μM) n=4

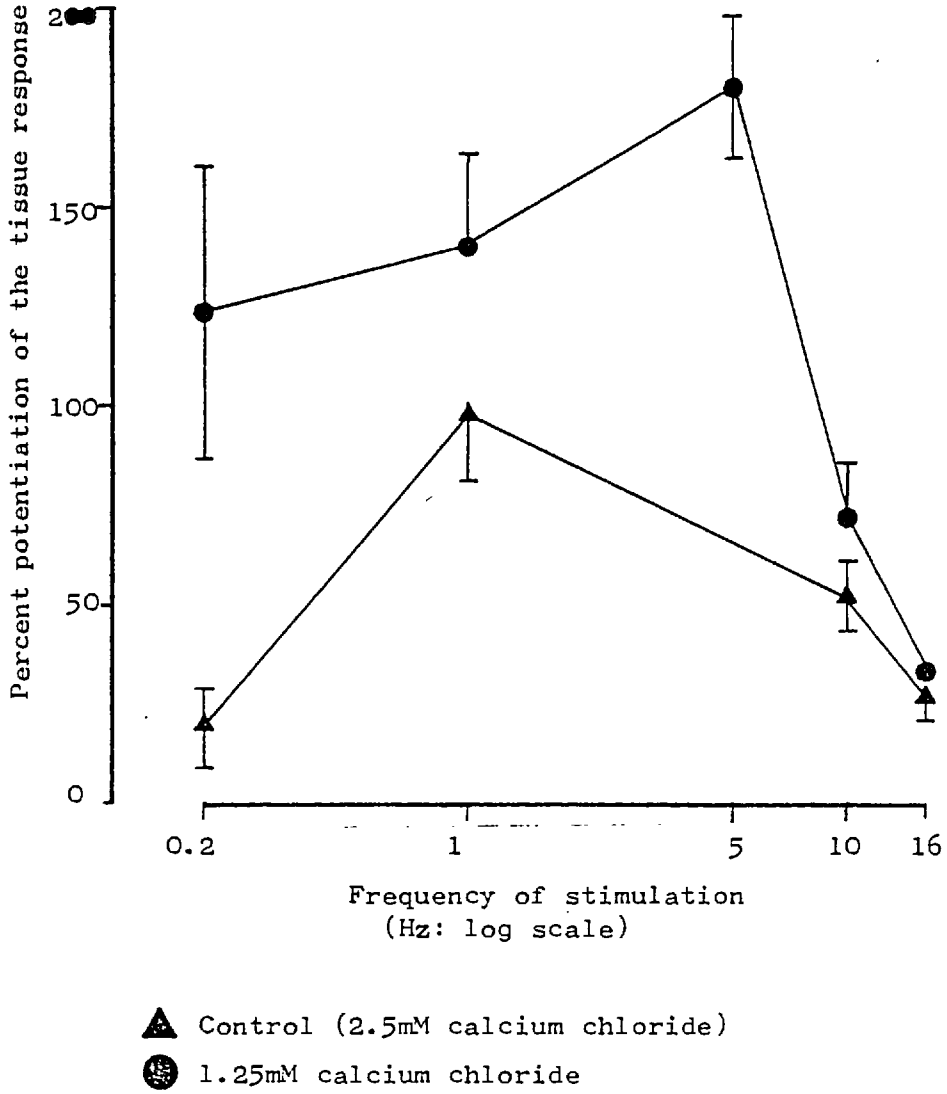


Fig. 35 The effect of halving the calcium ion concentration of the Krebs solution upon the potentiation of the tissue response by phentolamine (10μM) n=4

Effect of presynaptic alpha-adrenoceptors on the response of the tissue to constant stimulation

Addition of 100 nM yohimbine to the Krebs solution 20 minutes before stimulation decreased the decline in response during constant stimulation (2 ms, 0.1 Hz) after 10, 15 and 20 minutes (Table 17, $P < 0.05$).

Effect of depleting the noradrenaline content of the mouse vas deferens on the response to yohimbine

Despite the noradrenaline content of vasa deferentia from animals pretreated with reserpine being greater than that of those from animals pretreated with reserpine and αMept, the twitch response to electrical stimulation of the latter is greater than that of the former. Addition of yohimbine (128 nM but not 3.2 nM) to the organ bath two minutes before stimulation of these tissues, potentiates the response of vasa deferentia from animals pretreated with reserpine, but not the response of those from animals pretreated with reserpine and αMept. This potentiation is much less than that seen when vas deferens from untreated animals are stimulated in the present of yohimbine (table 18).

Table 17

The effect of yohimbine upon the decline of the tissue response on constant stimulation

DURATION OF STIMULATION (MINUTES)	PERCENT DECREASE IN RESPONSE	
	CONTROL	100 nM YOHIMBINE
5	27.3 ± 1.3	31.2 ± 8.2
10	41.4 ± 2.0	29.8 ± 6.8
15	52.1 ± 1.8	40.0 ± 4.5
20	62.2 ± 2.9	41.4 ± 3.4

Table 18

The effect of reserpine and reserpine plus α-Mept pretreatment upon the response of the stimulated mouse vas deferens to yohimbine

PRETREATMENT		PERCENT POTENTIATION OF THE TWITCH	
	n	3.2 nM YOHIMBINE	128 nM YOHIMBINE
Control	5	39.7 ± 8.2	129.8 ± 13.5
Reserpine	4	0	13.6 ± 7.3
Reserpine plus α-Mept	4	4.9 ± 4.0	0

DISCUSSION

Marshall et al (1978) have shown that clonidine and yohimbine act selectively at presynaptic alpha-adrenoceptors in this tissue. This selectivity has been used to investigate some of the properties of these receptors, namely their frequency and ion dependence previously described. The responses to other drugs have been analysed in terms of this dependency to find out if they too are mediated via presynaptic alpha-adrenoceptors. As it is well known that certain ions are required for the normal function of nerves and muscle, preliminary experiments were carried out to examine the effects of changing the ionic composition of the Krebs solution on the normal responses of the tissue.

Since the discovery of Hukovic and Muscholl (1962) that decreasing the external calcium ion concentration decreased the release of noradrenaline from adrenergic neurones, the role of ions in transmitter release has been extensively investigated using several methods. For example, Farmer and Campbell (1967), and Burn and Gibbons (1964) measured the tissue response in varying ionic conditions; Boullin (1967), and Kirpekar and Misu (1967), measured noradrenaline overflow; and Kuriyama (1964), measured membrane potentials. All these authors agree that calcium is required for noradrenaline release and that magnesium antagonises the effect of calcium. It has been proposed that magnesium competes for a calcium binding site involved in transmitter release. Decreasing the calcium ion concentration from the normal 2.5 mM found in physiological salt solutions decreases transmitter release. However the converse is not

true; Boullin (1967) reported that increasing the calcium concentration from 2.5 mM to 10 mM did not increase transmitter release.

The effect of ionic changes in the Krebs solution on the response of the mouse vas deferens to electrical stimulation is consistent with these findings. Decreasing the calcium concentration or the addition of magnesium decreased the response, while increasing the calcium to 12.5 mM had no significant effect. Though these results are consistent with what one would expect from changes in transmitter release, one must also consider the effect of these ionic changes on the response of the smooth muscle. Unlike skeletal or cardiac muscle, the spike potential leading to the contraction of smooth muscle may be due to calcium influx rather than sodium (Brading, Bulbring and Tomita, 1969). To check the effect of ionic manipulation on the smooth muscle response, the effect upon contractions due to exogenous acetyl choline were recorded in Krebs of varying compositions. Bulbring and Kuriyama (1963) have reported that in calcium free solutions acetyl choline did not contract the guinea-pig taenia coli. Increasing the calcium concentration from 2.5 mM to 7.5 mM potentiated contractions produced by acetyl choline. In the mouse vas deferens increasing the calcium concentration five-fold did not potentiate the response. Halving the calcium concentration did not affect the response to acetyl choline up to 1.1 μM but decreased the response to higher concentrations. The addition of 1.2 mM magnesium chloride to the Krebs had no effect on

the response to concentrations of acetyl choline up to 1.1 μM, but potentiated the response to higher concentrations. This synergistic action of magnesium with calcium in the muscle response as opposed to its antagonism in nerves, supports the suggestion by Bulbring and Tomita (1970) that both divalent cations may be involved in the generation of a spike potential in smooth muscle.

These experiments demonstrate that it is possible to manipulate the ionic composition of the Krebs solution and produce predictable and consistent changes in the normal responses of the tissue. However as already discussed, increasing the calcium concentration, in these experiments five fold, does not produce the predicted increase in response. If calcium influx is important to normal nerve function (as well as to the smooth muscle), its influx will be carefully controlled. Therefore while decreasing the external calcium concentration will produce a scarcity of calcium ions available for influx, giving the predicted decrease in response, the converse need not be true if the calcium influx is controlled. Hence a method for producing an increase calcium influx was sought. Baker, Blaustein, Hodgkin and Steinhardt (1969), reported that replacing some of the sodium chloride in the bathing media with lithium chloride increased the calcium influx into squid axon. In the mouse vas deferens this procedure produced a potentiation of the response to electrical stimulation at 0.2 and 5 Hz, whilst decreasing the response to acetyl choline at all concentrations. If this ionic change produced an increase in calcium influx into nerves

in response to stimulation, and into muscle in response to acetyl choline, one would expect both responses to increase. However this is unlikely as the increased calcium influx probably occurs all the time and not only in response to a given stimulus. There are several problems in interpreting the effects of this substitution. It did not prove possible to measure calcium influx into the neurones of the mouse vas deferens, so it is not known if the effect is the same as in the squid axon. Even if such an increased calcium influx occurs it is not known if it is into a 'pool' involved in transmitter release. That this was not the case is suggested by the fact that the response to electrical stimulation was not increased uniformly. If the concept of calcium being involved in the spike generation in smooth muscle is correct (Brading et al., 1969) the effect of an increased influx of calcium into smooth muscle must also be considered. The fact that the response to acetyl choline was decreased suggests that this ionic substitution affects systems which are involved in the contraction of smooth muscle other than those requiring calcium.

The selective presynaptic alpha-adrenoceptor agonist clonidine (Starke et al., 1974), inhibited the twitch response of the mouse vas deferens in a dose dependent manner (0.28 - 28 nM). This inhibition was reduced by the selective presynaptic alpha-adrenoceptor antagonist yohimbine (Starke et al., 1975). The addition of yohimbine alone potentiated the response to electrical stimulation. These results are consistent with the presence of a presynaptic alpha-adrenoceptor in this tissue, stimulation

of which produces an inhibition of the response to electrical stimulation. Furthermore this receptor is normally effective during stimulation of the preparation, controlling the magnitude of the twitch, as its blockade potentiated the twitch. Investigation of the properties of this receptor showed both inhibition by clonidine and potentiation by yohimbine to be frequency dependent, agreeing with the findings of Vizi et al.,(1973). Whilst inhibition by clonidine was simply inversely proportional to the frequency of stimulation, potentiation by yohimbine showed a peak at 1 Hz, declining at higher and lower frequencies. This effect could be interpreted as being due to a balance point between the increasing concentration of noradrenaline in the synaptic cleft, which increases presynaptic receptor stimulation as the frequency of stimulation increases; and the decreasing effectiveness of presynaptic alpha-adrenoceptors as the frequency increases. However this result is inconsistent with the explanation of Vizi et al.,(1973) for the frequency dependence as outlined in the introduction. Briefly these authors argued that exogenous presynaptic agonists were less effective at high frequencies because of stimulation of these receptors by endogenous noradrenaline; as the frequency of stimulation rises, so does the concentration of noradrenaline in the synaptic cleft. If this were so presynaptic antagonists would produce a greater potentiation as the frequency increases. As this is not the case, such an explanation for the frequency dependence may not be correct. According to the calcium availability explanation for this

frequency dependence as outlined in the introduction, if the calcium influx into the nerves is restricted then the effects of presynaptic alpha-adrenoceptor stimulation will be increased. When the calcium content of the Krebs is halved or when magnesium is added the inhibitory effect of clonidine is increased. The effect of magnesium may be due to its ability to compete with calcium for a binding site. Clonidine's inhibition is decreased in the lithium substituted Krebs and at low frequencies in the high calcium Krebs. This is consistent with the theory that increasing calcium influx, decreases the effectiveness of presynaptic alpha-adrenoceptors. It is logical to conclude that as the effect of presynaptic alpha-adrenoceptor stimulation is increased in low calcium solutions then the potentiation of the twitch response when these receptors are blocked would be greater than in normal Krebs, as the twitch is under greater presynaptic control. However in low calcium solutions the release of transmitter is also decreased, and therefore a higher frequency of stimulation is required to release enough noradrenaline to stimulate the presynaptic receptors. Hence the yohimbine frequency response curve is shifted to the right in low calcium Krebs solutions, peaking at 5 Hz instead of 1 Hz, 0.2 Hz and 1 Hz producing less potentiation than in normal Krebs, 5 - 16 Hz producing a greater potentiation. The addition of magnesium to the Krebs solution produces a potentiation of the response to yohimbine at all frequencies, with a peak at 5 Hz, the potentiation being greater than that seen when the calcium concentration was lowered. This greater effect of magnesium may be due to the fact that it does not reduce the amount of calcium that is available. These results are

consistent with the view that magnesium competes with calcium at some site involved in the presynaptic alpha-adrenoceptor regulatory mechanism. The shift in the peak of the response to yohimbine is consistent with calcium influx being dependent upon the frequency of stimulation. Raising the calcium concentration of the Krebs had no effect upon the response to yohimbine, once again possibly reflecting the control of calcium influx by a mechanism other than the presynaptic alpha-adrenoceptor. However in the lithium substituted Krebs the response to yohimbine is decreased as one would predict if the influx of calcium was increased thus negating the effect of presynaptic alpha-adrenoceptor stimulation.

These results support the existence of a presynaptic alpha-adrenoceptor in the mouse vas deferens which regulates the response to stimulation. Marshall (1978) and Marshall, Shepperson and Nasmyth (1979), have reported that histamine inhibits the twitch response of the mouse vas deferens. Histamine, unlike clonidine, inhibited the contractions of the vas deferens produced by exogenous noradrenaline, and did not alter the overflow of noradrenaline from the stimulated vas deferens preparation (see Part III). It appears therefore that histamine's inhibition was mediated via post-synaptic receptors. When its inhibition was examined at various frequencies of stimulation, unlike clonidine's inhibition, it remained constant at 0.2 Hz - 5 Hz decreasing at 10 Hz and 16 Hz. Furthermore histamine's inhibition was not calcium dependent. These results suggest that frequency and calcium dependence as reported for clonidine are characteristic of presynaptic inhibition, and

are not exhibited by drugs which inhibit the twitch response via post synaptic receptors. The effects of other compounds on the twitch response of this tissue were now examined in the light of the evidence for a presynaptic alpha-adrenoceptor which is characteristically both frequency and calcium dependent.

The inhibition of the twitch response produced by noradrenaline is antagonised by yohimbine, and is frequency and calcium dependent. The frequency response curve resembles that of clonidine and is shifted to the right in low calcium Krebs solutions. These results suggest that noradrenaline's inhibition of the twitch response is due to stimulation of a presynaptic alpha-adrenoceptor. This was confirmed by carrying out a pA_2 determination by the method of Schild (1947). The pA_2 of yohimbine versus clonidine was 9.4 ± 0.3, and yohimbine versus noradrenaline was 8.9 ± 0.3.

Blockade of neuronal and extra-neuronal noradrenaline uptake with cocaine and oestradiol produced a frequency and calcium dependent inhibition of the twitch response which was reversed to a potentiation by yohimbine. These results suggest that this inhibition is also mediated via presynaptic alpha-adrenoceptors. Cocaine alone also produces a frequency dependent inhibition of the twitch, which is greater at all frequencies than the inhibition produced by oestradiol and cocaine. As already shown in Part I, if extra-neuronal uptake is blocked the neuronal uptake of (^{3}H)-noradrenaline increases. Thus in the presence of oestradiol, cocaine produces a less effective blockade of neuronal uptake, probably because it is a competitive inhibitor and blockade of extra-neuronal uptake will

increase the concentration of noradrenaline in the synaptic cleft. If neuronal uptake were important for removal of noradrenaline from the vicinity of the presynaptic alpha-adrenoceptor, the more effective blockade by cocaine in the absence of oestradiol would produce a greater inhibition of the twitch than in its presence. Jenkins, Marshall and Nasmyth (1977) have reported that oestradiol alone produces only a small and variable inhibition of the twitch. This suggests that extra-neuronal uptake is less important than neuronal uptake for the removal of noradrenaline from the vicinity of the presynaptic alpha-adrenoceptor. It is notable that the inhibition produced by uptake blockade peaks at 1 Hz, resembling the potentiation produced by yohimbine. This peak probably reflects once again the balance between the release of sufficient noradrenaline to stimulate presynaptic alpha-adrenoceptors, and the lack of effectiveness of these receptors at higher frequencies. The inhibition of the twitch response produced by tyramine is also frequency and calcium dependent, the frequency response curve resembling those of clonidine and noradrenaline. The inhibition is antagonised by yohimbine. These results suggest that this inhibition is also due to stimulation of presynaptic alpha-adrenoceptors. As it is known that the noradrenaline releasing action of tyramine is independent of calcium (Chubb, De Potter and De Schaepdryver, 1972), the calcium dependence of its inhibition illustrates that it is the presynaptic alpha-adrenoceptor, activated by noradrenaline released by tyramine, that is calcium dependent and produces the inhibition of the twitch response. It has been said above that yohimbine reverses the inhibition

produced by uptake blockade to a potentiation of the twitch response. One would expect this potentiation if noradrenaline were the motor transmitter in this tissue, in which case exogenous noradrenaline and tyramine should also potentiate the response when presynaptic alpha-adrenoceptors are blocked. A complete blockade of these receptors was achieved by adding yohimbine to the Krebs (10 nM for noradrenaline, and 100 nM for tyramine). In the presence of yohimbine noradrenaline and tyramine potentiated the twitch response to stimulation at 0.2 Hz. This potentiation was reversed once more to an inhibition by 33 nM phenoxybenzamine or 10 μM phentolamine, both of which did not inhibit the twitch itself. This inhibition in the presence of yohimbine and phenoxybenzamine or phentolamine could be reduced by propranolol (33 nM) indicating that it is mediated via a beta-adrenoceptor. These results show that it is possible to potentiate the twitch response of the mouse vas deferens with noradrenaline or an indirectly acting amine, though the receptor mediating this potentiation is different or located in a different position from that mediating the twitch response. Also the twitch response is a resultant of the interaction of three or four receptors, pre- and post-synaptic alpha-adrenoceptors, a beta adrenoceptor, and possibly a further receptor mediating the twitch response. As it had already been shown that the twitch response was controlled by a presynaptic alpha-adrenoceptor it was possible that the 'fall off' in the response seen on constant stimulation was due to the influence of this receptor. Addition of 100 nM yohimbine to the Krebs solution reduced the fall off but did not prevent it. Thus the fall off is

probably due to a combination of presynaptic regulation and other factors.

The requirement of a minimum release of noradrenaline to stimulate the presynaptic alpha-adrenoceptor is demonstrated by the response of the vas deferens after noradrenaline depletion. When animals are pretreated with alpha methyl-p-tyrosine and reserpine the response of the isolated vas deferens to stimulation is larger than that of tissues from animals pretreated with reserpine alone, despite the noradrenaline content of the latter being greater than the former. Addition of yohimbine (128 nM) produces a potentiation of the response of the vas deferens to stimulation from reserpine pretreated but not from alpha methyl-p-tyrosine plus reserpine pretreated animals. This could be interpreted as being due to a larger noradrenaline release from the former group than the latter. The larger release being sufficient to stimulate presynaptic alpha-adrenoceptors producing a decrease in the response, the smaller release not being sufficient to stimulate these receptors.

The potentiation of the twitch response produced by phenoxybenzamine (15 μM) and phentolamine (10 μM) is frequency dependent, producing a frequency response curve resembling yohimbine, with a peak at 1 Hz. The curve for phentolamine was different from phenoxybenzamine, producing less potentiation at low frequencies and more at high frequencies. As phenoxybenzamine is known to block noradrenaline uptake the effect of noradrenaline uptake blocking agents on the phentolamine potentiation was examined and found to alter the frequency response curve to one more closely resembling that of phenoxybenzamine.

When the calcium content of the Krebs is halved the frequency response curve for phentolamine is shifted to the right and peaks at 5 Hz instead of 1 Hz, resembling the yohimbine curve. However in low calcium Krebs phenoxybenzamine produces its maximum potentiation at 0.2 Hz and this declines exponentially as the frequency increases. This change in the frequency response curve is similar to the change in the frequency response curve of noradrenaline uptake blocking agents, which peaks at 1 Hz in normal Krebs but becomes an exponential decline in half calcium Krebs. Phenoxybenzamine (15 μM) is the only alpha-adrenoceptor blocking agent examined which exhibits this changing frequency response curve, and it produces a greater inhibition of noradrenaline uptake than the other alpha-adrenoceptor blocking agents. These results suggest that this change in the frequency response curve is produced by blockade of noradrenaline uptake. One explanation of this could be as follows, uptake blockade increases the concentration of noradrenaline in the synaptic cleft and therefore the stimulation of presynaptic alpha-adrenoceptors. Halving the calcium content of the Krebs solution increases the effectiveness of these receptors, effectively moving the frequency response curve upwards (greater inhibition at each frequency). However the effect of presynaptic alpha-adrenoceptors is greater at low frequencies and therefore the upward shift at 0.2 Hz is greater than at 1 Hz, hence the change in shape of the curve.

Phenoxybenzamine (33 nM) which does not potentiate the twitch response has no effect on the inhibition produced by clonidine but abolishes contraction to exogenous noradrenaline,

15 μM phenoxybenzamine, which potentiates the twitch, abolishes the clonidine inhibition. These results are consistent with the theory that it is the blockade of presynaptic rather than post-synaptic alpha-adrenoceptors which produces the potentiation of the twitch response by phenoxybenzamine. It has already been shown that uptake blockade, another property of phenoxybenzamine, inhibits rather than potentiates the twitch response.

In conclusion, these results demonstrate the frequency and ionic dependence of the effects of clonidine, yohimbine, noradrenaline, tyramine, uptake blockade, phenoxybenzamine and phentolamine on the twitch response of the mouse vas deferens. Noradrenaline, tyramine and uptake blockade all stimulate or produces effects which result in stimulation of a receptor which can be selectively blocked by yohimbine. Phenoxybenzamine and phentolamine antagonise the effect of clonidine. These facts are consistent with the effects of all these drugs on the twitch response of the mouse vas deferens to electrical stimulation, being mediated via presynaptic alpha-adrenoceptors.

PART III

The effect of pre-synaptic alpha-adrenoceptors on the overflow of (^{3}H)-noradrenaline and its (^{3}H) metabolites from the mouse vas deferens.

INTRODUCTION

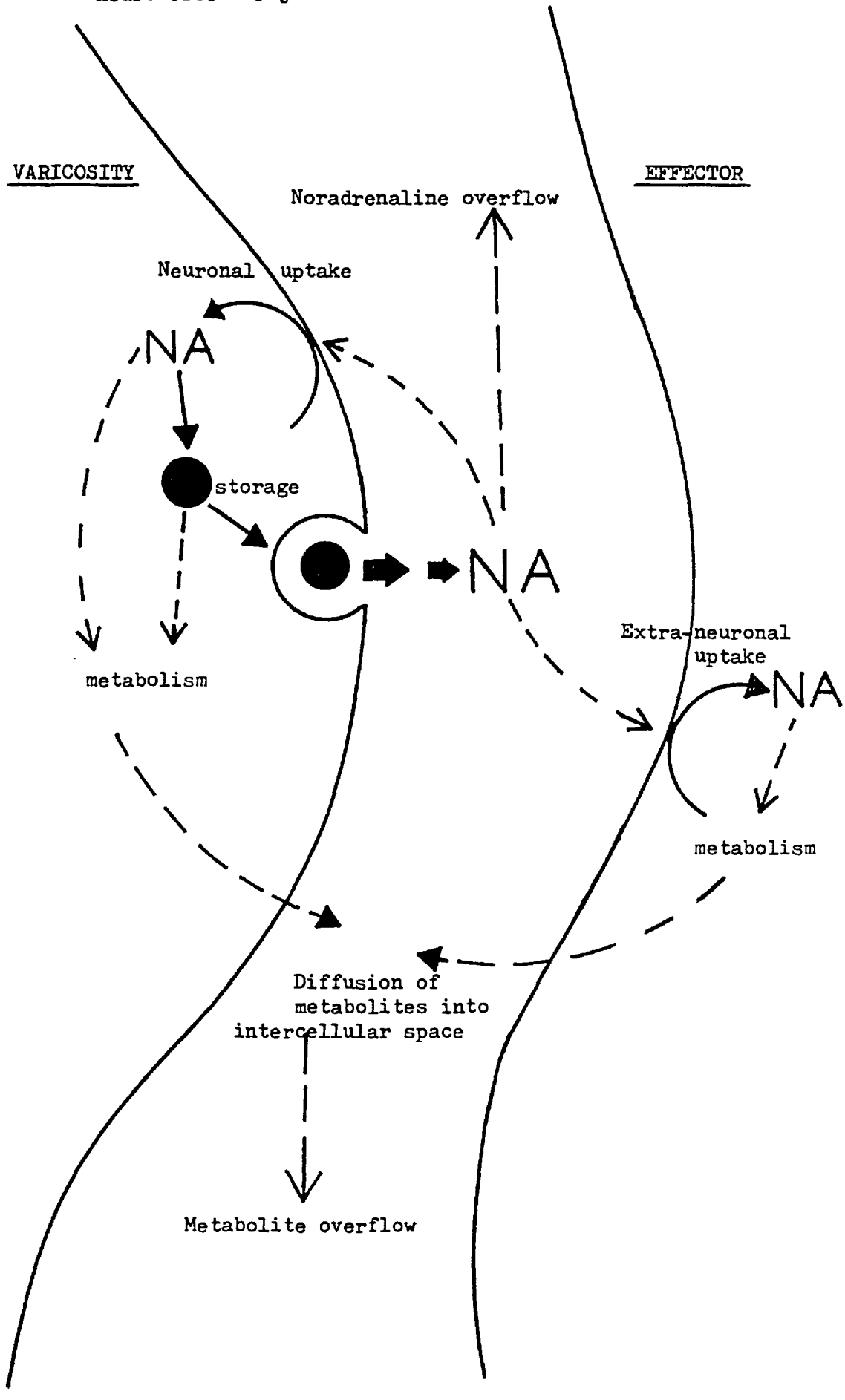
Since the development of the theory of a presynaptic control mechanism many workers have studied the role of presynaptic receptors in controlling transmitter overflow. Exogenous noradrenaline has been shown to decrease noradrenaline overflow from the fifteen adrenergically innervated tissues of the seven species so far studied (Starke, 1977). These tissues include the vasa deferentia of the rat and guinea-pig. The vas deferens of the mouse was examined by Farnebo and Malmfors (1971), who found that exogenous noradrenaline increased the basal overflow of tritium from tissues preloaded with (^{3}H)-noradrenaline, making interpretation of their results difficult. Clonidine decreased the overflow of noradrenaline in five of the six tissues so far examined (Starke, 1977). The exception was reported by Stjarne (1975), who found that clonidine increased the overflow of tritium from guinea-pig vasa deferentia preloaded with (^{3}H)-noradrenaline. In thirty-nine tissues (eight species) including the vasa deferentia of the rat, guinea-pig and rabbit, alpha-adrenoceptor blocking agents have been reported to increase noradrenaline overflow (Starke, 1977). It is predictable therefore, that drugs, which from the investigations in Part II of this thesis, have been suggested to act as presynaptic alpha-adrenoceptor agonists should decrease noradrenaline overflow. Conversely, drugs which are antagonists at these receptors should increase noradrenaline overflow. The effects of both types of drugs on the overflow of (^{3}H)-noradrenaline from the mouse vas deferens have been examined.

The selection of a satisfactory method for measuring noradren-

aline overflow has posed several problems. The radioenzymic method used in Part I to measure noradrenaline has some disadvantages as a routine procedure to measure changes in noradrenaline overflow. It is not sensitive enough to measure basal overflow, so changes such as those seen by Farnebo and Malmfors (1971) outlined above, would not be detected. Noradrenaline added to the organ bath to examine its effects on the overflow would also be converted to labelled adrenaline in the assay, thus confusing the results. Finally, the assay is both expensive and time consuming. A different method which is commonly used is the preloading of tissues with (^{3}H)-noradrenaline and measuring tritium overflow (e.g. work by Stjarne, Starke and, Farnebo and Malmfors). However the demonstration by Hertting and Axelrod (1961), that tritium released from stimulated cat's spleen preloaded with (^{3}H)-noradrenaline, contains labelled noradrenaline and normetanephrine, shows that released noradrenaline is collected as metabolites as well as unchanged noradrenaline. Collecting and measuring the tritium overlow measures both noradrenaline and metabolities, as well as any other tritiated product in the sample. As it is conceivable that the metabolites as well as the noradrenaline overflow may change on stimulation, measuring tritium overflow may give an incorrect view of what happens to noradrenaline overflow.

The realisation that it is overflow that is being measured, and not release of transmitter is important for the interpretation of results. Overflow being the product of release, uptake and metabolism of the transmitter, illustrated diagramatically in figure 36. To remove the problem of uptake it has become common to include noradrenaline uptake

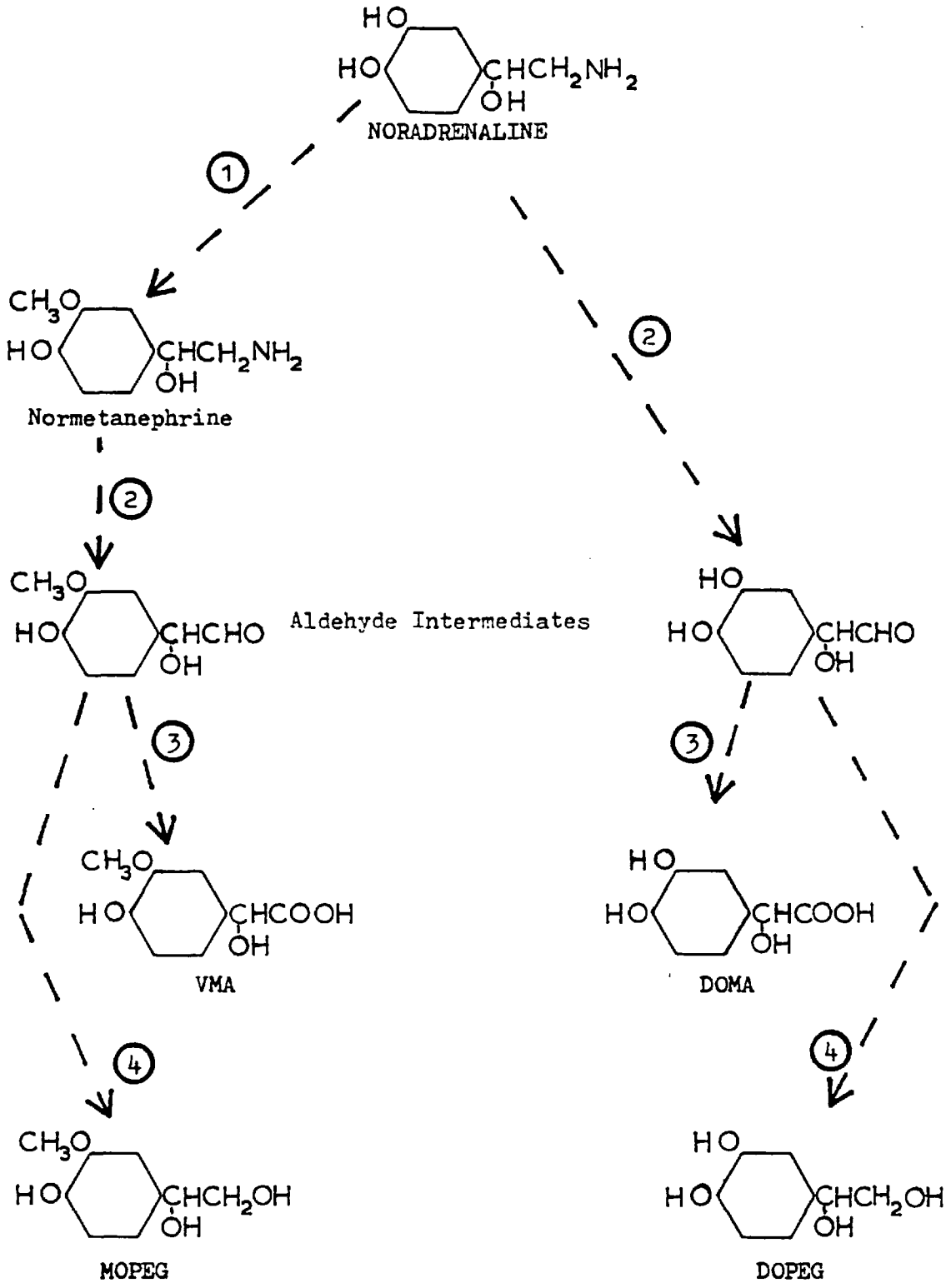
Fig. 36 The fate of released noradrenaline at an adrenergic neuro-effector junction.



inhibitors in the Krebs bathing the tissue. This procedure, particularly in the case of neuronal uptake inhibitors, may add to the problems of interpretation. These drugs may increase the concentration of noradrenaline in the synaptic cleft, which in turn will increase the stimulation of presynaptic alpha-adrenoceptors and thus depress noradrenaline release. In this situation one is faced with an abnormally low noradrenaline release, and will see less effect with presynaptic alpha-adrenoceptor agonists, than in the absence of uptake blockers. Some drugs commonly used to block uptake are known to effect presynaptic alpha-adrenoceptors, normetanephrine stimulating and desmethyl imipramine blocking these receptors (Starke, 1977). This confused situation can be improved by separating (^{3}H)-noradrenaline from its tritiated metabolites, using a method developed by Langer and his colleagues. Langer (1970) used two paper chromatographic systems to analyse the metabolism of (^{3}H)-noradrenaline released by stimulation of the cat nictitating membrane and rat vas deferens. This procedure was found to produce low recoveries of some metabolites and inadequate separation leading to difficulties in producing a quantitative result. Langer, Stefano and Enero (1972), used a column chromatographic method to investigate the pre- and post-synaptic origins of noradrenaline metabolites formed during transmitter release from the cat nictitating membrane. This procedure led to the contamination of the noradrenaline fraction with DOPEG. This contamination was avoided in the method used by Graefe, Stefano and Langer (1973), to investigate the metabolism of transmitter released from the rat vas deferens. It is a variation of this final

method that has been used to assay the overflow of (^{3}H)-noradrenaline and its metabolites from the mouse vas deferens in this section.

The overflow of (^{3}H)-noradrenaline and its metabolites from this tissue has been studied at 1 Hz and 10 Hz, and at 1 Hz in Krebs containing only half the normal calcium concentration. The overflow of (^{3}H)-noradrenaline has also been examined in the presence of some of the drugs used to investigate the nature of the twitch response in parts I and II. In fig. 37 the possible routes of metabolism of (^{3}H)-noradrenaline in an isolated preparation are represented diagramatically.



For abbreviations see Fig. 38.

Metabolic enzymes:- 1 catechol O-methyltransferase
2 monoamine oxidase
3 aldehyde dehydrogenase
4 alcohol dehydrogenase

Fig. 37. Possible pathways for the metabolism of noradrenaline.

Assay of tissue content and release of tritiated noradrenaline and its metabolites

MATERIALS

1. Dowex, 50 W x 4. prepared by the method of Graefe, Stefano and Langer (1973).
2. Alumina, prepared by the method of Anton and Sayre (1962).
3. (^{3}H)-noradrenaline (5.9 µM) in a solution of 1% sodium metabisulphite.
4. A standard solution of noradrenaline 115 µM, DOPEG 118 µM, and EDTA 30 mM.
5. Hydrochloric acid, 0.01 M and 2.0 M.
6. Acetic acid 0.2 M.
7. Sodium acetate 0.2 M, pH 8.6, adjusted to the correct pH immediately before use.
8. 2 M Tris buffer pH 9.4 containing 5% EDTA.
9. Chilled perchloric acid 0.1 M.
10. Krebs containing 110 µM ascorbic acid, 30 µM EDTA, and 3.7 µM oestradiol.
11. Packard 'Instagel'.

SAMPLE PREPARATION

Mice were killed by cervical dislocation and their vasa deferentia removed, cleaned of surrounding mesentery and blood vessels and placed in Krebs solution bubbled with 95% oxygen/5% carbon dioxide. 900 µl aliquots of the oxygenated Krebs were placed in flasks in a shaking water bath at 37°C. Two vasa deferentia were placed in each flask and 100 µl of (^{3}H)-noradrenaline solution added (to give a final concentration of 590 nM). After a forty-five minutes incubation period the vasa deferentia were removed either

for assay of their tritium content or suspended in an organ bath for overflow studies. Those removed for assay were washed and homogenised as described for the assay of (^{3}H)-noradrenaline uptake. Before assay an equal volume of Tris buffer and 0.5 ml of the noradrenaline standard solution were added to the perchloric acid supernatant. Vasa deferentia used for overflow studies were tied in parallel in groups of six and suspended in an organ bath containing oxygenated Krebs solution, and put under 1 gm tension. The bath contents were replaced every five minutes for forty-five minutes to wash the tissue. The preparation was then stimulated via platinum wire gutter electrodes at either 1 Hz or 10 Hz, and the bath contents discarded. The tissue was then washed every five minutes for a further fifteen minutes. Krebs solution was then left in contact with the preparation for a time period equal to the length of the stimulation period (2 minutes for 1 Hz or 12 seconds for 10 Hz, thus keeping the number of pulses constant). This Krebs sample was collected and called the prestimulation sample. The preparation was then stimulated (2 ms, 64v, 1 or 10 Hz) and the Krebs collected immediately after stimulation. This was called the stimulation sample. Two successive two minute collection periods were then taken and called post-stimulation samples. The preparation was then washed every five minutes for fifteen minutes, and the collection and stimulation cycle repeated twice more. The collected samples were frozen to await assay, which always took place within 24 hours.

When agonists were added the pre-stimulation and stimulation periods were lengthened to 2½ minutes, allowing the agonist

to be added 30 seconds before stimulation. In experiments in which tyramine or noradrenaline were added, after the two post-stimulation periods were taken, two further successive collections of five minutes each were made. Antagonists were added at the beginning of the pre-stimulation period and again immediately before stimulation. Phentolamine and cocaine were added to the Krebs and given a twenty minute contact time. Before assay, 0.5 ml of the noradrenaline standard solution and 1 ml of sodium acetate were added to the Krebs samples.

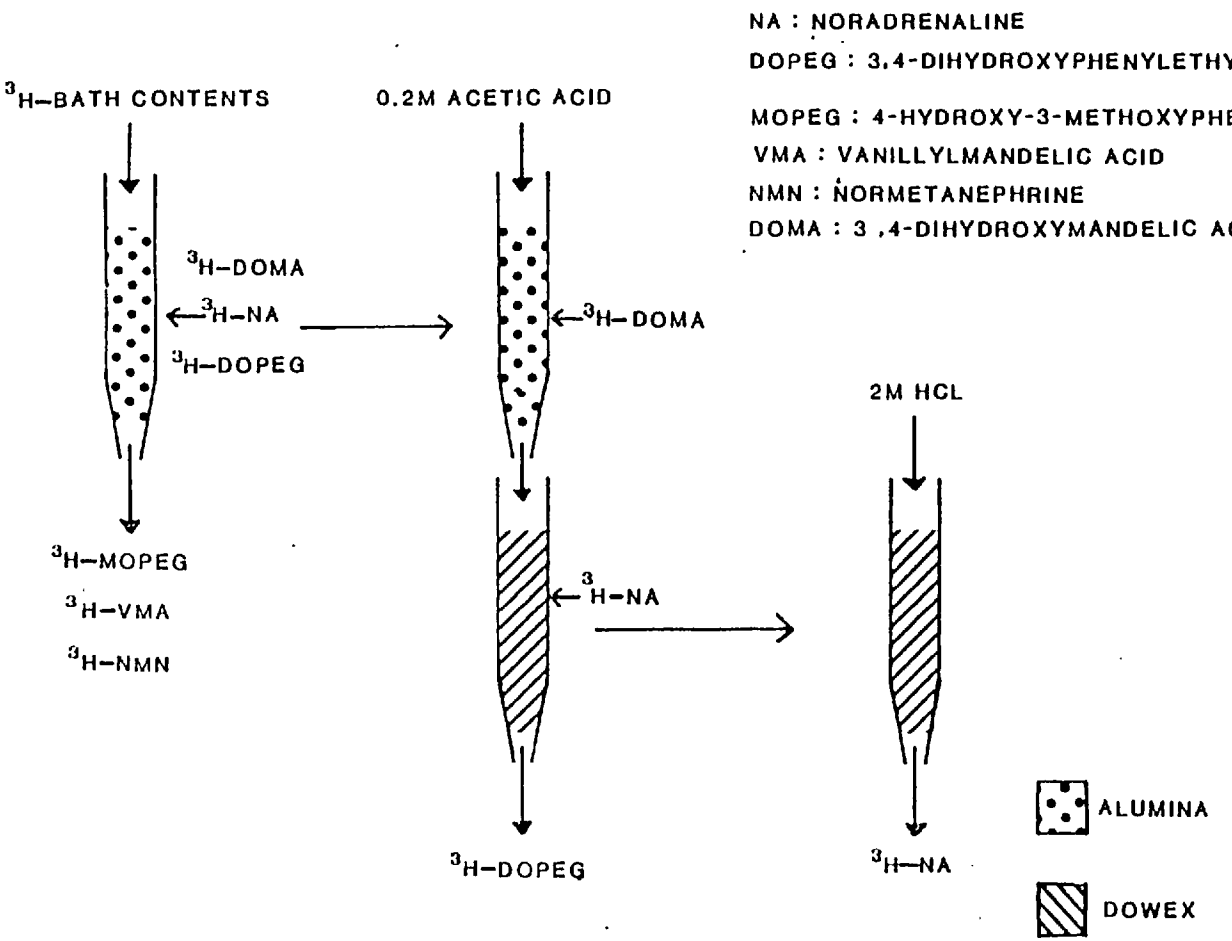
ASSAY

This has been based upon the method of Graefe, Stefano and Langer (1973). Glass columns, approximately 0.5 cms in diameter were plugged with glass wool and filled with either 225 mgs of alumina or to a depth of 1½ cms with Dowex. The alumina was equilibriated at pH 8.6 by passing 10 mls of sodium acetate through the column, the Dowex being equilibriated with approximately 15 mls 0.01 M HCl. The sample was then passed over the alumina and the effluent collected. Catechols, which were absorbed on to the alumina, were removed by eluting the column with 3.0 mls of acetic acid (0.2M). The effluent from the column was passed over a Dowex column, the effluent from this column being collected. Noradrenaline, trapped by the Dowex was eluted with 2.0 mls of 2 M HCl and collected. After either the sample or each reagent had been added to the column it was washed through with 1 ml distilled water which was then collected with the previous fraction. As the separation by these columns and elution from them was found to be inversely proportional to the rate of flow through

them, all samples and reagents were added in 1 ml aliquots. This procedure separated the tritium sample into noradrenaline, DOPEG, non-catechols and DOMA as shown in fig 38. The column effluents, Dowex and alumina were all suspended in 'Instagel' and counted by liquid scintillation spectrometry. No radioactivity was normally found remaining on the Dowex. While blockade of neuronal uptake by cocaine produces a large inhibition of the twitch response as pointed out in the introduction, Jenkins, Marshall and Nasmyth (1977) reported that oestradiol produced only a small inhibition. As it has been found that oestradiol increases neuronal uptake of (^{3}H)-noradrenaline, oestradiol (3.7 μM) was added to the Krebs solution bathing the vasa deferentia during the incubation with (^{3}H)-noradrenaline. In an attempt to decrease the number of metabolites overflowing from the tissue by preventing extra-neuronal metabolism oestradiol was also added to the Krebs during the overflow experiments. This drug unlike normetanephrine and desmethyl imipramine has not been shown to interfere with presynaptic alpha-adrenoceptors.

Fig. 38

Method for Separating ^{3}H-Noradrenaline and its ^{3}H-Metabolites



RESULTS

The average weight of the vas deferens used in these experiments was 78.6 ± 1.4 mg per group of six (n = 27). After incubation in 100 ng (^{3}H)-noradrenaline the vasa deferentia contained 8.27 ± 0.86 x 10^{4} d.min^{-1}mg^{-1}. At the time of the first collected stimulation the preparation contained 4.02 ± 0.13 x 10^{4} d.min^{-1}mg^{-1}. The twitch tension developed in response to stimulation of the preparation was recorded in all experiments and is given in table 19.

Table 19

Twitch tension developed by groups of six vasa deferentia in overflow experiments in response to field stimulation

STIMULATION PARAMETER AND DRUG USED	NUMBER OF EXPERIMENTS	TENSION (mg) mean ± s.e.
1 Hz, 2.5 mM $CaCl_2$		
Control	22	595.6 ± 35.8
Clonidine 2.8 nM	3	400.0 ± 83.9
Yohimbine 128 nM	5	1506.7 ± 146.9
Tyramine 20 µM	4	162.5 ± 50.2
Cocaine 10 µM	3	300.0 ± 38.5
Cocaine 10 µM and Phentolamine 10 µM	3	1011.0 ± 44.4
Noradrenaline 3 µM	3	1111.0 ± 109.4
Noradrenaline 3 µM and Yohimbine 128 nM	3	1799.8 ± 405.5
Noradrenaline 3 µM and Cocaine 10 µM	4	141.6 ± 43.8
Noradrenaline 3 µM and Yohimbine 128 nM and Cocaine 10 µM	4	558.3 ± 104.0
1 Hz, 1.25mM $CaCl_2$		
Control	12	255.0 ± 31.0
2.8 nM Clonidine	5	130.5 ± 28.8
128 nM Yohimbine	4	537.8 ± 27.6
20 µM Tyramine	3	8.8 ± 2.2
10 Hz, 2.5 mM $CaCl_2$		
Control	10	7,500.0 ± 870.0
2.8 nM Clonidine	3	7064.9 ± 1282.5
128 nM Yohimbine	3	12,998.7 ± 1677.6

Presentation of results obtained from the overflow experiments

Raw data obtained from the metabolite separation is counts per minute and this is routinely converted to disintegration per minute ($d.min^{-1}$) (see appendix 2). There is a limited store of (^3H)-noradrenaline in the tissue, as no (^3H)-noradrenaline can be synthesised by it. Therefore the amount available for release, will decrease after each stimulation. Thus, at each successive stimulation the release of (^3H)-noradrenaline decreases, due to the decreasing tissue store. This drop in release can be accounted for by expressing the (^3H)-noradrenaline overflow as a fraction of that in the tissue at the time of stimulation, this figure is known as fractional release. Theoretically when this procedure is carried out the release of (^3H)-noradrenaline will reflect the release of endogenous noradrenaline. Figures expressed as fractional release in this section can be defined as follows:-

Tritium fractional release This figure expresses the total amount of tritium released, before, during or after stimulation, as a fraction of the tritium content of the tissue at the time of release. This measures not only the overflow of (^3H)-noradrenaline, but also all of it's tritiated metabolites, and any other tritiated product.

Evoked (^3H)-noradrenaline fractional release This figure considers only the (^3H)-noradrenaline released by stimulation. The basal (^3H)-noradrenaline release is subtracted from the stimulated (^3H)-noradrenaline release and the resulting value divided by the total tritium content of the tissue at the time of stimulation. The use of total tritium content of

the tissue to determine (^{3}H)-noradrenaline fractional release is allowable as almost all the tissue tritium is (^{3}H)-noradrenaline (see table 9).

In studies of the overflow of labelled transmitter from stimulated preparations it is common to refer to the evoked tritium fractional release. To obtain this value the basal level of tritium overflow during stimulation is assumed to be equivalent to that in the absence of stimulation. Studies of the overflow from this preparation show this assumption to be incorrect in these experiments. On stimulation at 1 Hz the overflow of (^{3}H)-noradrenaline and (^{3}H)-DOMA always increased above basal levels. The overflow of tritiated non-catechol metabolites and (^{3}H)-DOPEG decreased below basal levels in 56.6% and 64.4% respectively of the samples studied. At 10 Hz, in 85.7% of the samples the overflow of tritiated non-catechol metabolites, and in all samples the overflow of (^{3}H)-DOPEG, decreased below basal levels. Therefore in these experiments it would be incorrect to use evoked tritium efflux i.e. stimulated efflux minus basal efflux, as a measure of transmitter release, due to the different composition of the tritium overflow in the basal and stimulated overflows.

In these experiments changes in the metabolism of (^{3}H)-noradrenaline on stimulation, or in the presence of drugs, can best be studied by considering the composition of the total tritium overflow, before, during and after stimulation. This is done by expressing the amount of each metabolite ($d.min^{-1}$) in the tritium overflow ($d.min^{-1}$) as a percentage of the tritium overflow.

Control Experiments

The percentage of the total (^{3}H)-noradrenaline released by stimulation at 1 Hz overflowing in the stimulation and post-stimulation periods is given in fig 39. The duration of the overflow was not significantly affected by halving the calcium content of the Krebs. In both cases the majority of the overflow occurred during the stimulation period. After stimulation at 10 Hz, though the majority of the (^{3}H)-noradrenaline overflow took place during the stimulation period, the proportion of the total was lower than that occurring at 1 Hz (fig 39).

In control experiments at 1 Hz and 10 Hz there was no significant decrease in the fractional release of tritium or (^{3}H)-noradrenaline between successive stimulation.

1 Hz - normal Krebs solutions (2.5 mM calcium chloride)

The fractional release of tritium was significantly increased upon stimulation from $3.38 \pm 0.22 \times 10^{-3}$ to $4.00 \pm 0.21 \times 10^{-3}$ ($P < 0.05$, fig 40). The tritium fractional release returned to the basal levels in the first post stimulation period. The evoked fractional release of (^{3}H)-noradrenaline was $5.95 \pm 0.62 \times 10^{-4}$.

On stimulation the percentage of (^{3}H)-noradrenaline in the tritium overflow rose 3.4 fold to $18.8 \pm 1.46\%$. The major metabolite in the pre-stimulation, stimulation and post-stimulation periods was (^{3}H)-DOPEG, which was the largest single component of the overflow (table 20). The percentages of (^{3}H)-noradrenaline and (^{3}H)-metabolites return to basal levels by the second post stimulation period.

1 Hz - the effect of halving the calcium content (to 1.25 mM) of the Krebs solution

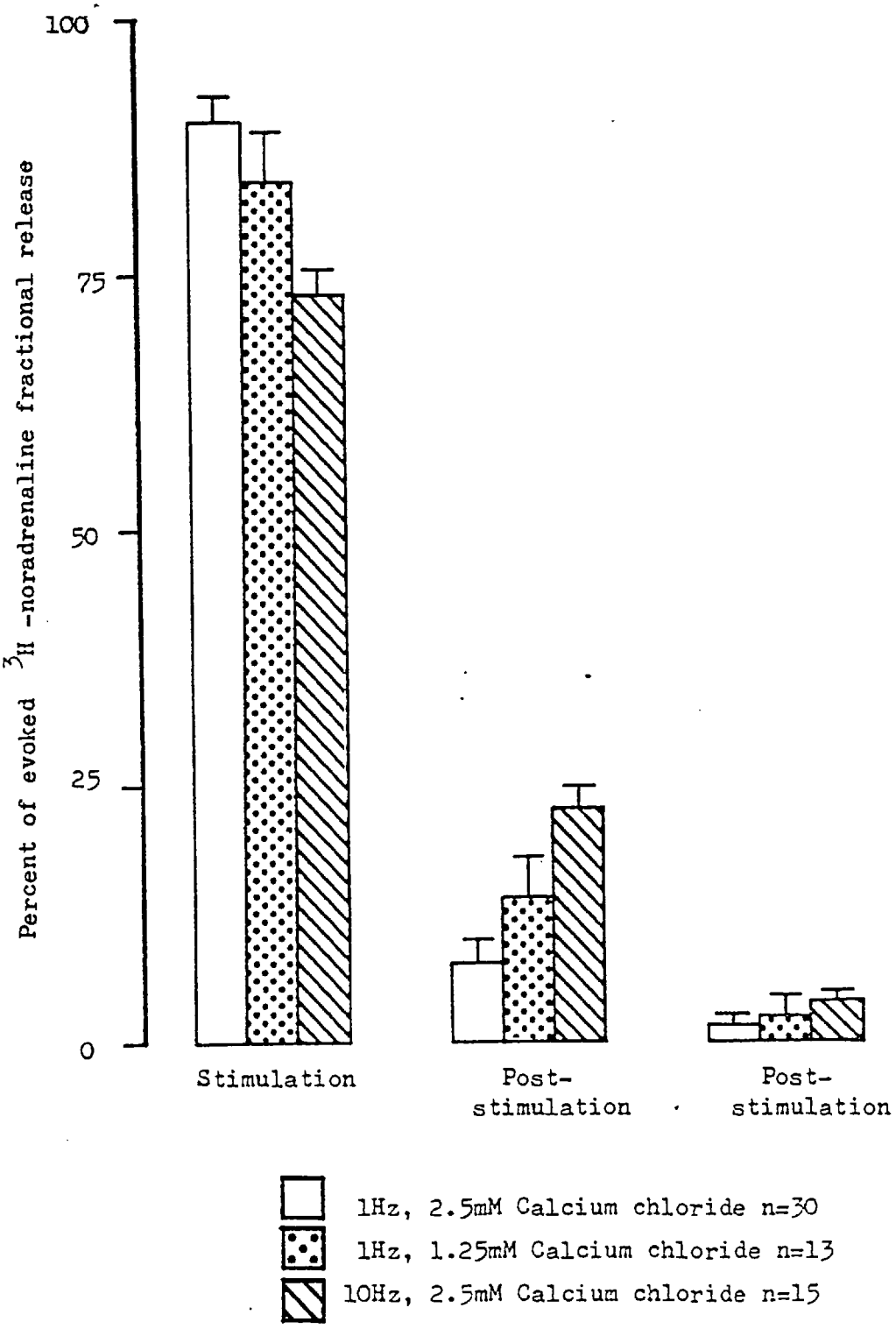


Fig. 39 Percentage of the evoked ^{3}H -noradrenaline fractional release overflowing in each of the collection periods following stimulation of mice vasa deferentia in control experiments.

Table 20

The composition of the tritium overflow from mice vasa deferentia stimulated at 1 Hz in Krebs containing 2.5 mM or 1.25 mM calcium chloride, and at 10 Hz in 2.5 mM calcium chloride

DRUG OR EXPERIMENT	COLLECTION PERIOD	PERCENT OF THE TOTAL TRITIUM IN EACH COLLECTION PERIOD			
		NORADRENALINE	NON-CATECHOLS	DOPEG	DOMA
1 Hz 2.5 mM $CaCl_2$ n = 18	pre-stimulation	5.6 ± 0.49	45.4 ± 0.83	47.7 ± 1.14	1.25 ± 0.34
	stimulation	18.8 ± 1.46	37.5 ± 0.93	41.7 ± 0.94	2.10 ± 0.21
	post-stimulation	6.1 ± 0.58	42.4 ± 0.80	49.0 ± 1.24	1.18 ± 0.11
	post-stimulation	5.2 ± 0.59	43.2 ± 0.82	50.6 ± 1.08	0.90 ± 0.17
1 Hz 1.25 mM $CaCl_2$ n = 11	pre-stimulation	4.3 ± 0.71	46.1 ± 2.04	48.3 ± 2.02	1.00 ± 0.12
	stimulation	8.5 ± 1.19	44.3 ± 3.13	45.4 ± 2.24	1.20 ± 0.18
	post-stimulation	5.4 ± 0.88	45.0 ± 2.09	49.1 ± 2.43	0.90 ± 0.13
	post-stimulation	4.4 ± 0.64	45.3 ± 2.52	49.1 ± 2.86	0.80 ± 0.08
10 Hz 2.5 mM $CaCl_2$ n = 8	pre-stimulation	4.2 ± 0.71	45.1 ± 1.96	48.6 ± 1.88	1.26 ± 0.04
	stimulation	46.3 ± 4.36	25.3 ± 2.41	23.7 ± 2.17	4.80 ± 0.90
	post-stimulation	14.5 ± 4.16	41.5 ± 2.85	45.8 ± 2.65	2.00 ± 0.18
	post-stimulation	5.3 ± 1.07	41.8 ± 1.30	51.9 ± 0.99	1.30 ± 0.16

The evoked fractional release of (^{3}H)-noradrenaline was significantly reduced in half calcium Krebs compared to normal Krebs, being 2.83 ± 0.35 x 10^{-4}. The fractional release of tritium was also decreased in the stimulation period compared with controls, the basal overflow was not affected (fig 40). The percentage of (^{3}H)-noradrenaline and its metabolites in the prestimulation period was not significantly different from those in normal Krebs, but the percentage of (^{3}H)-noradrenaline rose only two fold on stimulation (table 20). (^{3}H)-DOPEG remained the largest single component of the overflow. The composition of the overflow returned to basal levels during the first post-stimulation period. The duration of (^{3}H)-noradrenaline overflow was not significantly different (fig 39) from that in normal calcium.

<u>The effect of increasing the frequency of stimulation to 10 Hz on the overflow in normal Krebs solution (2.5 mM calcium chloride)</u>

At 10 Hz the fractional evoked release of (^{3}H)-noradrenaline was 11.30 ± 1.20 x 10^{-4}, which was significantly greater than at 1 Hz ($P < 0.001$). However both the prestimulation and stimulation overflow of tritium were less than at 1 Hz (fig 40). This decrease may reflect the shorter duration of the collection period at this frequency, (2 minutes at 1 Hz and 12 seconds at 10 Hz) used in order to maintain a constant number of stimuli (120). As the tritium overflow continues to rise in the post-stimulation periods it is probable that 12 seconds is not long enough for the diffusion of the evoked release from the tissue into the organ bath. This is also reflected in the longer duration of the (^{3}H)-noradrenaline

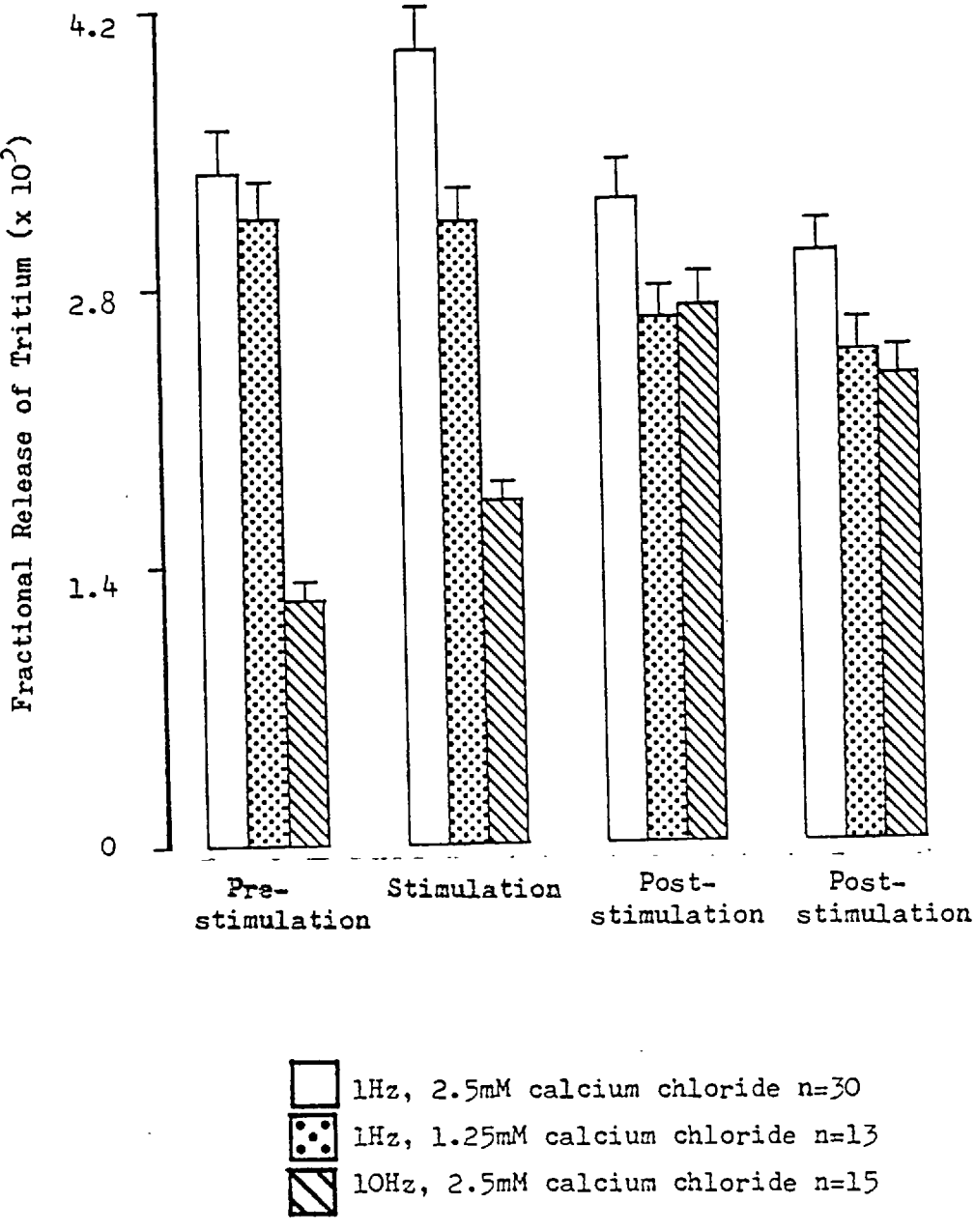


Fig. 40 The fractional release of tritium from mice vasa deferentia stimulated at 1Hz in 2.5mM or 1.25mM calcium chloride and at 10Hz in 2.5mM calcium chloride.

overflow compared with that at 1 Hz (fig 39). The percentage of (^{3}H)-noradrenaline and its metabolites in the tritium overflow during the pre-stimulation period was the same as 1 Hz, but on stimulation the percentage of (^{3}H)-noradrenaline rose eleven fold (table 20). The major single constituent of the stimulated overflow at 10 Hz was noradrenaline, there being a drop in the percentage of (^{3}H)-non catechols and (^{3}H)-DOPEG on stimulation. The composition of the tritium overflow returns to basal levels by the second post-stimulation period.

The effect of drugs on the overflow of (^{3}H)-noradrenaline and its metabolites from the mouse vas deferens

A. Clonidine (2.8 nM)

At 1 Hz in Krebs solution containing the normal calcium concentration clonidine had no significant effect on the tritium fractional release (fig 41). However evoked (^{3}H)-noradrenaline fractional release was significantly decreased by 70.8 ± 7.3% (table 21). This decrease was due to a fall in the percentage of (^{3}H)-noradrenaline in the tritium overflow compared with controls ($P < 0.05$, table 22). There were also significant increases in the percentages of (^{3}H)-non catechol metabolites and (^{3}H)-DOPEG ($P < 0.01$) compared with controls. The duration of (^{3}H)-noradrenaline overflow was not significantly affected by clonidine.

In Krebs containing only half the normal calcium concentration the tritium fractional release was not significantly reduced by clonidine (fig 42). The evoked (^{3}H)-noradrenaline fractional release was decreaded by 89.0 ± 4.6%, compared with controls carried out in half calcium Krebs (table 23). This was a significantly greater decrease than that seen in normal Krebs solution ($P < 0.05$). The percentage of (^{3}H)-noradrenaline in the tritium overflow was decreased from controls ($P < 0.05$), showing no discernable rise on stimulation (table 24). There was no significant affect on the percentage of any of the metabolites in tritium overflow compared with the pre-stimulation sample (table 24).

At 10 Hz, as at 1 Hz, clonidine was added to the organ bath thirty seconds before stimulation. The stimulation period was only twelve seconds to produce the same number of stimuli (120) as at 1 Hz, thus the stimulation period overflow sample

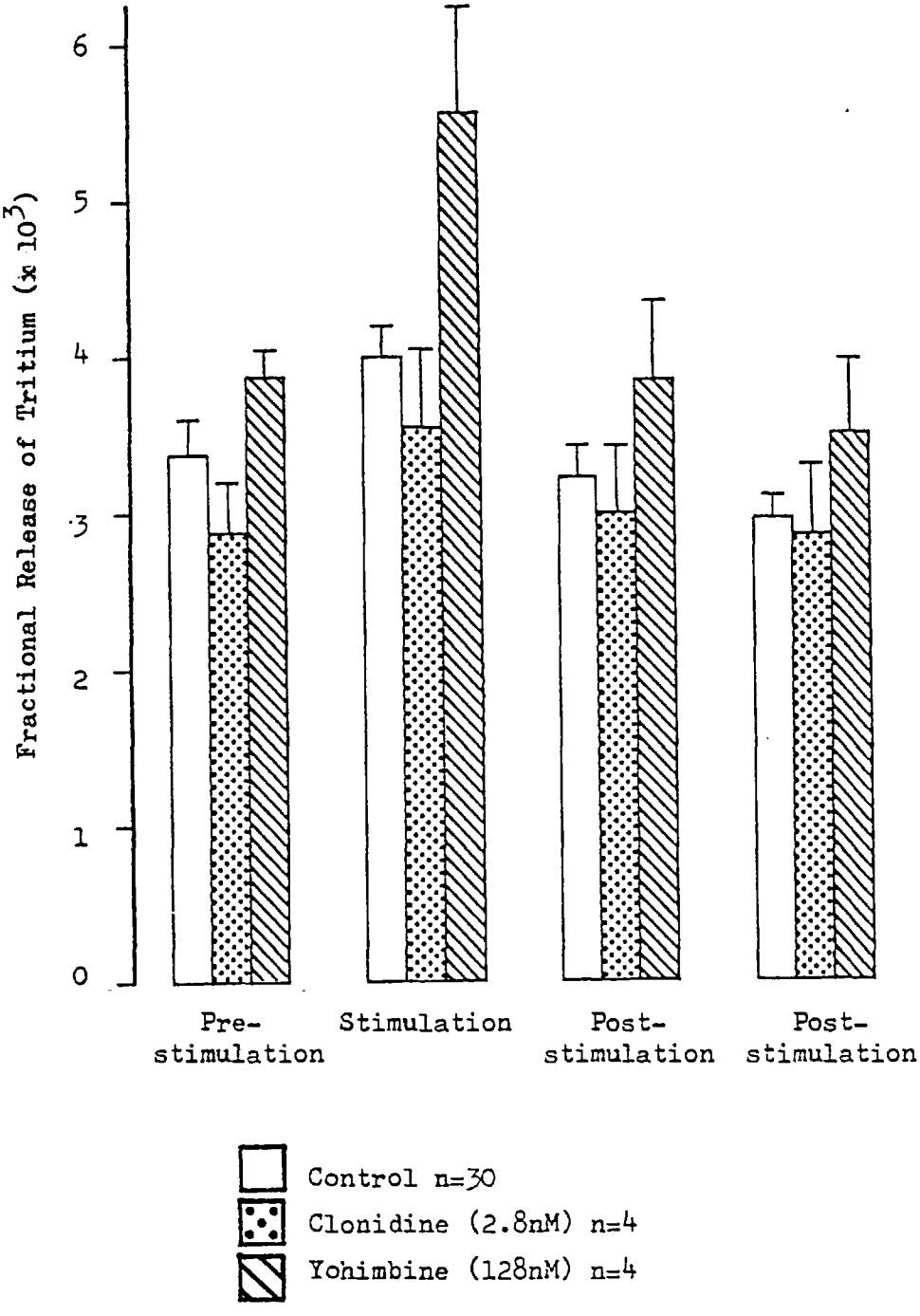


Fig. 41 The effect of clonidine and yohimbine on the fractional release of tritium from mice vasa deferentia stimulated at 1Hz in Krebs containing 2.5mM calcium chloride.

Table 21

The effect of drugs on the evoked ^{3}H-noradrenaline and total tritium fraction release at 1 Hz in normal Krebs solution (2.5 mM $CaCl_2$)

Drug	(^{3}H)-noradrenaline x 10^{4}	Tritium x 10^{3}	n
Control	5.95 ± 0.62	4.00 ± 0.21	31
Clonidine (2.8 nM)	1.74 ± 0.43	3.54 ± 0.54	4
Yohimbine (128 nM)	12.64 ± 2.56	5.59 ± 0.69	4
Tyramine (20 µM)	72.70 ± 6.40	16.10 ± 1.20	4
Tyramine (20 µM), unstimulated prep.	74.40 ± 10.90	13.80 ± 0.40	4
Cocaine (10 µM)	9.23 ± 2.17	3.70 ± 0.14	6
Cocaine (10 µM) plus Phentolamine (10 µM)	49.10 ± 8.70	8.21 ± 1.01	3
Noradrenaline (3 µM)	15.30 ± 1.80	13.20 ± 1.20	4
Noradrenaline (3 µM) plus Yohimbine (128 nm)	14.60 ± 4.90	15.20 ± 2.30	4
Cocaine (10 µM) plus Noradrenaline (3 µM)	0.85 ± 0.03	3.75 ± 0.17	3
Cocaine (10 µM) plus Noradrenaline (3 µM) plus Yohimbine (128 nM)	1.57 ± 0.13	4.06 ± 0.32	3

Table 22.

The effect of clonidine (2.8 nM) on the composition of the tritium overflow from mice vasa deferentia stimulated at 1 Hz in Krebs containing 2.5 mM calcium chloride

DRUG OR EXPERIMENT	COLLECTION PERIOD	PERCENT OF THE TOTAL TRITIUM IN EACH COLLECTION PERIOD			
		NORADRENALINE	NON-CATECHOLS	DOPEG	DOMA
Control n = 18	pre-stimulation	5.6 ± 0.49	45.4 ± 0.83	47.7 ± 1.14	1.25 ± 0.34
	stimulation	18.8 ± 1.46	37.5 ± 0.93	41.7 ± 0.94	2.10 ± 0.21
	post-stimulation	6.1 ± 0.58	42.4 ± 0.80	49.0 ± 1.24	1.18 ± 0.11
	post-stimulation	5.2 ± 0.59	43.2 ± 0.82	50.6 ± 1.08	0.90 ± 0.17
2.8 nM Clonidine n = 4	pre-stimulation	4.5 ± 1.0	47.0 ± 0.9	47.7 ± 1.1	0.90 ± 0.1
	stimulation	7.8 ± 1.4	42.6 ± 1.6	48.4 ± 2.2	1.30 ± 0.1
	post-stimulation	4.6 ± 0.9	45.2 ± 1.8	48.9 ± 2.3	1.00 ± 0.1
	post-stimulation	3.4 ± 1.2	43.8 + 0.8	52.4 ± 0.8	0.60 ± 0.1

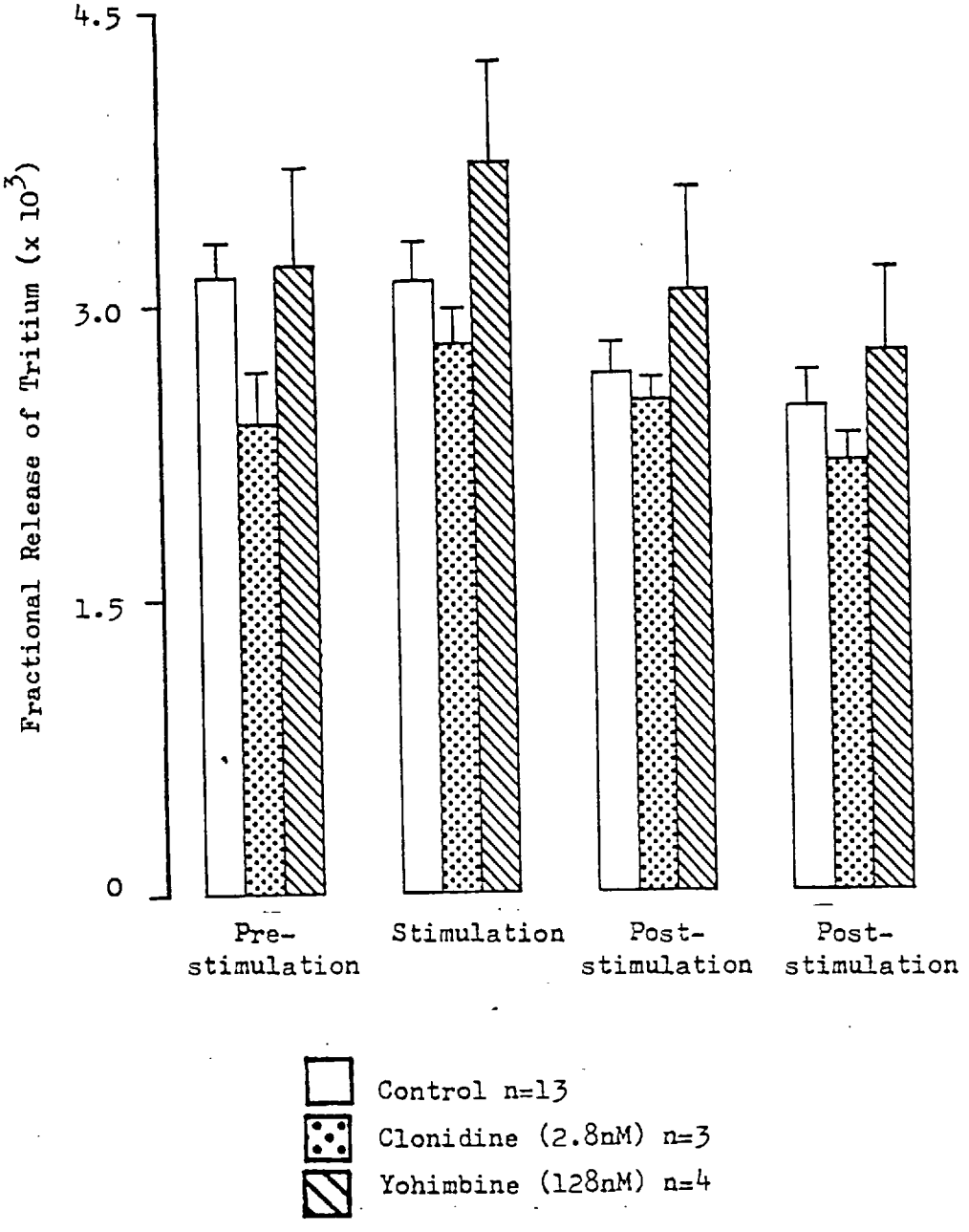


Fig. 42 The effect of clonidine and yohimbine on the fractional release of tritium from mice vasa deferentia stimulated at 1Hz in Krebs containing 1.25mM calcium chloride.

Table 23

The effect of drugs on the evoked ^{3}H-noradrenaline and total tritium fraction release at 1 Hz in half calcium Krebs (1.25 mM)

Drug	(^{3}H)-noradrenaline x 10^{4}	Tritium x 10^{3}	n
Control	2.83 ± 0.35	3.13 ± 0.17	18
Clonidine (2.8 nM)	0.312 ± 0.13	2.80 ± 0.17	3
Yohimbine (128 nM)	4.40 ± 1.64	3.72 ± 0.52	4
Tyramine (20 μM)	81.40 ± 0.40	16.10 ± 1.20	4

Table 24

The effect of clonidine (2.8 nM) on the composition of the tritium overflow from mice vasa deferentia stimulated at 1 Hz in Krebs containing 1.25 mM calcium chloride

DRUG OR EXPERIMENT	COLLECTION PERIOD	PERCENT OF THE TOTAL TRITIUM IN EACH COLLECTION PERIOD			
		NORADRENALINE	NON-CATECHOLS	DOPEG	DOMA
Control n = 11	pre-stimulation	4.3 ± 0.71	46.1 ± 2.04	48.3 ± 2.02	1.0 ± 0.12
	stimulation	8.5 ± 1.14	44.3 ± 3.13	45.4 ± 2.24	1.2 ± 0.18
	post-stimulation	5.4 ± 0.88	45.0 ± 2.09	49.1 ± 2.43	0.9 ± 0.13
	post-stimulation	4.4 ± 0.64	45.3 ± 2.52	49.1 ± 2.86	0.8 ± 0.08
2.8 nM Clonidine n = 4	pre-stimulation	5.4 ± 0.8	39.9 ± 1.6	54.9 ± 1.9	0.7 ± 0.2
	stimulation	5.5 ± 0.6	38.8 ± 1.1	54.4 ± 1.7	1.1 ± 0.3
	post-stimulation	4.7 ± 0.4	38.2 ± 2.0	56.3 ± 1.6	0.8 ± 0.03
	post-stimulation	2.8 ± 1.0	37.8 ± 1.9	58.5 ± 2.9	0.9 ± 0.4

Table 25

The effect of clonidine on the evoked (^{3}H)-noradrenaline and total tritium fractional release at 10 Hz in normal Krebs (2.5 mM $CaCl_2$)

Drug	(^{3}H)-noradrenaline x 10^4	Tritium x 10^3	n
Control	6.64 ± 0.33	1.75 ± 0.17	3
Clonidine (2.8 nM)	3.79 ± 0.70	1.57 ± 0.12	3

consisted of thirty seconds basal overflow and twelve seconds stimulated overflow. This prolonged collection period significantly decreased the evoked (^{3}H)-noradrenaline fractional release compared with controls, and therefore a new series of controls, including the thirty second non-stimulation period were undertaken (table 25). The tritium fractional release was not affected by this prolonged collection period, but was significantly decreased by clonidine (fig 43, $P < 0.05$). Addition of clonidine decreased the evoked (^{3}H)-noradrenaline fractional release by 42.9 $\pm$ 10.6% (table 25, $P < 0.025$). There was no significant change in the composition of the tritium overflow in the presence of clonidine, compared with controls (table 26). The duration of (^{3}H)-noradrenaline overflow was also not affected.

B. Yohimbine (128 nM)

Yohimbine was added to the organ bath at the beginning of the pre-stimulation period to provide a two minute contact time with the tissue before stimulation. However it had no effect on the magnitude or the composition of the basal tritium fractional release in either normal or low calcium Krebs solution (fig 41 and 42). On stimulation at 1 Hz in normal Krebs (2.5 mM calcium), yohimbine increased the tritium fractional release 1.4 fold ($P < 0.025$, fig 41) and the evoked (^{3}H)-noradrenaline fractional release 2.1 fold ($P < 0.01$, table 21). There was no significant change in the percentage of (^{3}H) noradrenaline in the tritium overflow, non-catechols being the only metabolites that were affected, being decreased ($P < 0.01$, table 27) in the presence of yohimbine compared with controls. The duration of (^{3}H)-

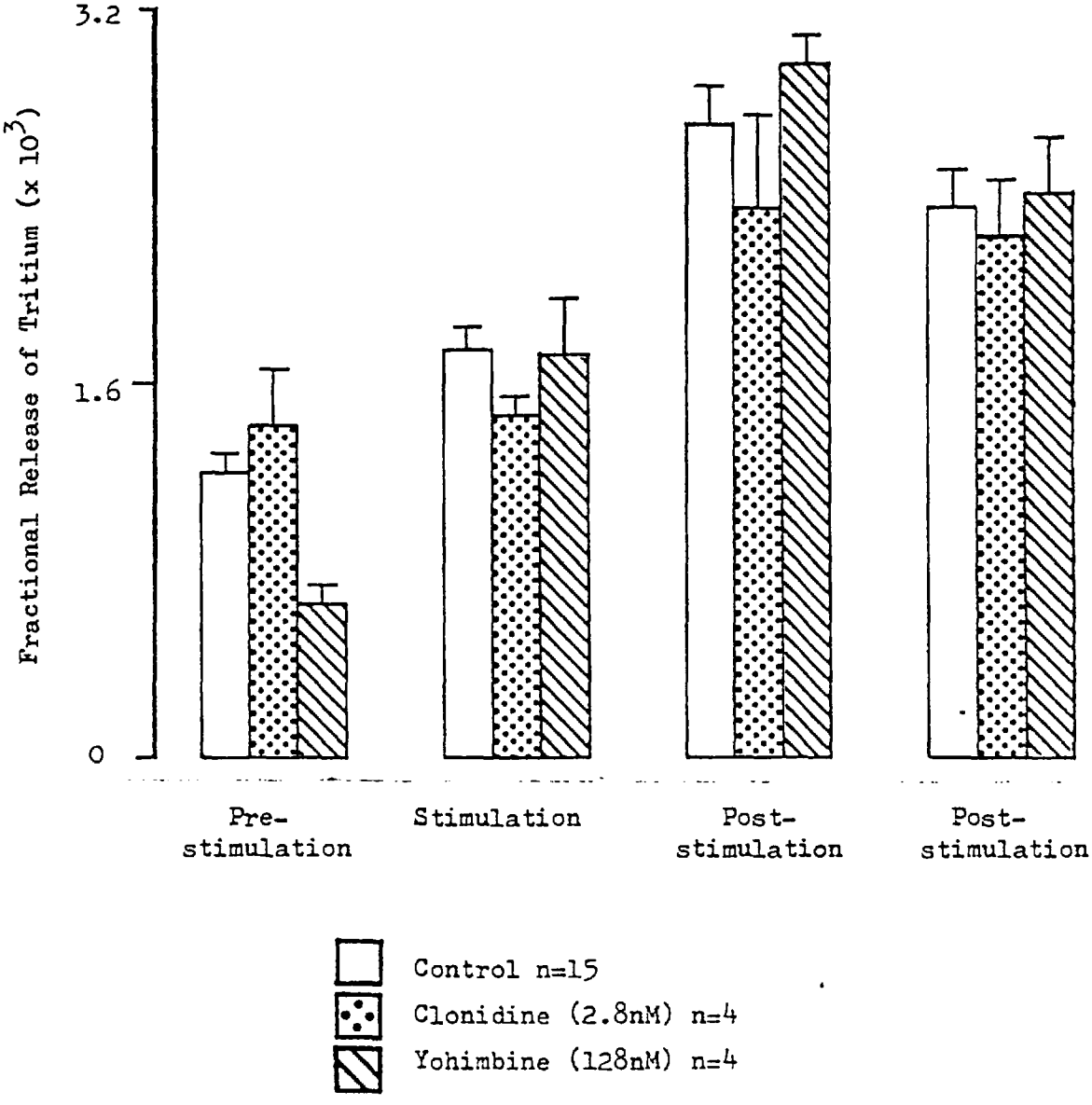


Fig. 43 The effect of clonidine and yohimbine on the fractional release of tritium from mice vasa deferentia stimulated at 10Hz in Krebs containing 2.5mM calcium chloride.

Table 26

The effect of clonidine (2.8 nM) on the composition of the tritium overflow from mice vasa deferentia stimulated at 10 Hz in Krebs containing 2.5 mM calcium chloride

DRUG OR EXPERIMENT	COLLECTION PERIOD	PERCENT OF THE TOTAL TRITIUM IN EACH COLLECTION PERIOD			
		NORADRENALINE	NON-CATECHOLS	DOPEG	DOMA
Control n = 8	pre-stimulation	48.3 ± 2.2	47.6 ± 3.1	3.2 ± 1.1	1.0 ± 0.0
	stimulation	32.6 ± 2.2	33.1 ± 2.0	30.8 ± 3.1	3.6 ± 1.0
	post-stimulation	41.3 ± 2.3	48.6 ± 3.4	8.4 ± 1.3	1.8 ± 0.3
	post-stimulation	42.0 ± 1.6	53.4 ± 1.2	2.7 ± 0.2	1.6 ± 0.3
2.8 nM Clonidine n = 3	pre-stimulation	4.5 ± 0.8	49.5 ± 2.5	45.6 ± 1.4	0.4 ± 0.1
	stimulation	24.3 ± 3.7	37.5 ± 2.6	34.2 ± 2.4	4.0 ± 0.8
	post-stimulation	6.0 ± 0.9	36.9 ± 1.2	55.2 ± 0.4	1.8 ± 0.3
	post-stimulation	1.7 ± 0.4	40.6 ± 1.3	56.8 ± 1.5	0.8 ± 0.3

Table 27

The effect of yohimbine (128 nM) on the composition of the tritium overflow from mice vasa deferentia stimulated at 1 Hz in Krebs containing 2.5 mM calcium chloride

DRUG OR EXPERIMENT	COLLECTION PERIOD	PERCENT OF THE TOTAL TRITIUM IN EACH COLLECTION PERIOD			
		NORADRENALINE	NON-CATECHOLS	DOPEG	DOMA
Control n = 18	pre-stimulation	5.6 ± 0.49	45.4 ± 0.83	47.7 ± 1.14	1.25 ± 0.34
	stimulation	18.8 ± 1.46	37.5 ± 0.93	41.7 ± 0.94	2.10 ± 0.21
	post-stimulation	6.1 ± 0.58	42.4 ± 0.80	49.0 ± 1.24	1.18 ± 0.11
	post-stimulation	5.2 ± 0.59	43.2 ± 0.82	50.6 ± 1.08	0.90 ± 0.17
128 nM Yohimbine n = 4	pre-stimulation	5.1 ± 1.3	44.7 ± 0.8	49.8 ± 1.7	0.60 ± 0.08
	stimulation	22.5 ± 2.0	32.4 ± 1.0	41.0 ± 3.2	3.70 ± 1.3
	post-stimulation	11.0 ± 2.3	39.2 ± 1.9	48.9 ± 2.8	1.00 ± 0.13
	post-stimulation	5.8 ± 1.0	41.7 ± 0.8	51.8 ± 0.7	0.80 ± 0.06

noradrenaline overflow was not significantly affected.
On stimulation in Krebs containing only half the normal calcium concentration (1.25 mM), yohimbine had no significant effect on the tritium fractional release, or the evoked (^{3}H)-noradrenaline release compared with controls (table 23, fig 42). However there was a significant rise in the percentage of (^{3}H)-noradrenaline, and a decrease in the percentage of non-catechols in the tritium overflow during stimulation compared with controls (table 28). The duration of (^{3}H)-noradrenaline overflow was not significantly affected.
During stimulation at 10 Hz yohimbine had no significant affect on the evoked (^{3}H)-noradrenaline, or tritium fractional release compared with 10 Hz controls (table 29, fig 43). There was a significant increase in the percentage of (^{3}H)-noradrenaline and a significant fall in the percentage of non-catechols and DOPEG in the tritium overflow compared with controls ($P < 0.05$, table 30). There was no significant effect on the duration of (^{3}H)-noradrenaline overflow.

C. Cocaine (10 μM)

Addition of cocaine to the Krebs solution did not significantly affect the magnitude of the basal or stimulated tritium fractional release (table 21, fig 44). The composition of the basal overflow of tritium was not affected by cocaine, but during stimulation there was a significant decrease in the non-catechol fraction ($P < 0.025$) and a significant increase in the (^{3}H)-noradrenaline fraction ($P < 0.05$ table 31). There was a small but non-significant rise in the evoked (^{3}H)-noradrenaline fractional release on stimulation in the

Table 28

The effect of yohimbine (128 nM) on the composition of the tritium overflow from mice vasa deferentia stimulated at 1 Hz in Krebs containing 1.25 mM calcium chloride

DRUG OR EXPERIMENT	COLLECTION PERIOD	PERCENT OF THE TOTAL TRITIUM IN EACH COLLECTION PERIOD			
		NORADRENALINE	NON-CATECHOLS	DOPEG	DOMA
Control n = 11	pre-stimulation	4.3 ± 0.71	46.1 ± 2.04	48,3 ± 2.02	1.00 ± 0.12
	stimulation	8.5 ± 1.19	44.3 ± 3.13	45.4 ± 2.24	1.20 ± 0.18
	post-stimulation	5.4 ± 0.88	45.0 ± 2.04	49.1 ± 2.43	0.90 ± 0.13
	post-stimulation	4.4 ± 0.64	45.3 ± 2.52	49.1 ± 2.86	0.80 ± 0.08
128 nM Yohimbine n = 4	pre-stimulation	4.5 ± 1.2	42.1 ± 1.7	52.7 ± 2.6	0.60 ± 0.2
	stimulation	13.5 ± 2.3	36.5 ± 1.1	48.3 ± 1.2	1.70 ± 0.3
	post-stimulation	3.4 ± 1.0	39.3 ± 1.5	56.3 ± 2.5	0.90 ± 0.1
	post-stimulation	5.7 ± 1.2	41.4 ± 1.0	52.2 ± 1.5	0.70 ± 0.2

Table 29

The effect of yohimbine on the evoked ^{3}H-noradrenaline and total tritium fractional release at 10 Hz in normal Krebs (2.5 mM $CaCl_2$)

Drug	(^{3}H)-noradrenaline x 10^{4}	Tritium x 10^{3}	n
Control	11.30 ± 1.20	1.73 ± 0.11	15
Yohimbine (128 nM)	16.40 ± 2.70	1.73 ± 0.25	3

Table 30

The effect of yohimbine (128 nM) on the composition of the tritium overflow from mice vasa deferentia stimulated at 10 Hz in Krebs containing 2.5 mM calcium chloride

DRUG OR EXPERIMENT	COLLECTION PERIOD	PERCENT OF THE TOTAL TRITIUM IN EACH COLLECTION PERIOD			
		NORADRENALINE	NON-CATECHOLS	DOPEG	DOMA
Control n = 8	pre-stimulation	4.2 ± 0.71	45.1 ± 1.96	48.6 ± 1.88	1.26 ± 0.04
	stimulation	46.3 ± 4.36	25.3 ± 2.41	23.7 ± 2.17	4.80 ± 0.90
	post-stimulation	14.5 ± 4.16	41.5 ± 2.85	45.8 ± 2.65	2.00 ± 0.18
	post-stimulation	5.3 ± 1.07	41.8 ± 1.30	51.9 ± 0.99	1.30 ± 0.16
128 nM Yohimbine n = 3	pre-stimulation	6.6 ± 0.7	40.9 ± 1.7	51.4 ± 2.3	1.10 ± 0.7
	stimulation	63.4 ± 7.7	14.4 ± 3.2	14.1 ± 2.8	8.20 ± 1.7
	post-stimulation	15.9 ± 1.6	28.6 ± 2.2	53.7 ± 2.8	1.80 ± 0.2
	post-stimulation	6.7 ± 0.8	37.3 ± 0.6	54.3 ± 1.1	1.30 ± 0.03

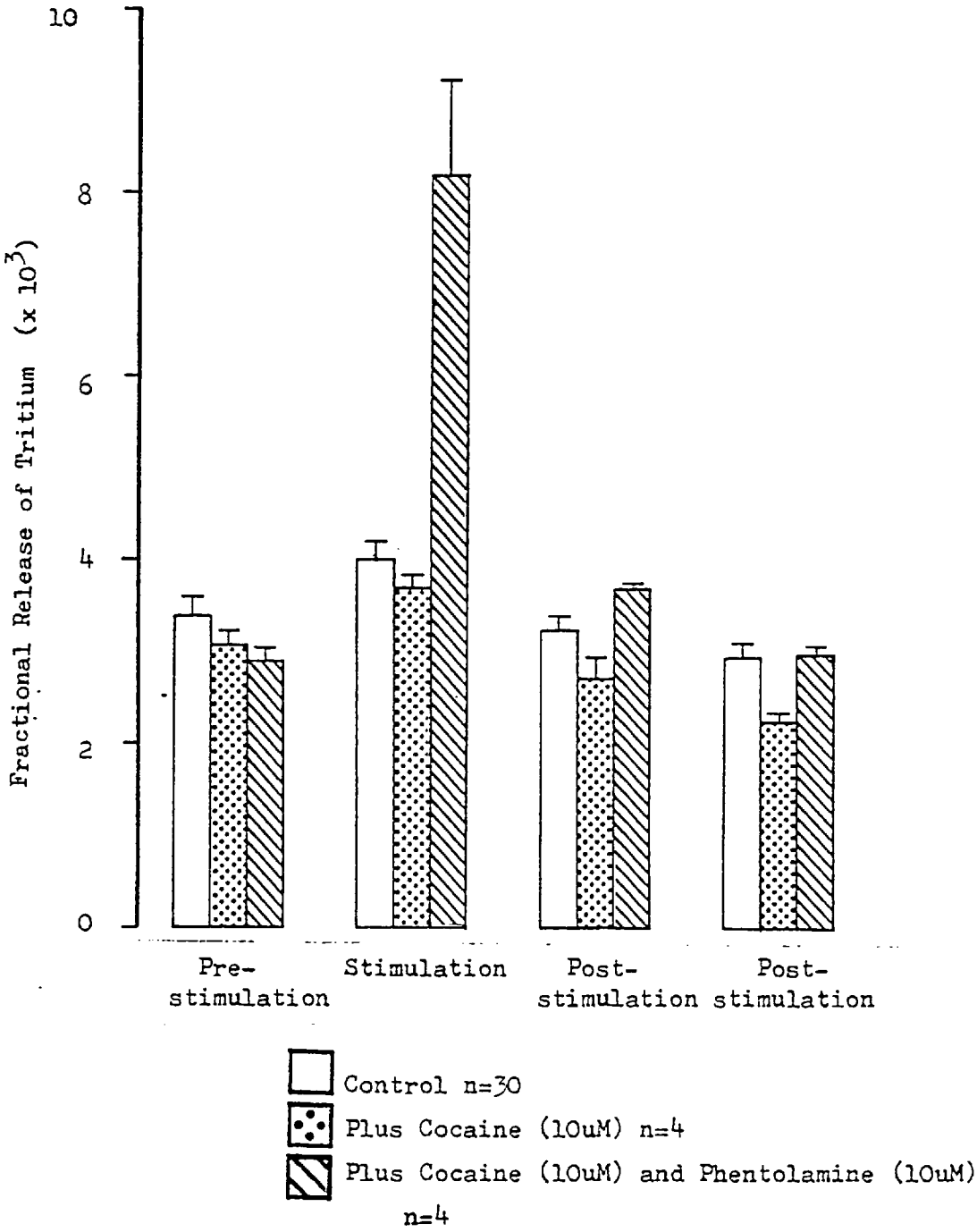


Fig. 44 The effect of cocaine and cocaine plus phentolamine on the fractional release of tritium from mice vasa deferentia stimulated at 1Hz in Krebs containing 2.5mM calcium chloride.

Table 31

The effect of cocaine, and cocaine plus phentolamine on the composition of the tritium overflow from mice vasa deferentia stimulated at 1 Hz in Krebs containing 2.5 mM calcium chloride

DRUG OR EXPERIMENT	COLLECTION PERIOD	PERCENT OF THE TOTAL TRITIUM IN EACH COLLECTION PERIOD			
		NORADRENALINE	NON-CATECHOLS	DOPEG	DOMA
Control n = 18	pre-stimulation	5.6 ± 0.49	45.4 ± 0.83	47.7 ± 1.14	1.25 ± 0.34
	stimulation	18.8 ± 1.46	37.5 ± 0.93	41.7 ± 0.94	2.10 ± 0.21
	post-stimulation	6.1 ± 0.58	42.4 ± 0.80	49.0 ± 1.24	1.18 ± 0.11
	post-stimulation	5.2 ± 0.59	43.2 ± 0.82	50.6 ± 1.08	0.90 ± 0.17
10 μM Cocaine n = 6	pre-stimulation	6.0 ± 1.50	40.6 ± 1.10	52.8 ± 1.50	0.50 ± 0.20
	stimulation	26.8 ± 4.10	30.3 ± 1.70	40.7 ± 3.20	2.20 ± 0.70
	post-stimulation	10.5 ± 0.90	38.4 ± 0.90	50.1 ± 1.10	0.90 ± 0.30
	post-stimulation	4.8 ± 0.60	39.5 ± 1.20	55.3 ± 1.40	0.40 ± 0.10
10 μM Cocaine and 10 μM Phentolamine n = 3	pre-stimulation	4.9 ± 1.60	30.4 ± 0.70	63.4 ± 2.00	1.0 ± 0.10
	stimulation	53.9 ± 3.30	14.4 ± 1.40	21.6 ± 1.10	7.9 ± 1.40
	post-stimulation	23.4 ± 3.00	28.4 ± 2.30	44.6 ± 0.60	3.6 ± 0.20
	post-stimulation	7.0 ± 1.40	37.3 ± 2.80	54.4 ± 2.60	1.2 ± 0.20

presence of cocaine compared to controls (table 21). The duration of (^{3}H)-noradrenaline overflow was not significantly affected.

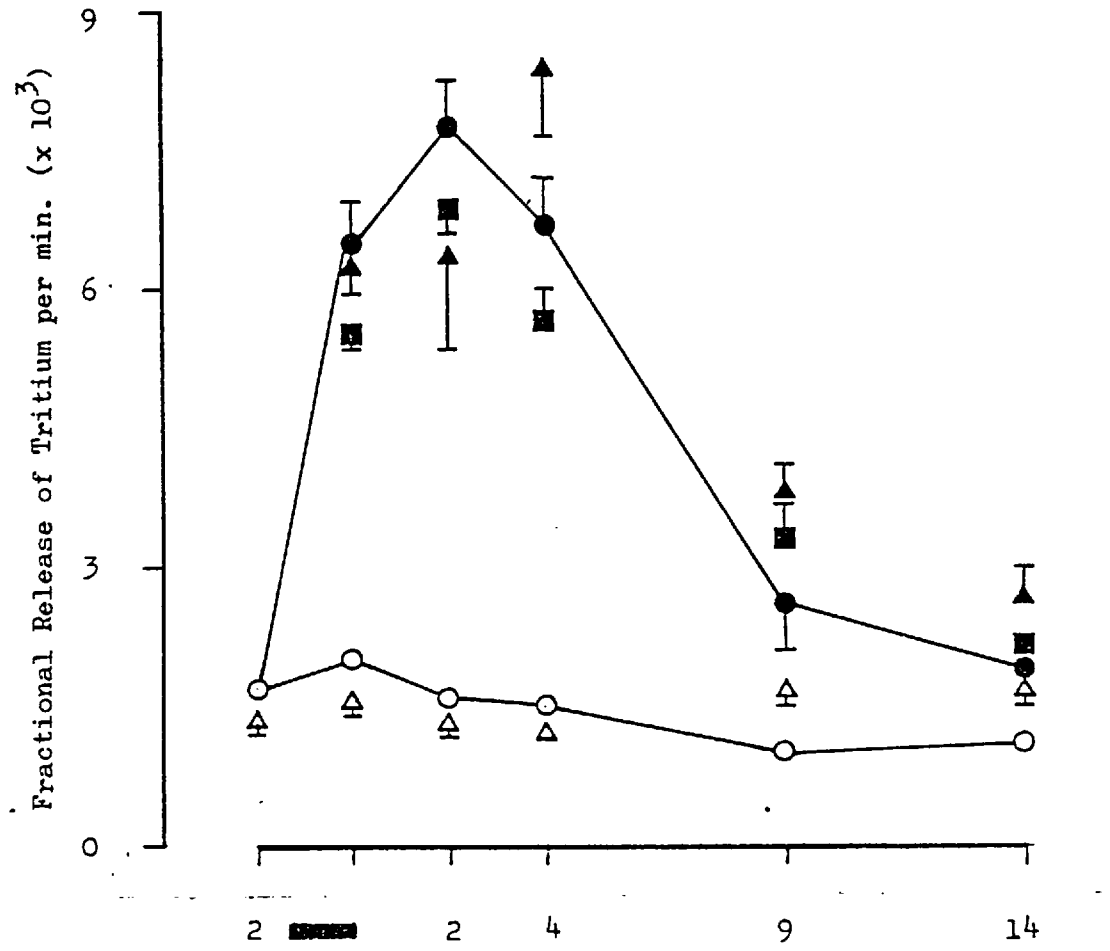
D. Blockade of alpha-adrenoceptors in the presence of cocaine

Addition of phentolamine (10 μM) to the Krebs solution in the presence of cocaine (10 μM) had no significant effect on the magnitude of the basal tritium fractional release, compared with that in the presence of cocaine alone (fig 44). There was a significant fall in the percentage of non-catechols and a significant rise in the percentage of DOPEG in the basal tritium overflow compared with that in the presence of cocaine alone ($P < 0.05$, table 31). Addition of phentolamine caused a significant increase in the tritium and evoked (^{3}H)-noradrenaline fractional release during stimulation (fig 44, table 21). The percentage of (^{3}H)-noradrenaline in the tritium overflow during the stimulation period was significantly increased compared with that in the presence of cocaine alone, the percentages of both DOPEG and non-catechols being significantly reduced ($P < 0.05$, table 31). Phentolamine does not significantly alter the duration of (^{3}H)-noradrenaline overflow.

E. Tyramine

1) Stimulated preparations

Tyramine (20 μM) added thirty seconds before stimulation produced a significant increase in the overflow of tritium and the fractional release of (^{3}H)-noradrenaline during stimulation compared with controls (fig 45 and table 21). The tritium and (^{3}H)-noradrenaline overflows remained



The tissues were stimulated for 2min at the bar

- ○ Control 2.5mM calcium chloride n=30
- △ Control 1.25mM calcium chloride n=13
- ● Tyramine added prior to stimulation in 2.5mM calcium chloride n=4
- ▲ Tyramine added prior to stimulation in 1.25mM calcium chloride n=4
- ■ Tyramine in the absence of stimulation in 2.5mM calcium chloride n=4

Fig. 45 The effect of tyramine (20μM) on the fractional release of tritium from mice vasa deferentia stimulated at 1Hz in Krebs containing 2.5mM or 1.25mM calcium chloride, and in the absence of stimulation.

elevated for over fourteen minutes after the tyramine was washed from the organ bath (fig 45 and 46). During stimulation there was no significant change in the percentage of (^{3}H)-noradrenaline in the tritium overflow compared with controls, however it remained elevated above basal levels for a longer period than in controls (table 32). There was a significant rise in the percentage of (^{3}H)-DOPEG and a corresponding fall in the percentage of (^{3}H)-non-catechols in the stimulated overflow, compared with controls (table 32).

Halving the calcium content of the Krebs solution did not significantly alter the effect of tyramine on the magnitude or duration of the tritium and (^{3}H)-noradrenaline overflow compared with its effect in normal Krebs solution (fig 45 and 46, table 21). In the presence of tyramine in half calcium Krebs the percentage of (^{3}H)-noradrenaline in the tritium overflow during the stimulation and post-stimulation periods were not significantly different from those in the presence of tyramine in normal Krebs. However in the low calcium solution the percentage of (^{3}H)-DOPEG in the tyramine induced tritium overflow tended to be lower than in normal Krebs, while the percentage of (^{3}H)-non-catechols tended to be higher (table 33).

2) Unstimulated preparations

In these experiments tyramine was left in contact with the tissue for 2½ minutes without stimulating the tissue, the bath contents being collected after this period. There was no significant difference in the magnitude or duration of the tritium or (^{3}H)-noradrenaline overflows compared with the stimulated preparations (fig 45 and 46, table 21). The

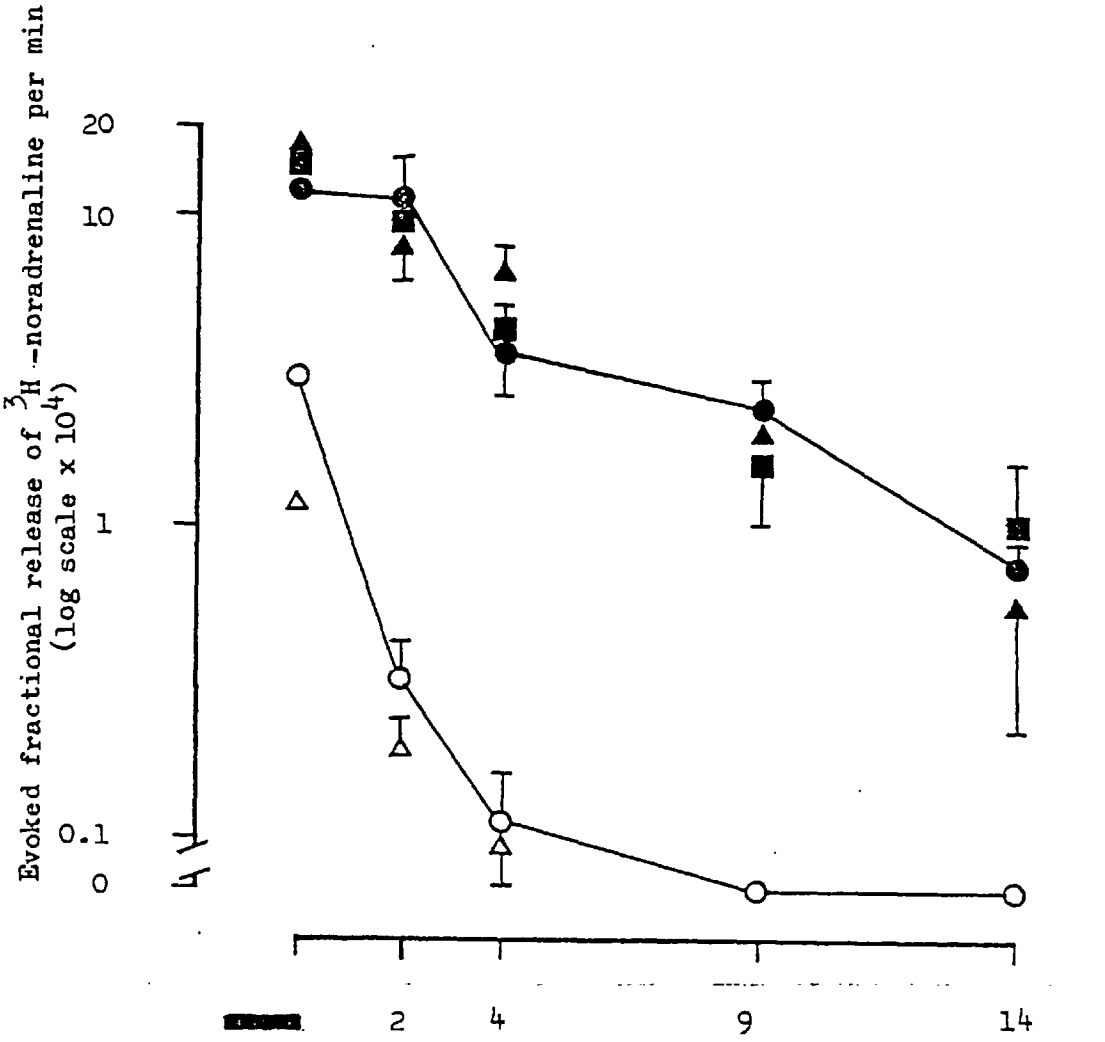


Fig. 46 The effect of tyramine (20μM) on the evoked fractional release of ^{3}H -noradrenaline from mice vasa deferentia stimulated at 1Hz in Krebs containing 2.5mM or 1.25mM calcium chloride, and in the absence of stimulation.

Table 32

The effect of tyramine (20 μM) on the composition of the tritium overflow from mice vasa deferentia stimulated at 1 Hz, and from unstimulated preparations in Krebs containing 2.5 mM calcium chloride

DRUG OR EXPERIMENT	COLLECTION PERIOD	PERCENT OF THE TOTAL TRITIUM IN EACH COLLECTION PERIOD			
		NORADRENALINE	NON-CATECHOLS	DOPEG	DOMA
Control n = 18	pre-stimulation	5.6 ± 0.49	45.4 ± 0.83	47.7 ± 1.14	1.25 ± 0.34
	stimulation	18.8 ± 1.46	37.9 ± 0.93	41.7 ± 0.94	2.10 ± 0.21
	post-stimulation	6.1 ± 0.58	42.4 ± 0.80	49.0 ± 1.24	1.18 ± 0.11
	post-stimulation	5.2 ± 0.59	43.2 ± 0.82	50.6 ± 1.08	0.90 ± 0.17
	post-stimulation	4.3 ± 0.71	37.3 ± 1.79	57.8 ± 1.63	0.70 ± 0.17
	post-stimulation	4.4 ± 1.41	34.5 ± 3.17	60.6 ± 4.44	0.50 ± 0.16
20 μM Tyramine stimulated preparations n = 4	pre-stimulation	6.0 ± 1.5	40.5 ± 0.6	52.3 ± 0.97	1.10 ± 0.1
	stimulation	23.8 ± 3.5	11.5 ± 0.7	60.4 ± 4.7	4.40 ± 0.6
	post-stimulation	18.6 ± 4.2	13.1 ± 2.0	65.7 ± 3.1	2.80 ± 0.2
	post-stimulation	8.8 ± 2.4	17.2 ± 0.8	71.9 ± 1.8	2.00 ± 0.2
	post-stimulation	9.0 ± 2.2	33.1 ± 3.5	56.4 ± 3.4	1.50 ± 0.1
	post-stimulation	6.3 ± 1.6	34.8 ± 1.7	57.5 ± 3.3	1.10 ± 0.3
20 μM Tyramine unstimulated preparations n = 4	pre-stimulation	7.5 ± 1.1	43.2 ± 0.7	48.5 ± 0.4	0.90 ± 0.2
	stimulation	27.7 ± 6.6	13.7 ± 0.4	56.0 ± 7.7	3.30 ± 0.1
	post-stimulation	16.6 ± 3.6	14.4 ± 1.4	66.9 ± 5.0	2.00 ± 0.03
	post-stimulation	11.8 ± 1.0	20.1 ± 1.0	66.5 ± 0.4	1.50 ± 0.1
	post-stimulation	5.5 ± 0.9	29.4 ± 3.2	63.9 ± 3.5	1.30 ± 0.3
	post-stimulation	7.0 ± 1.5	39.4 ± 1.9	52.5 ± 1.0	1.10 ± 0.07

Table 33

The effect of tyramine (20 μM) on the composition of the tritium overflow from mice vasa deferentia stimulated at 1 Hz in Krebs containing 1.25 mM calcium chloride

DRUG OR EXPERIMENT	COLLECTION PERIOD	PERCENT OF THE TOTAL TRITIUM IN EACH COLLECTION PERIOD			
		NORADRENALINE	NON-CATECHOLS	DOPEG	DOMA
Control n = 11	pre-stimulation	4.3 ± 0.71	46.1 ± 2.04	48.3 ± 2.02	1.00 ± 0.12
	stimulation	8.5 ± 1.19	44.3 ± 3.13	45.4 ± 2.24	1.20 ± 0.18
	post-stimulation	5.4 ± 0.88	45.0 ± 2.09	49.1 ± 2.43	0.90 ± 0.13
	post-stimulation	4.4 ± 0.64	45.3 ± 2.52	49.1 ± 2.86	0.80 ± 0.08
	post-stimulation	3.4 ± 0.40	56.8 ± 1.20	36.2 ± 1.60	3.50 ± 3.00
	post-stimulation	4.4 ± 0.60	60.2 ± 1.80	35.2 ± 2.40	0.40 ± 0.07
20 μM Tyramine n = 4	pre-stimulation	2.5 ± 0.3	56.9 ± 1.8	40.0 ± 2.0	0.50 ± 0.0
	stimulation	21.7 ± 0.6	20.8 ± 1.5	56.7 ± 0.7	1.80 ± 0.5
	post-stimulation	18.9 ± 5.2	29.6 ± 3.8	49.7 ± 8.4	1.70 ± 0.1
	post-stimulation	7.2 ± 1.3	28.7 ± 1.8	58.9 ± 3.7	0.80 ± 0.09
	post-stimulation	7.3 ± 0.5	37.6 ± 5.4	56.6 ± 2.1	0.60 ± 0.3
	post-stimulation	4.9 ± 0.5	53.1 ± 3.4	42.2 ± 2.6	0.40 ± 0.01

composition of the tritium overflow was not significantly different from stimulated preparations (table 32).

F. Noradrenaline

Noradrenaline (3 μM, added 30 seconds before stimulation) significantly increased the evoked (^{3}H)-noradrenaline ($P < 0.005$) and the total tritium ($P < 0.005$) fractional release during stimulation at 1 Hz (2.5 mM calcium) compared with controls (table 21). These increases lasted for over fourteen minutes after the exogenous noradrenaline was washed from the organ bath (fig 47 and 48). Noradrenaline did not alter the percentage of (^{3}H)-noradrenaline in the tritium overflow during stimulation compared with controls, though that of (^{3}H)-DOPEG was increased, and that of (^{3}H)-non-catechols decreased (table 34). Exogenous noradrenaline also produced a variable increase in the basal overflow of (^{3}H)-noradrenaline (1.2 to 26.1 fold), and of tritium (1.1 to 3.2 fold). Yohimbine (128 nM) did not have any significant affect on the increases during stimulation when added two minutes before the noradrenaline (tables 21 and 34, fig 47 and 48).
Addition of cocaine (10 μM) to the Krebs solution greatly reduced or abolished the increases in basal overflow of tritium and (^{3}H)-noradrenaline produced by exogenous noradrenaline. During stimulation in the presence of cocaine (10 μM) exogenous noradrenaline decreased the fractional evoked release of (^{3}H)-noradrenaline by 91.8 ± 4.1% (table 21). The overflow of tritium was not significantly altered during the stimulation and first post stimulation period (0-2 minutes after stimulation). However the tritium fractional release was significantly increased in the second post stimulation

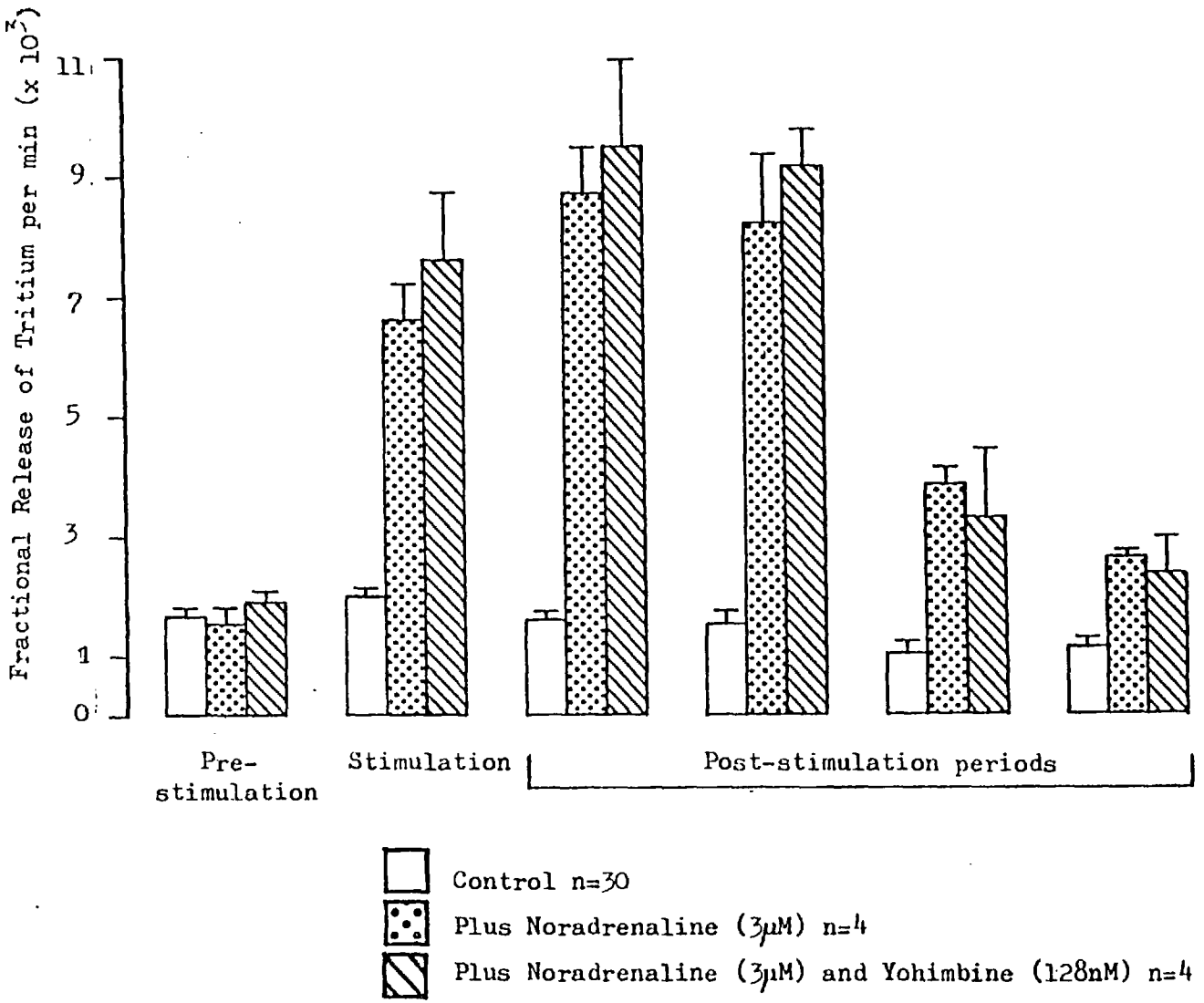


Fig. 47 The effect of noradrenaline and noradrenaline in the presence of yohimbine, on the fractional release of tritium from mice vasa deferentia stimulated at 1Hz in Krebs containing 2.5mM calcium chloride.

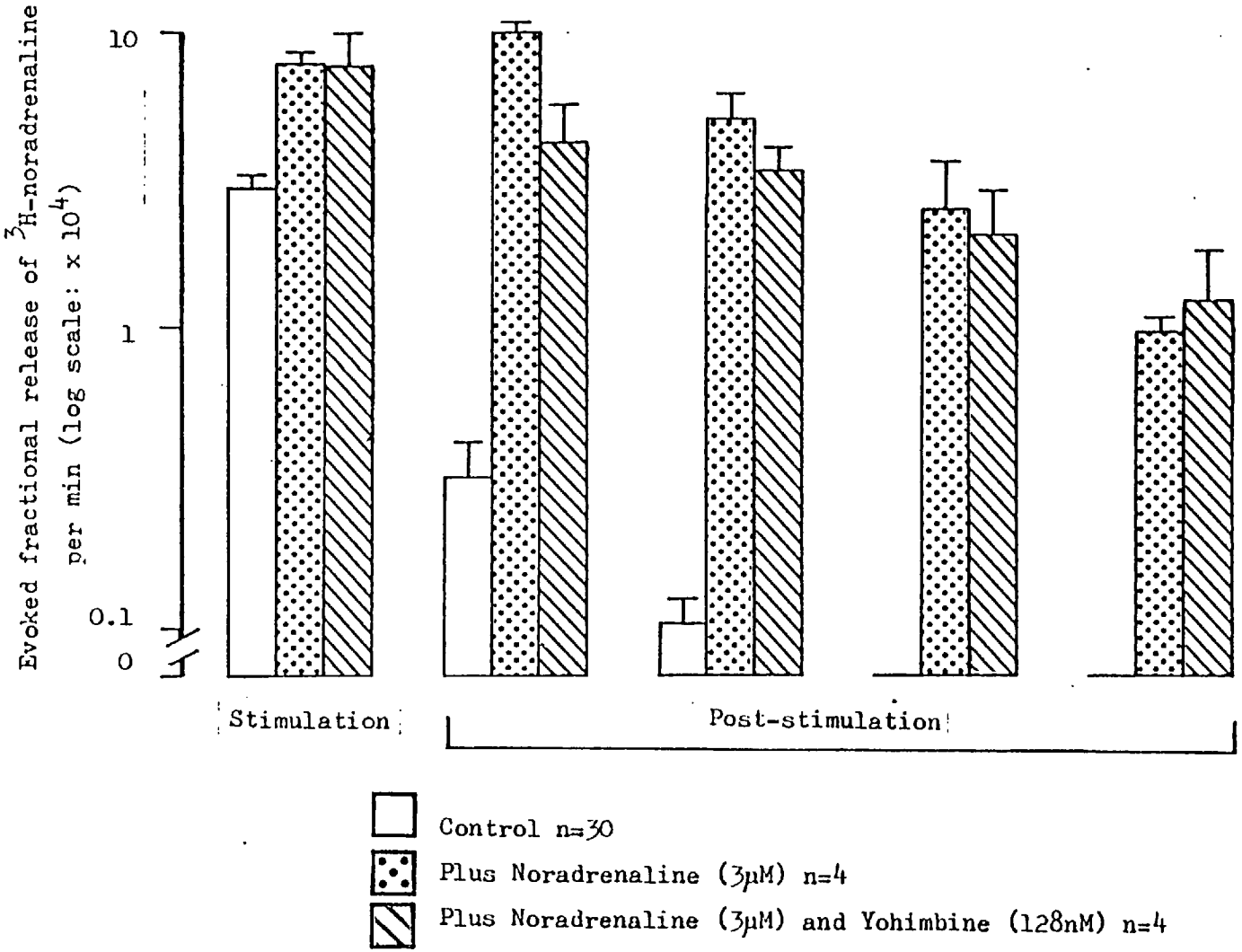


Fig. 48 The effect of noradrenaline, and noradrenaline in the presence of yohimbine, on the evoked fractional release of ^{3}H-noradrenaline from mice vasa deferentia stimulated at 1Hz in Krebs containing 2.5mM calcium chloride

Table 34

The effect of noradrenaline (3 μM) and noradrenaline (3 μM) in the presence of yohimbine (128 nM) on the composition of the tritium overflow from mice vasa deferentia stimulated at 1 Hz in Krebs containing 2.5 mM calcium chloride

DRUG OR EXPERIMENT	COLLECTION PERIOD	PERCENT OF THE TOTAL TRITIUM IN EACH COLLECTION PERIOD			
		NORADRENALINE	NON-CATECHOLS	DOPEG	DOMA
Control n = 18	pre-stimulation	5.6 ± 0.49	45.4 ± 0.83	47.7 ± 1.14	1.25 ± 0.34
	stimulation	18.8 ± 1.46	37.5 ± 0.93	41.7 ± 0.94	2.10 ± 0.21
	post-stimulation	6.1 ± 0.58	42.4 ± 0.80	49.0 ± 1.24	1.18 ± 0.11
	post-stimulation	5.2 ± 0.59	43.2 ± 0.82	50.6 ± 1.08	0.90 ± 0.17
	post-stimulation	4.3 ± 0.71	37.3 ± 1.79	57.7 ± 1.63	0.70 ± 0.17
	post-stimulation	4.4 ± 1.41	34.5 ± 3.27	60.4 ± 4.44	0.50 ± 0.16
3 μM Noradrenaline n = 4	pre-stimulation	7.8 ± 3.94	37.3 ± 4.95	54.3 ± 4.84	0.25 ± 0.05
	stimulation	13.6 ± 2.18	13.5 ± 2.27	72.1 ± 2.48	0.83 ± 0.25
	post-stimulation	10.5 ± 1.98	13.8 ± 1.32	74.4 ± 2.51	1.30 ± 0.57
	post-stimulation	7.4 ± 1.23	17.2 ± 1.77	74.8 ± 1.59	0.60 ± 0.17
	post-stimulation	7.9 ± 2.22	21.2 ± 0.83	70.5 ± 2.09	0.28 ± 0.13
	post-stimulation	5.1 ± 2.39	25.4 ± 1.27	69.1 ± 2.86	0.37 ± 0.67
3 μM Noradrenaline and 128 nM Yohimbine n = 4	pre-stimulation	5.5 ± 0.50	31.2 ± 1.91	62.4 ± 2.29	0.33 ± 0.05
	stimulation	11.9 ± 1.80	18.1 ± 1.40	69.1 ± 3.37	0.93 ± 0.26
	post-stimulation	8.0 ± 0.49	16.4 ± 1.38	75.0 ± 1.59	0.65 ± 0.11
	post-stimulation	6.3 ± 0.65	19.2 ± 2.24	74.0 ± 2.15	0.58 ± 0.09
	post-stimulation	7.4 ± 0.96	31.3 ± 5.16	61.0 ± 4.37	0.33 ± 0.09
	post-stimulation	7.5 ± 0.38	34.1 ± 3.38	58.0 ± 3.39	0.40 ± 0.06

period (2-4 minutes after stimulation, $P < 0.05$, fig 49). Exogenous noradrenaline reduced the percentage of (^3H)-noradrenaline in the tritium overflow but the percentage of (^3H)-DOPEG was increased compared with controls. (table 35). The duration of (^3H)-noradrenaline overflow varied greatly between preparations but was generally increased compared with controls in the presence of cocaine. Addition of yohimbine (128 nM) to the organ bath two minutes before the exogenous noradrenaline significantly reduced the decrease in the evoked (^3H)-noradrenaline fractional release produced by noradrenaline ($P < 0.005$, table 21). Yohimbine did not significantly effect the magnitude or composition of the tritium overflow in the presence of exogenous noradrenaline (fig 49, table 35).

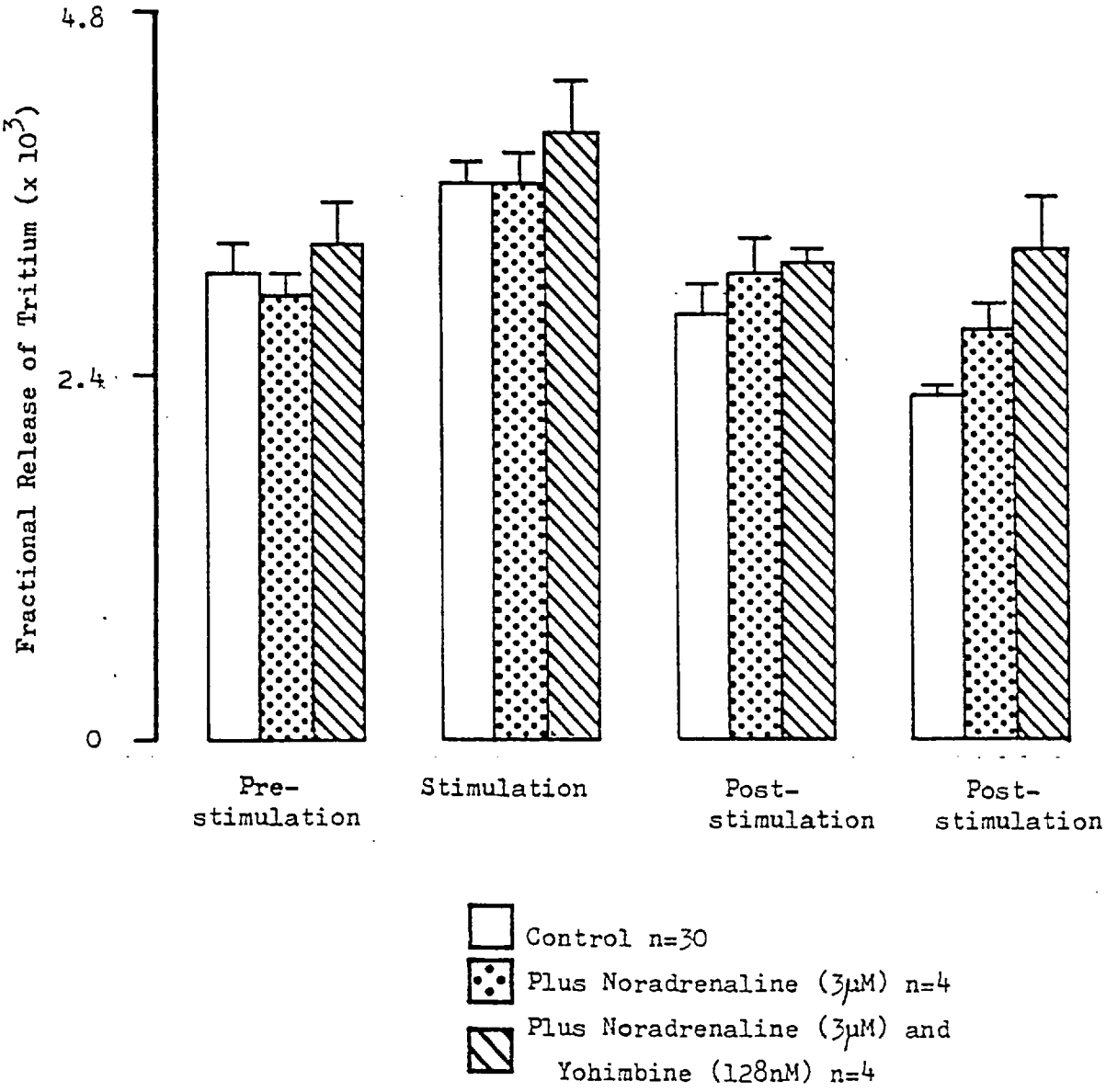


Fig. 49 The effect of noradrenaline, and noradrenaline in the presence of yohimbine, on the fractional release of tritium from mice vasa deferentia stimulated at 1Hz in Krebs containing cocaine (10μM).

Table 35

The effect of noradrenaline (3 μM) and noradrenaline (3 μM) in the presence of yohimbine (128 nM) on the composition of the tritium overflow from mice vasa deferentia in Krebs containing 10 μM cocaine (2.5 mM calcium chloride)

DRUG OR EXPERIMENT	COLLECTION PERIOD	PERCENT OF THE TOTAL TRITIUM IN EACH COLLECTION PERIOD			
		NORADRENALINE	NON-CATECHOLS	DOPEG	DOMA
Control n = 3	pre-stimulation	7.5 ± 2.96	41.4 ± 1.96	50.8 ± 1.99	0.23 ± 0.09
	stimulation	18.6 ± 3.17	33.5 ± 2.09	47.3 ± 1.88	0.63 ± 0.03
	post-stimulation	9.9 ± 1.63	38.0 ± 1.89	51.6 ± 0.40	0.23 ± 0.03
	post-stimulation	5.9 ± 0.50	39.7 ± 2.24	54.2 ± 2.56	0.18 ± 0.11
3 μM Noradrenaline n = 3	pre-stimulation	7.4 ± 2.56	35.9 ± 2.20	56.6 ± 1.52	0.09 ± 0.006
	stimulation	7.2 ± 1.69	27.0 ± 2.34	64.8 ± 3.48	0.40 ± 0.100
	post-stimulation	6.8 ± 0.87	22.1 ± 2.82	70.8 ± 2.06	0.33 ± 0.03
	post-stimulation	8.2 ± 0.12	26.4 ± 1.53	65.3 ± 1.56	0.17 ± 0.07
3 μM Noradrenaline and 128 nM Yohimbine n = 3	pre-stimulation	5.5 ± 0.53	35.5 ± 3.69	58.9 ± 3.68	0.13 ± 0.03
	stimulation	8.43 ± 0.13	24.7 ± 1.31	66.5 ± 1.37	0.33 ± 0.03
	post-stimulation	7.53 ± 1.45	22.3 ± 2.17	69.8 ± 1.25	0.30 ± 0.04
	post-stimulation	7.30 ± 1.38	24.2 ± 0.07	68.1 ± 1.35	0.18 ± 0.07

In the preceding section the overflow of (^{3}H)-noradrenaline and its (^{3}H)-metabolites from mice vasa deferentia preloaded with (^{3}H)-noradrenaline has been studied. The effect of varying the frequency of stimulation, the calcium content of the Krebs solution, and of several drugs, upon this overflow have been examined. This method of studying noradrenaline overflow provides an indirect estimation of the overflow of the endogenous transmitter, unlike the radioenzymic method used in part I. However the tracer method proved to be more reproducable and more sensitive (allowing measurement of basal overflow) than the radioenzymic assay. Due to the routine addition of oestradiol (3.7 μM) to the Krebs solution in the tracer method (in an attempt to decrease the post-synaptic uptake and metabolism of (^{3}H)-noradrenaline), the results obtained by the two methods are only directly comparable in two sets of experiments. These are when the vasa deferentia were stimulated at 1 Hz in the presence of oestradiol and cocaine, or in the presence of oestradiol, cocaine and phentolamine. In each situation the fractional release of noradrenaline (produced by 120 stimulation pulses), measured by either method was not significantly different. (oestradiol and cocaine, tracer method = $9.23 \pm 2.17 \times 10^{-4}$, radioenzymic = $10.5 \pm 1.30 \times 10^{-4}$; cocaine, oestradiol and phentolamine, tracer = $49.1 \pm 8.7 \times 10^{-4}$, radioenzymic = $41.7 \pm 11.8 \times 10^{-4}$). These results would suggest that the tracer method is a valid one to measure the overflow of endogenous noradrenaline. One important difference between the results obtained by the two methods however, was the effect of increasing the frequency of stimulation on the noradrenaline overflow. Using the radio-

enzymic method, the overflow of noradrenaline per stimulus pulse remained constant as the frequency of stimulation was increased from 1 to 16 Hz. However using the tracer method, the overflow of (^{3}H)-noradrenaline was approximately doubled by increasing the frequency of stimulation from 1 to 10 Hz. Henderson, Hughes and Kosterlitz (1975), and Henderson and Hughes (1976), have used biological and fluorimetric methods to measure endogenous noradrenaline overflow. These authors found that the noradrenaline overflow per stimulus pulse from the cat nictitating membrane rose only 1.3 fold, and that from the mouse (T.O strain) vas deferens remained constant, when the frequency of stimulation rose from 0.5 to 15 Hz. This constancy is apparently a characteristic of morphine sensitive neuroeffector junctions. In those vas deferens preparations which are not sensitive to morphine (e.g. the rabbit and C57/BL strain of mice) and other non-sensitive tissues such as the rabbit portal vein, the overflow of endogenous noradrenaline increases at least ten fold as the frequency of stimulation is increased from 0.5 to 15 Hz (Hughes 1972, Hughes and Roth 1974). The apparent difference in the present results obtained by the two methods employed to measure overflow in these experiments is probably due to the inclusion of oestradiol in the Krebs for the (^{3}H)-noradrenaline overflow estimations. In the absence of this drug the overflow of (^{3}H)-noradrenaline at 1 and 10 Hz could be constant. Addition of oestradiol then raises the concentration of noradrenaline in the synaptic cleft due to uptake blockade, and this leads to increased stimulation of presynaptic alpha-adrenoceptors. Due to the frequency dependent nature of these receptors they would then

produce a greater decrease in (^{3}H)-noradrenaline release at 1 Hz than 10 Hz, hence the overflow is less at 1 Hz than 10 Hz. This concept is supported by the fact that when yohimbine is added (blocking the presynaptic alpha-adrenoceptor) the overflow of (^{3}H)-noradrenaline is constant when the frequency of stimulation is increased from 1 to 10 Hz. Measurement of noradrenaline or tracer overflow is only an approximation of what is released, and Langer (1970) concluded that it was necessary to consider both noradrenaline and its metabolites when calculating actual evoked transmitter output. In the present experiments the evoked (^{3}H)-noradrenaline fractional release, the total tritium fractional release and the metabolic composition of the tritium overflow have been considered. The evoked (^{3}H)-noradrenaline fractional release was calcium dependent, as was the total tritium fractional release, both decreasing when the calcium concentration of the Krebs was halved. Increasing the frequency of stimulation from 1 to 10 Hz increased the evoked (^{3}H)-noradrenaline fractional release but decreased the total tritium fractional release during the stimulation period. The decrease in the tritium is probably due to the decreased collection period of the sample, as the tritium overflow continued to rise in the post-stimulation periods, and there was also a significant decrease in the amount of tritium in the basal overflow period prior to stimulation. The percentage of (^{3}H)-noradrenaline in the total tritium overflow during stimulation was significantly greater at 10 Hz than at 1 Hz. The main metabolite in the overflow at either frequency was (^{3}H)-DOPEG. This preferential metabolism of (^{3}H)-noradrenaline to (^{3}H)-DOPEG has been reported to occur in the rat vas deferens (Langer, 1970),

and the rat and guinea-pig atria (Roffler-Tarlov and Langer, 1971). The origin of the metabolites in the tritium overflow could be both pre- and post-synaptic as it has been shown that MAO and COMT are present at both sites (Langer, 1970). However in these experiments the majority of metabolism will take place presynaptically as the extraneuronal noradrenaline uptake inhibitor oestradiol has been included in the Krebs solution. Whether these metabolites are produced from noradrenaline released from the neurones then taken up and subsequently metabolised; or whether the noradrenaline is metabolised in the neurone without being released, or if both processes occur, is not known. As exocytotic release is low during non-stimulated periods it seems likely that the basal release consists of metabolites occuring as a result of intra-neuronal metabolism. On stimulation exocytosis increases, and any increase in metabolites is probably due to the re-uptake and subsequent intra-neuronal metabolism of noradrenaline. In the majority of the experiments with the mouse vas deferens stimulation leads to a decrease in the overflow of metabolites compared with the basal overflow, and this decrease is greater at 10 Hz than at 1 Hz. On stimulation, therefore, the amount of intra-neuronal metabolism decreases, though how much of the released noradrenaline is taken up and metabolised is not known. At both 1 and 10 Hz the (^{3}H)-non-catechol and (^{3}H)-DOPEG fraction of the tritium overflow decrease in parallel, providing no evidence for any redirection of metabolism during stimulation as opposed to basal metabolism. Prevention of neuronal uptake of the (^{3}H)-noradrenaline released on stimulation should provide evidence as to how much of the released noradrenaline is taken

up and metabolised and hence the proportion of the metabolites due to this process. As reported for the cat spleen (Dubocovich and Langer, 1976), and nictitating membrane (Langer, Stefano and Enero, 1972), in the mouse vas deferens cocaine (10 μM) had no significant effect on the fractional evoked release of (^{3}H)-noradrenaline. Langer et al.(1972), found that cocaine (880 nM) had no significant effect on the percentage of (^{3}H)-noradrenaline in the total tritium overflow, ^{3}H-NMN being the only metabolite significantly increased above controls. Dubocovich and Langer (1976) found that cocaine (2.9 μM) significantly increased the percentage of (^{3}H)-noradrenaline, decreased the ^{3}H-OMDA metabolites and abolished the ^{3}H-DOPEG release. In the mouse vas deferens the percentage of (^{3}H)-noradrenaline in the tritium overflow was significantly increased by cocaine, while that of ^{3}H-non-catechols was significantly decreased. There was no significant change in the percentage of ^{3}H-DOPEG. From these results it is apparent that the effect of cocaine varies between tissues. This may be due to the differences in the width of the synaptic cleft in different tissues. The uptake mechanism is thought to be more important for the removal of noradrenaline from the cleft in tissues where the cleft is narrow, thus allowing less opportunity for diffusion. The cleft in the mouse vas deferens has been reported to be very narrow (Merrillees, Burnstock and Holman, 1963) and therefore uptake may play an important role in this tissue. However the effect of cocaine on the overflow of (^{3}H)-noradrenaline and its metabolites from this tissue may be complicated by another factor, presynaptic alpha-adrenoceptors. Uptake blockade leads to inhibition of the twitch response to

field stimulation, as a result of interaction of noradrenaline with these receptors, this may decrease noradrenaline release. The selective presynaptic alpha-adrenoceptor agonist clonidine decreased the evoked (^{3}H)-noradrenaline fractional release, but as reported by Stjärne (1975), did not affect the total tritium fractional release. This can be explained by the increase in the percentage of (^{3}H)-metabolites offsetting the decrease in (^{3}H)-noradrenaline. This alteration in the metabolism of (^{3}H)-noradrenaline may be intra-neuronal, as clonidine has no effect on the neuronal uptake of (^{3}H)-noradrenaline. Thus uptake blockade and presynaptic alpha-adrenoceptor stimulation have opposite effects on overflow. If therefore, the presynaptic alpha-adrenoceptors are blocked and then cocaine is added one should see the true effect of uptake blockade. However under the present experimental conditions presynaptic alpha-adrenoceptors are stimulated by released endogenous noradrenaline and thus their blockade results in an increase in (^{3}H)-noradrenaline overflow. In the presence of cocaine and phentolamine (plus oestradiol) which as well as blocking alpha-adrenoceptors will contribute to uptake blockade, there is an increased evoked (^{3}H)-noradrenaline fractional release and total tritium fractional release compared with controls. The percentage of (^{3}H)-noradrenaline in the tritium overflow is increased approximately three fold above controls, the ^{3}H-DOPEG and ^{3}H-non-catechol metabolites being greatly reduced. That these changes in the metabolic composition of the overflow are due to uptake blockade and not due to presynaptic alpha-adrenoceptor blockade can be seen by reference to the effect of the selective presynaptic alpha-adrenoceptor antagonist yohimbine.

Yohimbine (128 nM) increased the evoked (^{3}H)-noradrenaline and total tritium fractional release at 1 Hz stimulation frequency in normal Krebs solution. These increases are what would be expected if the overflow of (^{3}H)-noradrenaline were under the control of presynaptic alpha-adrenoceptors, stimulated by endogenous noradrenaline released on field stimulation of the tissue. This effect of endogenous noradrenaline leads to a decrease in the maximum possible overflow of (^{3}H)-noradrenaline at this frequency. This concentration of yohimbine had no significant effect on the (^{3}H)-noradrenaline and ^{3}H-DOPEG fractions of the tritium overflow, but slightly decreased the ^{3}H-non-catechol fraction. All these results demonstrate that in this tissue the study of the effect of uptake blockade on (^{3}H)-noradrenaline and its ^{3}H metabolites is complicated by the effects of presynaptic alpha-adrenoceptors on the overflow and metabolism of (^{3}H)-noradrenaline. However taking these facts into account it seems that uptake blockade per se produces an increase in the percentage of (^{3}H)-noradrenaline and a decrease in the percentage of ^{3}H-DOPEG in the tritium overflow, the ^{3}H-non-catechols probably not being affected. This result would be expected if in the absence of uptake blockade noradrenaline were released, then taken up and subsequently metabolised intra-neuronally.

The effect of clonidine in normal Krebs solutions has been outlined above. Halving the calcium concentration of the Krebs solution increased its inhibition of the evoked (^{3}H)-noradrenaline fractional release, as in normal Krebs clonidine had no effect on the tritium fractional release. The metabolic changes were similar to those produced in normal

Krebs. Increasing the frequency of stimulation to 10 Hz decreased the inhibition of the evoked (^{3}H)-noradrenaline fractional release produced by clonidine and its metabolic effects were also reduced but still apparent. Both these results reflect changes seen in the response of the tissue to field stimulation outlined in part II. The affect of clonidine on overflow is consistent with the concept that pre-synaptic modulation of the twitch response is mediated via control of transmitter release. Furthermore the potentiation of the effect of clonidine in low calcium solutions and its decreased effect at high frequencies of stimulation are consistent with the hypothesis that transmitter release is modulated by presynaptic alpha-adrenoceptors via changes in the availability of calcium ions.

The effects of yohimbine at 1 Hz stimulation frequency in normal Krebs solution have been outlined above. Halving the calcium concentration of the Krebs solution abolished the potentiation of the evoked (^{3}H)-noradrenaline and total tritium release produced by yohimbine. Increasing the frequency of stimulation from 1 Hz to 10 Hz in normal Krebs solution decreased the potentiation of the evoked (^{3}H)-noradrenaline fractional release elicited by yohimbine and abolished its effect on the total tritium fractional release. As with clonidine these results reflect the effect of yohimbine on the response of the tissue to field stimulation. The decreased effect of yohimbine in low calcium and at 10 Hz compared with its effect at 1 Hz in normal Krebs are also consistent with the calcium availability hypothesis of the mode of action of presynaptic alpha-adrenoceptors, as outlined in part II.

The effect of exogenous noradrenaline on the overflow of (^{3}H)-noradrenaline was difficult to assess. Farnebo and Malmfors (1971) showed that exogenous noradrenaline (10^{-6}M) increased the basal overflow of tritium from the mouse vas deferens. Similar results were obtained in the present experiments. Noradrenaline (3 μM) increased the basal and stimulated overflow of tritium and the evoked fractional release of (^{3}H)-noradrenaline and these increases were not affected by yohimbine (128 nM). Contrary to the experiments with single vasa reported in part II, in these experiments noradrenaline potentiated the twitch response of the groups of six vasa deferentia to stimulation at 1 Hz. This potentiation of the twitch was not affected by yohimbine. The increase in basal overflow produced by noradrenaline could be prevented by the addition of cocaine (10 μM) to the Krebs solution. This suggests that the increase in basal overflow was due to displacement of (^{3}H)-noradrenaline from its storage sites by the unlabelled exogenous noradrenaline taken up into the neurone by the uptake one process. In the presence of cocaine (10 μM) exogenous noradrenaline decreased the evoked (^{3}H)-noradrenaline fractional release during stimulation at 1 Hz, but as with the inhibition produced by clonidine, the total tritium fractional release was not affected. In this situation the twitch response was also inhibited. As seen with clonidine, exogenous noradrenaline decreased the percentage of (^{3}H)-noradrenaline in the tritium overflow and increased the percentage of (^{3}H)-DOPEG and (^{3}H)-non-catechols. All these changes were antagonised to some extent by yohimbine (128 nM), suggesting that they were mediated via presynaptic alpha-adrenoceptors.

From these results it is possible to suggest that the increased

(^{3}H)-noradrenaline release in the presence of exogenous noradrenaline (in the absence of cocaine) is due to displacement of (^{3}H)-noradrenaline and not an indication of an increased release of endogenous noradrenaline. The fact that the tissue response was increased argues against this and suggests that there was a real increase in noradrenaline overflow. Why this occurs is not apparent but it is presumably related to the grouping of six vasa deferentia together, as a single vas deferens preparation is always inhibited by noradrenaline. The potentiation however is not directly related to the large increase in (^{3}H)-noradrenaline overflow as is demonstrated by the effect of tyramine. The indirectly acting amine, tyramine (20 μM) produced a prolonged increase in evoked (^{3}H)-noradrenaline and the total tritium fractional release. The evoked (^{3}H)-noradrenaline fractional release was approximately twelve fold that of controls and almost five fold that produced by noradrenaline (3 μM). However, tyramine inhibited the twitch response of the 6 vasa preparation as it did the single vas deferens. The effects of tyramine have been well documented and as in other tissues (Chubb et al 1972, Stjärne 1961) its release of noradrenaline in this preparation was independent of electrical stimulation of the tissue and the calcium concentration of the Krebs solution. The tritium released by both noradrenaline (in the absence of cocaine) and tyramine contained a high percentage of (^{3}H)-DOPEG, which would suggest that a high proportion of the (^{3}H)-noradrenaline was released and metabolised intraneuronally. This similarity between the effects of tyramine and noradrenaline suggests that at least part of the action of noradrenaline is to displace endogenous

noradrenaline from its storage site (Trendelenburg, 1961).

In conclusion, clonidine decreases and yohimbine increases the evoked (^{3}H)-noradrenaline fractional release. These effects are calcium and frequency dependent, supporting the theory that presynaptic alpha-adrenoceptor stimulation decreases the tissue response by decreasing transmitter release as a result of limiting the amount of calcium available for exocytosis. Exogenous noradrenaline can decrease the evoked (^{3}H)-noradrenaline fractional release and this can be antagonised by yohimbine. These results suggest that exogenous noradrenaline inhibits the tissue response via presynaptic alpha-adrenoceptors. Cocaine has little effect on the transmitter overflow which may be due to the opposing factors of uptake blockade increasing overflow, and presynaptic alpha-adrenoceptor stimulation by noradrenaline decreasing further release. Addition of phentolamine in the presence of cocaine produces a large increase in the evoked (^{3}H)-noradrenaline release as expected if phentolamine blocked presynaptic alpha-adrenoceptors. Tyramine, as in other tissues, produces a large increase in the evoked (^{3}H)-noradrenaline and total tritium fractional release.

GENERAL DISCUSSION AND CONCLUSIONS

The isolated vas deferens preparation from several species responds to electrical stimulation with a contraction composed of two distinct phases (Swedin, 1971; Anton, Duncan and McGrath 1977). While the mouse vas deferens does not respond with two such distinct phases, it is apparent that the responses to 5 - 16 Hz as examined in these experiments, are composed of at least two components. The first phase, or twitch component, which has been the subject of this work, is potentiated by alpha-adrenoceptor blocking agents and inhibited as a result of noradrenaline uptake blockade. Apparently therefore this phase is not adrenergically mediated. The second component is more variable in magnitude than the first, but is reduced by post synaptic alpha-adrenoceptor blocking agents, and potentiated by noradrenaline uptake blockade. Therefore this phase is adrenergically mediated. The first component is also inhibited by exogenous noradrenaline and by tyramine. The initial aim of these experiments was to explain these apparent discrepancies in the response of a tissue which is anatomically sympathetically innervated. Investigation has shown that this tissue contains presynaptic alpha-adrenoceptors which modulate both the noradrenaline overflow and the twitch response of the tissue.

The inhibition of the twitch response to electrical stimulation produced by exogenous noradrenaline, tyramine and noradrenaline uptake blockade by oestradiol and cocaine, or cocaine alone; can be explained by the preferential action of noradrenaline (exogenous and endogenous) upon presynaptic alpha-adrenoceptors. This would suggest that noradrenaline has an inhibitory role in this tissue (Jenkins et al. 1977) modulating the release of another motor transmitter. It would appear contradictory

to suggest that noradrenaline were the motor transmitter, and upon its release would act preferentially to inhibit further release, rather than acting upon post synaptic receptors. However the argument for a purely inhibitory role for noradrenaline can be attacked on several points. The precise location of presynaptic alpha-adrenoceptors on the neurone is not known. They may be located at the terminal varicosities in the synaptic cleft, or at any site or sites along the axon. The former location is preferable if the presynaptic feedback mechanism is considered to be a means of modulating noradrenaline release. The latter is more suitable if the feedback mechanism is a method of preventing large increases in release which may influence other receptor sites. Both distributions may occur on the same neurone, thus mediating both functions. If the receptors were present on the axon it is possible that they would be stimulated in preference to post synaptic receptors, which may lie within the so called 'narrow junctions' in this tissue (Furness and Iwayama 1971 and 1972). The inhibitory effect of cocaine on the twitch is interesting as a similar argument can be applied as to the location of the neuronal uptake process, is it solely in the terminal varicosities, or present along the whole axon? However the fact that neuronal uptake blockade produces a greater inhibition of the twitch response than blockade of extraneuronal uptake, suggests that the former, rather than the latter, has an important role in preventing stimulation of presynaptic receptors by excess noradrenaline. This has two important consequences. Firstly it supports the ascribing of a pre-synaptic location to the receptor mediating the feedback

mechanism. Secondly it infers upon the neuronal uptake mechanism not only the role of conserving and terminating the effect of released transmitter; but also of maintaining a constant level of transmitter release by preventing excess accumulation of noradrenaline in the synaptic cleft and hence presynaptic receptor stimulation. If the neuronal uptake mechanism is blocked and synaptic concentrations of noradrenaline rise, the presynaptic alpha-adrenoceptor will be stimulated and decrease release.

Consideration of the noradrenaline overflow results shows that overflow parallels the twitch response in all but two instances. Uptake blockade as explained above inhibits the twitch response but does not decrease the overflow. This increased overflow may in fact represent an increase recovery of released noradrenaline, which is no longer taken up and therefore diffuses into the bathing fluid. This process may mask a decrease in noradrenaline release, paralleling the decreased twitch. Tyramine releases noradrenaline by a calcium independent mechanism, possibly from different areas of the neurone than exocytosis. The massive release of noradrenaline due to tyramine's displacement of it from it's storage sites (Burn and Rand 1958 and 1960), may be occurring mainly at sites distant from the postsynaptic receptor, and proximal to the presynaptic receptor. This may produce a decrease in the exocytocic release of noradrenaline which is masked by the releasing action of tyramine. With these two exceptions why should it be necessary for noradrenaline release to parallel the twitch response if its role is an inhibitory one? Logically the inhibitory compound's release would be inversely related to the twitch size. Unless that

is, the motor neurone is also inhibited via a presynaptic alpha-adrenoceptor by the noradrenaline released on stimulation. If this were not so addition of a presynaptic agonist would decrease noradrenaline release and increase the twitch size. By the same argument the depletion of noradrenaline by reserpine, or alpha methyl-p-tyrosine would be expected to increase the twitch size by removing the inhibitory substance. As in fact the twitch is decreased one must presume that the motor transmitter is also depleted by these drugs. Clearly the description of the 'other transmitter substance' supposedly under the modulation of noradrenaline, is approaching a description of noradrenaline itself, which is released on stimulation and acts via presynaptic alpha-adrenoceptors to modulate its own release, and post synaptically to contract the tissue. This description is complete when the fact that the response to stimulation is blocked by the adrenergic neurone blocking agent guanethidine is considered. An explanation of the preferential action of noradrenaline upon presynaptic alpha-adrenoceptors under these circumstances might be a consequence of the physiological role of this tissue. It is presumably not a tonically active muscle and is required to contract only at specific instances and should remain quiescent at other times. A well developed feedback mechanism may be required to prevent 'stray' impulses causing contraction of the tissue. A high frequency burst of nerve impulses would, by the nature of presynaptic receptors overcome their inhibitory influence and allow a large release of noradrenaline and hence contraction of the tissue. The question of the frequency of stimulation of this tissue is most important

when the results of pretreating animals with alpha methyl-p-tyrosine are considered. Depletion of the noradrenaline content of the vas deferens by this drug was the same as the depletion of the heart. As the vas deferens is unlikely to receive the same amount of stimulation as the heart it would seem probably that the vas deferens loses noradrenaline from its storage sites by some other process. This is apparent when the results of the (^{3}H)-noradrenaline overflow studies are considered. There proved to be a high basal release of tritium from this tissue, composed mainly of metabolites. The main effect of stimulation of this tissue was to increase the percentage of (^{3}H)-noradrenaline in the overflow, there being only a small increase in the total tritium overflow. As the ultimate source of the overflowing metabolites must be (^{3}H)-noradrenaline, it is apparent that the vas deferens loses amounts of (^{3}H)-noradrenaline which are comparable to those released by nerve stimulation, by metabolism during periods when it is not being electrically stimulated. Presumably this (^{3}H)-noradrenaline 'leaks' from storage vesicles into the cytoplasm where it is metabolised.

The one, as yet unexplained feature of this tissue's response to stimulation is the inability of alpha and/or beta adrenoceptor blocking agents to inhibit the twitch response. It is possible, as suggested in an earlier section, that the noradrenaline receptor in this tissue is different from conventional adrenoceptors. The concept of a spectrum of receptors rather than individual receptor types is well recognised. This has been recently emphasized by the finding that the affinity of antagonists and agonists for pre- and postsynaptic alpha-adrenoceptors is different, and therefore

that these receptors are different (Dubocovich and Langer, 1974). This has led to the suggestion that these receptors should be referred to as alpha$_1$ (post-synaptic) and alpha$_2$ (pre-synaptic) (Langer, 1974). It is also becoming apparent that the alpha$_2$ receptor varies between tissues and species (Starke, Tauke and Borowski, 1977). The use of more specific alpha$_1$ adrenoceptor blocking agents, such as prazosin, (Cambridge, Davey and Massingham, 1977) to investigate the twitch response of this tissue may prove useful in elucidating the nature of the receptor mediating it.

Despite the problems that the inability to block the twitch response with adrenoceptor blocking agents may provide for interpreting the role of noradrenaline in this tissue, it is a very useful property from the point of view of investigating presynaptic receptor mechanisms. In other adrenergically innervated tissues where the post synaptic receptor is of the alpha type e.g. spleen, cat nictitating membrane and blood vessels, addition of an alpha adrenoceptor blocking agent increases the noradrenaline overflow, but decreases the tissue response. In the vas deferens however, it is possible to correlate the effect of these drugs on overflow and tissue response. Also, as the twitch response is not blocked by other post synaptic blocking agents (to date) this tissue can be used to detect drugs that act as agonists either pre or post synaptically to depress the twitch response. Presynaptic agonists can be selected by their frequency and calcium dependency, their effect on noradrenaline overflow, and in the case of those acting via alpha-adrenoceptors, their blockade by yohimbine. Employing this type of screen has, for example, identified ergometrine as an agonist at presynaptic

alpha-adrenoceptors (Marshall, Nasmyth, Russell and Shepperson, 1977), and histamine as a post-synaptic agonist (Marshall et al., 1979) in this tissue.

The effects of presynaptic alpha adrenoceptor stimulation on the overflow of (^{3}H)-noradrenaline are somewhat unusual. The decrease in (^{3}H)-noradrenaline overflow produced by stimulation of these receptors is expected, but the fact that this decrease is mirrored by an increase in metabolites thus maintaining the tritium overflow is not. While this effect can be considered from a positive point of view i.e. clonidine increasing the metabolism of noradrenaline, it could probably be explained more easily on the basis of the large noradrenaline loss through metabolism as discussed earlier. In the absence of stimulation there is a large overflow of tritium from this tissue in the form of (^{3}H)-noradrenaline metabolites. Stimulation causes only a small rise in the total tritium overflow but a large increase in (^{3}H)-noradrenaline overflow, presumably due to an increase in exocytotic release as opposed to leakage of (^{3}H)-noradrenaline from the storage vesicles. This causes a rise in the fraction of the tritium overflow that is (^{3}H)-noradrenaline. The effect of presynaptic alpha-adrenoceptor stimulation may be to prevent the exocytocic release (due to lack of calcium) of (^{3}H)-noradrenaline from vesicles which are in some way mobilised by neuronal stimulation. This (^{3}H)-noradrenaline 'leaks' from the vesicles and is metabolised instead of released, thus maintaining the tritium overflow and decreasing the (^{3}H)-noradrenaline content. The increased metabolism could thus be a consequence rather than the direct result of presynaptic alpha-adrenoceptor stimulation. It is unlikely

that presynaptic alpha-adrenoceptor stimulation increases metabolism of the released (^{3}H)-noradrenaline, as clonidine, for example, does not affect neuronal uptake, which would need to be increased to increase metabolism. These speculations must also be considered in terms of yohimbine, which theoretically acts by blocking presynaptic alpha-adrenoceptors. However yohimbine does not produce changes in overflow that are exactly opposite to those of adding clonidine or noradrenaline. Yohimbine increases both tritium and (^{3}H)-noradrenaline overflow without altering the percentage of (^{3}H)-noradrenaline in the overflow. The percentage of (^{3}H)-noradrenaline may not be raised due to neuronal uptake of some of the increased (^{3}H)-noradrenaline overflow. Addition of phentolamine in the presence of cocaine produced an increase in the percentage of (^{3}H)-noradrenaline in the overflow, with a corresponding decrease in metabolites, but as with yohimbine, there is an increase in the tritium overflow. These results suggest that yohimbine and phentolamine may well have actions other than blockade of the presynaptic alpha-adrenoceptor normally stimulated by exogenous and endogenous noradrenaline and drugs such as clonidine.
The three characteristics differentiating pre from post-synaptic inhibitory agonists in this tissue, calcium and frequency dependence, and causing a decrease in noradrenaline overflow are a consequence of the supposed mode of action of presynaptic adrenoceptors, i.e. restriction of calcium availability for exocytosis. Evidence obtained in this tissue show that agonists acting presynaptically via other types of receptors e.g. morphine (Henderson, Hughes and Kosterlitz, 1975), also exhibit these characteristics. (Marshall et al., 1979).

Langer (1977), has proposed that the neuronal membrane may contain several types of receptor. Prostaglandin E, muscarine, dopamine, adenosine and morphine and/or enkephalin receptor sites inhibiting noradrenaline release; beta-adrenoceptor, nicotine and angiotensin receptors increasing noradrenaline release. It would be interesting to know if all these inhibitory receptors possessed similar characteristics to those of the alpha-adrenoceptor, and if they all influence noradrenaline release by decreasing calcium availability via a common final pathway. The concept of this common pathway is prompted by observations on this tissue. Marshall et al. (1979), have reported that vasa deferentia from animals made tolerant to morphine are less sensitive to the inhibitory effects of morphine and clonidine. The tolerance to morphine has therefore developed at some point after the recognition site of the receptor (the opiate and alpha-adrenoceptor being separate entities in the guinea-pig ileum, Kosterlitz and Watt, 1968 and mouse vas deferens, Jenkins, Marshall and Nasmyth 1975) which is common to both opiate and alpha-adrenoceptors. Hughes, Kosterlitz, Robson and Waterfield (1978), have also reported an interaction between morphine and clonidine. These authors showed that electrical stimulation of isolated ilea from morphine dependent guinea-pigs are not inhibited by clonidine unless morphine is present in the bathing media. These authors have suggested an interpretation of their results based upon adenylate cyclase. It has been shown that acutely morphine decreases adenyl cyclase activity in neuroblastoma and glioma hybrid cell lines (Sharma, Klee and Nirenberg 1975). In morphine tolerant animals adenyl cyclase activity may be increased, this could be nullified by the presence of opiates,

and clonidine would be normally effective. However, when morphine is removed from the bathing solution, the increased adenyl cyclase activity would be 'unmasked'. In this situation more clonidine would be required to depress the adenyl cyclase activity below normal levels than in the control situation (naive animals, or tolerant animals in the presence of morphine). This interpretation of course, implies a depression of adenyl cyclase is a result of presynaptic alpha-adrenoceptor stimulation. If this were so one could postulate a control mechanism for noradrenaline release based upon adenyl cyclase activity, cAMP levels and noradrenaline release being increased by facilitatory presynaptic receptors, and depressed by inhibitory presynaptic receptors. An alternative concept involving cyclic nucelolides is suggested by the work of Dubocovich, Langer and Pelayo (1978). Their experiments in the pineal gland were consistent with the stimulation of beta-adrenoceptors increasing cAMP levels, and stimulation of alpha-adrenoceptors increasing cGMP levels. Evidence that presynaptic beta-adrenoceptor stimulation may lead to an increase in cAMP has also been found in the cat spleen by Celuch, Dubocovich and Langer (1978). Thus it may be that these two nucleotides have oppositve effects on noradrenaline release and thus mediate the response to stimulation of these receptors. The above discussion provides only two alternative theories of the role of cyclic nucleotides as 'second messengers' between receptor stimulation and the modulation of transmitter release. The elucidation of the exact role of cyclic nucleotides will be, as in other systems, hampered by the question of compartmentalisation of the adenyl cyclases within the tissue, and it may be that their role is

not the primary one suggested above. But the interaction between morphine and clonidine suggests that there is a common pathway somewhere between the receptor recognition sites and the resulting modulation of calcium influx. There is now substantial evidence in favour of the calcium availability hypothesis as being the mode of action of presynaptic alpha-adrenoceptors. In this tissue the effect of lowering the calcium content or addition of magnesium to the Krebs solution upon the responses to clonidine and yohimbine support this theory, and similar results have been reported by other authors in other tissues (Langer, Dubocovich and Geluch 1975, Drew 1978). The site of action of these receptors is not yet known. It may be at the stage of calcium crossing the neuronal membrane or at a later stage in its utilisation.

In conclusion, the mouse vas deferens is an adrenergically innervated tissue in which the release of noradrenaline and the twitch response to field stimulation are modulated via presynaptic alpha-adrenoceptors. These receptors form a negative feedback loop which may have an important physiological role in preventing unwanted contractions of the tissue. The vas deferens also contains other presynaptic receptors, such as opiate receptors, whose physiological function, if they have any, is unknown. The fact that the twitch response of this tissue to electrical stimulation cannot be blocked by post-synaptic receptor blocking agents, makes it a particularly useful tissue for the investigation of presynaptic receptors.

APPENDIX 1

The effects of reserpine treatment on mice

When mice were dosed with reserpine as described in methods their body weight decreased after 24 hours, and decreased further 48 hours after the first dose (fig 50). At this time the mice were very sedated and had ptosis. Mice not exhibiting these effects were not used in experiments.

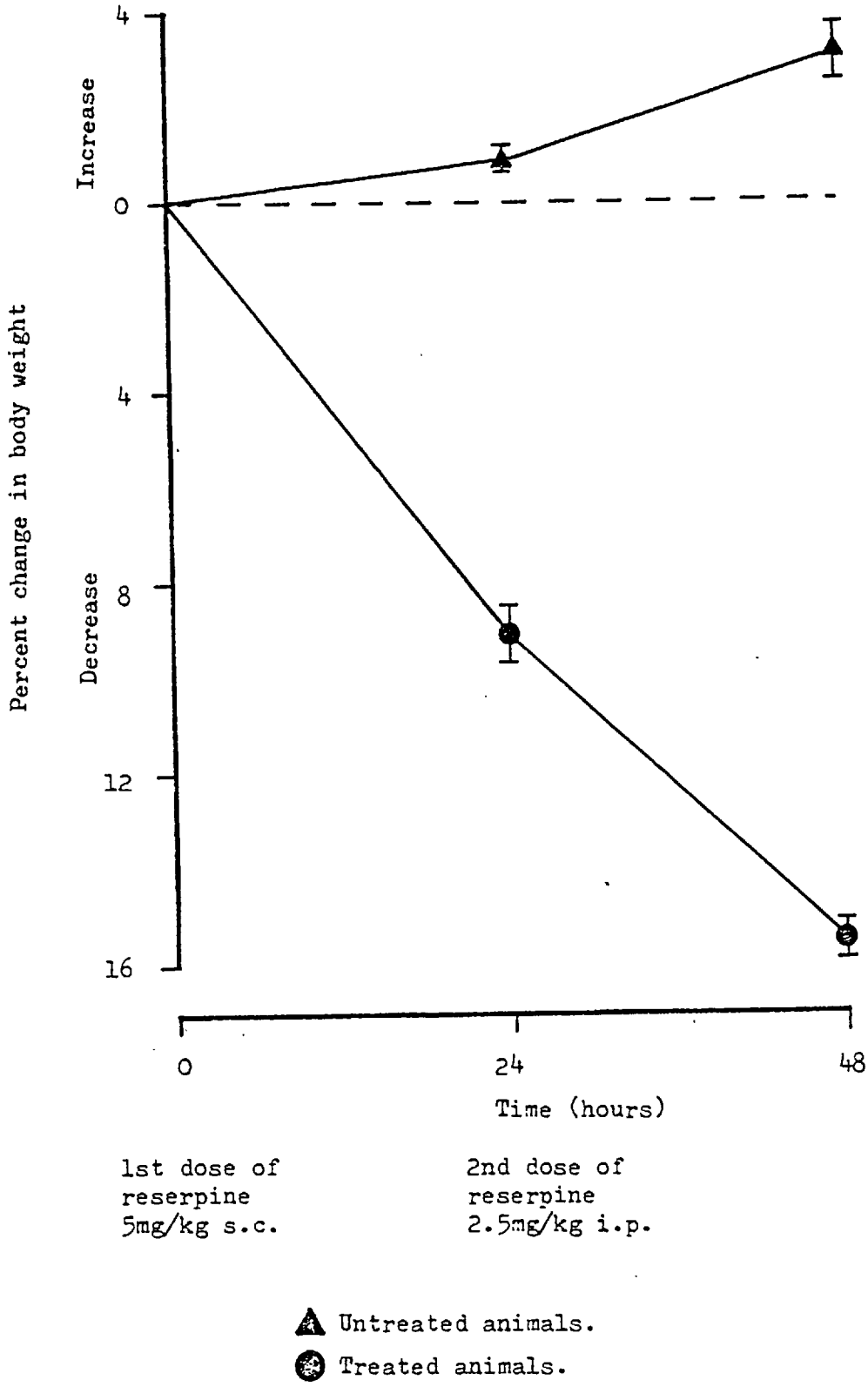


Fig. 50 The effect of reserpine treatment on mice body weights.

APPENDIX 2

Radio-labelled compounds and liquid scintillation spectrometry

Compounds

All radio-labelled compounds were suppled by The Radiochemicals Centre Ltd., Amersham.

S-adenosyl-L-(methyl-^{3}H) methionine, specific activity, 18.7 - 25 m Ci/mg, radioactive concentration 0.9 - 1.0 m Ci/ml stored as delivered. 2.5 µCi used per assay tube.

L-(7-^{3}H)-noradrenaline, specific activity 29 - 89 m Ci/mg, radioactive concentration 1.0 m Ci/ml. Stored in a 1% sodium metabisulphite solution. 100 ng added to each flask for preloading tissues, and 10 ng per flask for uptake studies, giving final concentrations of 590 nM and 59 nM respectively.

Scintillants

Liquifluor - 50 gms 2,5-diphenyloxazole, 625 mg 1,4-di(2-(5-phenyloxazolyl)) benzene, in 500 mls of toluene. 400 µl of this mixture per 9 mls of toluene.

Instagel - supplied by Packard Ltd. Added to aqueous samples. A 1:1 mixture forms a solid gel for counting.

Spectrometry

Samples were contained in either glass counting vials (20 ml capacity) or in plastic mini-vials (5 ml capacity). Samples were counted for at least five minutes in a Packard Tricarb liquid scintillation counter. The results (c.p.m.) were corrected for the counting efficiency by the channels ratio method. Quench correction curves being produced by using standard solutions of (^{3}H)-water for Instagel, or (^{3}H)-toluene. These standards were supplied by Packard Ltd.

APPENDIX 3

DRUGS USED

DRUG	SOURCE	TRADE NAME
Acetyl Choline	Sigma	
Clonidine	Boehringer-Inglheim	Catapress
Cocaine	St. Mary's Hospital	
Guanethidine	Ciba	Ismelin
Beta-oestradiol	Koch-Light	
Alpha Methyl-p-tyrosine	Sigma	
Noradrenaline	Koch-Light	
	Winthrop	Levophed
Phenoxybenzamine	Smith, Kline and French	Dibenyline
Phentolamine	Ciba	Rogitine
Propranolol	I.C.I.	Inderal
Reserpine	Halwood Chemicals Ltd.	
Tyramine	Sigma	
Yohimbine	Sigma	

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