THE INHIBITORY AND MOTOR INNERVATION OF THE

ANOCOCCYGEUS MUSCLE

A thesis presented for the degree of

Doctor of Philosophy

by

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December 1973

ProQuest Number: 10760464

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INTRODUCTION

The description of the nervous system began in the 2nd century A.D.
when Galen introduced the "experimental method" into the neurological
sciences. His method of combining anatomical findings, as revealed
by dissection, with physiological observations was so successful that
it resulted in a description of the nervous system which remained
virtually unchanged for 15 centuries. Subsequent developments
have still been based on this correlation of anatomy with physiological
function, although both have been examined at an increasingly fine
level. In anatomy this has involved the use of the light microscope,
the development of histochemistry and most recently the electron
microscope. The identification of the individual tissue components
which this revealed has paralleled and assisted the development of
sophisticated methods of measuring physiological function. These
techniques employed the industrial technology which was gradually
developed throughout the 19th and 20th centuries to examine the
electrical and chemical properties of tissues.

It has been the application of these techniques of micro - anatomy,
electrophysiology and biochemistry together with the powerful effects
of certain chemical substances on physiological processes which has led
to the present state of knowledge of neurological function.

The modern concept of the autonomic nervous system began in the second
half of the 19th century when it was recognised that this was a
discrete, anatomically distinct part of the nervous system,
characterised by an unusual efferent pathway (see Gaskell, 1916).
It was subsequently realised, mainly due to the work of Gaskell (1916)
and Langley (1921) that this consisted of two separate but related
systems which differed in their anatomical nature, produced essentially
opposite physiological responses and were differently affected by

blocking drugs. Following the demonstration of chemical transmission (Loewi, 1921) it was recognised that the difference in the two systems extended to the transmitters involved, a recognition which led Dale (1933) to suggest a functional classification into adrenergic and cholinergic nerves to replace the existing anatomical distinction of sympathetic and parasympathetic. The interplay of these two divisions was recognised to provide a wide range of homeostatic control mechanisms (see Cannon, 1939; Dale, 1965).

From this emerged the present day conventional picture of the autonomic nervous system as a control system, with afferent, central and efferent components. The efferent part innervates smooth muscle, most exocrine secretory cells and cardiac muscle. It consists of two major divisions, the sympathetic and parasympathetic. The sympathetic consists of a preganglionic thoracico - lumbar outflow which emerges from the corresponding ventral roots of spinal nerves to synapse with postganglionic neurons in the paravertebral or prevertebral ganglia, which are sited at locations distant from the end - organs. These postganglionic neurons then run to and supply the peripheral organs. The transmitter liberated from the preganglionic nerves is acetylcholine (cholinergic) whereas from the postganglionic nerves it is noradrenaline (adrenergic). The parasympathetic consists of cranial and sacral preganglionic outflows which emerge from either certain cranial nerves or the ventral roots of sacral spinal nerves and run uninterrupted to peripheral tissues where they synapse with postganglionic nerves in ganglia lying in or near the innervated organ. The preganglionic parasympathetic nerves are cholinergic and the postganglionic

parasympathetic fibres are also cholinergic. When both systems innervate the same organ, the two systems usually cause opposite responses.

Before the development of the theory of chemical transmission, the division between the two branches was based mainly on the spinal origin of the preganglionic fibres. With the advent of chemical transmission and the realisation that this transmitter differed in different nerves, each branch became identified with its postganglionic nerves. Thus pathways with postganglionic cholinergic fibres became synonymous with parasympathetic and vice versa.

It became clear very early in the life of this theory, however, that exceptions to this universal concept existed. These exceptions came to notice largely due to the use of specific drugs. In the course of developing the theory of chemical transmission, drugs played a central role both as agents producing effects similar to those of nerve stimulation and as blockers of the effects of nerve stimulation. This assisted greatly in the early identification of the type of transmission involved in the different branches of the autonomic system but eventually produced anomalies which required modification of the classical picture. The first conclusive example of this was demonstrated by Dale & Feldberg (1934). Starting from the observation that sweating, although initiated by stimulation of sympathetic nerves, was blocked by atropine, they were able to demonstrate that secretory nerve fibres originating in ganglia of the sympathetic chain, and therefore classified as postganglionic sympathetic nerves, were cholinergic. Together with other comparable deviations from the classical picture, which came to light at around the same time (e.g. cholinergic sympathetic nerves to the blood vessels of dogs'

lips, Euler & Gaddum, 1931; in iris, Young, 1932; and in the
vasculature of skeletal muscle, Bulbring & Burn, 1935), this led
to the view that the general rules still held but that a recognition
that there were exceptions to the rules would clear up many
apparent anomalies (Dale & Feldberg, 1934).

Subsequent developments have shown that deviations from the
conventional system can take two main forms, viz. (1)   unconventional
transmission   (2) direct anatomical anomalies.

Anomalous transmission arose first as examples like that shown by
Dale & Feldberg (1934) where the transmission was still adrenergic
or cholinergic but the transmitter was inappropriate for its
anatomical origin.   This could be readily accommodated into the
existing system, but later findings have suggested that transmitters
other than noradrenaline or acetylcholine might exist, e.g. 5 -
hydroxytryptamine (Bulbring & Gershon, 1967), adenosine triphosphate
(Burnstock, 1972), prostaglandins (see Campbell, 1970).   If a
multiplicity of transmitters does exist, then serious re - appraisal
of the division of the autonomic nervous system may become necessary.
The further factor of more than one transmitter from a single nerve
is suggested by the theory of Burn & Rand (1959, 1960), which states
that adrenergic nerves may liberate acetylcholine as well as
noradrenaline and the related finding that during the development of
sympathetic nerves they may release acetylcholine as transmitter
before they obtain the capacity to release noradrenaline (Day & Rand,
1961).

Examples of the kind of direct anatomical anomalies which have been

uncovered are to be found in the innervation of the secondary
sexual organs and of the gastro - intestinal tract. The
sympathetic pathway to the vas deferens and seminal vesicles via
the hypogastric nerve has its ganglia located along the nerve trunk
itself very near to the organs innervated. This produces a system
which is therefore similar to the parasympathetic in the positioning
of its ganglia, although the spinal origin of preganglionic nerves is
thoracico - lumbar and the postganglionic nerves are adrenergic (for
review see Sjostrand, 1965; Ferry, 1967). In the gastro - intestinal
tract a further dimension has been added to the whole system with
the discovery that the adrenergic nerves may not make direct neuro -
muscular contact with the smooth muscle but may act by synapsing with
a further neuron or neurons which may then act on the muscle (Norberg,
1964, 1967; Jacobowitz, 1965; Aberg & Eranko, 1967; Gabella & Costa,
1968; Read & Burnstock, 1969).

These anatomical features, together with the possibility of several
transmitters, thus present a considerable challenge to the classical
framework and have indeed led some people to call for a re - appraisal
of the whole system, free from attempts to fit in deviant phenomena
as aberrations from the norm but which would consider them rather in
their own right (e.g. Campbell, 1970).

This call has been partly answered by Burnstock (1972) who has
suggested a third component to the autonomic nervous system whose
postganglionic neurons are neither cholinergic nor adrenergic but
utilise ATP or a related compound as transmitter and which he has
thus tentatively called "purinergic" (Burnstock, 1971). Just as

Langley's (1921) original division of the autonomic nervous system
into two parts was based on comparative studies of the innervation
in different organs and in different species, so this latter division
into at least three parts also rests largely on the evidence of
comparative studies, not only in different tissues in mammals,
but also in invertebrate species (Burnstock, 1969).

With this background, it is not surprising that each previously
untested preparation which is now studied provides further fuel
to this debate on the classification of the autonomic nervous
system. The subject of this thesis, the neural control of the
anococcygeus muscle, provides an excellent example of this, since
the anococcygeus is a smooth muscle tissue receiving both a
powerful inhibitory innervation, whose transmitter is unknown,
and also an anatomically conventional motor adrenergic innervation
distributed throughout the muscle. The purpose of the present thesis
was therefore to study this preparation in three ways –

(1)   To investigate the inhibitory innervation and determine
whether it represented a separate nerve pathway distinct from the
sympathetic and whether it was organised on the efferent pattern of
the autonomic nervous system, i.e. with an interrupting ganglion.

(2)   To compare the characteristics of the motor innervation in
the vas deferens and in the anococcygeus to see what, if any, differences
were introduced by the "short" postganglionic neurons in the vas.

(3)   To compare the properties of the anococcygeus muscle in two
species, the rat and cat, in order to determine which features were
common and whether this comparative study would shed more light on the
unknown inhibitory innervation.

The history of the anococcygeus preparation illustrates, in a small
way, the inter - relationship between form and function and the role
of drugs in the development of any neurological study.   The
anococcygeus came to light during a histochemical investigation of
the density of adrenergic innervation of smooth muscle at different
levels in the alimentary canal of the rat (Gillespie & Maxwell, 1971).
It was the dense adrenergic innervation, as shown by the fluorescence
technique of Falck & Hillarp which drew attention to the presence of
this strap - like muscle which had not previously been reported in the
rat and had been little more than noted to be present in other species
(Straus - Durckheim, 1845;   Langley & Anderson, 1895;   Miller,
Christensen & Evans, 1964).   Further histological examination showed
it to be composed of a thin, flat sheet of smooth muscle making it
"particularly suitable for in vitro studies, electrophysiologic
measurements of membrane potentials and the effects of adrenergic nerve
stimulation on these" (Gillespie & Maxwell, 1971).

Subsequent in vitro studies (Gillespie, 1972) demonstrated that the
adrenergic innervation could be excited by electrical field stimulation
and that this produced contraction of the muscle;   a motor response.
Further investigation demonstrated that, in the presence of a large dose
of guanethidine, this adrenergic innervation was blocked and the tone
of the muscle raised and that, under these conditions, field stimulation
now produced relaxation of the muscle;   an inhibitory response.   This
inhibitory response could be elicited with short pulses of 1 m sec or
less and was abolished by low doses of tetrodotoxin thus showing the
characteristics of a nerve mediated response.   It could not, however,
be blocked by the conventional blocking agents which prevent responses

to noradrenaline or acetylcholine in other tissues and so the nature of the transmission process remained unknown.

In the anococcygeus, therefore, the histochemical discovery of the adrenergic nerves within the muscle led to the finding that this tissue received a powerful motor adrenergic innervation. While further investigating its physiological / pharmacological properties in vitro , this in turn led to the discovery of an unusual type of inhibitory innervation.

The first objective of the present investigation was to further extend the anatomical study of this system by determining whether the inhibitory as well as the motor pathway originated in the spinal cord and if so whether it was interrupted by a ganglion synapse, a condition which would allow it to be classified within the autonomic nervous system. This was approached using the pithed rat preparation of Gillespie, MacLaren & Pollock,(1970) in which the pithing rod can be used as a moveable electrode to selectively stimulate the spinal outflow at any desired level. By recording the resultant mechanical responses of the anococcygeus muscle in situ, it was thus possible to delineate the spinal origin of both motor and inhibitory pathways and then pharmacologically analyse the nature of these pathways.

Several possible explanations of a dual response to field stimulation or to stimulation of the extrinsic nerve exist. These can be summarised as follows -

(1)   If both responses are produced by a single type of fibre, the nature of the response might be determined by (a) the "peripheral mechanism"; the response depends on the "state" of the nerve and

muscle at the periphery (McCrea, McSwiney & Stopford, 1925)

(b) Wedensky inhibition; block of the nerve impulse somewhere in the pathway to the effector muscle without diminution of the ability of the muscle to respond, i.e. the inhibitory response results from the loss of the motor response (e.g. Veach, 1925; Cannon, Raule & Schaefer, 1954) (c) variable effect of a single transmitter (e.g. Bozler, 1940; Burn & Robinson, 1951; Gardner & Kandel, 1972).

(2)   Two functionally distinct fibre groups exist, producing opposite responses.   These are usually adrenergic and cholinergic and can be organised as (a) according to the conventional autonomic pattern with anatomically discrete sympathetic and parasympathetic nerves (b) sympathetic and parasympathetic fibres with separate anatomical origins but which may come together peripherally to reach the target organ in a common nerve bundle (e.g. McSwiney & Robson, 1929; Finkleman, 1930; Gillespie & Mackenna, 1961) (c) fibres share a common anatomical origin but the sympathetic may be cholinergic (Dale & Feldberg, 1934) (d) one or both of the nerves might be neither adrenergic or cholinergic and have as its transmitter some other substance, e.g. ATP (Burnstock, 1972) (e) one of the responses might be due to antidromic stimulation of afferent nerves producing liberation of some substance at the peripheral endings (e.g. dorsal root afferents, Bayliss, 1902; Lewis & Marvin, 1927; Wybauw, 1936; Holton & Perry, 1951).

Delineation of the spinal origins of the nerves responsible for the responses together with the effects of selective blocking drugs obviously eliminates most of these possibilities; an analysis impossible by _in vitro_ experiments alone.

Once the in vivo preparation of the anococcygeus was developed, it provided the means for the second part of the study, the comparison with the vas deferens. By appropriate selection of the level of spinal stimulation, the sympathetic outflows to the anococcygeus and vas deferens and the vasomotor outflow could be stimulated simultaneously and the resultant responses compared. This enabled comparison of the new preparation, the anococcygeus, with the known but anatomically unconventional preparation, the vas deferens, and both of these with the known but anatomically more conventional vasomotor system.

This comparison was carried out in two separate parts.

Firstly, the effects of agonist and antagonist drugs which normally mimic or modify adrenergic responses were studied. This permitted the assessment of any differences in the effects on the vas deferens from those on the other two test organs. In recent years many of the effects of such drugs on the vas have been shown to be different from those found on other tissues and have led to a debate over whether motor transmission is indeed adrenergic.

When the isolated guinea - pig vas deferens - hypogastric nerve preparation was introduced by Hukovic (1961) it seemed to be a suitable preparation for demonstration of adrenergic neurotransmission. Two glaring anomalies, however, emerged from the start. Adrenergic α - blocking agents did not block the mechanical response to nerve stimulation except in extremely high concentrations and could, in fact, potentiate the response in the moderate concentrations which were sufficient to block responses in other tissues (Boyd, Chang & Rand, 1960).

In addition, the response could be potentiated by cholinesterase inhibitors and inhibited by hemicholinium (Rand & Chang, 1960), evidence which was taken to support the cholinergic link hypothesis (Burn & Rand, 1959, 1960). It gradually became apparent from the work of several authors that these latter anomalies, which implicated a cholinergic component, arose from the fact that the ganglia in the motor pathway were distributed along the hypogastric nerve close to its junction with the vas deferens, so that stimulation of the hypogastric nerve was initiating impulses in preganglionic nerves which therefore had to pass through the cholinergic synapse of the ganglion before reaching the postganglionic adrenergic nerve and its junction with the smooth muscle of the vas (Bentley & Sabine, 1963; Birmingham & Wilson, 1963; Ferry, 1963, 1967; Kuriyama, 1963; Ohlin & Stromblad, 1963; Falck, Owman & Sjostrand, 1965). This largely accounted for the ability of drugs affecting the cholinergic system to influence the response and explained why such drugs were ineffective if the tissue was stimulated postganglionically by transmural field stimulation (Birmingham & Wilson, 1963). The resistance to α - blockers, however, remained whether stimulation was pre - or postganglionic.

More recently, this paradox has been more closely investigated by Ambache and co - workers who have linked this to the finding that noradrenaline itself does not produce contractions except in high concentrations and can inhibit the response to stimulation of the motor nerves in lower concentrations (Ambache & Zar, 1970) and that this latter effect is prevented by phentolamine. Together with the finding that the motor response in the vas to nerve stimulation is not

prevented by reserpine pretreatment, this has led Ambache & Zar (1971)
to suggest that this motor transmission is non - adrenergic and that
if the adrenergic nerves have any function in this tissue, it is
likely to be an inhibitory one. To further support this contention,
they have subsequently demonstrated that the sympathomimetic agents
tyramine and cocaine also inhibit the motor response (Ambache, Dunk,
Verney & Zar, 1972). This hypothesis has not yet deflected the main -
stream of opinion from the conventional view that motor transmission in
the vas deferens is noradrenergic (see Euler, 1972) and evidence has been
produced which upholds this conventional view arguing that the
resistance to α - blockers is due to the morphological arrangement
of the neuro - muscular junction (Swedin, 1971) and that complete
sympathectomy using 6OHDA can abolish the motor nerve response (Wadsworth,
1973). In addition, these nerve responses are abolished by guanethidine,
a finding acknowledged by Ambache & Zar (1971). Nevertheless, the
results produced by Ambache et al present a considerable challenge to
the contention that transmission in the vas is adrenergic, and it was
hoped that by comparison with the anococcygeus some further light might
be thrown on this paradox.

The second comparison of the vas deferens with the anococcygeus was
undertaken in order to clarify the reasons for the relative resistance
of the former tissue to the noradrenaline depleting effect of reserpine
(Sjostrand & Swedin, 1968). Since Holzbauer & Vogt (1956) first showed
that tissue catecholamines were depleted by reserpine, it has been shown
that the rate of depletion is dose - dependent (e.g. Carlsson, Rosengren,
Bertler & Nilsson, 1957; Bertler, 1961) and is similar for most
adrenergically innervated peripheral organs tested (Bertler, 1961).

Sjostrand & Swedin (1968) found, however, that the vas deferens and
seminal vesicles were depleted of their noradrenaline content by
reserpine more slowly than other peripheral tissues such as the heart
and submaxillary glands and that decentralisation of the former organs
did not affect the rate of depletion, whereas it slowed the rate of
depletion of submaxillary glands in the same rats. Two possible
explanations of this resistance are that either the rate of depletion
is dependent on the firing frequency in the nerves and therefore
reduced in the intermittently active nerves of the vas deferens and
seminal vesicles or that the "short" adrenergic neurons, which are
found in the seminal vesicles and vas deferens and have their cell
bodies located close to the target organs (Owman & Sjostrand, 1965;
Sjostrand, 1965), are like the adrenal medullary cells less easily
depleted than the "long" adrenergic neurons found elsewhere.

The first hypothesis is supported by the finding that the rate of
depletion by reserpine in various organs is reduced if impulse flow
is reduced by decentralisation of the adrenergic nerves before
reserpine (Holzbauer & Vogt, 1956; Hertting, Potter & Axelrod, 1962;
Benmiloud & Euler, 1963; Sedvall, 1964) or administration of ganglion
blocking drugs (Karki, Paasonen & Vanhakartano, 1959; Mirkin, 1961;
Hertting, Potter & Axelrod, 1962).

The second explanation is suggested by the evidence that "short" adrenergic
neurons have a lower sensitivity to 6OHDA (Malmfors & Sachs, 1968) as
well as to reserpine, react differently to immunosympathectomy
(Hamberger, Levi - Montalcini, Norberg & Sjoquist, 1965; Zaimis, Berk
& Callingham, 1965; Iversen, Glowinski & Axelrod, 1966) and their
transmitter granules, when isolated, have different properties compared
with those from splenic nerve (Euler & Lishajko, 1966; Stjarne &

Lishajko, 1966).

These hypotheses were tested by contrasting the effect of abolishing neuronal activity completely by pithing and reinforcing that activity by maximal artificial stimulation of the sympathetic nerves by the same pithing rod on the rate of depletion of noradrenaline in the heart, vas deferens and anococcygeus muscles. Since an earlier part of the study shows that the anococcygeus receives conventional "long" adrenergic neurons, any difference linked to the length of the neuron should become obvious. The dose - dependence and time course of noradrenaline depletion by reserpine in vivo was first determined and then the effect on the rate of depletion of simultaneous stimulation of the nerves to the anococcygeus and vas deferens was examined using pithed rats.

The third part of the investigation was the comparative study of the cat and rat anococcygeus in vitro. This was intended primarily as a direct comparison of the effects of field stimulation on the muscle from the two species in order to determine whether the dual innervation and especially the inhibitory component found in the rat was also to be found in a second species. Having established that the dual response was present in the cat, several putative transmitter substances and specific blockers were tested on both species in an attempt to categorise the inhibitory nerves.

This study was given further impetus when Garrett, Howard & Lansdale (1972) examined the cat anococcygeus histologically, showing that it had, like the rat, a dense adrenergic innervation but, unlike the rat, also stained for cholinesterase. It is tempting to interpret this staining as

evidence of a cholinergic innervation and to associate this with the
inhibitory innervation.

The possibility that the inhibitory nerves might be cholinergic had
been rejected by Gillespie (1972) on the grounds that acetylcholine
was motor on the rat anococcygeus, so in addition to any putative
role as a transmitter, other possible roles for acetylcholine in the
tissue had to be considered.

In addition to transmitter at "cholinergic" nerve terminals, acetylcholine
has in recent years been ascribed several roles in transmission at
adrenergic nerve terminals (for reviews see Muscholl, 1970; Kosterlitz
& Lees, 1972). These were embodied into the "cholinergic link hypothesis"
of Burn & Rand (1959, 1960) which states that when the nerve impulse
reaches the adrenergic nerve terminal there is a release of acetylcholine
which, by activation of nicotinic receptors, in turn releases noradrenaline
from its stores. The evidence for and against this hypothesis was
reviewed by Ferry (1966). Whatever the physiological role, if any, of
acetylcholine turns out to be, cholinomimetic agents certainly have effects
on the release of noradrenaline from adrenergic nerve terminals which
must be considered when investigating the role of acetylcholine in an
adrenergically innervated tissue, e.g. facilitation of noradrenaline release
at low doses, inhibition of noradrenaline release at intermediate doses
and release of noradrenaline from the nerves at high doses (Malik & Ling,
1969; Loffelholz & Muscholl, 1969). The possible effects of acetylcholine
on both motor and inhibitory nerves as well as on the muscle itself were
therefore investigated in the anococcygeus.

A further property of the anococcygeus muscles from both species which
was investigated in vitro was the ability of several substances to

produce sympathomimetic effects. Guanethidine, cocaine, LSD and 5HT all produced contraction of the muscles from both species which were inhibited by the adrenergic α - receptor blocker phentolamine.

Direct actions on smooth muscle have been reported previously for guanethidine (Maxwell, 1965; Maxwell, Wastila & Eckhardt, 1966; Bevan & Verity, 1967), cocaine (Daniel & Wolowyck, 1967; Maxwell, Wastila & Eckhardt, 1966; Cliff, 1968), LSD (Gant & Dyer, 1971; Dyer & Gant, 1973) and 5HT (Gaddum & Hameed, 1954; Gaddum & Picarelli, 1957; Innes, 1962). Indirect action via release of noradrenaline from adrenergic nerves has also been reported for guanethidine (Kadzielawa, 1962; Bhagat & Shideman, 1963; Pluchino, Muskus & Pluchino, 1969), cocaine (Kukovetz & Lembeck, 1962; Maengwyn-Davies & Koppanyi, 1966; O'Donnell & Hecker, 1967; Trendelenburg, 1968) and 5HT (Furchgott, 1955; Innes, 1962; Pluchino, 1972).

It was thus of interest to determine whether the responses produced by these substances on the anococcygeus were direct effects on the muscle or indirect effects produced by the release of noradrenaline from adrenergic nerves. Early distinctions between the actions of tyramine and of adrenaline were that both cocaine and denervation potentiated the effects of adrenaline but antagonised those of tyramine (Tainter & Chang, 1927; Burn & Tainter, 1931; Bulbring & Burn, 1938). More recently, additional evidence for the release of noradrenaline by tyramine and similar drugs was obtained by demonstrating that tyramine lost its effectiveness after depletion of noradrenaline stores by reserpine (Burn & Rand, 1958). At the present time these three methods, cocaine, denervation and reserpine pretreatment are the main experimental approaches to

classification of sympathomimetic effects as direct or indirect (Trendelenburg, 1972).

In the present study, specific chemical denervation of the adrenergic nerves using 6OHDA was chosen as a means of making this distinction (see Malmfors & Thoenen, 1971). It has recently been shown by Gibson & Gillespie (1973) that pretreatment of rats with 6OHDA will selectively destroy the motor but not the inhibitory nerves. In the case of the cat anococcygeus, sympathectomy was produced by incubating the tissue in vitro with 6OHDA since the normal in vivo pretreatment was impracticable. This method has previously been shown to be successful in various tissues by Sachs (1971) and Wadsworth (1973).

A final aspect of the cat anococcygeus muscle which was investigated was the ability of the nerves and muscle to survive cold storage. This was necessitated since the tissues were obtained from cats primarily intended for experiments by other workers with the perfused spleen. Due to the timing of these experiments, the anococcygeus muscles, although removed fresh from the cats, were sometimes stored in Krebs' solution at $4^{o}C$ for 24 - 48 hrs before use. It was therefore of interest to determine whether this storage caused any change in the properties of the tissue which would make it unsuitable for experimental use. Another aspect of cold storage which might also have been of interest would have been if one or other of the sets of nerves had preferentially survived storage. This property has been employed in the past to differentiate between sets of nerves. For instance, nerves with their ganglion cell body included within the isolated preparation will survive longer than nerve endings cut off from the cell body (Holman & Hughes, 1965; Hattori, Kurahashi, Mori & Shibata, 1972).

The results of these three separate aspects of the study are presented according to the techniques employed. Thus the first section includes all the experiments where the mechanical responses in the tissues to stimulation of the autonomic outflow were examined, viz. (a) Origin and properties of the anococcygeus inhibitory and motor nerves (b) Comparison of the properties of the anococcygeus, vas deferens and cardiovascular responses in the pithed rat. The second section consists of the study of the effect of nerve stimulation on the depletion of tissue noradrenaline by reserpine, and the third part includes all the in vitro experiments comparing cat and rat anococcygeus muscles.

By this combination of anatomical, biochemical and pharmacological techniques it was hoped to further our understanding of the unusual dual innervation of the anococcygeus while simultaneously investigating some pharmacological problems of current interest.

METHODS
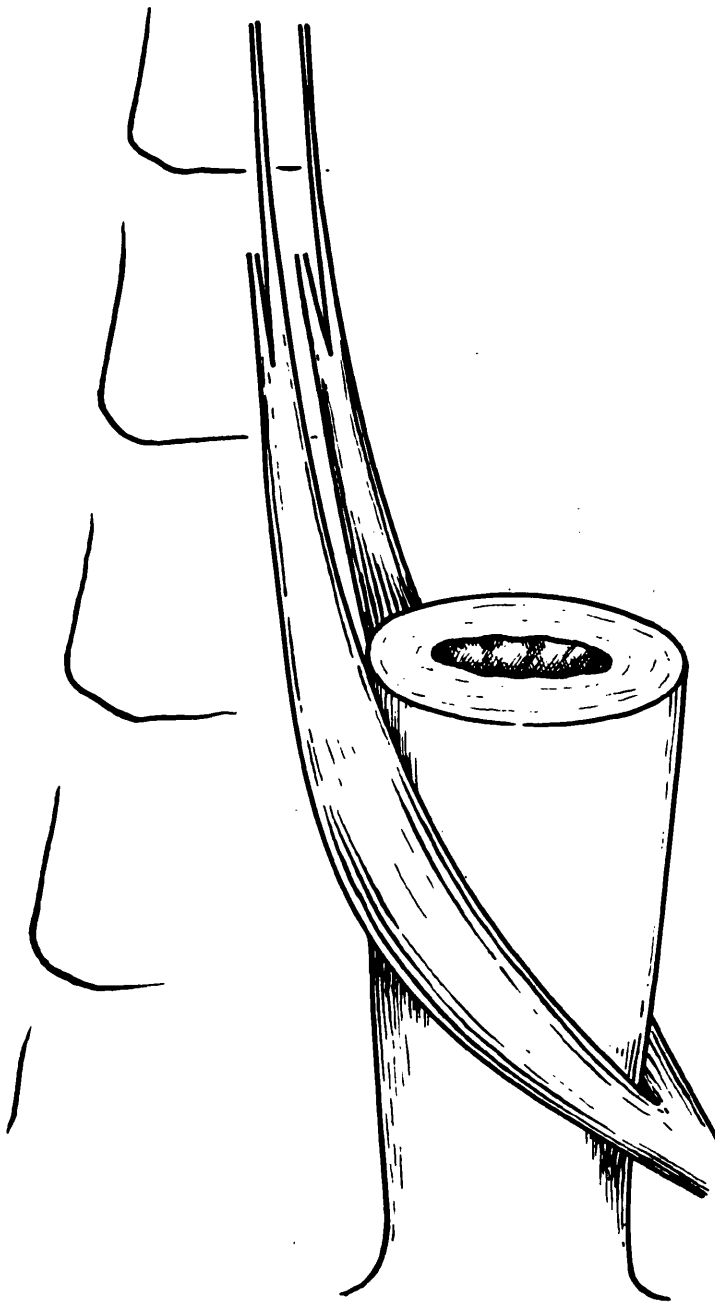
Fig. 1   Position of anococcygeus muscles in the male rat.   The
muscles lie deep to the colon in the pelvic cavity, originating
from a tendinous origin attached to the coccygeal vertebrae and
running around either side of the colon to join together on its
ventral surface just short of the anal margin.
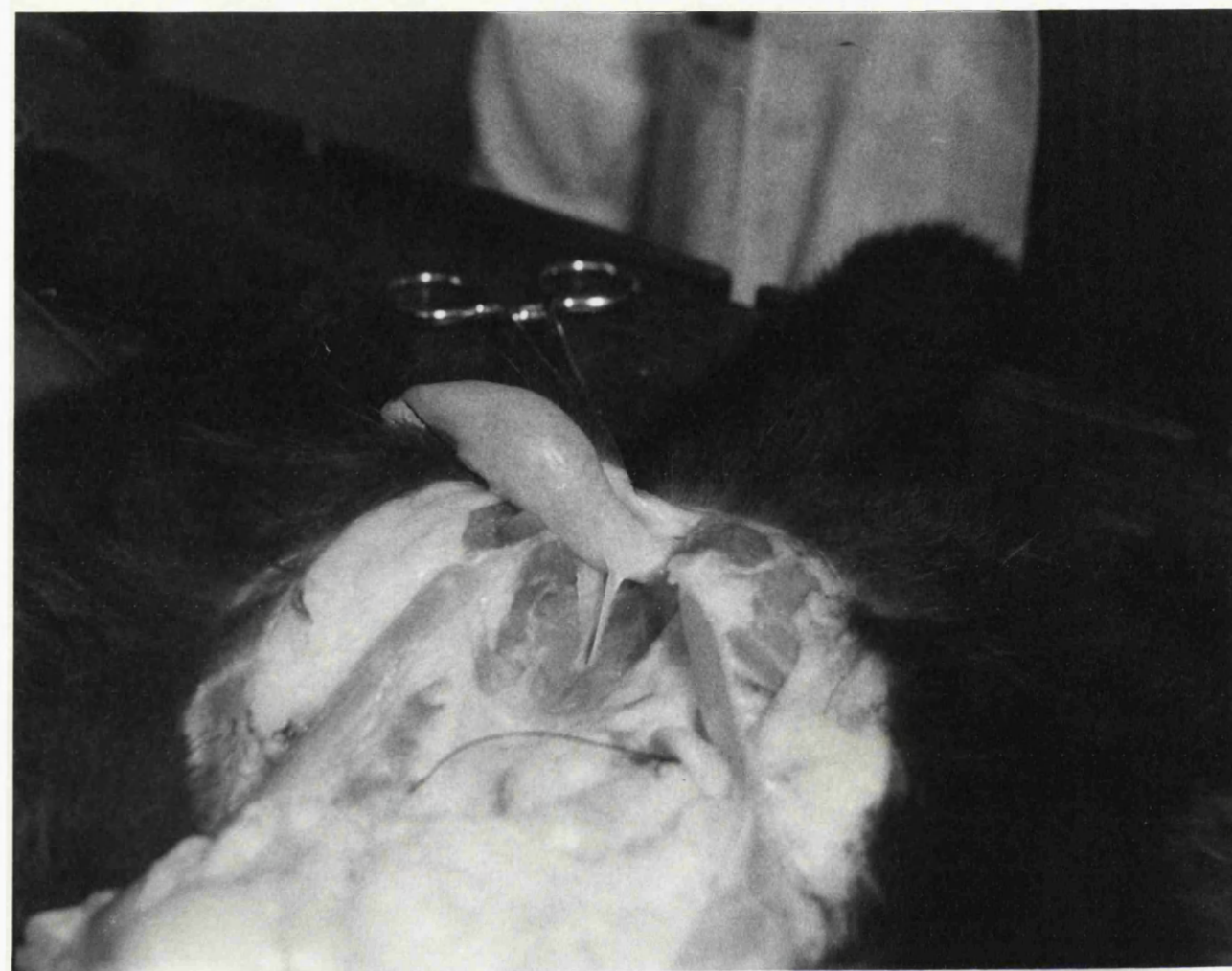
Fig. 2   Position of the anococcygeus muscles in the female

cat.   The two muscles can be seen running from the pelvic

floor to join either side of the rectum.
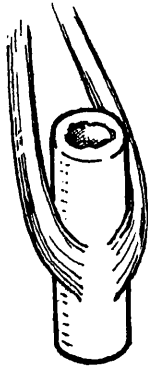
RAT   CAT
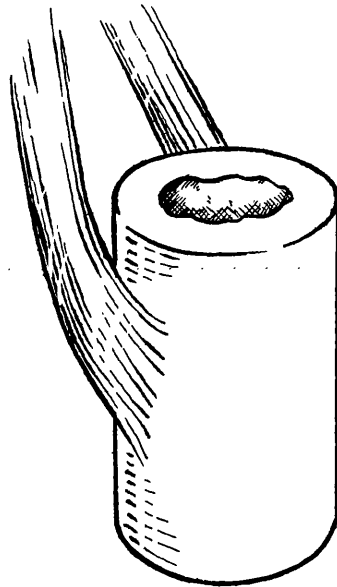
Fig. 3   Diagram of the position of the anococcygeus

in the female rat and cat.   Compare with male in Figs. 1

& 5.   In the female the muscles do not meet on the ventral

surface of the colon to form a bar, but merge with the

longitudinal muscle of the colon itself.   This is more

marked in the cat than in the rat.   In the rat the

contralateral muscles almost meet.   Approx. x 2 actual size.

Anatomy of the anococcygeus muscle

The anococcygeus muscles in the rat lie deep to the colon in the pelvic cavity. They consist of parallel bundles of smooth muscle fibres which arise from a tendinous origin attached to coccygeal vertebrae 1 and 2. They run caudally first behind the colon and then passing ventrally around either side of the terminal colon to join together in front to form a ventral bar (Fig. 1). In the male, this ventral bar continues in the mid-line of the perineum as the retractor penis muscle. The muscle is surrounded by a thin, loose sheath of connective tissue but is otherwise free from both the colon and the pelvic floor along its entire length except where it comes into close contact with the side of the colon. Here some fibres may merge with the longitudinal muscle of the colon as is found in the dog and cat (Fig. 2). In the male this is the only close contact between anococcygeus and colon, but in the female, where the ventral extension is absent, the muscles remain in close contact with the colon till they join (Fig. 3).

Each muscle in the male is 25-30 mm in length, 4-5 mm in breadth, and less then 1 mm in thickness <u>in situ</u>. On removal from the animal, they contract to approximately half this length. The average weight for a single muscle is 19.1 ± 2.7 mg (n=12). Muscles from females are shorter due to the absence of the ventral extension.

In the cat, the anococcygeus muscles lie in a similar position to those in the rat. The muscles interlock with the rectococcygeus which is absent in the rat but in the cat runs caudally from the dorsal surface of the colon (Fig. 5). The anococcygeus muscles lie on either side of the rectococcygeus and run in the opposite direction. The muscles are broader and thicker than those in the rat but generally of a similar length, although in large male cats they can be up to 50 mm long. Weights for single muscles are male 51 mg (n=4); female 29 mg (n=8).
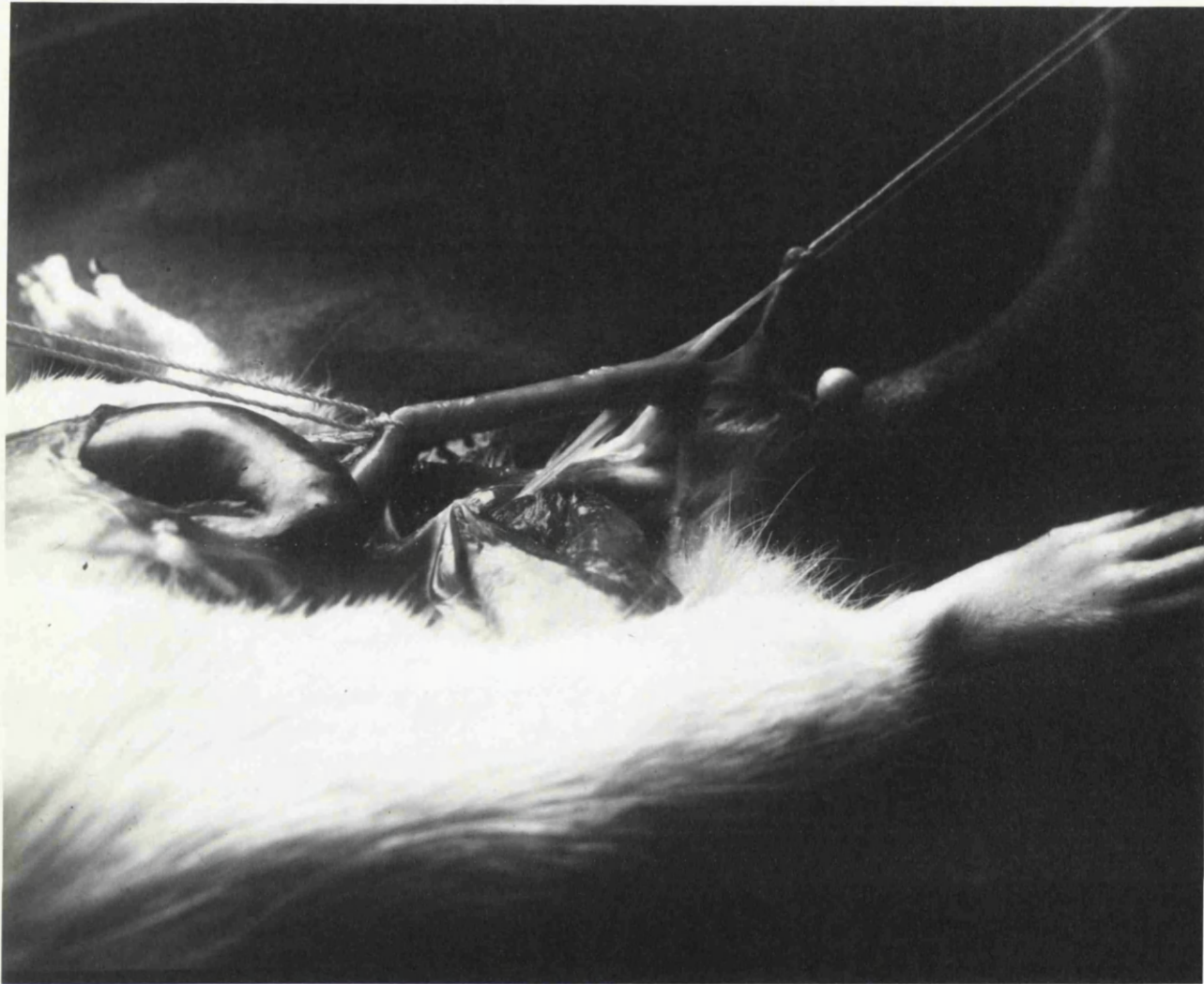
Fig. 4   Position of the anococcygeus muscles in the male

rat.   The muscles are pulled straight by the thread running

to the top right of the picture.   The two muscles run from

the pelvic floor and pass around either side of the colon

to join together to form the ventral bar which is here

attached to the thread.

In the rat, two nerve bundles enter the muscle on the deep surface at the level where the anococcygeus and the colon come into close contact. These nerves originate from the posterior scrotal branch of the perineal nerve (MacLellan, 1973). The origin of the extrinsic nerves has not been traced in the cat.

Isolation and removal of the rat anococcygeus muscles

Adult male albino Wistar strain rats were used in the weight range 200-300 g. Below 200 g the rats were not fully grown and above 300 g excess fat impeded dissection. Rats were killed by a blow on the head and immediately exsanguinated by cutting both carotid arteries. The abdomen was then opened along the mid-line to just beyond the penis and the intestines reflected to one side. The bladder, urethra, vasa deferentia, seminal vesicles and testicles were removed, exposing the descending colon to the point where it passed into the pelvic cavity. The symphysis pubis was then split taking care to avoid damaging the ventral bar of the anococcygeus muscle which lies over the colon in this area. The two cut ends of the pubic arch were forced apart subluxing the sacroiliac joints and exposing the contents of the pelvic cavity. The anococcygeus muscles could then be seen emerging from under the colon and joining together on its ventral surface (Fig. 4). When the colon was then cut through at the level of the pelvic brim and the pelvic portion pulled ventrally, the full length of the anococcygeus muscles running from the colon to the coccygeal vertebrae became visible. The delicate connective tissue surrounding the muscles was cleared and the muscles separated from the colon by blunt dissection. It was now possible to remove the muscles by cutting through the ventral bar at the caudal end and the tendons at the rostral end which were now the muscles' only connections to the rat. In experiments where the tension in the

Ano.
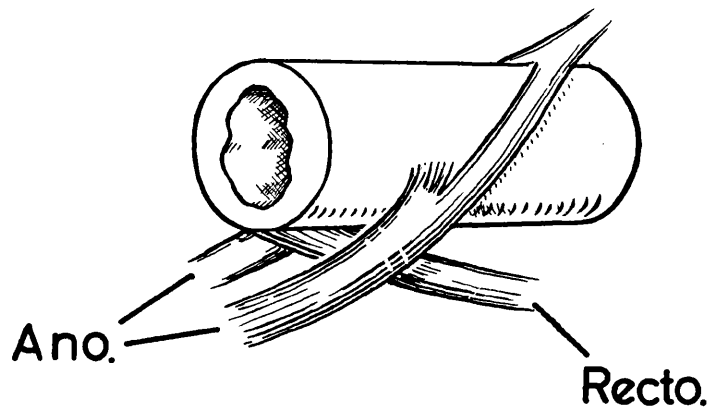
Recto.

Fig. 5   Diagram of the relative positions

of the anococcygeus muscles and the rectococcygeus

muscle in the cat.   The rectococcygeus interlocks

with the two anococcygeus muscles and runs from the

dorsal surface of the colon in the anal direction.

muscles was to be recorded _in vitro_, braided silk (40) threads were tied round each end of the muscle.

The entire dissection from the time of killing the animal took 5-10 min. From the point where the pelvis was split, the tissues were kept cool and moist by the liberal application of cold saline.

Isolation and removal of the cat anococcygeus muscles

The dissection of the cat anococcygeus was similar to that for the rat anococcygeus, but was more easily accomplished if the muscle was approached from the anus rather than via the abdomen. This was necessitated by the larger size of the rectum of the cat relative to the anococcygeus and the greater abundance and toughness of the connective tissue surrounding the muscles (Fig. 5).

The cats were both males and females and were obtained after either their spleen or heart had been removed for perfusion experiments by other workers. For their purpose, the cats were anaesthetised with a mixture of nitrous oxide and halothane; induction with 4% halothane in a mixture of 1 vol O_2 to 2 vol N_2O; maintenance with 1.0-1.5% halothane in the same gas mixture in a "semi-open circuit" system. 70 μg of PGE_1 and 20,000 International Units of heparin was administered intravenously 30 min prior to removal of the spleen (Blakeley, Brown, Dearnaley & Woods, 1969). The PGE_1 produced a temporary fall in blood pressure which lasted, however, only about 2 min. It is therefore unlikely that any PGE_1 would be left in the circulation by the time the anococcygeus muscles were removed. After removal of the spleen, the animal was killed by cutting the diaphragm and cardiac incision. After removal of the heart, this was of course unnecessary.

The dissection of the anococcygeus was then immediately started. The cat was placed on its back, the skin overlying the pubic arch was removed and

the pelvis split by cutting through the symphysis pubis with bone clippers. The pelvic cavity was then opened up by forcing apart the pubic bones. The urethra and bladder, together with the vagina and uteri in female cats were removed and the rectum tied and cut at the level of the pelvic brim. The rectum was then displaced ventrally and the connective tissue between it and the pelvic floor cleared by blunt dissection until the anococcygeus muscles became visible. This latter process required care since this connective tissue was sometimes very tough, unlike the delicate tissue in the rat, so the anococcygeus muscles could easily be torn. The portion of the anococcygeus muscles running from their coccygeal tendinous origin to the rectum could be easily freed from the surrounding connective tissue by blunt dissection. In female cats, the muscle appears to merge with the longitudinal muscle at the sides of the rectum and can be dissected free only with difficulty and with probable damage to the muscle. For this reason, in females only the free part of the muscle terminating at the rectum was removed for in vitro studies. In males, on the other hand, the part of the muscle running round the side of the rectum could be freed by blunt dissection and in this case the entire length of the muscle was taken from the tendon to the point where the two muscles join on the ventral surface of the rectum. Surgical threads were then tied round each end of the muscles and the remaining connections to the animal, beyond these ties, were severed enabling removal.

The dissection from the time the cat was killed took 10-15 min. After splitting the pelvis, the tissues were kept cool and moist by the application of cold saline.

Tension
Transducer
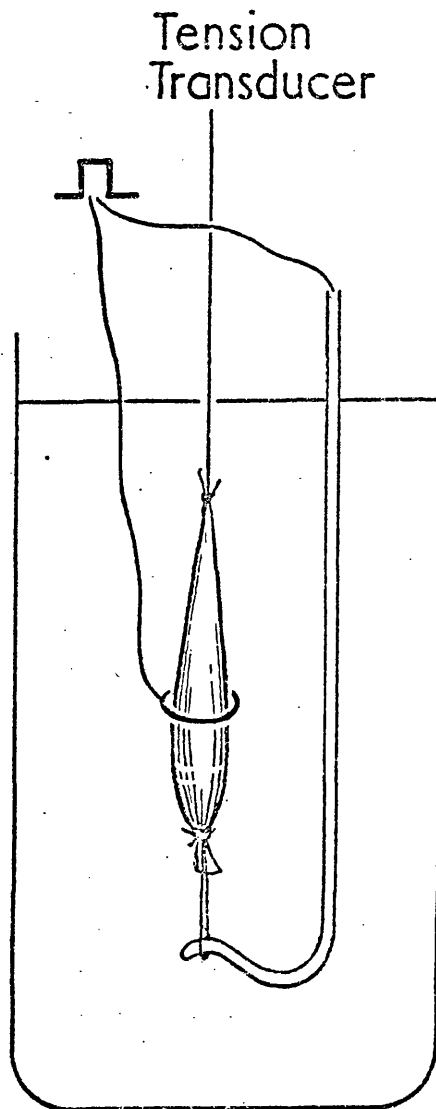
Fig. 6   Diagram of the electrode
assembly used for the field
stimulation of in vitro
anococcygeus muscles.

In vitro preparation of rat or cat anococcygeus muscle

For _in vitro_ experiments, the rat and cat anococcygeus muscles were treated similarly. The muscles were threaded through the ring electrode assembly shown in Fig. 6. This electrode, which is an adaptation of the one used by Burn & Rand (1960) for stimulation of the sympathetic chain, was originally designed for use with the rat anococcygeus but due to the similar dimensions of the cat anococcygeus, it was equally well suited to this muscle.

The electrode assembly was then lowered into Krebs solution in a 10 ml organ bath and clamped into position. The Krebs solution was maintained at $36^{o}C$ by a thermostatically controlled water circulator and gassed with a mixture of 95% O_2 and 5% CO_2. The physiological solution used throughout was Krebs' bicarbonate solution as described by Krebs u Henseleit (1932), with composition as follows - NaCl 6.9g/l; KCl 0.35g/l; $CaCl_2$ 0.29g/l; KH_2PO_4 0.16g/l; $MgSO_4.7H_2O$ 0.29g/l; $NaHCO_3$ 2.1g/l; glucose 2g/l.

This solution was made up daily from concentrated stock solutions which were sufficient for 5 experiments apart from glucose which was added solid to the mixture. To avoid precipitation of the calcium content, calcium chloride was added last to the mixture and the pH maintained at 7.4 by continuous gassing with 95% O_2; 5% CO_2.

The electrode assembly was positioned so that the muscles ran vertically through the ring electrode and were free to contract. The long thread on the tendinous end was attached to a Grass FT03 isometric transducer which was positioned vertically above the bath and mounted on a rack and pinion clamp. The muscle was first set up in a relaxed condition and then the transducer was racked up to stretch the muscle to a tension of 1 g. Following this, the muscle was left to equilibrate for a variable period of at least 20 min before any further operations were carried out. During

this period, the tension fell within the first 10 min to a resting level of 0.3-0.6 g in both species which was maintained from then on. Tension was displayed on a Grass model 7, four channel Polygraph. In most experiments, the two muscles were set up and recorded simultaneously in separate but identical baths.

Field stimulation of the intramural nerve fibres was by a Tektronix type 161 pulse generator triggered by a Tektronix type 162 waveform generator, and monitored by a Tektronix type 360 indicator oscilloscope. This gave the capacity to deliver pulses of 0.01-100 msec duration and 0-50 V intensity at frequencies of 0.1-10,000 Hz for gated periods of up to 10 sec. Longer periods were gated manually and lower frequencies applied manually as single pulses. Two separate stimulators were used enabling either independent or simultaneous stimulation of the two muscles. A pulse width of 1 msec was used except in experiments whose object was to examine the effects of pulse duration. Pulses of 1 msec duration stimulated nerves but not muscle as shown by the results and in agreement with experience with other tissues.

In order to determine the voltage to be used in each experiment, the threshold voltage to produce a detectable response was first found. This value was then doubled and a standard response obtained (usually the motor response to 10 Hz for 10 sec) and this voltage further increased to ensure that it was supra-maximal. Typical values were 3-4 V threshold; with a supra-maximal voltage of 10-15 V.

Drugs were added to the bath in volumes of 0.1-0.3 ml from graduated glass syringes. Mixing occurred rapidly due to the bubbling gas mixture and all concentrations are expressed as the final dilution in the bath. The bath was emptied from below and filled from above by gravity from a 1 L reservoir. Drugs were washed out by emptying and refilling the bath three times. In cases where one drug was required to be present continuously

throughout several washes, it was added to the reservoir to give the final concentration required.

Removal of rat heart and vasa deferentia

In experiments on the effects of reserpine, the heart, vas deferens and anococcygeus were removed for assay of their NA content. Rats were killed by a blow on the head followed by exsanguination, or, in the case of animals previously pithed, by exsanguination by removal of the carotid cannula. Tissues were removed in the order: heart, vas deferens, anococcygeus. The abdomen was opened along the mid-line and the heart exposed by cutting through the costochondral joints and removing a flap of chest wall. The pericardium was opened and the heart removed from the body by cutting the major blood vessels where they enter the heart. After removal, the ventricles were cut open, washed with saline and blotted to remove any blood that remained.

Dissection of the vasa deferentia started by pushing the testicles out of the scrotum into the abdominal cavity. Holding the epididymus with forceps, the connection between the epididymus and scrotum was severed and a thread tied round the epididymal end of the vas. It was then possible to dissect the vas free of mesenteric fold and fat along each side before severing the organ at the prostatic end. If a thread is tied round the prostatic end, the vas can be used as an in vitro preparation similar to that described by Hukovic (1961) or Birmingham & Wilson (1963) for measurement of the responses of the longitudinal smooth muscle. After removal for assay, the vasa were blotted and excess secretory products in the lumen were gently extruded before weighing, since it has been suggested by Sjostrand & Swedin (1968) that reserpine may cause an increase in such contents, thus causing an apparent decrease in the NA content per unit weight.

Removal of the heart and vasa deferentia took less than 2 min so there was no significant delay before dissection of the anococcygeus which followed immediately after. Tissues for NA assay were weighed immediately after removal in beakers cooled to $4^{\circ}C$ then quickly cooled in liquid nitrogen in order to eliminate any possible chemical or enzymic breakdown of the tissue NA.

The pithed rat spinal electrode preparation

Experiments in vivo on the anococcygeus and vas deferens were carried out on pithed rats where stimulation of the autonomic outflows to the organs was accomplished by using the pithing rod as the stimulating electrode. The use of the pithing rod as a means of stimulating the complete sympathetic outflow was first described by Gillespie & Muir (1967). They coated the pithing rod with varnish on those parts which lay in the sacral and cervical regions, thus restricting stimulation to the thoraco-lumbar region. Stimulation between this electrode within the vertebral canal and an indifferent electrode inserted underneath the skin, activated the entire sympathetic outflow. Since this method of stimulation inevitably stimulated the motor nerve fibres in the ventral roots and also vagal and sacral parasympathetic fibres, tubocurarine and atropine had to be given in order to see only pure sympathetic effects. This method was further refined by Gillespie, MacLaren & Pollock (1970), who developed an adjustable pithing rod electrode which could be used to stimulate specific, restricted levels within the vertebral canal enabling selective stimulation of the autonomic outflows to particular organs or groups of organs. In this way, the effects of stimulation of sympathetic or parasympathetic nerves to an organ could be observed in the absence of generalised autonomic stimulation thus eliminating the need to administer blocking drugs to eliminate unwanted effects. It was still necessary to give a neuro-muscular blocker since the
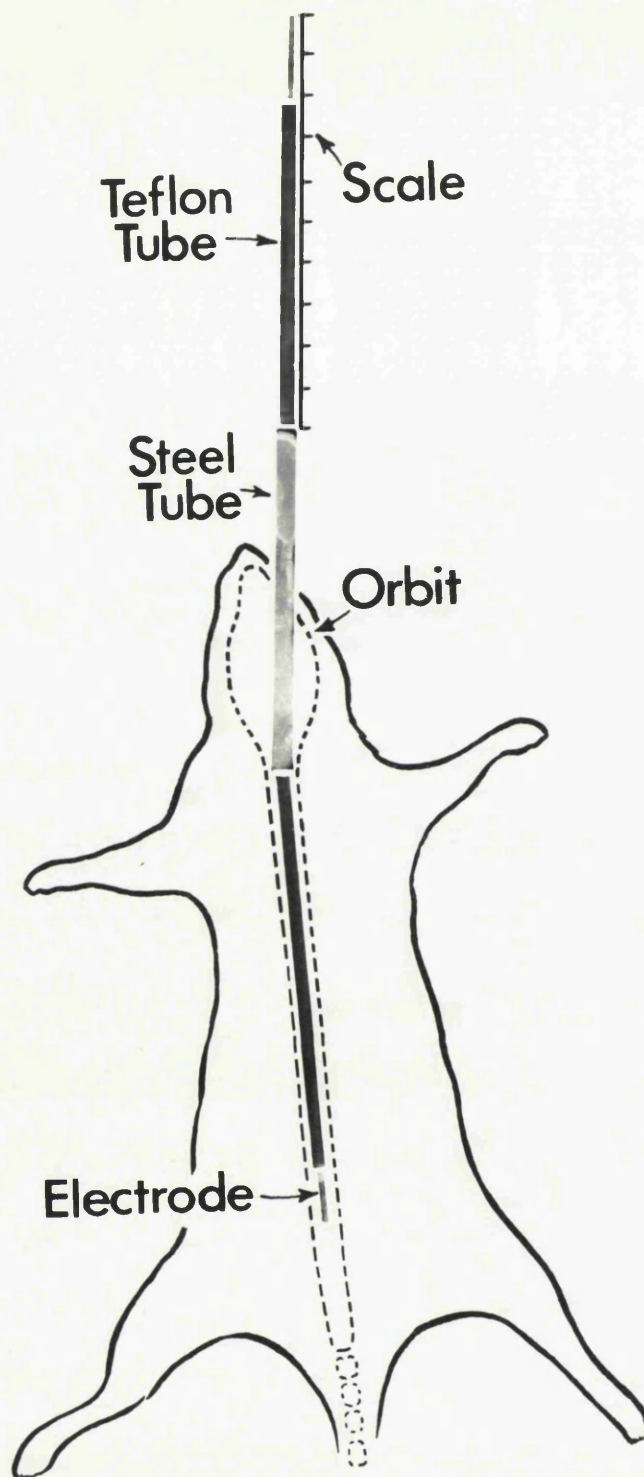
Fig. 7   Arrangement of the pithing rod electrode assembly in the pithed rat.   The animal is initially pithed via the orbit and foramen magnum by inserting a steel tube down into the spinal column to the level of the sixth cervical vertebrae.   Through this trocar is passed successively a teflon tube and inside that a fine steel tube. The position of this steel electrode within the canal and the length exposed can be determined by reference to a simple scale.

ventral roots adjacent to the electrode were still stimulated, but since specific drugs are now available such as gallamine and pancuronium which are relatively free of the autonomic side effects of tubocurarine, this was no serious disadvantage. This is the preparation which has now been used and adapted for stimulation of the nerves to the anococcygeus·and vas deferens.

Male Wistar rats 250-300 g were used. Gillespie, MacLaren & Pollock (1970) found that in this weight range the dimensions of the vertebrae were constant, enabling a standard size of pithing rod to be used and the location of the electrode within the canal to be known by reference to a simple scale.

The rats were anaesthetised with a mixture of 5% Halothane in 1 vol O_2 to 2 vol N_2O. The gas mixture was administered to the rats in an anaesthetic box to minimise any stress or excitement caused by induction which could lead to fatalities on pithing. When the animal no longer showed withdrawal reflexes, its trachea was intubated and then it was pithed and immediately respired artificially at 90 per min with a Palmer respiration pump.

The arrangement of the pithing rod is shown in fig. 7. A short steel tube (13 s.w.g.) was inserted through the orbit and foramen magnum and down into the spinal column as far as the sixth cervical vertebrae. Through this trocar were passed successively a teflon tube (0.16 mm O.D.) and inside that a fine steel tube (26 s.w.g.) which was extruded at the sacral end to complete the pithing. The most important features for survival of the preparation were

    1)   Deep anaesthesia beforehand which reduces the large autonomic discharge caused by pithing,

    2)   immediate and adequate ventilation after pithing since the respiration centre was destroyed, and

3)	maintenance of body temperature since control of this was also lost.

The temperature of each pithed animal was, in most experiments, maintained at 34-37°C by means of a tungsten lamp, and monitored by a rectal thermometer, but in some of the later experiments it was maintained automatically by a Homoeothermic Blanket (Epil) placed underneath the rat and regulated by a rectal probe thermistor.

After pithing, one carotid artery was cannulated with a polythene cannula filled with heparinised saline to record the blood pressure via a Statham P23A pressure transducer.   This pressure, together with the heart rate derived from it by a Grass Tachograph Pre-amplifier, was displayed on a Grass Polygraph.   A polythene cannula was inserted into the left femoral vein for administration of drugs.   No heparin was necessary to keep the venous cannula clear, so no heparin was introduced into the circulation. Drugs were administered in volumes of 0.05-0.2 ml from a glass syringe and washed in with 0.9% saline from a gravity feed to make up a total volume of 0.3 ml.   In some experiments, the left femoral vein was also cannulated in order to give a slow infusion of tyramine.   A Palmer slow injection apparatus was used to administer the drug at a rate of 5-40 µl/min.   A silver wire which acted as the indifferent electrode was inserted under the skin parallel to, and extending the full length of, the spinal column.

The position of the electrode within the vertebral canal was determined by means of the scale shown in fig. 7.   Since the lengths of the outer steel tube, teflon tube and inner steel electrode were known, if the lengths of the teflon and electrode outside the rat were measured, the length of the teflon and electrode within the vertebral canal could be arrived at by subtraction. Since the distances inside the vertebral canal to each segment were known

from the standard animals measured by Gillespie, MacLaren & Pollock (1970), the scale could be calibrated to show the position of the electrode within the canal. At the end of each experiment, these positions were checked by X-ray.

The level of stimulation was therefore determined by the depth of insertion of the teflon tube and the number of segments affected, regulated by varying the length of central steel electrode exposed beyond the teflon. This arrangement confined stimulation to the nerves leaving the vertebral canal in the region adjacent to this electrode and possibly extending a few millimetres beyond its ends. It was therefore possible to explore successive regions along the entire length of the canal from the cervical region to the second sacral segment with a 5 or 10 mm electrode. This enabled the level of origin of the nerves giving rise to any particular response to be determined.

There were some restrictions to this exploration. The vertebral canal was too narrow to accept the teflon shield beyond S2 and at the other end the presence of the guide trocar as far as C6 imposed an upper limit. This trocar could, however, be slightly withdrawn to extend the exploration.

Stimulation between the spinal and indifferent electrodes was achieved using the same stimulator described above for the in vitro anococcygeus experiments. A pulse width of 1 msec was used throughout since this was found adequate by Gillespie & Muir (1967) and Gillespie, MacLaren & Pollock (1970). The autonomic outflow could be stimulated with shorter pulses but no direct stimulation of muscle was found unless the pulse width exceeded 10 msec, so 1 msec was taken as a supramaximal stimulus for the excitation of nerves. The voltage used was supramaximal for the response being observed. Supramaximal voltage is a difficult parameter to define as well
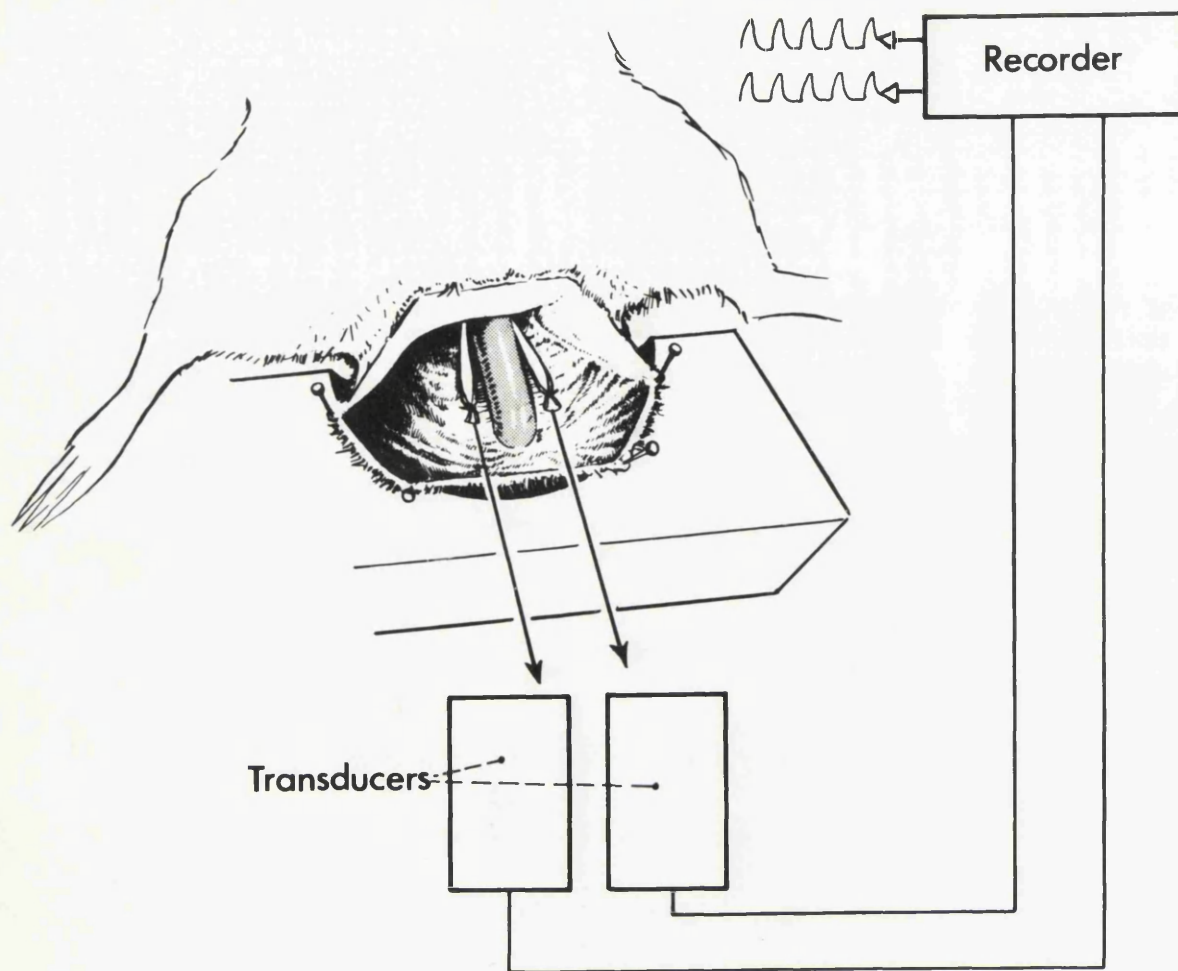
Fig. 8   The arrangement to record _in vivo_ the tension
response of the anococcygeus muscles in the rat.   The scrotum
is opened and pinned to a shaped wax block to form a pouch
which is filled with liquid paraffin.   Each anococcygeus is
attached by thread to an isometric transducer.

as determine in this preparation since if the outflow to a particular organ extends over a greater number of segments than are covered by the stimulating electrode then increasing the strength of stimulation beyond what is maximal for adjacent nerve fibres will continue to cause an increase in response by an extension of the region stimulated. What was required, however, was supramaximal stimulation of the autonomic outflows within the region of the electrode. In early experiments, it was found that 20 V was always supramaximal while still leaving the electrode specific to within 5 mm, so this parameter was used in each subsequent experiment with the proviso that double or half this voltage did not produce a significantly larger or smaller response. If they did, this normally was a pointer to an electrical fault since the electrical properties of the preparations were so similar.

Muscle twitching as a result of stimulating the ventral roots was prevented by giving Pancuronium bromide (1 mg/kg) intravenously at the start of each experiment as soon as the venous cannula was in place.

The rat anococcygeus _in vivo_ preparation

The tension of the male anococcygeus muscles can easily be recorded with a minimum of operative interference since the ventral bar lies superficially just under the skin of the scrotum a few mm short of the anal margin. If this ventral bar is split, a thread can be attached to each muscle without interference with the nerves and their individual responses to drugs administered intravenously or, in the case of pithed rats, from stimulation of the autonomic outflows, can be recorded.

Dissection of the anococcygeus began after pithing and insertion of cannulae.

The muscles were exposed by an incision in the scrotum just anterior to the anal margin and the edges of the incision pinned on to a shaped wax block placed across the tail as shown in fig. 8. The pouch so formed was filled

with liquid paraffin (sp. gr. 0.85) to prevent drying of the exposed tissue. The rectal thermometer or probe could be inserted underneath this block.

The muscle layers overlying the colon were then cleared by blunt dissection for approximately 25 mm from the anal margin in order to free more of the anococcygeus, the ventral bar was split and a thread tied round the cut end of each muscle.

Each thread was attached to a Grass FT03 isometric transducer mounted on a rack and pinion clamp, and the transducer positioned to apply an initial tension of 1 g to each muscle at an angle of approximately 30° to the horizontal. This held the muscle in its normal in situ position. There was no need for further dissection of the anococcygeus muscle since it was effectively anchored at the rostral end by the tendon attached to the vertebrae.

This preparation responded to drugs and spinal stimulation for as long as the rat remained viable as indicated by the presence of a blood pressure and heart beat, i.e. up to 10 hrs, and so presumably receives an adequate blood perfusion and intact innervation. Responses to nerve stimulation and drugs were comparable in size to those obtained in vitro, so the muscles appeared to be free to develop tension. Movement of the colon might have been expected to interfere with the tension in the anococcygeus, but, in fact, spontaneous, visible movements of the colon had no effect, and when the sacral parasympathetic outflow was stimulated, the resultant contraction of the longitudinal smooth muscle of the colon had no effect on the tension of the anococcygeus.

This dissection was at first performed with the help of a Zeiss dissection microscope but as it became more routine was carried out using the naked eye. The dissection took less than 10 min.
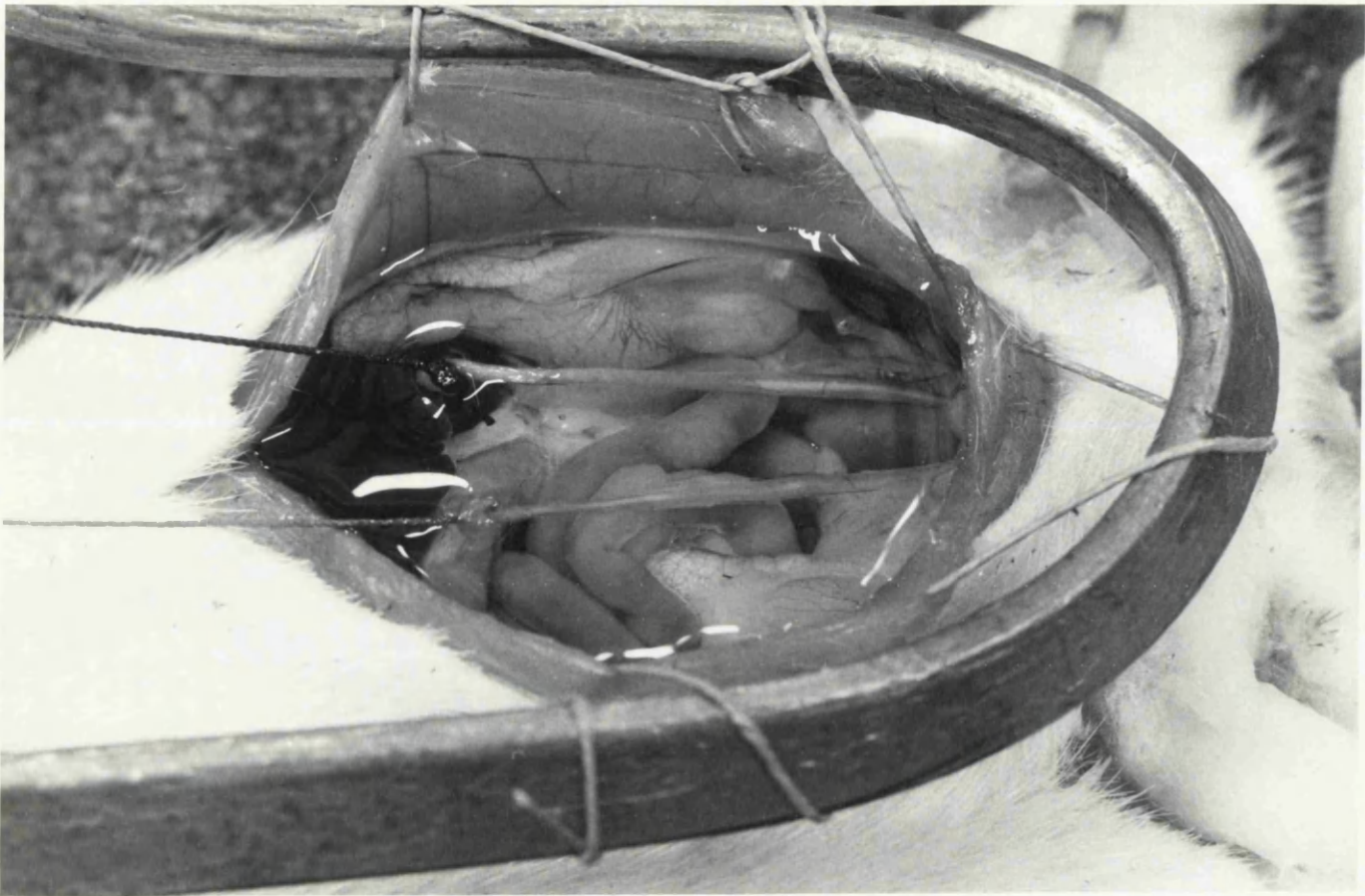
Fig. 9   In situ recording of the longitudinal tension in the

vas deferens.   Each vas is attached to a transducer at the

epididymal end.   The abdomen is filled with liquid paraffin

to prevent drying.

The rat vas deferens _in vivo_ preparation

Since Hukovic (1961) first introduced the guinea pig isolated hypogastric nerve - vas deferens preparation as an example of a sympathetically innervated smooth muscle, the response of this tissue to nerve stimulation has been extensively investigated (Sjostrand, 1962, 1965; Bentley & Sabine, 1963; Birmingham & Wilson, 1963; Ferry, 1963, 1967; Merrilees, Burnstock & Holman, 1963). In the rat, although the isolated hypogastric nerve-vas deferens preparation (Graham, Al Katib & Spriggs, 1968) and hypogastric denervation (Sjostrand & Swedin, 1967) have been described, relatively little work has been done with extrinsic stimulation, possibly because of the greater difficulty in preserving the extrinsic nerve. By comparison, there is an extensive body of work on the effect of drugs on the isolated vas in this species (Chang & Chang, 1965; Swedin, 1971; Furness & Burnstock, 1969; Birmingham, 1970). In addition, the effects of stimulating the nerves to the vas deferens _in vivo_ have rarely been described (Blakeley, Dearnaley & Harrison, 1970), and this only in the guinea pig. With the pithed rat, however, stimulation of the autonomic outflow offers an opportunity to examine the properties of the innervation of the rat vas deferens _in vivo_.

The dissection of the vas deferens was carried out after the rat had been pithed, cannulae inserted and, where both vas and anococcygeus were recorded in the same animal, after dissection of the anococcygeus.

The abdomen was opened in the mid-line for approximately 60 mm rostral from the penis and the edges of the incision sewn round a curved bar to form a a sac as shown in fig. 9. In a few initial experiments, the gastro-intestinal tract between the stomach and descending colon was removed, but this was discontinued since it was found to make no difference to the ease

of recording the vas deferens, which was the intention of the manoevre, and also shortened the survival time of the preparation, presumably due to loss of blood volume.

On first opening the abdomen, a few ml of liquid paraffin was run over the intestines to prevent drying. When the dissection of the vas was complete, the abdominal cavity was filled with liquid paraffin (approximately 20 ml) pre-warmed to 36°C to provide a pool in which the vas could function over a period of hours. The initial dissection was as for removal but leaving the prostatic end attached. Since the nerves enter and the blood vessels enter and leave at the prostatic end (Sjostrand, 1965), the vas can be tied off at the epididymal end and dissected free of connective tissue along its entire length without any loss of functional capacity. Two adjacent ties were made at the epididymal end and the vas cut between these, leaving it attached to the rat only at the prostatic end. The thread attached to the vas was then attached to a Grass FT03 isometric transducer which was positioned to pull the vas straight and apply an initial tension of 0.5 g (fig. 9). There was no need to fix the vas at the prostatic end since it was very effectively anchored by its attachment to the urethra which is firmly held in place by the ischio-cavernosus and pyramidalis muscles. Both left and right vas deferens were set up in this way.

This preparation enabled recording of the longitudinal tension developed by the smooth muscle of the vas.

Another way of recording the response of the vas was to perfuse physiological solution at a constant rate through the lumen from the epididymal end and measure the perfusion pressure (Gillespie, MacLaren & Pollock, 1970). This may be nearer to a measure of the physiological response of the organ in much the same way that the perfused ear artery is a more physiological response than the aortic spiral strip. Since no one

has ever suggested that the function of the vas is to move something by virtue of its shortening its length, it is surprising that so many examinations of this hollow organ have concentrated on its capacity to develop tension isometrically or shorten its length isotonically, when perfusion is so simple.

With the abdomen opened as above, the vas was cannulated at the epididymal end in the prostatic direction with a polythene cannula, taking care to avoid the artery which runs superficially along the surface. Krebs' solution was perfused at a constant rate of 20 or 40 μl/min using a slow injection apparatus and perfusion pressure was recorded from a side branch 1 cm from the cannula using a Statham P23A pressure transducer. The perfusate usually escaped via the penis without obstruction, the vas itself providing almost all of the resistance, but in a few cases a gradual build up of pressure occurred due to some obstruction distal to the vas, and in these instances an incision was made at the base of the adjacent seminal vesicle to allow egress of the perfusate. No further dissection was necessary.

The dissection of the vas took approximately 15 min for both organs.

The total dissection time to set up a pithed rat, insert cannulae and record both the anococcygeus and vasa eventually took 1 hr.

Administration of Reserpine – Protocol

In the experiments to determine the effects of reserpine on tissues, the reserpine was administered intra-peritoneally. Rat tissues are depleted of their catecholamines by reserpine whether it is administered intra-peritoneally (Graham, Al Katib, & Spriggs, 1968; Jane, Planas & Bonaccorsi, 1970; Haggendal & Dahlstrom, 1971) or sub-cutaneously (Sjostrand & Swedin, 1968). In the present experiments, reserpine could not be obtained as the

commonly used "Serpasil" injectable solution so the alkaloid was dissolved

in an aqueous solution of ascorbic acid (0.4% w/v) in which it is readily

soluble (Carlsson 1966). This solution was, however, acidic (pH 4) and

therefore the intra-peritoneal route was selected as being more humane and

less traumatic for the animals. The drug was administered in volumes of

1 ml/kg i.e. 0.25 ml/250 g rat. The rats showed no signs of being in any

discomfort after these injections.

The experiments with reserpine pretreatment could be split into 3 groups.

1) Variation of reserpine dose

Reserpine was given to groups of 6 rats, 24 hrs before sacrifice in doses

of 50, 100, 200, and 1,000 µg/kg.

2) Variation of time of pretreatment

Reserpine 200 µg/kg was given to groups of 6 rats at the following times

before sacrifice; 3, 6, 12, 24, and 48 hrs, 21, 28, and 35 days.

3) Pretreatment before pithing

Rats were given 200 µg/kg reserpine 3 hrs before pithing.

SOURCE OF RESERPINE - The reserpine used throughout these experiments

was Reserpine (pure) (Koch - Light). Due to an error on one day, reserpine

from a different source was used and it was noticed that the extent of

depletion of NA from the tissues was quite different. A comparison was

therefore made of reserpine from 3 different sources. The NA content of rat

heart was measured 6 hrs after a nominal 200 µg/kg dose and the content in

µg/g expressed as a percentage of the values obtained from control rats.

Results were as follows -

	NA % CONTROL
Reserpine (pure, crystalline) Koch - Light	9, 5, 10, 7
Reserpine (Serpasil, pure alkaloid) CIBA	39, 48, 61
Reserpine (crystalline) Sigma	57

It is clear from these results that the source of reserpine used is an important factor in any experiments performed.

CIRCADIAN CONSIDERATIONS - Rats in groups 1) and 2) were sacrificed as near as possible to 12.00 hrs except for the 6 hr pretreated rats which were treated at 9.00 hrs and therefore sacrificed at 15.00 hrs. The rats in group 3) were treated at 9.00 hrs, pithed at 12.00 hrs and their tissues taken at 15.00 hrs. This made direct comparison possible between the 3 and 6 hr pretreatment and pithed animals.

There is some evidence of circadian variation in peripheral catecholamine content in the submaxillary glands (Wurtman & Axelrod, 1966) and in the adrenals (Scheving, Harrison & Pauley, 1968), but there has been no suggestion of such a factor in the tissues used here. This arrangement of the experiment should, however, eradicate any such problems.

Assay of tissue noradrenaline

The main steps in the chemical assay of catecholamines are extraction, purification and concentration of the amines and thereafter the final estimation. Before assay of tissue catecholamines, an extract must be made and the proteins precipitated. The reaction utilised in this assay is the trihydroxyindole reaction. Since Loewi (1918) first reported the appearance of a yellow - green fluorescence after addition of strong alkali to an adrenaline solution, the reactions involved have been worked out and developed into a sensitive and specific assay for adrenaline and noradrenaline (Ehrlen, 1948; Lund, 1949). It was subsequently shown that noradrenaline and adrenaline could be determined differentially spectrophotometrically (Bertler, Carlsson & Rosengren, 1958; Euler & Lishajko, 1959) and this method further refined by stabilisation of the end-products (Euler & Lishajko, 1961). It is this method of Euler & Lishajko which I have used, with purification of the

noradrenaline on alumina columns as described by Gillespie, Hamilton & Hosie (1970).

Extraction   After removal, tissues were weighed and frozen in liquid nitrogen.   Tissues from 2 animals were pooled at this stage.   The tissues were then placed in a stainless steel mortar and pestle (also pre-cooled in liquid nitrogen) and pulverised for 20 secs using a power hammer driven from a compressor.   This procedure reduced the tissues to a fine powder.   The powdered tissue was transferred to a cooled beaker and 5 ml of 0.4 M perchloric acid added to precipitate the protein.   At this stage 1 µg NA aliquots (2) were added to perchloric acid to give control samples for the recovery from subsequent procedures.   After 30 min, on ice, the mixture was centrifuged in a bench centrifuge to remove the precipitate and the supernatant decanted for adsorption onto alumina columns.   The alumina ("CAMAG" Emanuel, pH of aqueous suspension 4.5 ± 0.5, 504 - C - 1) columns (15 mm high, 10 mm diameter) were prepared with a flow rate of at least 5 ml/min in order to shorten the time that the NA is on the columns at a pH at which it is unstable.   To each supernatant, EDTA was added to give a final concentration of 1% and the pH was adjusted to 8.2 - 8.3 with 2 M & 0.5 M NaOH.   At pH 8.2 the extract was added immediately to the alumina and the column washed with cold distilled water till the pH of the effluent reached 5.8 - 6.0.   The columns were dried using compressed nitrogen and the catecholamines eluted with 2 consecutive 5 ml aliquots of 0.25 M acetic acid.   The extract was then centrifuged for 10 min in a bench centrifuge to remove any alumina particles and the supernatant decanted for assay.   During these procedures, all materials were kept at $0^{o}C$ to reduce breakdown of catecholamines.

Assay   The trihydroxyindole reaction was carried out in two stages. Oxidation of the catecholamines to chrome derivatives was performed with potassium ferricyanide.   An advantage of potassium ferricyanide is that

dopamine, normetanephrine and metanephrine, if present, will hardly interfere (Haggendal, 1966). Oxidation was then interrupted and the chrome derivatives converted into fluorescent lutines by the addition of a mixture of ascorbic acid and sodium hydroxide. Ethylene diamine was also present in this latter mixture to stabilise the lutines (Euler & Lishajko, 1961). The composition of this alkaline ethylene diamine - ascorbic acid mixture was as follows - per 100 ml, ethylene diamine 2 ml; 5 M NaOH 88 ml; 2% ascorbic acid 10 ml.

The pH of the eluates was adjusted to 6.2 - 6.3 with 2 M NH_4OH and 3 ml aliquots reacted as follows -

Time 0 min   0.1 ml 0.25% potassium ferricyanide added

    3 min   4 ml alkaline ethylene diamine - ascorbic acid added

Another 3 ml was treated as a reaction blank as follows -

Time 0 min   4 ml alkaline ethylene diamine - ascorbic acid added

    3 min   0.1 ml 0.25% potassium ferricyanide added

An assay standard of 0.1 µg NA was also prepared as follows -

    2 ml pH 6.5 phosphate buffer

    1 ml 0.1 µg/ml NA

Two such NA standards were reacted and two acted as blanks, as above. The fluorescence in the samples and blanks was then read at 500 nm in an Aminco - Bowman Spectrophotofluorometer using an excitation wavelength of 400 nm. These are the emission and excitation peaks for the NA product. The adrenaline product fluorescence can be read with an excitation wavelength of 450 nm, but initial experiments showed that no adrenaline was present in any of the tissues used, so only NA was estimated in the subsequent experiments. The fluorescent product is stable for up to 2 hrs if kept in the dark (Euler & Lishajko, 1961; Haggendal, 1966) but fading can occur due to exposure to

fluorescent lighting, so the reacted samples were kept covered and read within 15 min of the reaction at which time fading was insignificant. Initial calibration experiments showed that the relationship between NA and fluorescence (test minus blank) was linear over the range necessary for the experiments, so all NA contents were calculated as a proportion of the mean assay standard after allowance for volumes.

The NA content of the various alumina eluates was thus calculated and the tissue extracts corrected for recovery from the columns using the 1 μg NA column standard. This column recovery never varied by more than 2% between the two standards in any single experiment. It varied between experiments from 70 - 85% and averaged 78%.

The NA content of tissues was then expressed as μg NA/g tissue, i.e. μg/g.

Histochemistry - Falck Technique

The histochemical method currently in use for the demonstration of catecholamines and indoleamines at the cellular level was developed in Sweden in the early sixties (Carlsson, Falck, Hillarp & Thieme, 1961; Falck, Hillarp, Thieme & Torp, 1962; Falck, 1962; Falck & Torp, 1962 a & b).

The method is based on the principle that amines can be transformed into intensely fluorescent isoquinoline derivatives by condensation with formaldehyde. This was first applied to tissues by Eranko (1952, 1955) who used liquid formaldehyde to demonstrate the location of catecholamines in the adrenals. The more sensitive technique involving the use of formaldehyde vapour on freeze-dried tissue was then later developed by Hillarp, Falck and co-workers. The reactions involved have been categorised (Corrodi & Hillarp, 1963, 1964; Corrodi, Hillarp & Jonsson, 1964; Corrodi & Jonsson, 1965 a & b, 1966; Jonsson, 1967) and developed into a quantitative microspectrofluorometric method permitting differentiation between noradrenaline, adrenaline, dopamine and 5 - hydroxytryptamine (Caspersson, Hillarp & Ritzen, 1966; Bjorklund, Ehinger & Falck, 1968).

The method used here is a modified version of this technique by Gillespie & Kirpekar (1966).

It is necessary to freeze-dry the tissues to achieve rapid fixation which prevents diffusion of amines out of nerves and also to keep the tissues dry through all subsequent procedures since water converts the fluorescent end-product to a non - fluorescent form.

Tissue blocks were frozen in liquid nitrogen cooled isopentane and freeze-dried in a Speedivac - Pearse tissue drier at $-40^{\circ}C$, 0.01 torr for 18 hrs. The temperature in the freeze-drier was then increased to $+40^{\circ}C$ to ensure that on removal the tissues were not exposed to condensation of water vapour from the atmosphere due to being below the ambient temperature. The tissues were then placed in a closed jar containing paraformaldehyde (Merck) in an oven and thus exposed to formaldehyde vapour at $80^{\circ}C$ for 1 hr. Following this, they were re-dried at 0.01 torr for 1 hr before being embedded in degassed wax under vacuum at $65^{\circ}C$. Wax blocks were subsequently made, sliced at 6 μ, mounted on heated glass slides and covered with liquid paraffin and a cover glass for examination in the fluorescence microscope. These sections were kept in the dark at all times to prevent fading of the fluorescence which occurs rapidly under daylight or the fluorescent lighting in the laboratory.

Tissues were examined and photomicrographs taken with a Leitz (Wetzlar) Orthoplan microscope with dark field illumination: HBO 200 Mercury vapour lamp; 3 mm BG12 exciter filter; Condensers - D 0.80, D 1.20 A oil immersion; K530 barrier filter; Objectives - Pl 10/0.25, Pl 25/0.50, Pl 40/0.65, Fl Oel 54/0.95 oil immersion.
Photomicrographs were taken on Ilford FP4 with a 3 or 5 min exposure.

Drugs used were -

Acetylcholine chloride (Koch - Light);   L - Adrenaline bitartrate (B.D.H.);

Atropine sulphate (B.D.H.);   Dexamphetamine sulphate;   Adenosine 5' -

triphosphate (Sigma);   Carbachol (Burroughs Wellcome);   Cocaine

hydrochloride;   Guanethidine sulphate (CIBA);   Hexamethonium bromide

(Koch - Light);   Histamine acid phosphate (B.D.H.);   6 - Hydroxydopamine

(kindly donated by Dr. H. Thoenen of Hoffmann La Roche);   5 - hydroxy-

tryptamine creatinine sulphate (Koch - Light);   DL - Isoprenaline

hydrochloride (Sigma);   D - Lysergic acid diethylamide tartrate (Sandoz);

Mecamylamine hydrochloride (Merck, Sharp & Dohme);   Mepyramine maleate

(May & Baker);   Methysergide bimaleate (Sandoz);   Neostigmine methylsulphate

(Roche);   L - Noradrenaline bitartrate (Koch - Light);   Oxymetazoline

(kindly donated by Dr. D. Jack of Allen & Hanbury);   Pancuronium bromide

(Organon);   Phentolamine mesylate (CIBA);   Propranolol sulphate (Sigma);

Prostaglandins E_1, E_2 & $F_{2\alpha}$ (kindly donated by Prof. E.W. Horton);

Reserpine (crystalline) (Koch - Light);   Tyramine hydrochloride (Sigma);

Vasopressin (Parke - Davis).

Doses in in vivo experiments are expressed in grams of the base and in

in vitro experiments as molar concentration of the base with the

exceptions of 6 - hydroxydopamine, prostaglandins (g/ml) and vasopressin

(International Units / ml).

Abbreviations used throughout the text are noradrenaline - NA;

5 - hydroxytryptamine - 5HT; Lysergic acid diethylamide - LSD;

6 - hydroxydopamine - 6OHDA; adenosine 5' - triphosphate - ATP.

RESULTS

Fig. 10   The effect of stimulating at different levels
of the vertebral canal on the response of the anococcygeus muscle
and the blood pressure in the pithed rat.   Stimulation was at
30 Hz for 10 sec with supramaximal voltage and is indicated
by a dot below the trace.   (a) In this rat the tension in
the left (L) and right (R) muscles was recorded separately.
A 5 mm exposure of the stimulating electrode was used, and
levels from S 1 to T 11 - 12 examined.   Motor responses were
obtained from two regions, at L 6 and S 1 and again at and
above L 3.   Between L 3 and L 5 no response was obtained from
the anococcygeus.   (b) In this rat the combined tension of
the two anococcygeus muscles was recorded at higher levels of
the vertebral canal explored with a 10 mm electrode exposure.
At T 8 - 10 a second, delayed component appears in the response,
and at higher levels this completely replaces the fast response
coincident with stimulation seen at lower levels.   The delay
averages about 15 sec so that the response begins after the
end of stimulation and is presumably due to catecholamine
release from the adrenals.   Time trace mins.

PART I - THE RAT ANOCOCCYGEUS IN VIVO

The Motor Response

Spinal origin of the motor nerves

The spinal origin of the motor nerves to the rat anococcygeus muscles was traced by obtaining responses in the muscles to stimulation at successive 5 mm intervals along the spinal canal from S 2 to C 7, as shown in Fig. 10.

Exploration began in the sacral region and the electrode was withdrawn between stimulation periods so as to stimulate progressively higher regions.

Two types of motor response were obtained. The first type consisted of contractions whose latent period was short (less than 1 sec), whose development of tension was rapid (reaching a maximum in less than 10 sec) and which declined rapidly when stimulation ceased. Rises in blood pressure accompanying these responses varied according to the region being explored but were also rapid and declined on cessation of stimulation. These anococcygeus responses have been interpreted as due to direct stimulation of the neural pathway to the muscles. A second type of response was observed when stimulating in the upper thoracic region. This response had a long latent period (10 - 15 sec), in some instances longer than the stimulation period (Fig. 12) developed and decayed less rapidly than the first type of response and unlike it was insensitive to the adrenergic blocking agent guanethidine. Accompanying this second type of response was a marked rise in blood pressure and cardiac acceleration. For these reasons it has been attributed to stimulation of the secreto-motor fibres to the adrenal medulla.

Direct neurally mediated motor responses in the anococcygeus were obtained at two positions; first between S 2 and L 6 and again between L 3 and T 10, with a gap between L 6 and L 3 (Figs. 10a and 11). The maximal response

Fig.11 EFFECT OF VARYING THE POSITION OF THE SPINAL ELECTRODE ON THE MOTOR RESPONSE OF THE ANOCOCCYGEUS IN THE PITHED RAT.
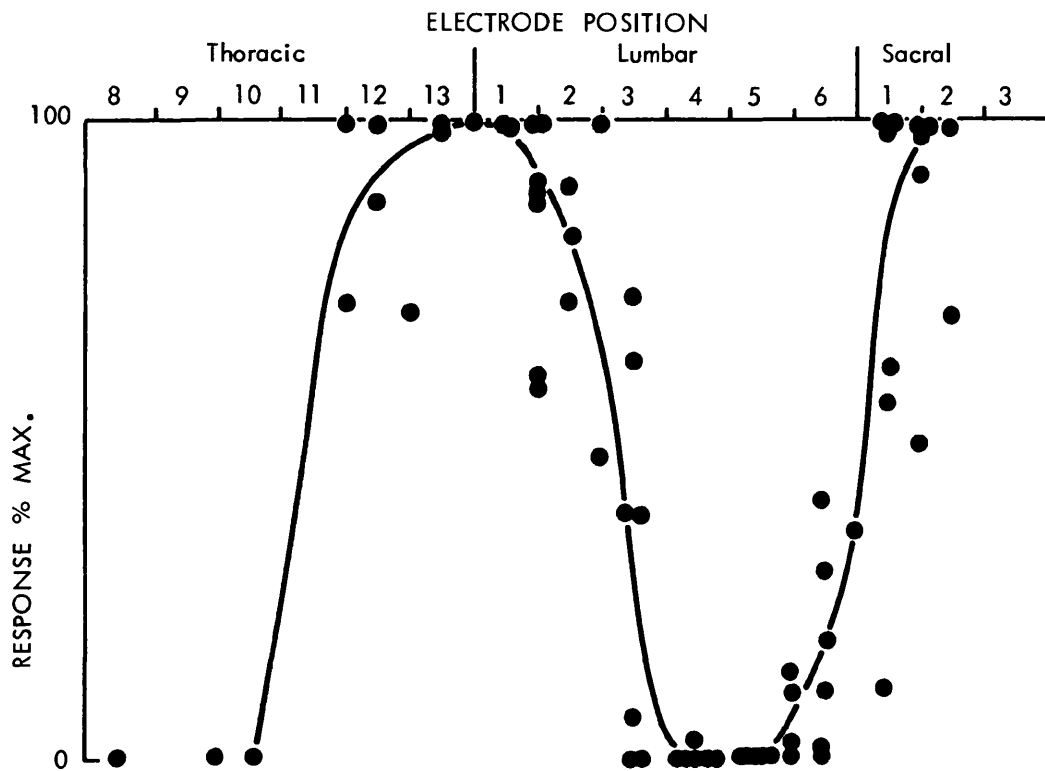
Fig. 11   Responses obtained in the anococcygeus

muscle to stimulation of the autonomic outflow at

5 mm intervals along the vertebral canal.   10 sec trains,

30 Hz, 1 msec pulses.   The response at each position is

expressed as a percentage of the maximum response

obtained in each rat.   Two optimum positions are

apparent at T 11 - L 3 and L 6 - S 2 with a gap

at L 3 - L 6.   (9 rats)
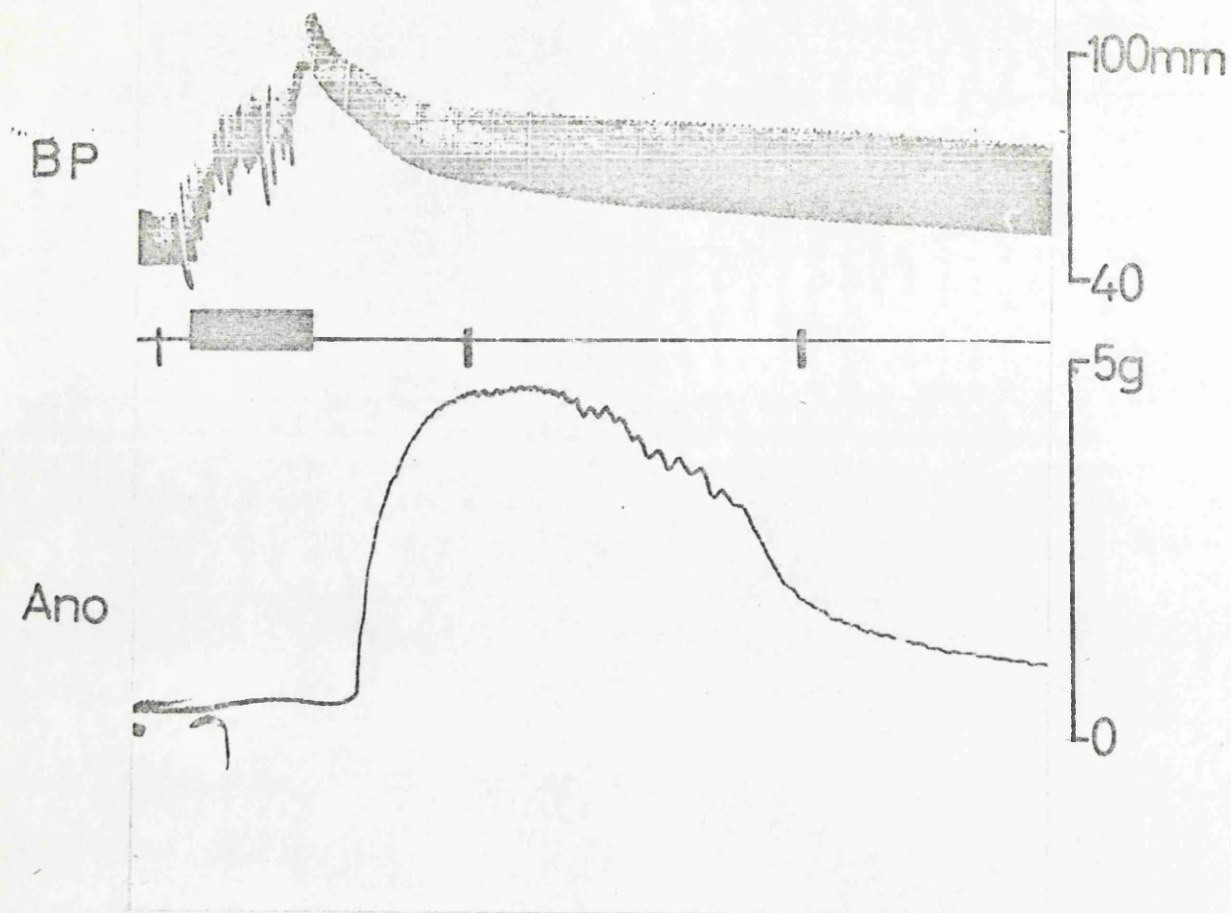
Fig. 12   Responses of the blood pressure and anococcygeus muscles (recorded jointly) of a pithed rat to stimulation at the bar on the time trace for 10 sec, 30 Hz at T 6 - 8.   A very small direct response was obtained in the anococcygeus, but after cessation of stimulation a large response was obtained due to catecholamines liberated from the adrenals. Time marker 30 sec.

was similar in size in both regions (4.9 ± 0.2 g per single muscle, n = 10).
At T 10 - 11 delayed responses appeared in addition to the direct responses
and beyond T 10 the rapid responses disappeared to be replaced by the delayed
adrenal responses (Figs. 10b and 12). The optimal position for the adrenal
response lay from T 10 to T 4. No responses were obtained higher than T 2.

Regions below S 2 could not be separately stimulated since the narrowness of
the spinal canal prevented entry of the teflon tube which isolates the
stimulating electrode. The precise lower limits of the lower motor outflow
could not, therefore, be defined, but experiments with finer teflon and the
electrode extruded to the end of the vertebral canal suggest that outflows to
the anococcygeus do not extend much beyond S 2 since responses to stimulation
with an electrode completely below S 2 were very small.

Frequency characteristics of the motor response

Three methods were used to determine the frequency/response characteristics
of the responses obtained from stimulation in the two motor regions, S 2 - L 6
('lower') and L 3 - T 10 ('upper').

(1)   Stimulation for a fixed 10 sec period at varying
   frequencies.

(2)   Stimulation until the maximum response was obtained
        at varying frequencies.

(3)   Stimulation with a fixed number of pulses at varying
        frequencies.

The frequency/response curves obtained with these different techniques are
shown in Fig. 13. Clearly the optimal frequency, whichever method is
chosen, is 30 Hz, but the efficacy of low frequency is very dependent on the
method of comparison used.

(1)   Stimulation for 10 sec periods at frequencies from 0.1 to 100 Hz
produced motor responses at all frequencies with a maximum at 10 - 30 Hz.

Fig. 13

FREQUENCY/RESPONSE CURVE FOR MOTOR RESPONSE IN ANOCOCCYGEUS IN PITHED RAT
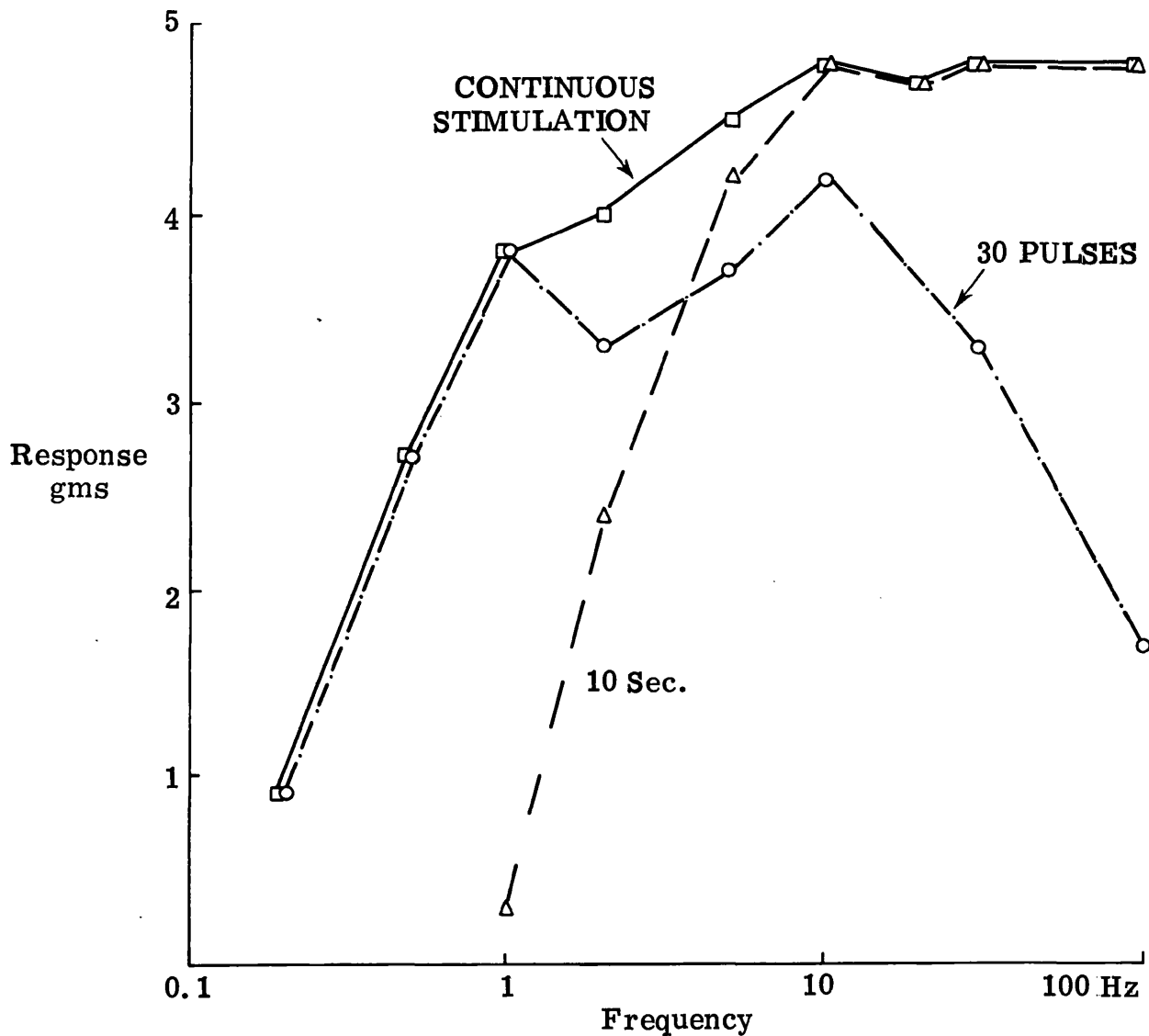
Stimulation with a 10 mm electrode at L 1.   Responses obtained in

a   single anococcygeus muscle to stimulation at frequencies indicated

for trains of 30 pulses, 10 sec or continuous stimulation till a

plateau was reached.
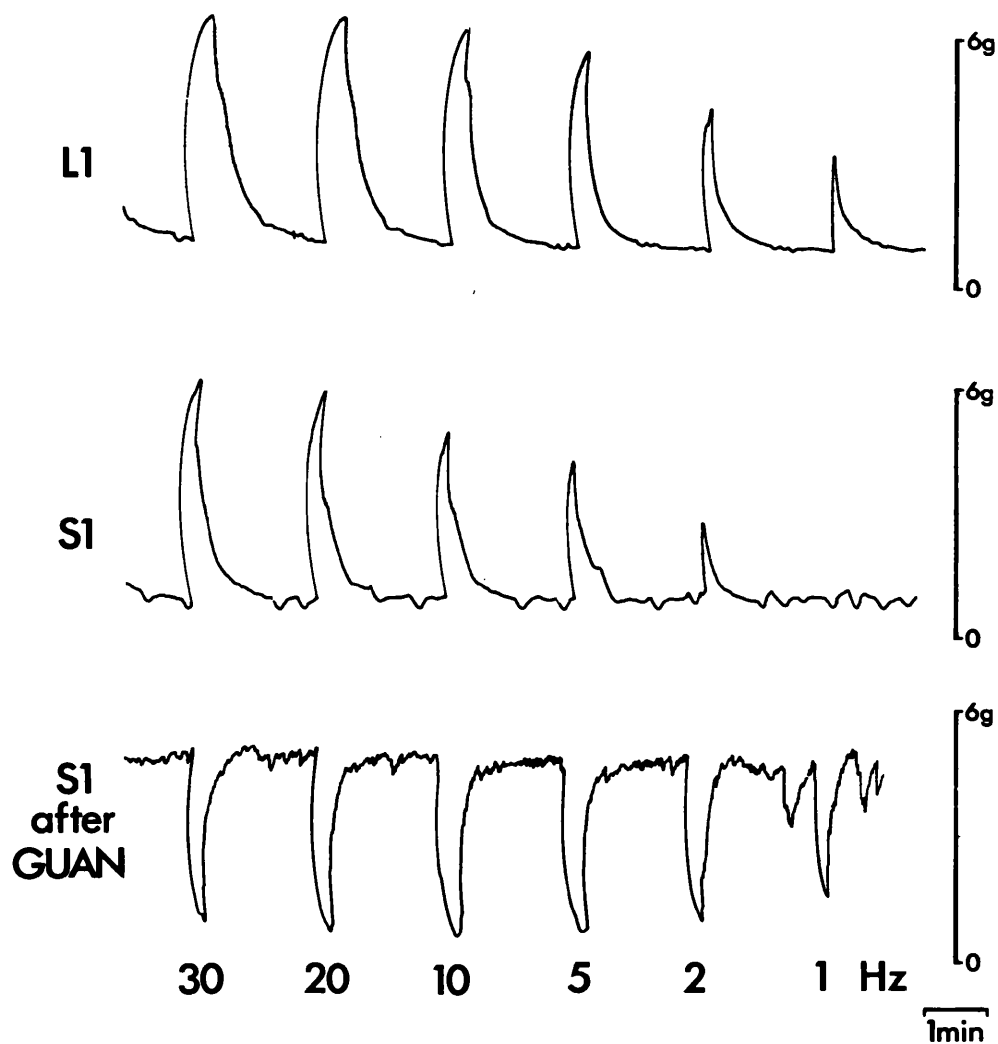
Fig. 14 Frequency characteristics of the motor and inhibitory responses in the anococcygeus muscle of a pithed rat from stimulation in the vertebral canal. The upper record shows the motor response from stimulation at L 1, the middle the motor response from stimulation at S 1, and the lower the inhibitory response from stimulation at S 1 in the presence of guanethidine (10 mg/kg). The frequency of stimulation is shown on the bottom record but applies to it all. The duration of stimulation was 10 sec and the electrode exposure 5 mm.

BP

0.5   30 Hz

Ano

Ano
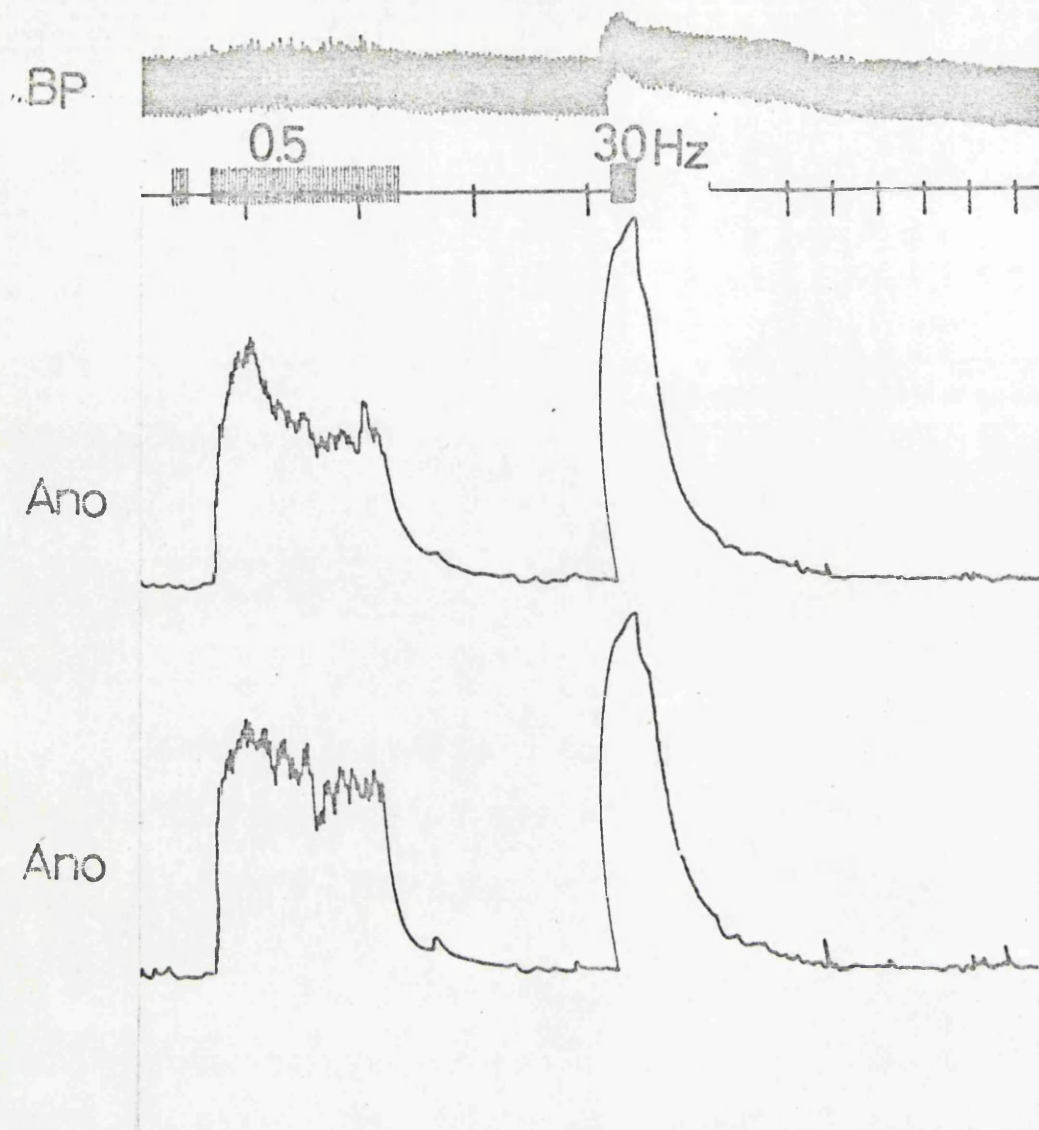
Fig. 15   Responses in two contralateral
anococcygeus muscles of a pithed rat to
stimulation at 0.5 Hz and 30 Hz at L 1.
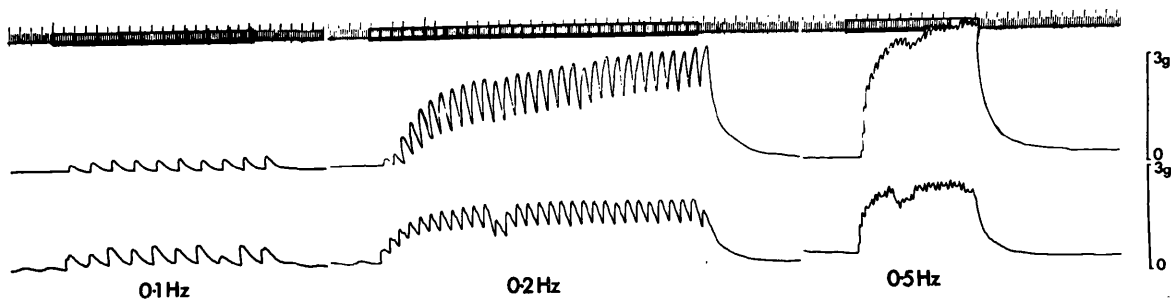
Fig. 16   The separate responses of the left and right

anococcygeus muscles in the pithed rat to stimulation in the

vertebral canal at L 1 - 2, at the frequencies shown at the

bottom of each panel.   At 0.1 Hz the responses to individual

stimuli do not summate;   at 0.2 Hz and 0.5 Hz summation occurs

and if sufficient time is allowed, large tensions are developed.

Time marker 1 and 5 sec.
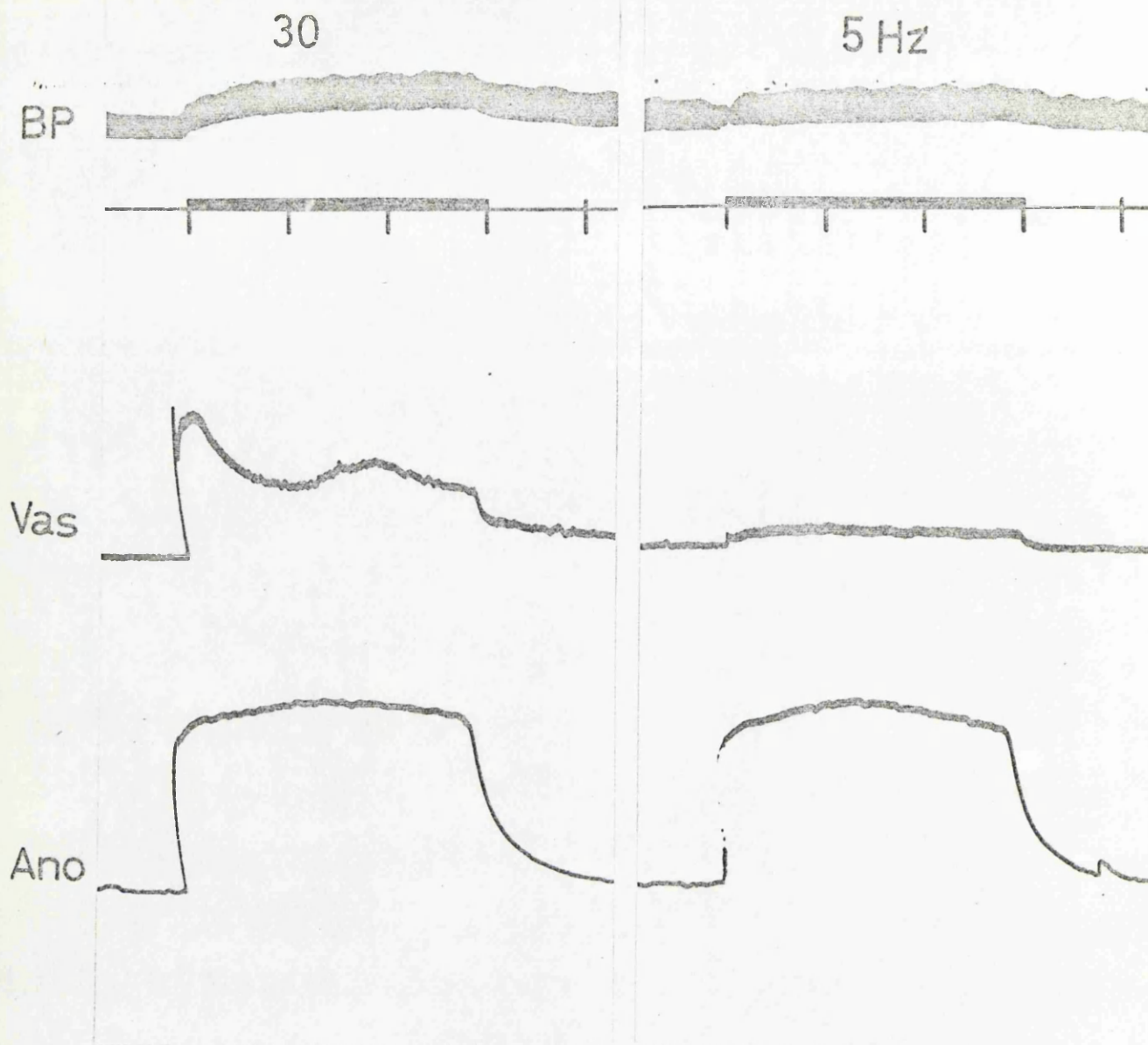
Fig. 17   Comparison of the responses of the anococcygeus
and vas deferens of the pithed rat to continuous stimulation.
10 mm electrode, 30 Hz and 5 Hz for 3 min.   Anococcygeus
maintained raised tone at both frequencies.   At 30 Hz the vas
deferens exhibited the customary initial "spike" and secondary
responses and then maintained a lower tone.   At 5 Hz the vas
showed a small but well maintained response.   Time 1 min.

BP

Ano
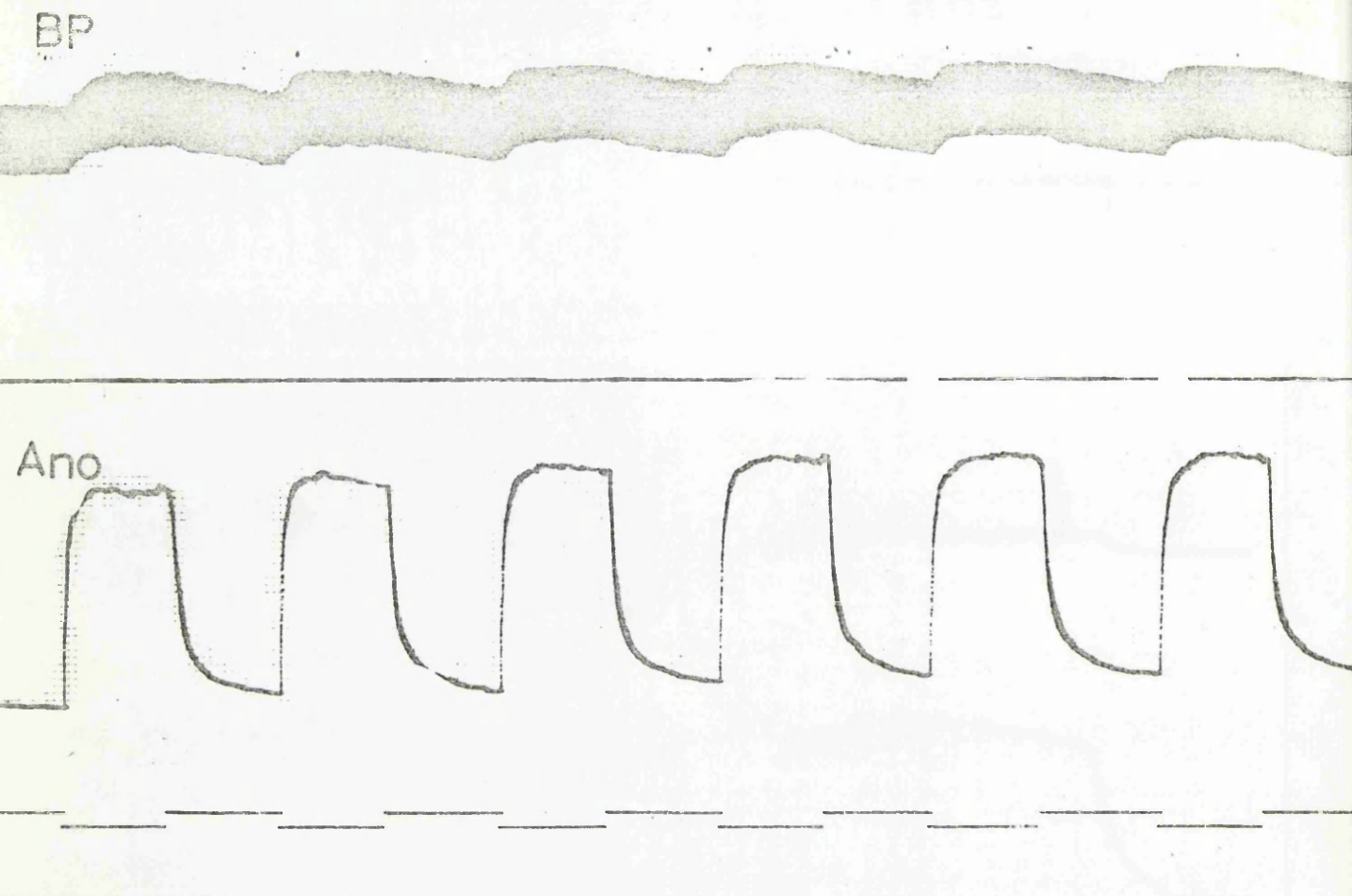
Fig. 18   Responses of the anococcygeus in the pithed
rat to stimulation for alternate minutes at 30 Hz.
10 mm electrode at L 1.   Muscles recorded jointly.
Muscles maintain their tone during stimulation and
show no signs of fatigue after 6 x 1 min periods.
Time trace indicates beginning and end of stimulation
every 60 sec.

At frequencies above 10 Hz, the responses from the upper and lower regions were equal and had reached their maximum amplitude within the 10 sec period of stimulation. At low frequencies, responses were often larger from stimulation in the upper than in the lower region and had not reached their maximum amplitude within the 10 sec stimulation period (Fig. 14). In several other experiments, however, the frequency/response characteristics in the two motor regions were similar.

(2)   Continuous stimulation until the maximum was reached showed that the most effective frequency remained at 30 Hz, as in (1), but frequencies as low as 0.5 Hz could eventually produce a plateau tension of some 70% of the maximum at 30 Hz (Fig. 15).

Single pulses produced responses of approximately 10% of the maximum tension and summation of tension was seen at frequencies at or above 0.1 Hz (Fig. 16). At higher frequencies, up to 50 Hz, repetitive stimulation produced a smooth contraction well maintained for a 3 min period (Fig. 17).

Responses were not only maintained within a single period of stimulation, but prolonged and repetitive stimulation at 30 Hz for alternate minutes did not affect the maintenance of responses (Fig. 18).

Single pulses and continuous stimulation produced similar responses from stimulation in either spinal region in most experiments. As with (1), in one experiment responses were smaller from the lower region. It is possible that simultaneous stimulation of the inhibitory nerves to the anococcygeus itself or mechanical interference from stimulation of the parasympathetic nerves to the colon could have affected the response to stimulation in this lower region in these experiments. Since in the majority of experiments responses were similar from stimulation in either region these possibilities were not further investigated.

(3)   Stimulation with trains of 30 pulses demonstrated that frequencies as low as 1 Hz could produce as large a response as 30 Hz emphasising the importance of the number of pulses as opposed to the duration of the stimulation

period (Fig. 13). No difference was found in these experiments between responses from the two vertebral regions.

In the pithed rat, therefore, stimulation of the nerves to the anococcygeus muscles produces mechanical responses in the muscles over a wide range of frequencies from single pulses to 100 Hz. The maximum tension which may be developed is produced by continuous stimulation at 30 Hz, but much lower frequencies are capable of producing large responses if stimulation is continued long enough for the individual pulses to summate to a plateau level. At all frequencies, the response is well maintained over a period of at least 3 min and there is no evidence of fatigue even after prolonged stimulation at high frequency.

Effects of blocking drugs on the motor response

The effects of an ∝- blocking drug, phentolamine, of an adrenergic neurone blocking agent, guanethidine, of two ganglion blocking agents, hexamethonium and mecamylamine, and of a cholinergic blocking agent, atropine, were examined on the motor response. The intention was to analyse the nature of the motor pathway to see whether it was indeed adrenergic as the in vitro experiments suggested, whether it was interrupted by a ganglion and so anatomically belonged to the autonomic nervous system and finally whether there was any possibility of a cholinergic contribution since acetylcholine also causes contraction of the muscle in vitro. The effect of LSD - 25 was also examined since it inhibits motor nerve responses of the vas deferens in vitro (Ambache, Dunk, Verney & Zar, 1973).

Phentolamine Responses from both motor regions were abolished by phentolamine (2 mg/kg) (Fig. 19). Complete blockade occurred within 2 min of administration and lasted for 15 - 20 min. Responses had recovered to 80% of their previous size within 30 - 45 min.
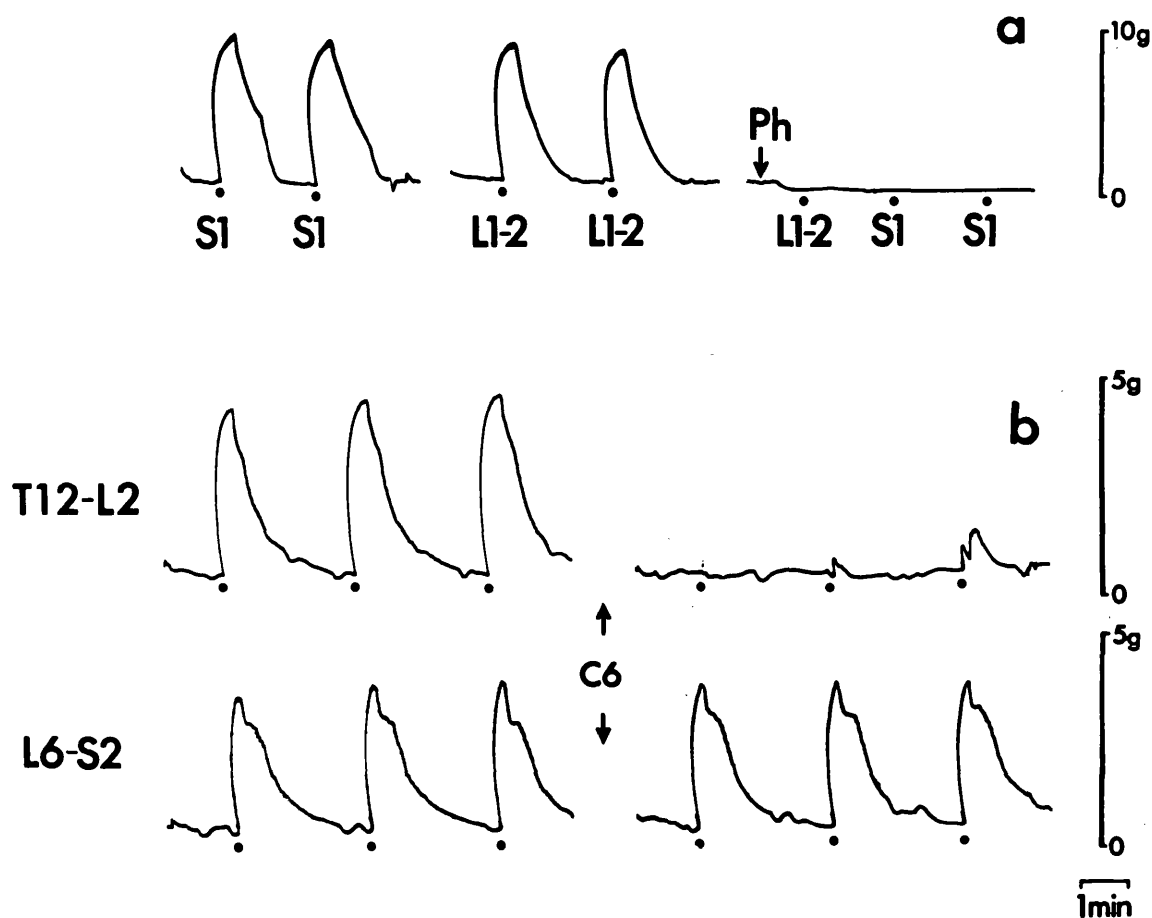
Fig. 19   (a) This shows the effect of phentolamine

(Ph: 2 mg/kg) on the motor response of the rat anococcygeus to

stimulation in the vertebral canal at S 1 and at L 1 - 2.

Phentolamine blocks both responses.   (b) This shows the effect

of hexamethonium (C6: 5 mg/kg) on the motor response of the

anococcygeus in another rat to stimulation in the vertebral

canal in two similar positions, L 6 - S 2 and T 12 - L 2.

Hexamethonium blocks the motor response from the rostral

stimulation without affecting that from the caudal.

Stimulation was for 10 sec at 30 Hz.   Electrode exposure,

(a) 5 mm, (b) 10 mm.
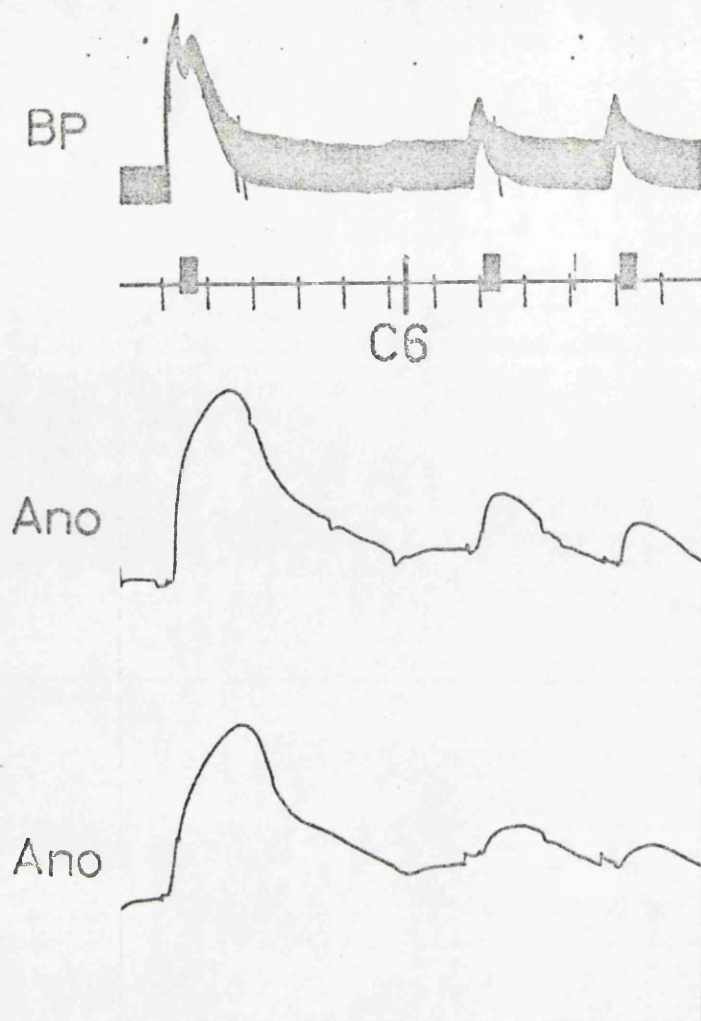
Fig. 20   Effect of hexamethonium (C 6) (1 mg/kg)
on the adrenal response of the anococcygeus muscles and
blood pressure.   Stimulation at T 6 - 8, 30 Hz for
20 sec.   Response in anococcygeus muscles is delayed
and therefore indirect.   Response in blood pressure
includes a direct vasopressor component in addition to
the delayed adrenal response.   After C 6, the
anococcygeus response is reduced and the indirect
component of the B.P. response is abolished leaving only
the direct component.   Time 1 min.

Guanethidine (0.1 mg/kg) blocked responses from both regions within 10 min. This block could be temporarily reversed within the first 20 min by the intravenous injection of NA (500 ng).

Ganglion blockers   A short lived inhibition of the motor response to stimulation from the upper (L 3 - T 11) region was produced by 1 mg/kg hexamethonium, and the response was completely abolished by 5 mg/kg (Fig. 19).   Recovery from even 5 mg/kg hexamethonium was rapid, the response being back to 30% of its previous size after 15 min.   After responses had recovered from blockade by hexamethonium they could again be blocked by a similar dose (5 mg/kg). Another ganglion blocker, mecamylamine (5 mg/kg), also blocked responses from the upper region.

The motor response from the lower (S 2 - L 6) region was unaffected by these doses of hexamethonium or mecamylamine (Fig. 19).

Atropine & LSD   Atropine (1 mg/kg) and LSD (0.5 - 400 μg/kg) had no effect on the motor responses from either region.

The motor responses from stimulation higher in the spinal canal and due to liberation of catecholamines from the adrenals were abolished by phentolamine (1 mg/kg) and reduced by 70% by hexamethonium (5 mg/kg) (Fig. 20).   Adrenal responses were not blocked by guanethidine (5 mg/kg) (Fig. 20).

From the effects of these blocking drugs it appears that stimulation within the vertebral canal at the upper motor region activates preganglionic sympathetic nerves which can be blocked at the ganglion by hexamethonium or mecamylamine and at the neuro - muscular junction by guanethidine or phentolamine, while stimulation in the lower region activates postganglionic sympathetic nerves which can be blocked by guanethidine or phentolamine, but which are unaffected by ganglion blockers.

Stimulation in the upper region thus corresponds with the previous findings that the pithing rod electrode stimulates the preganglionic autonomic outflows
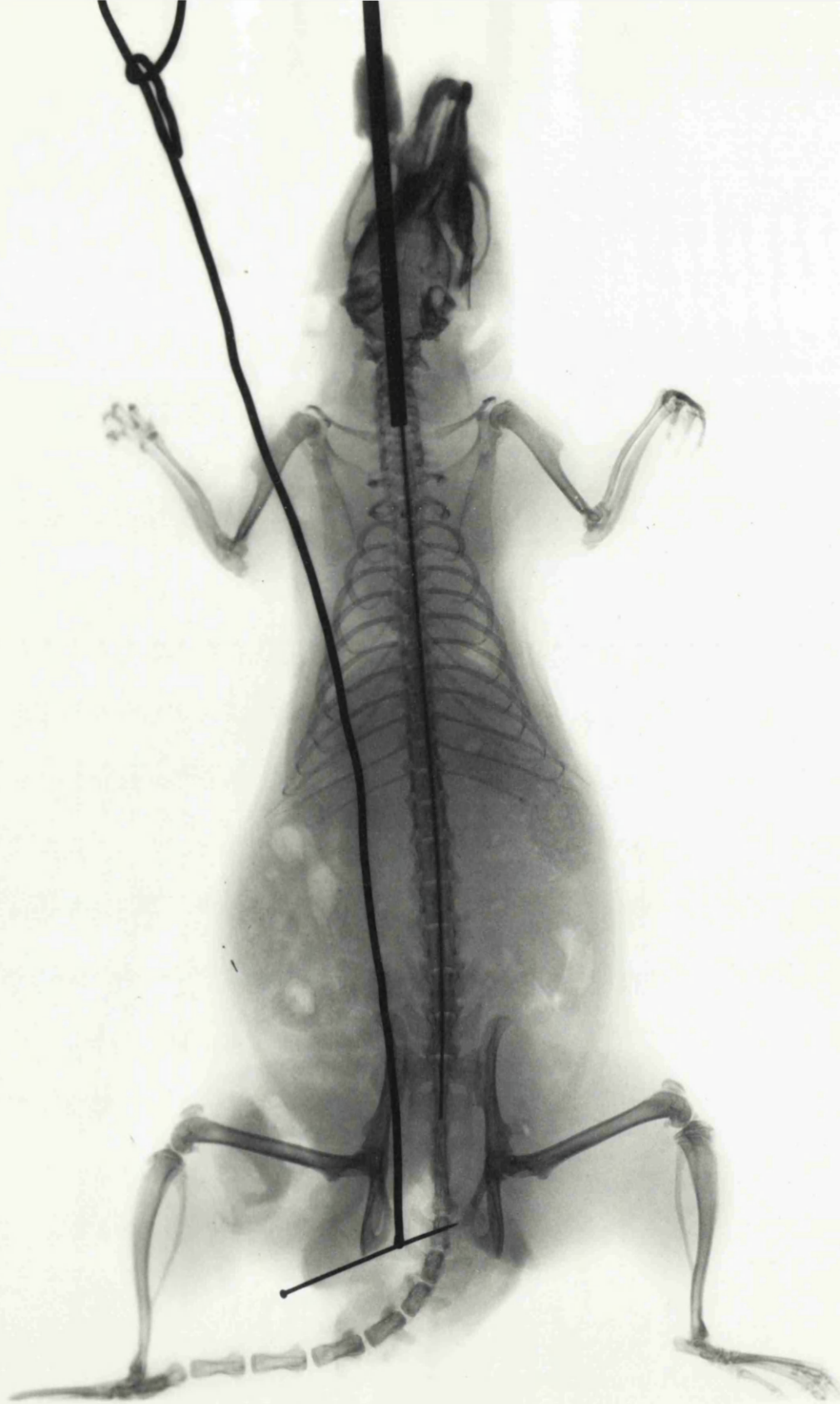
Fig. 21   Position of stimulating electrode with respect to the
anococcygeus muscle.   In this contact print of an X - ray photograph,
the rostral end of the anococcygeus is marked by the intersection of
the pin with the vertebrae.   The electrode is visible within the
vertebral canal with its tip at S 2, the nearest it ever comes to
the anococcygeus.   The dark line on the left is the indifferent electrode.

at the point where they leave the vertebral canal (Gillespie & Muir, 1967; Gillespie, MacLaren & Pollock, 1970), but the ability to stimulate post-ganglionic fibres in the lower region is unique. One explanation for this could have been the position of the anococcygeus muscle directly in front of the vertebral column and having a tendinous origin from the upper coccygeal vertebrae. These conditions might have been particularly favourable for direct stimulation of intramural nerves in a muscle attached to bone overlying the stimulating electrode. This possibility was excluded by marking the position of the upper tendinous origin of the anococcygeus muscle with a metal marker and comparing by X-ray the relative positions of the marker and the spinal electrode (Fig. 21). There was always a vertical displacement of at least 20 mm and up to 40 mm when the electrode was in the lower motor region, so it is unlikely that motor intramural nerves could be stimulated by an electrode so far away from the muscle. The present experiments do not pin-point the feature responsible for the ability to stimulate postganglionic fibres to this muscle. One possibility, however, could be that if the ganglia on the motor pathway to the anococcygeus were para-vertebral rather than pre-vertebral, as in the case of several of the other systems studied, then this would lead to postganglionic fibres being nearer to the vertebral column and therefore to the stimulating electrode.

Another factor which might have interfered with the interpretation of the isometric tension responses in the _in situ_ anococcygeus was movements of the descending colon. When the colon was visibly, spontaneously active, small disturbances on the anococcygeus baseline could be detected and it was feared that stimulation, especially in the sacral region, would involve the sacral parasympathetic outflow and so cause contraction of the longitudinal muscle of the colon, and this might in turn indirectly influence the tension record from the anococcygeus muscles. To test this, the tension in the longitudinal muscle of the colon was recorded isometrically by sewing a thread through the
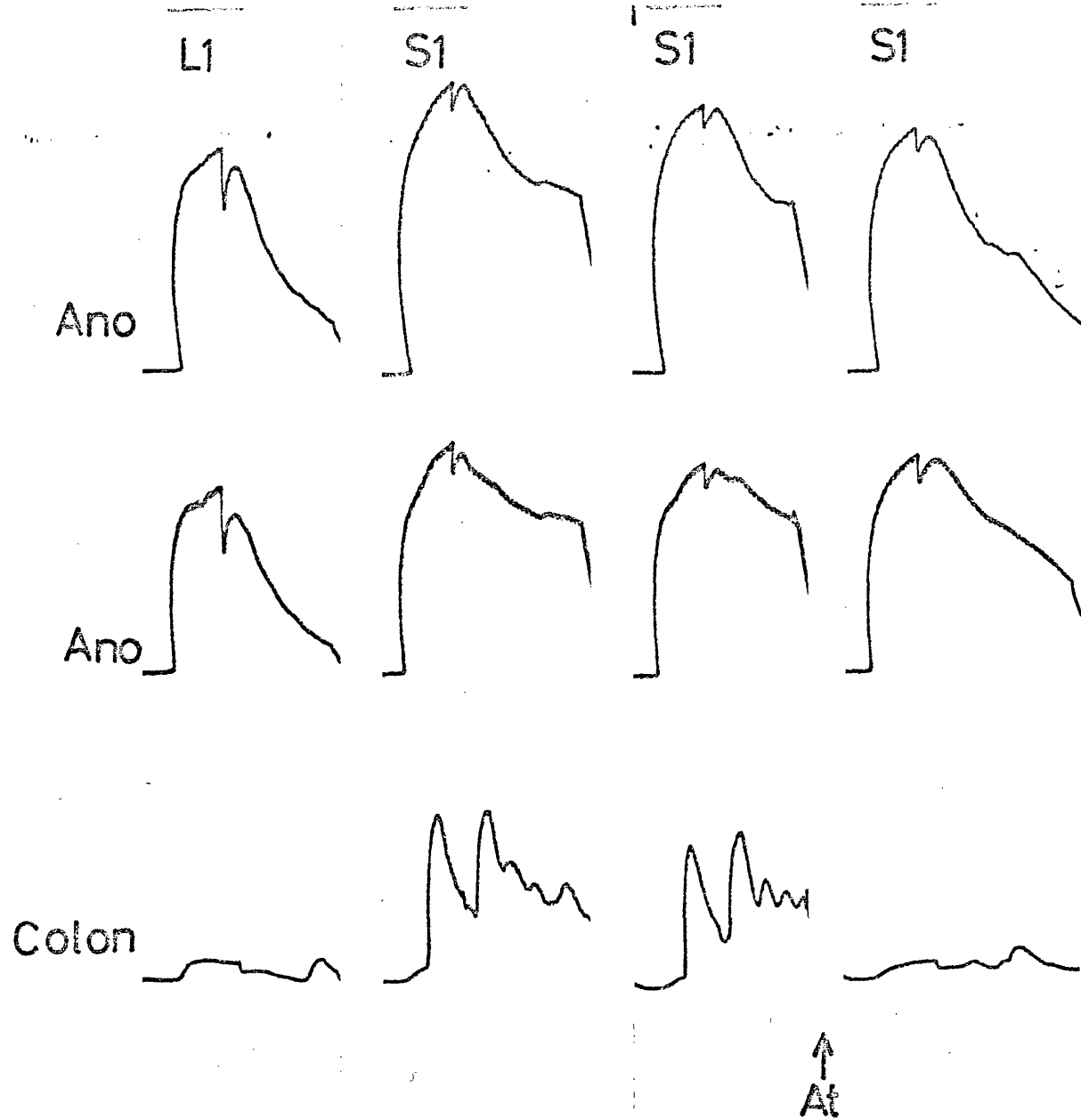
Fig. 22   Effect of atropine 1 mg/kg on the anococcygeus
muscles and on the longitudinal muscle of the colon in a
pithed rat.   Stimulation (30 Hz, 10 sec) at L1 produced
responses in the anococcygeus muscle and a small response
in the colon due to mechanical interference from the
anococcygeus.   Similar stimulation at S1 produced responses
in the anococcygeus muscles, and, in addition, a complex
response in the colon.   After atropine (At), the
anococcygeus responses were unaffected but the response
in the colon was abolished, only the small response from
interference from the anococcygeus remaining. Time 1 sec,
5 sec.

ventral surface of the colon midway between the two anococcygeus muscles and attaching it to an FT03 transducer so that it was parallel with the direction of recording of the anococcygeus muscle (i.e. approximately 30° to the horizontal). Recording in this way, the spontaneous activity in the colon could be monitored and compared with the anococcygeus tension (Fig. 22). Spontaneous contractions of the colon resulted in small relaxations of the anococcygeus which did not interfere with the recording of responses to spinal stimulation. Stimulation at S 1 but not L 1 produced contractions of the colon at the same time as the anococcygeus response. Atropine (2 mg/kg) abolished this colon response without affecting the anococcygeus response. It was thus concluded that stimulation at S 1 stimulated the sacral para-sympathetic nerves which in turn stimulated the longitudinal muscle of the colon, but this did not interfere with the response in the anococcygeus muscles.

Drugs producing motor responses

In vitro the inhibitory response in the anococcygeus can only be seen after raising the tone in the muscle. In order to establish a similar situation in vivo for examination of possible inhibitory responses, the action of several drugs capable of raising the tone in vitro were examined on the pithed rat.

Noradrenaline Single injections of NA produced dose - related motor responses (Fig. 23) which reached a peak within 2 min and had returned to baseline within 5 - 10 min. Threshold for the response was 0.2 ug/kg but maximal responses could not be determined since the dose necessary would have killed the rat due to its effects on the blood pressure and heart.

Comparison with responses obtained in vitro (Fig. 23) shows that the maximum response was not produced until the concentration of NA in the organ bath reached 10^{-4} M. The largest dose which was examined in the pithed rat was 800 µg/kg. Expressed as a molar concentration in the whole rat, this is equivalent to 4×10^{-6} M or, assuming that the NA is restricted to the blood

Fig. 23

NA AND TYRAMINE DOSE/RESPONSE ON RAT ANOCOCCYGEUS
COMPARISON OF IN VITRO WITH IN VIVO

(a) IN VITRO

10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} M

Response gms IN VITRO

▲ TYR
○ NA

(b) IN VIVO

40μg/min Tyr

Mean max to nerve stim.

NA

TYR

Response gms IN VIVO

0.01 0.1 1 10 100 1000 μg/Kg
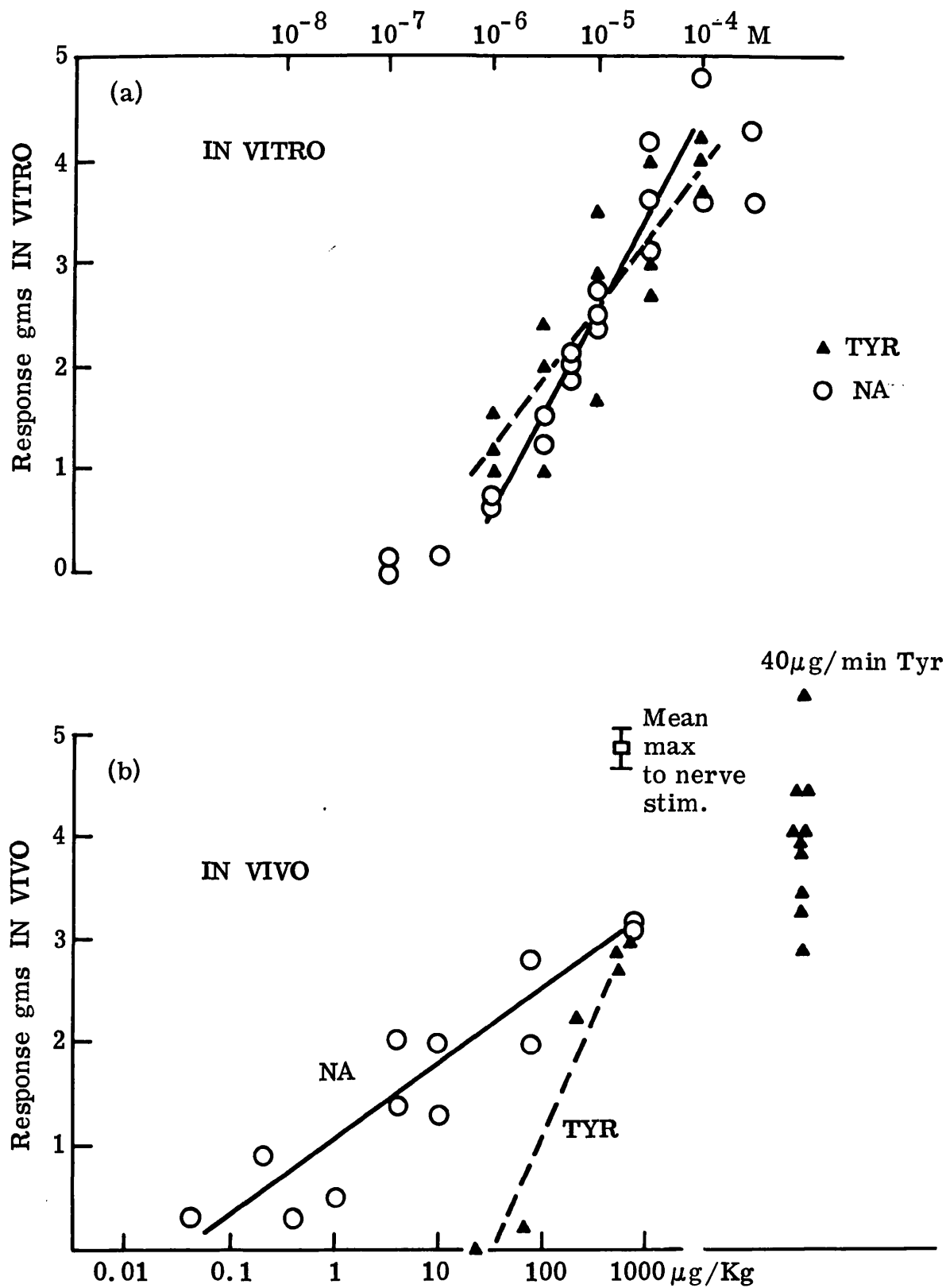
(a) Responses in single rat anococcygeus muscles in vitro to concentrations in the bath of NA (circles) and tyramine (triangles) between 10^{-7} M and 3×10^{-4} M (n = 3). Solid line indicates slope of dose / response curve for NA, dotted line for tyramine.

(b) Responses in single anococcygeus muscles in pithed rats to intravenous injections of NA and tyramine. Symbols as for (a). Dose / response curves were not obtained in each rat. Individual symbols represent single injections to different rats. It is likely that none of these responses are maximal. This can be seen from the mean response to supramaximal nerve stimulation in these rats, 30 Hz, 20 sec at L 1 (n = 5 rats) and from the plateau responses obtained in different rats to intravenous infusion of tyramine at 40 µg/min and shown on the right (n = 10 rats).
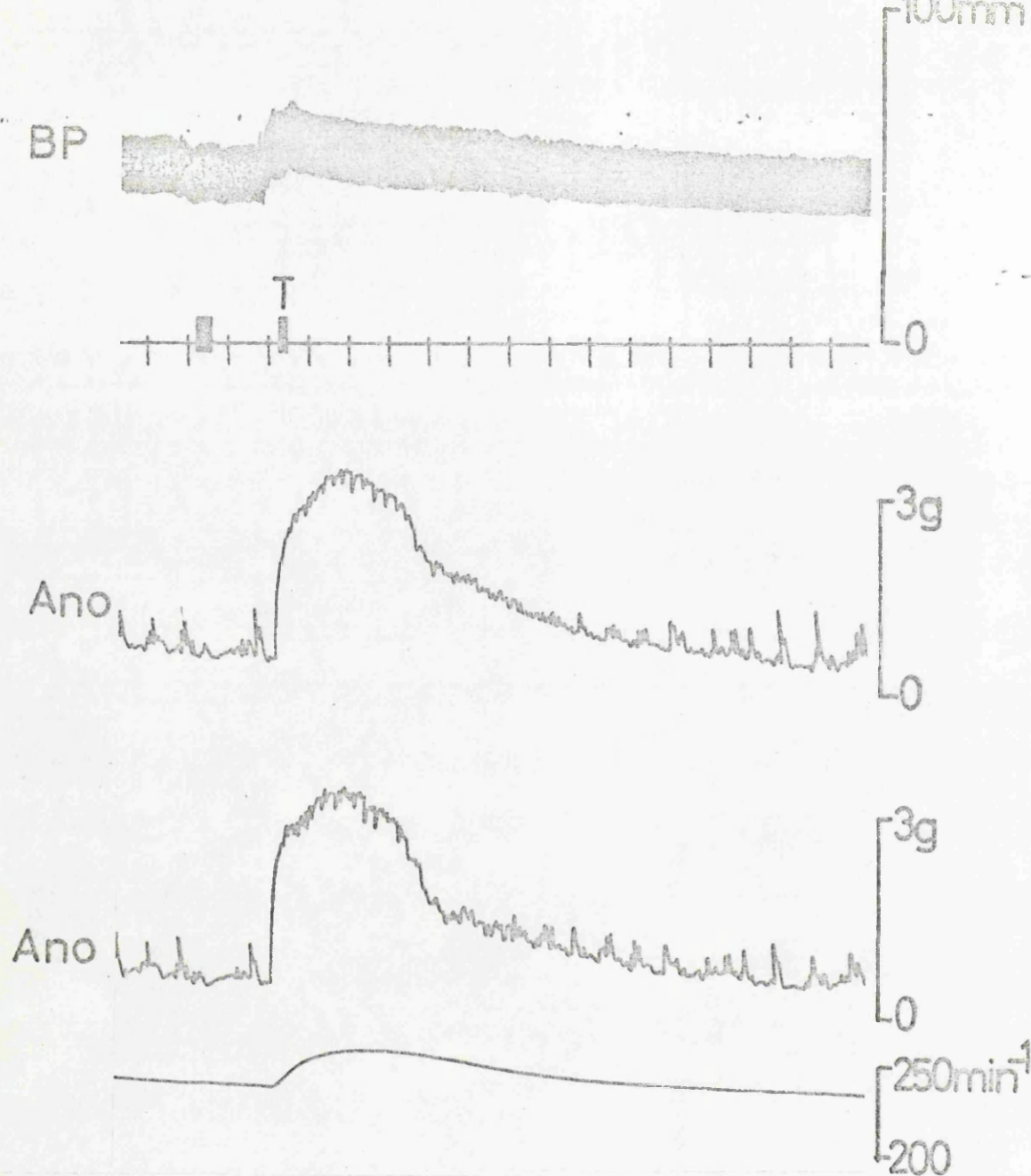
Fig. 24   Responses to tyramine (250 ug/kg)

(T) of the pithed rat blood pressure, anococcygeus

muscles and heart rate.   Time 1 min.
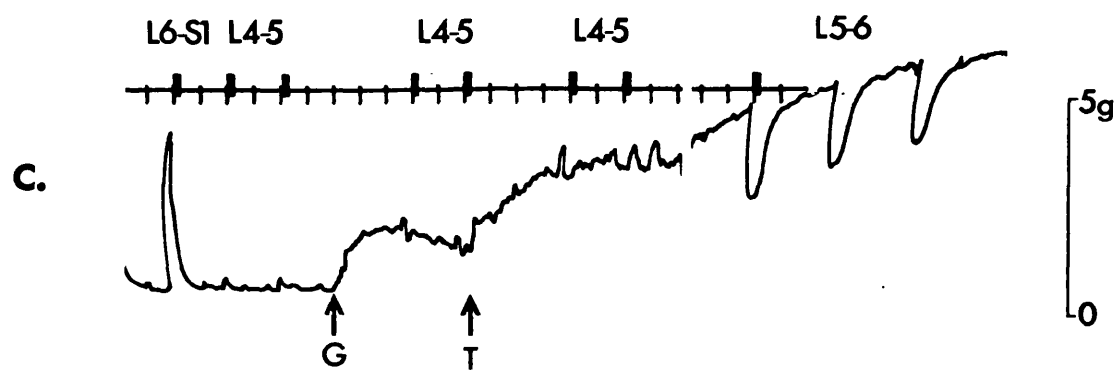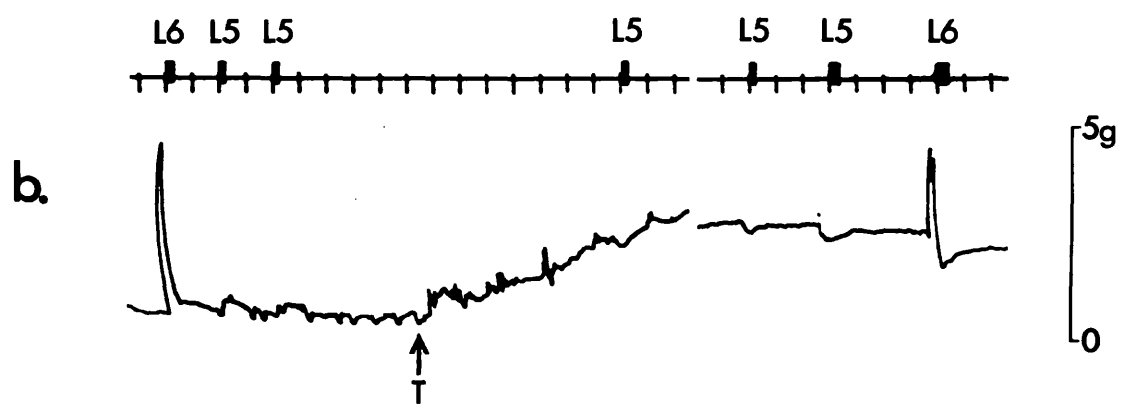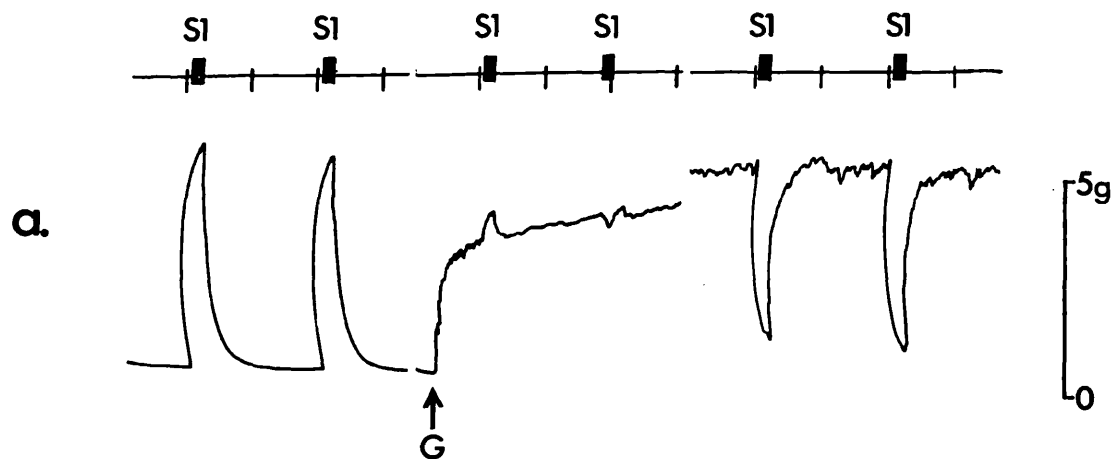
Fig. 25　　　　　　　　Effect of guanethidine and tyramine on the
anococcygeus muscle of the rat, and its response to nerve stimulation
in the vertebral canal at the position shown above the time trace on
each record.　Time / min.　(a) The motor response to nerve
stimulation before guanethidine (G: 10 mg/kg) was converted to
inhibition in the presence of that drug which itself produced a
prolonged rise in muscle tone.　Tension was recorded from both
muscles.　Electrode exposure 5 mm.　(b) In another rat a slow
infusion of tyramine (T: 20 ug/min) caused a maintained rise in tone
but did not reverse the motor response to stimulation at L 6.
Stimulation at L 5, however, which before tyramine was ineffective,
caused inhibition in the presence of that drug.　Tension was
recorded from a single muscle.　Electrode exposure 5 mm.
(c) In a third rat guanethidine (G: 5 mg/kg) caused only a
small rise in tone;　a tyramine infusion (T: 20 ug/min) further
increased this.　This record also shows the sharp boundary of
the inhibitory outflow.　Stimulation at L 4 - 5 produced little
inhibition whereas at L 5 - 6 large inhibitory responses were
obtained.　Record from a single muscle.　Electrode exposure
10 mm.
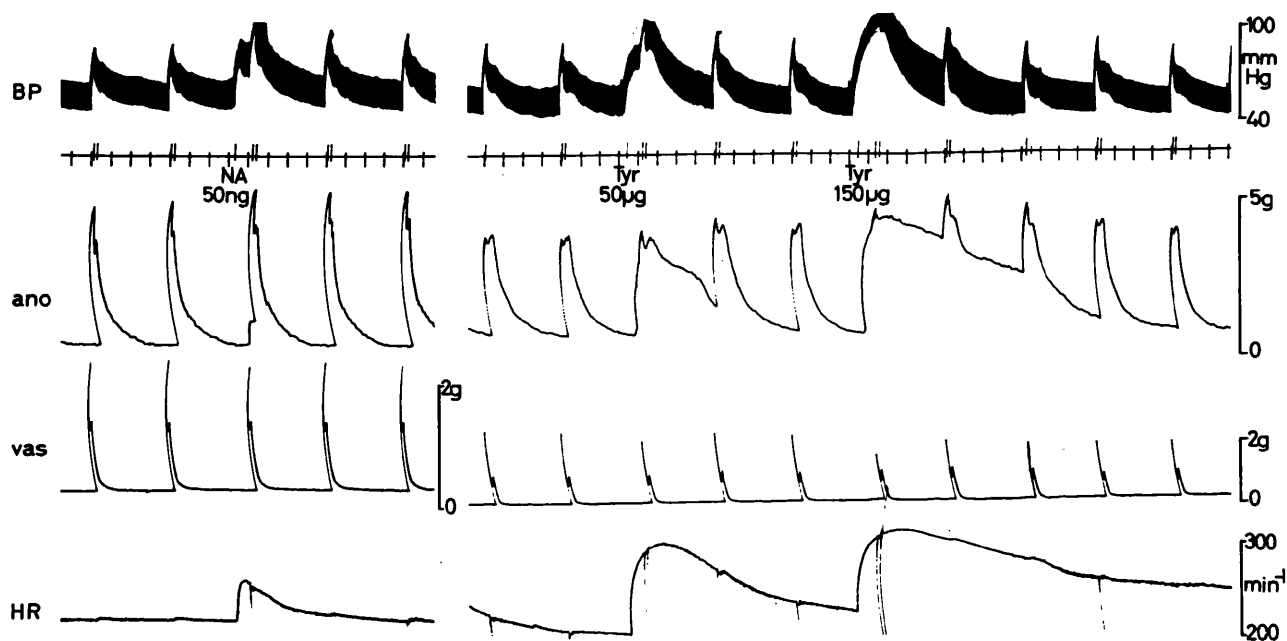
Fig. 26   Effects of NA and tyramine on the anococcygeus, vas deferens, blood pressure and heart rate of the pithed rat. Stimulation at L 1 - 3, 30 Hz, 10 sec every 4 min.   NA produced a motor response on the anococcygeus which summated with the motor response to nerve stimulation.   On the vas deferens, NA produced inhibition of the "spike" phase of the response.   On another rat, tyramine (Tyr) produced motor responses in the anococcygeus which summated with the motor response to nerve stimulation.   On the vas deferens, however, tyramine inhibited the "spike" phase of the response.   Time 1 min.

volume alone, i.e. approximately 6% of body weight, is equivalent to 6×10^{-5} M. Since the actual concentration reaching the anococcygeus is likely to be between these two figures, comparison with the responses in vitro to NA 4×10^{-6} M - 6×10^{-5} M shows that the responses in the two situations are of the same order of magnitude. On the other hand, making similar assumptions, the threshold dose is much lower in the pithed rat than in vitro. Doses of 1 ug/kg (5×10^{-9} M - 8×10^{-8} M) are consistently supra-threshold in the pithed rat, but in vitro the threshold lies between 3×10^{-8} M and 3×10^{-7} M. This high sensitivity to NA in the pithed rat anococcygeus is similar to and may be produced by the same factors which lead to the sensitivity of the pithed rat blood pressure to NA. Since a dose of 50 ng NA can easily be detected in a 250 g rat, the anococcygeus preparation might indeed be developed as a sensitive assay for NA.

Tyramine Single injections of tyramine produced dose - related motor responses similar to those for NA, reaching a peak within 2 min and declining to baseline within 5 - 10 min (Figs. 23 and 24). The threshold for tyramine was , however, higher at approximately 50 µg/kg. The highest single dose given at 600 µg/kg did not produce a maximal response, but if the tyramine was given by slow infusion into the femoral vein at 40 µg/min the response plateaued at a level equivalent to both the maximum responses in vitro and the maximum response to motor nerve stimulation in vivo (Fig. 23). Slower rates of infusion of tyramine (20 µg/min and 10 µg/min) produced sub-maximal motor responses which were also well maintained (Fig. 25).

Both NA and tyramine responses summated with the motor response to nerve stimulation from within the vertebral canal. Neither drug had a significant effect on motor nerve responses at doses which were sub-threshold for contraction, but in doses which did produce motor responses (Fig. 26) the drug + nerve response was larger than the pre-drug nerve response (provided that the nerve response was sub-maximal; maximal responses could not be increased in this way). The increase in tension above that produced by the

agonist drug, produced by nerve stimulation was, however, smaller than in the absence of the agonist, illustrating the difficulty of interpreting the effects of a drug on nerve responses when that drug itself alters the baseline from which contractions are measured. It was clear, however, that neither drug reduced the ability of the motor nerves to produce responses when given by single injection.

After prolonged, i.e. greater than 1 hr, infusion of tyramine, the motor nerve responses were reduced in size.

<u>Guanethidine</u> in doses at or above 0.1 mg/kg blocked the motor responses to nerve stimulation within the vertebral canal. At doses at and above 0.5 mg/kg, in addition to blocking nerve responses, guanethidine also raised the tone of the muscle. At 0.5 mg/kg these responses were small and short lived, but became increased in size and more prolonged with increasing dose until at 10 mg/kg a maximal motor response was produced (Fig. 25) which was well maintained for 25 - 40 min, after which time tension gradually declined and spontaneous activity started. Further increases in dose beyond 10 mg/kg produced responses which were less well maintained.

Once spontaneous activity and tension decline had started, the response could not be restabilised by an additional dose of guanethidine. 30 min after spontaneous activity had ceased, however, a further dose of guanethidine (10 mg/kg) could again produce a stable response. The ability to produce motor responses by nerve stimulation returned approximately 30 min after the end of spontaneous activity, coinciding with the return of the ability of guanethidine to itself produce a motor response. This may reflect the mechanism of development of tachyphylaxis to guanethidine whereby the capacity of guanethidine to release NA is blocked by the same mechansim which blocks the release of NA by nerve impulses.

Phentolamine (2 mg/kg) abolished motor responses to NA, tyramine and guanethidine. Together with the evidence from <u>in vitro</u> experiments it is reasonable to assume,
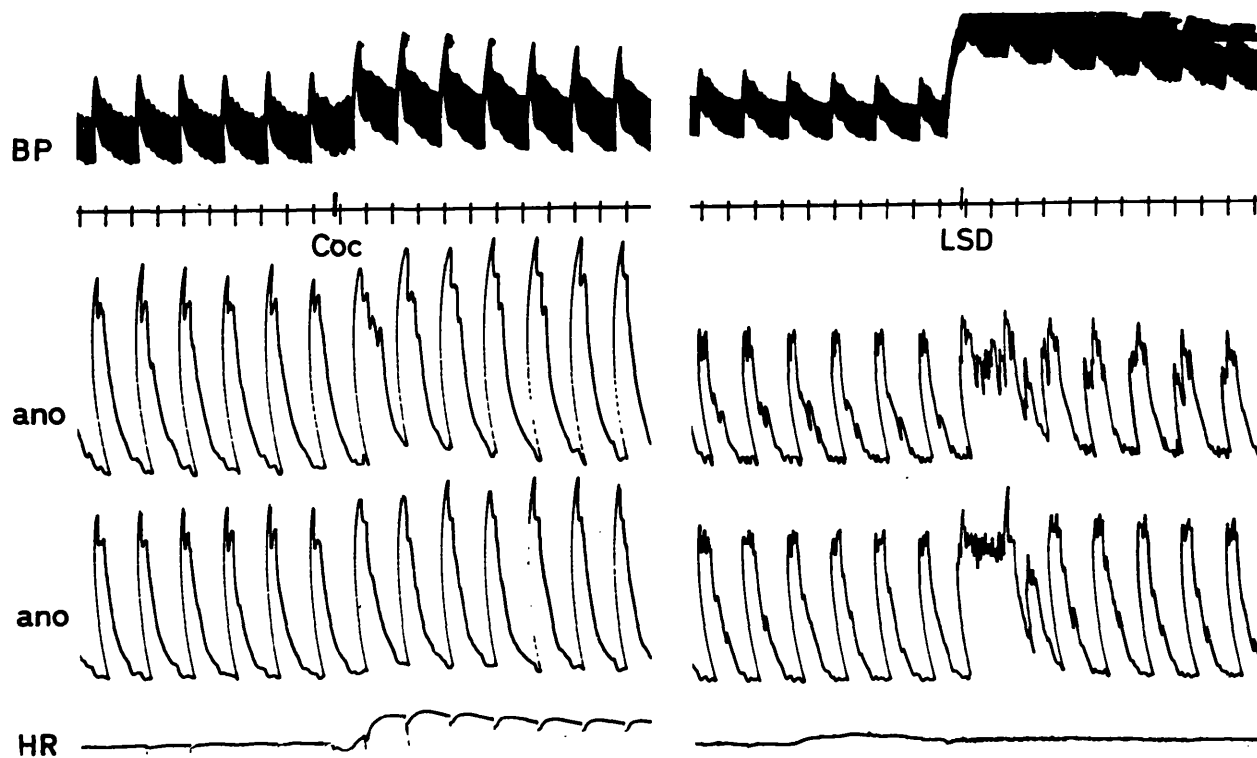
Fig. 27   Effect of cocaine and LSD on the responses to

spinal stimulation in the pithed rat anococcygeus muscles.

Stimulation at L 1 - 2, 10 mm electrode, 10 Hz for 10 sec

every 90 sec.   Cocaine 4 mg/kg produced no direct response

in the anococcygeus but potentiated and prolonged the response

to nerve stimulation.   In another rat, LSD 200 ug/kg

produced a motor response in the anococcygeus muscles but

made no significant difference to the responses to nerve

stimulation.   Time 1 min.

therefore, that tyramine and guanethidine are acting as indirect sympathomimetics.

Other indirect sympathomimetics   Cocaine (4 mg/kg), which produces motor responses in vitro, produced a small motor response in vivo.   At this dose, it also potentiated the height of sub-maximal motor nerve responses (10 Hz, 5 sec at L 1) by 5% and prolonged their duration (Fig. 27).

Large doses of LSD (100 - 800 ug/kg) produced motor responses which lasted 2 - 5 min but did not affect the responses to motor nerve stimulation (Fig. 27). Both LSD and cocaine are believed to produce these contractions by an indirect sympathomimetic action as will be shown later in vitro.

Other agonists   Other drugs which raise the tone of the muscles in vitro include acetylcholine and other muscarinic agonists and 5-hydroxytryptamine (5HT).   Neither of these proved to be a useful means of raising the tone in vivo due to their other widespread systemic effects.

Acetylcholine and carbachol did not consistently produce any response in doses (1 - 10 mg/kg) which stopped the heart and therefore killed the rat. Furmethide produced irregular responses in doses of 2 - 10 mg/kg which eventually killed the rat.   These responses were prevented by atropine (2 mg/kg).

5HT (0.2 - 2 mg/kg) produced small responses but also produced dramatic effects on the blood pressure and heart.   High blood pressure and arrythmia quickly terminated these experiments.

It was thus apparent that the most suitable drugs for producing maintained motor responses in the in vivo anococcygeus were indirect sympathomimetics which produced a stable and viable preparation.

The Inhibitory Response

Spinal origin of the inhibitory nerves

When the tone of the muscles was raised by a single injection of guanethidine (10 mg/kg), 10 sec periods of stimulation at 30 Hz in the region L 5 - S 2 produced inhibitory responses.   In individual experiments this inhibition could amount to 90% of the induced tone (Fig. 25a).   The average maximal inhibition in all experiments was 62 ± 5% of induced tone (n = 12).

When the tone was raised with tyramine (20 μg/min), which did not abolish motor responses, stimulation in the upper end of this region (L 5) produced small inhibitory responses, whereas stimulation in the lower part of the same region (L 6 - S 2) produced a biphasic response;   contraction followed by relaxation (Fig. 25b).

These experiments showed that inhibitory nerves did arise in the spinal cord and could be stimulated in one region only, in the lower lumbar and upper sacral region, overlapping with, but extending one segment further rostrally than, the lower motor region.

To examine the origin and properties of the inhibitory nerves systematically, however, required that the tone be raised to a steady plateau and that the action of the motor nerves be blocked.   Guanethidine was clearly the better drug for this purpose, because of its neuronal blocking action on the adrenergic motor nerves in addition to its indirect sympathomimetic action in raising tone. Guanethidine had, however, the disadvantage that after 25 - 40 min the tone began to fall and rythmic activity appeared.   In these circumstances it was difficult to demonstrate inhibition.   The possibility was, therefore, investigated of reinforcing the motor action of guanethidine with a slow infusion of tyramine, thus combining the neuronal blocking action of the former with the steady induced tone of the latter.

b
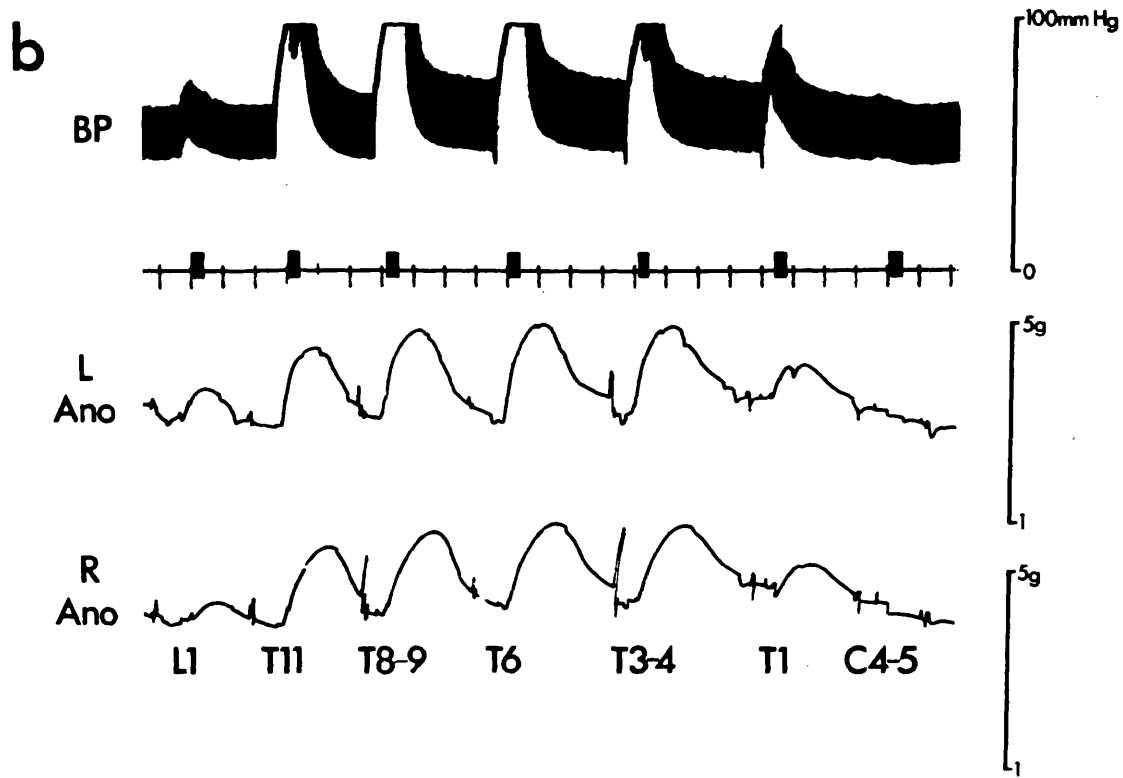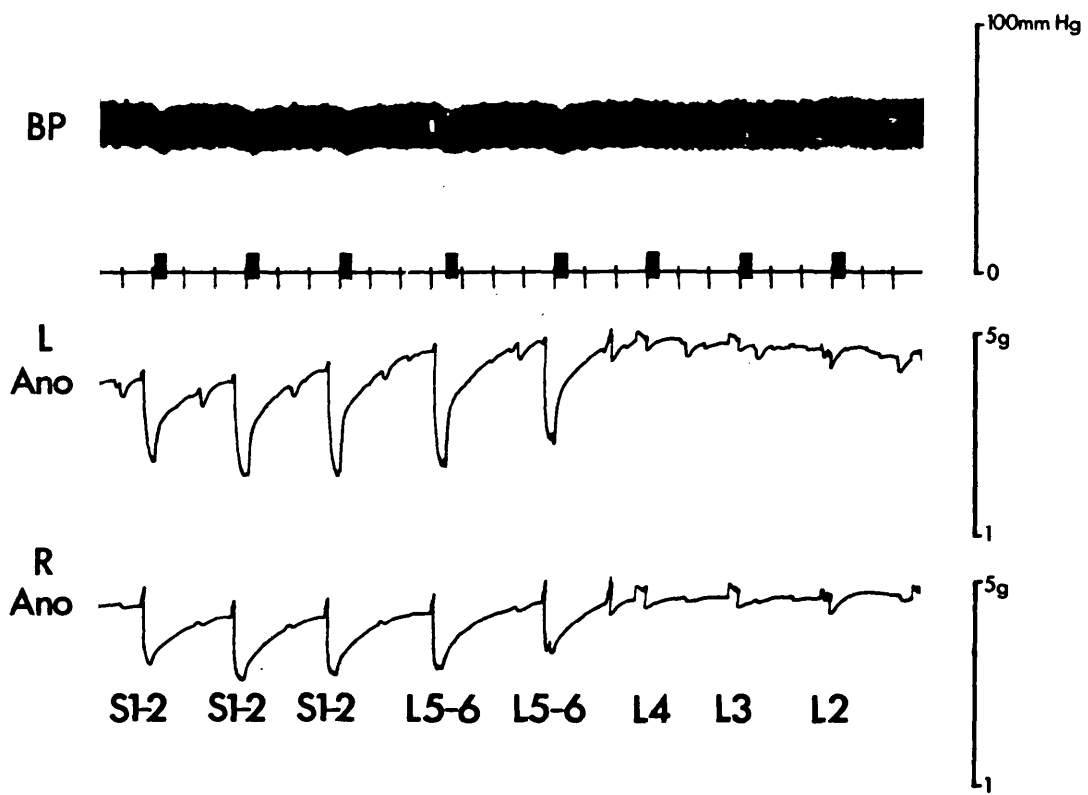
Fig. 28   Responses of the anococcygeus and the

blood pressure in the pithed rat to stimulation in the vertebral

canal at 30 Hz at the levels shown below each record.   An

initial injection of guanethidine (G: 5 mg/kg) was given to

reverse the response to stimulation to inhibition.   The two

sets of records are successive and represent exploration

of the response to stimulation between S 1 - 2 and C 4 - 5.

(a) shows that the inhibitory response is confined to

stimulation in the region S 2 - L 5.   (b) shows that more

rostral stimulation in the thoracic region can restore

the motor response as a consequence of liberating

catecholamines from the adrenal gland.   At these levels

there is also a large vasopressor response.   Time / min.

Guanethidine (5 mg/kg) was given and a slow infusion of tyramine (20 µg/min) started 5 min later. Tone in the anococcygeus was raised and could be maintained for 2 hr, producing optimum conditions for studying the inhibitory response (Fig. 25c). Under these conditions, the spinal origin of the inhibitory fibres was determined by stimulating at successive 5 mm intervals along the spinal canal, as shown in Fig. 28.

Inhibitory responses were obtained only between S 2 and L 5. No inhibitory responses were found at the upper region giving motor responses or at any level up to C 4. Stimulation in the region corresponding to the efferent fibres to the adrenals still produced a delayed contraction from catecholamine liberation. This could not, however, have disguised an inhibitory nerve response from this region since the latter would have started immediately on stimulation.

It could thus be demonstrated in either the presence or absence of adrenergic blockade that the region giving inhibitory responses (S 2 - L 5) overlapped with the lower motor region (S 2 - L 6), but the former extended one segment more rostrally.

As with the motor responses, the lower limit of the inhibitory outflow could not be properly defined, but again experiments with a finer teflon shield and the electrode extruded to the end of the vertebral canal suggested that the outflows of inhibitory as well as motor nerves do not extend much beyond S 2.

Frequency characteristics of the inhibitory response

The inhibitory response in vivo showed the same high sensitivity to low frequency as was shown in vitro.

A more limited investigation of the frequency characteristics of the inhibitory response was carried out than in the case of the motor response. Stimulation
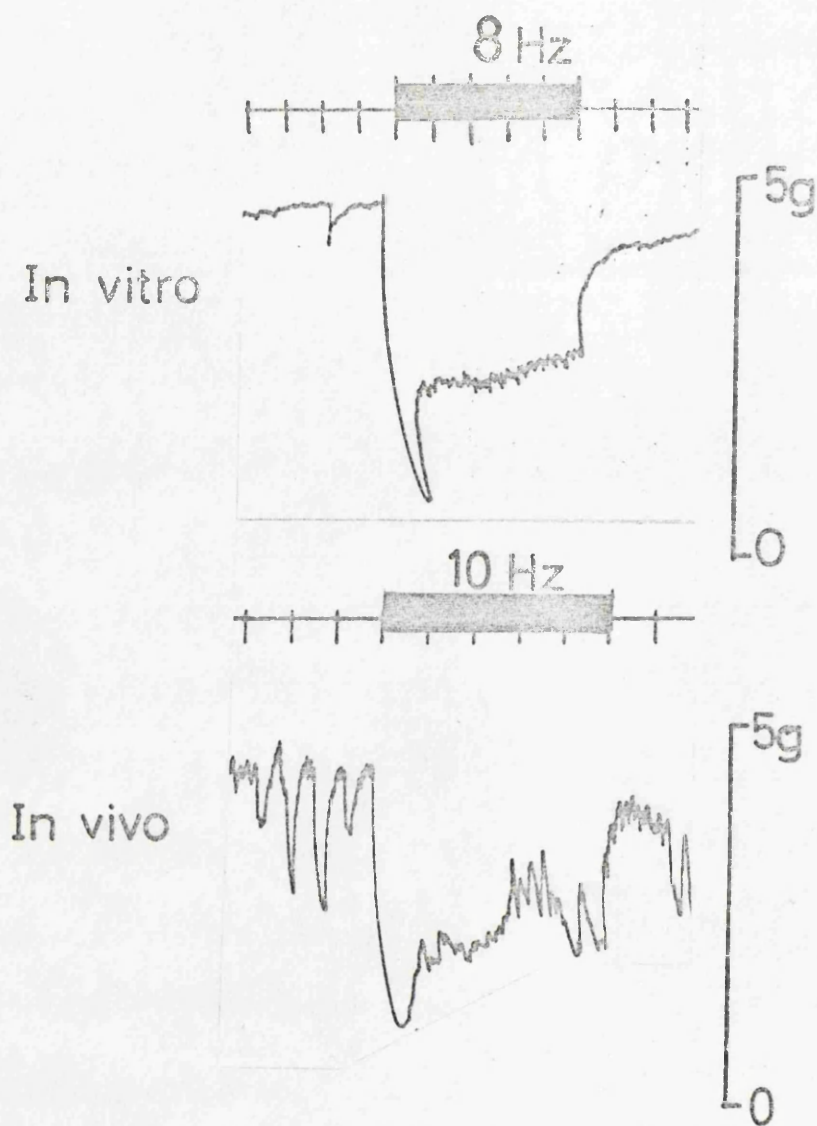
Fig. 29   Comparison of the inhibitory response in the
rat anococcygeus to continuous stimulation in vitro and
in vivo.   Upper trace shows response of a single anococcygeus
muscle in vitro to field stimulation at 8 Hz, 1 msec pulses for
5 min in the presence of guanethidine 3×10^{-5} M.   Lower trace
shows response of a single anococcygeus muscle in a pithed rat
to stimulation at S 1, 10 Hz, 1 msec pulses for 5 min after
administration of guanethidine 10 mg/kg.   Note presence of
spontaneous activity in the lower trace.   The in vivo and
in vitro responses both show an initial response which
declines after 15 - 30 sec to a plateau which is then
maintained until stimulation stops.   Time 1 min.
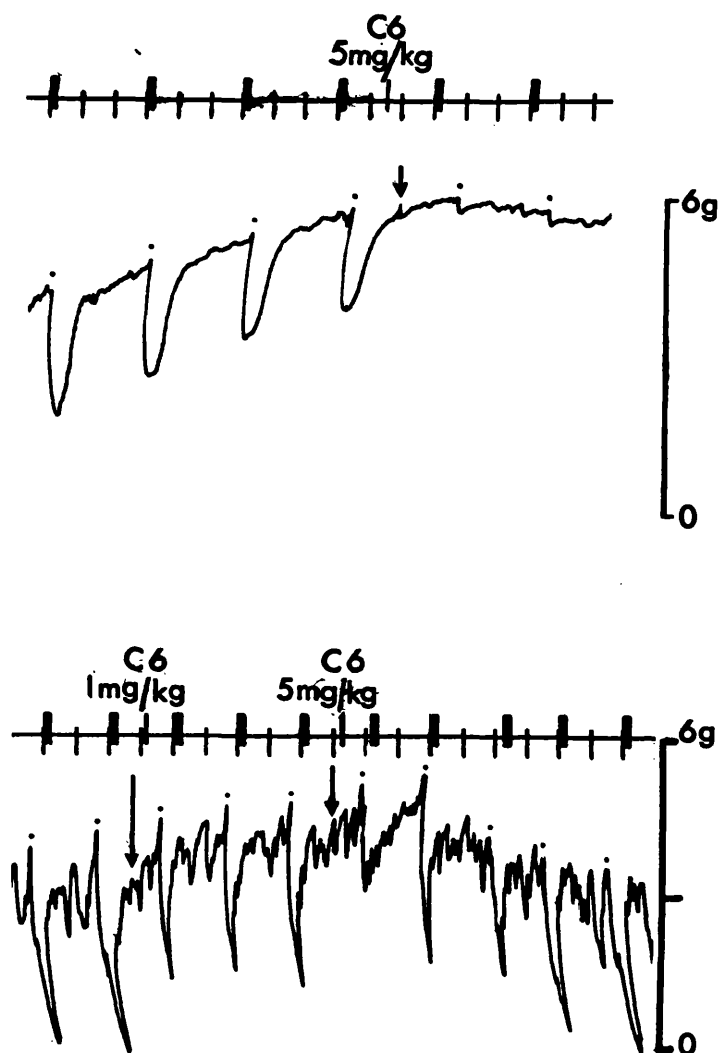
Fig. 30   Effects of hexamethonium (C 6) on the inhibitory

responses of the anococcygeus of the pithed rat to stimulation

at 30 Hz for.10 sec in the vertebral canal at L 6 - S 1.   The

start of each stimulation period is indicated by a dot and the

injection of hexamethonium by an arrow.   In the upper record

a single dose of hexamethonium (5 mg/kg) almost completely

abolishes the inhibitory response.   In the lower trace a smaller

dose of hexamethonium (1 mg/kg) caused a small reduction in

the size of the inhibitory response and a subsequent larger

dose (5 mg/kg) produces only a transient block of the response.

Time 1 min.

for a fixed time with varying frequency showed that maximal responses could be obtained with 10 sec periods of stimulation at 2 - 5 Hz (Fig. 14).

Responses could be produced to single pulses, but these were very small and transient and summation did not occur till 0.5 Hz. With continuous stimulation, in the region 1 - 10 Hz, large responses were obtained which were rapid in onset and well maintained over a 20 sec period. If stimulation was continued for several minutes, the response declined slightly after 20 - 30 sec to a plateau which could then be maintained for up to 8 min. This was similar to the shape of the response in vitro (Fig. 29).

The frequency characteristics of the inhibitory innervation thus show a slightly different pattern from that of the motor nerves. The motor nerves can produce significant responses to single pulses, can maintain responses with low frequencies and the maximum response which can be produced by continuous stimulation increases with increasing frequency up to 30 Hz. The inhibitory nerves on the other hand cannot produce readily detectable responses to single pulses or very low frequencies but are highly effective in the region 2 - 10 Hz in which maximal responses can be produced.

Effects of blocking drugs on the inhibitory response

Clearly nerve fibres mediating inhibition in the anococcygeus were located in the spinal cord. The question of whether this pathway to the muscle was interrupted by a ganglion relay was investigated by examining the effects of hexamethonium and mecamylamine.

Hexamethonium or mecamylamine (5 mg/kg) completely abolished the inhibitory response. This is shown for hexamethonium in Fig. 30. When recovery from this inhibition occurred, after 30 - 60 min, a second similar dose had little effect in renewing the block. If initially, a small (1 mg/kg) dose of hexamethonium was given, a transient reduction in the response was observed. On recovery from this, the tissue was insensitive to subsequent large (5 mg/kg +)

doses of the drug. Similar desensitisation was found with mecamylamine.

Ganglion blockers did not, therefore, present a clear cut effect on the inhibitory pathway. Rapid desensitisation occurred which is in contrast to their effects on the motor pathway. A similar desensitisation was, however, found in the motor pathway to the vas deferens where a ganglion synapse has been convincingly demonstrated (for review see Sjostrand, 1965) and in the pathway producing depressor responses on blood pressure (see following two sections). Taken together with this evidence, the present results are consistent with the presence of a ganglion synapse in the inhibitory outflow to the anococcygeus muscles. Since ganglion blockers have no effect on inhibitory responses <u>in vitro</u>, the location of this ganglion remains undefined.

<u>Atropine</u> (2 mg/kg) did not block inhibitory responses, but caused a transient and unexplained reduction in guanethidine tone. This argues against a role for muscarinic receptors at either the ganglion or at the nerve – muscle junction.

Cardiovascular Responses

During these experiments on the pithed rat, aortic blood pressure was recorded at all times and heart rate was usually monitored.

Responses on blood pressure and heart rate from stimulation within the vertebral canal and to administration of most of the drugs used have been previously studied (Gillespie & Muir, 1967 a, b, c; Gillespie, MacLaren & Pollock, 1970). It was thus possible to correlate cardiovascular and anococcygeus responses without systematically studying the cardiovascular aspects of the preparation.

<u>Drugs producing pressor responses</u>

<u>Noradrenaline, tyramine and guanethidine</u> in doses which produced motor responses in the anococcygeus also produced dose – related vasopressor responses

and increases in heart rate.   A single dose of guanethidine (10 mg/kg) or a slow infusion of tyramine (10 - 40 ug/min) which maintained a steady tone in the anococcygeus also maintained an elevated blood pressure and heart rate.

5 - hydroxytryptamine   (0.2 mg/kg) produced a large pressor response and increased heart rate.   Higher doses led to arrythmia.

LSD   (0.4 - 100 ug/kg) produced dose - related vasopressor responses without affecting heart rate.

Cocaine   (4 mg/kg) increased blood pressure and heart rate.

Spinal origin of cardiovascular responses

The blood pressure responses obtained to stimulation within the vertebral canal in the absence of pressor drugs are shown in Fig. 10.   Small pressor responses were obtained from S 1 to L 3 and larger pressor responses from L 2 to T 12.   These were direct, adrenergic nerve responses which started immediately on stimulation and were not accompanied by any increase in heart rate.   From T 11 to T 2 a direct vasopressor response was followed by a delayed pressor response which was accompanied by an increase in heart rate and was due to release of catecholamines from the adrenals (see also Fig. 22).

When the blood pressure was elevated by tyramine, guanethidine or a combination of both, depressor responses were found from stimulation in the region S 2 - L 5 (Fig. 28).   No changes in heart rates accompanied these responses.   It was not necessary to block the adrenergic pressor nerves to obtain these depressor responses since the vasopressor response at S 2 - L 5 was small. When the adrenergic nerves were blocked by guanethidine, no depressor responses were found outside of the S 2 - L 5 region.   There thus appears to be a depressor outflow originating from S 2 - L 5 which might be considered analogous to the inhibitory pathway to the anococcygeus.   The depressor responses were small when viewed against the total systemic blood pressure, but became quite distinct and reproducible if the polygraph sensitivity was increased and
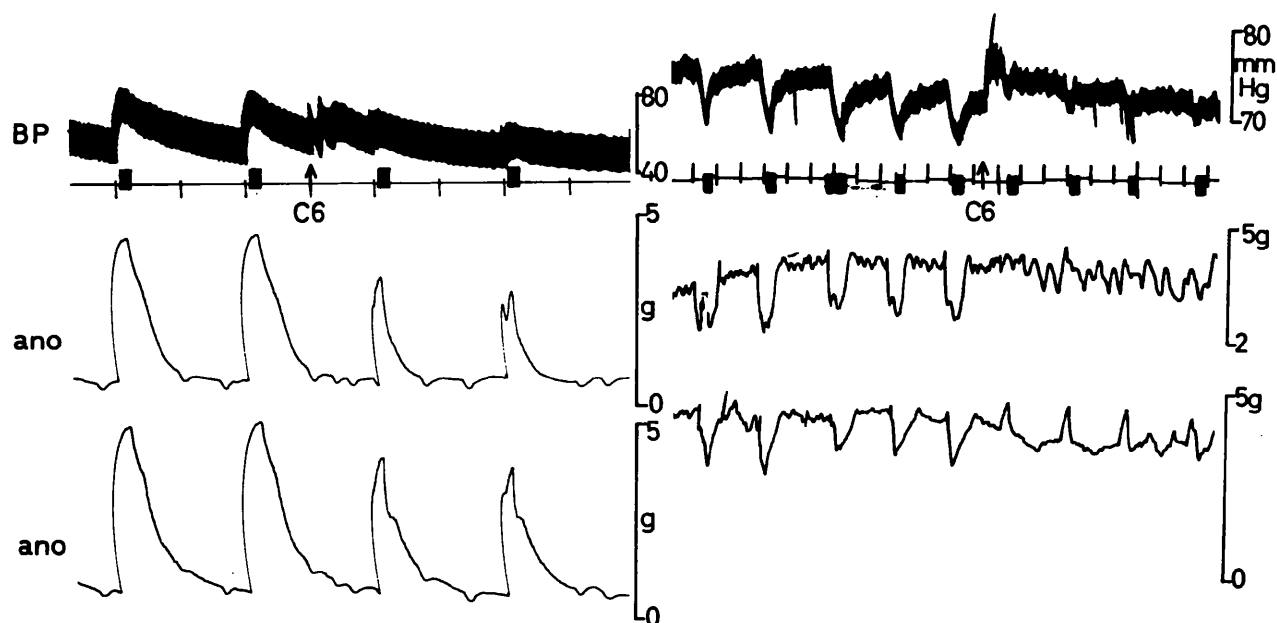
Fig. 31   Comparison of the effects of hexamethonium (C6)

on the responses to spinal stimulation of the blood pressure and

anococcygeus of the pithed rat.   In the left hand panel,

pressor responses and motor responses in the anococcygeus

to stimulation at L 1   (30 Hz, 10 sec) were inhibited by

C6 1 mg/kg.   In the right hand panel, depressor responses and

inhibitory responses in the anococcygeus, obtained from

stimulation at S 1   (10 Hz, 20 sec) after administration

of guanethidine 5 mg/kg and continuous infusion of tyramine

20 ug/min, were almost completely abolished by C6 5mg/kg.

Note that in the lower anococcygeus muscle, the response

to stimulation was reversed from inhibitory to motor by

C6.   Time 1 min.

damped to give a mean blood pressure reading (Fig. 31).

Effects of drugs on nerve or drug mediated cardiovascular responses

(1)   The pressor response

Phentolamine   (2 mg/kg) which blocked the effects of NA and sympathetic
nerves on the anococcygeus, also blocked the effects of NA and pressor nerves
on the blood pressure as well as the adrenal response on blood pressure.

Guanethidine   (0.1 - 10 mg/kg) which blocked the effects of adrenergic nerves
but not NA on the anococcygeus, also blocked the effects of the pressor nerves
without preventing the action of NA on blood pressure or blocking the adrenal
response.

Hexamethonium and mecamylamine   in doses which blocked the responses in the
anococcygeus to preganglionic stimulation blocked both the pressor nerves and
adrenal responses (Figs. 31 and 20).

Noradrenaline, tyramine and cocaine   in doses which potentiated the
anococcygeus sympathetic nerve response also potentiated the pressor nerve
responses (Figs. 26 and 27).

(2)   The depressor response

The depressor nerve response from S 2 - L 5 was unaffected by guanethidine
(10 mg/kg), propranolol (1 mg/kg) or atropine (4 mg/kg).   Its sensitivity to
ganglion blockers was, however, similar to that of the anococcygeus inhibitory
nerves.

Hexamethonium and mecamylamine   (5 mg/kg) which blocked the anococcygeus
inhibitory nerves also blocked this depressor response (Fig. 31).   After
recovery, a second dose did not block, and in the experiments where a small
dose was given first, parallel desensitisation occurred in the blood pressure
and anococcygeus responses.

It therefore appears that the preganglionic fibres in a pathway capable of
producing depressor responses originate from the same region within the

vertebral canal as the preganglionic inhibitory nerves to the anococcygeus.
These depressor fibres are organised in the pattern of the autonomic nervous
system with a ganglion relay and so are unlikely to be posterior root
vasodilators.

In summary, the effects of several drugs are similar on the adrenergic
vasopressor nerves and the adrenergic nerves to the anocccccygeus; the
effect of drugs on a depressor pathway originating in the vertebral canal is
similar to that on the anococcygeus inhibitory pathway.

Comparison of the motor responses in the rat anococcygeus
and vas deferens

The vas deferens has been studied in the past as a convenient example of a
smooth muscle preparation receiving a dense adrenergic motor innervation and
whose longitudinal tension responses could be recorded in vitro to stimulation
of these nerves or to drugs. The motor responses of the vas to nerve
stimulation are, however, complex e.g. they are inhibited by noradrenaline or
tyramine, and on the basis of this and similar evidence, it has been suggested
that the motor transmitter is not noradrenaline (Ambache & Zar, 1971).
Since the anococcygeus has a dense adrenergic innervation and produces motor
responses to nerve stimulation, it was of interest to compare simultaneously
the responses of these two superficially similar preparations in the pithed
rat.

The optimal spinal position for stimulation of the sympathetic nerves to the
vas deferens in the pithed rat has been shown by Gillespie, MacLaren &
Pollock (1970) to be L 2 - 3. In the present work, some characteristics of the
response to spinal stimulation and the effects of drugs on this response have
been examined simultaneously on the vas deferens and on the anococcygeus
muscles.

Fig.32 COMPARISON OF THE SPINAL ORIGINS OF THE MOTOR NERVE PATHWAYS
TO THE ANOCOCCYGEUS AND VAS DEFERENS.
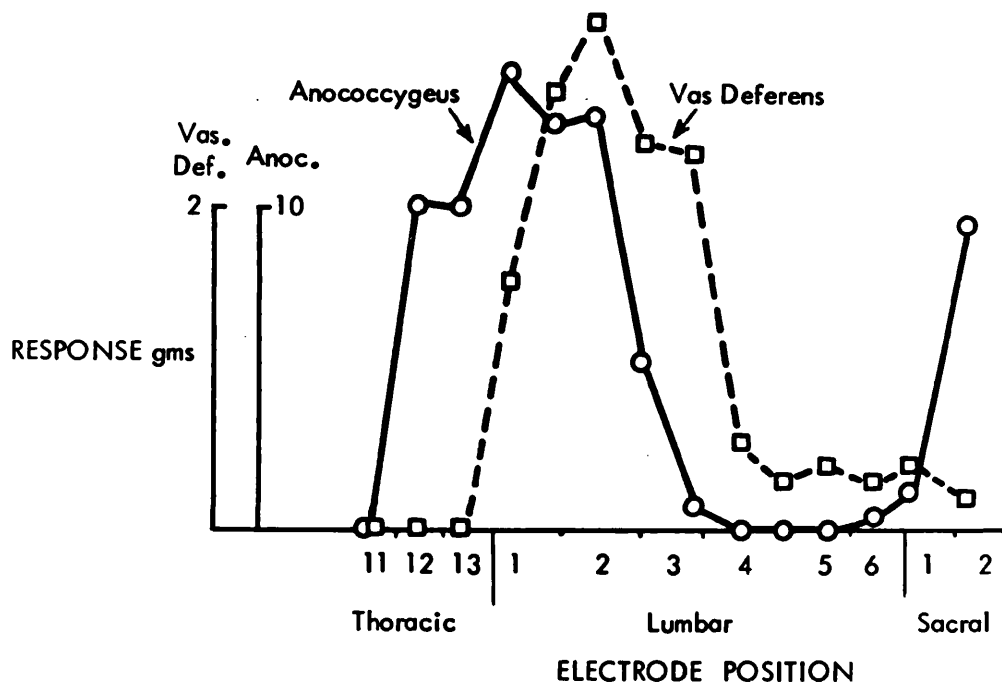
Longitudinal tension responses obtained simultaneously

in the anococcygeus muscles (recorded jointly) and one

vas deferens of a pithed rat. Stimulation at 5 mm

intervals along the vertebral canal. 10 sec trains,

30 Hz, 1 msec pulses. The optimal position for the

anococcygeus was more rostral than for the vas. This

arrangement permits either selective stimulation of

each of the tissues or of both simultaneously.

BP

L1-T12   T8-10

Vas
ressure

Vas
Tension

HR
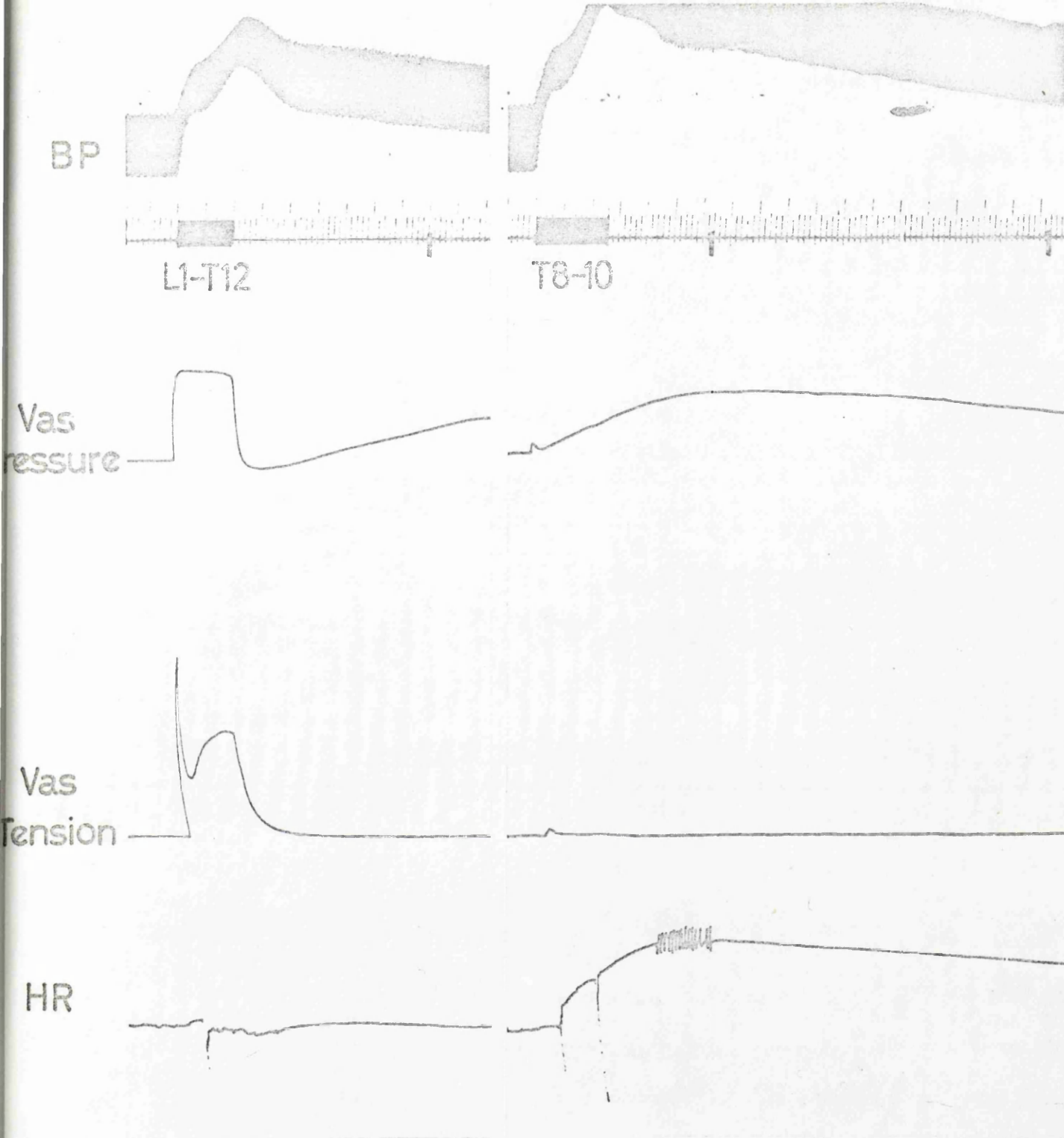
Comparison of the time course of the motor response in the smooth muscle
of the vas deferens measured as the perfusion pressure in the right vas
and longitudinal tension in the left vas.   Stimulation at L 2
produced a direct response in both vasa, a monophasic response in the
pressure recording and a biphasic response in the longitudinal tension.
At T 8 ~ 10 only an adrenal response is obtained in the pressure
recording with a time course similar to the heart rate response.   No
response was obtained in the longitudinal tension to stimulation
in this position.   Time 1 sec, 5 sec.

BP

Tension

vas
pressure

↑
LSD

↑
C6
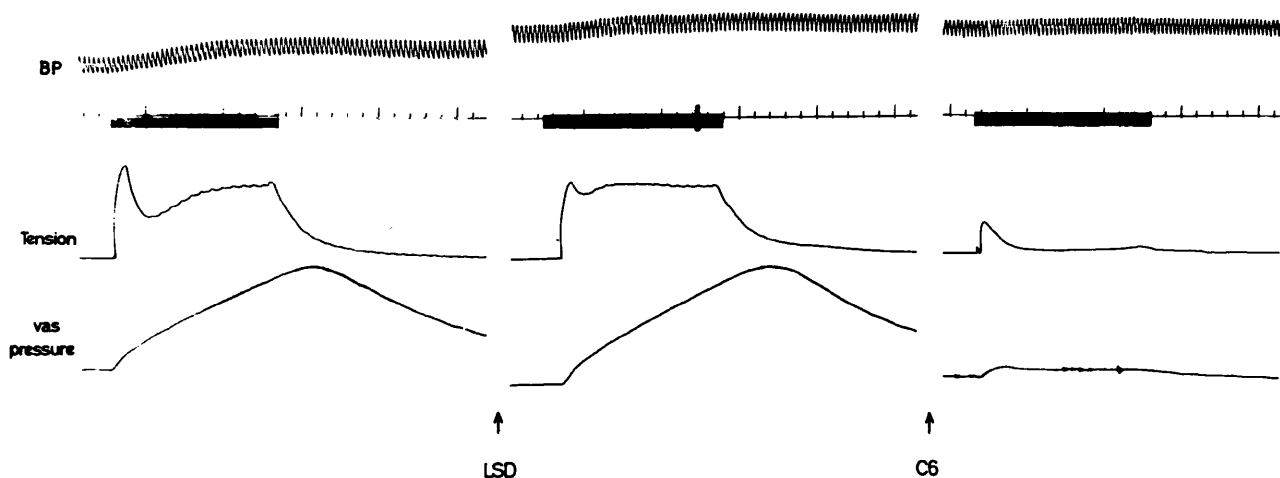
Fig. 33   Comparison of the effects of LSD and

hexamethonium on the response of the pithed rat vas deferens

measured as longitudinal tension or constant volume perfusion

pressure.   Stimulation at L 1 - 3, 30 Hz for 10 sec.   The

response, measured in the left vas deferens as longitudinal tension

shows a biphasic response but in the right vas deferens as perfusion

pressure, only a monophasic response is found.

While LSD 200 ug/kg inhibits the "spike" phase of the tension response,
it has no visible effect on the pressure response.   Subsequent
administration of hexamethonium (C6) (5 mg/kg) completely abolished
the secondary response of the tension and almost completely abolished
the pressure response.   Time 1 sec, 5 sec.

Fig. 34

FREQUENCY /RESPONSE CURVE FOR MOTOR RESPONSE IN VAS DEFERENS IN PITHED RAT
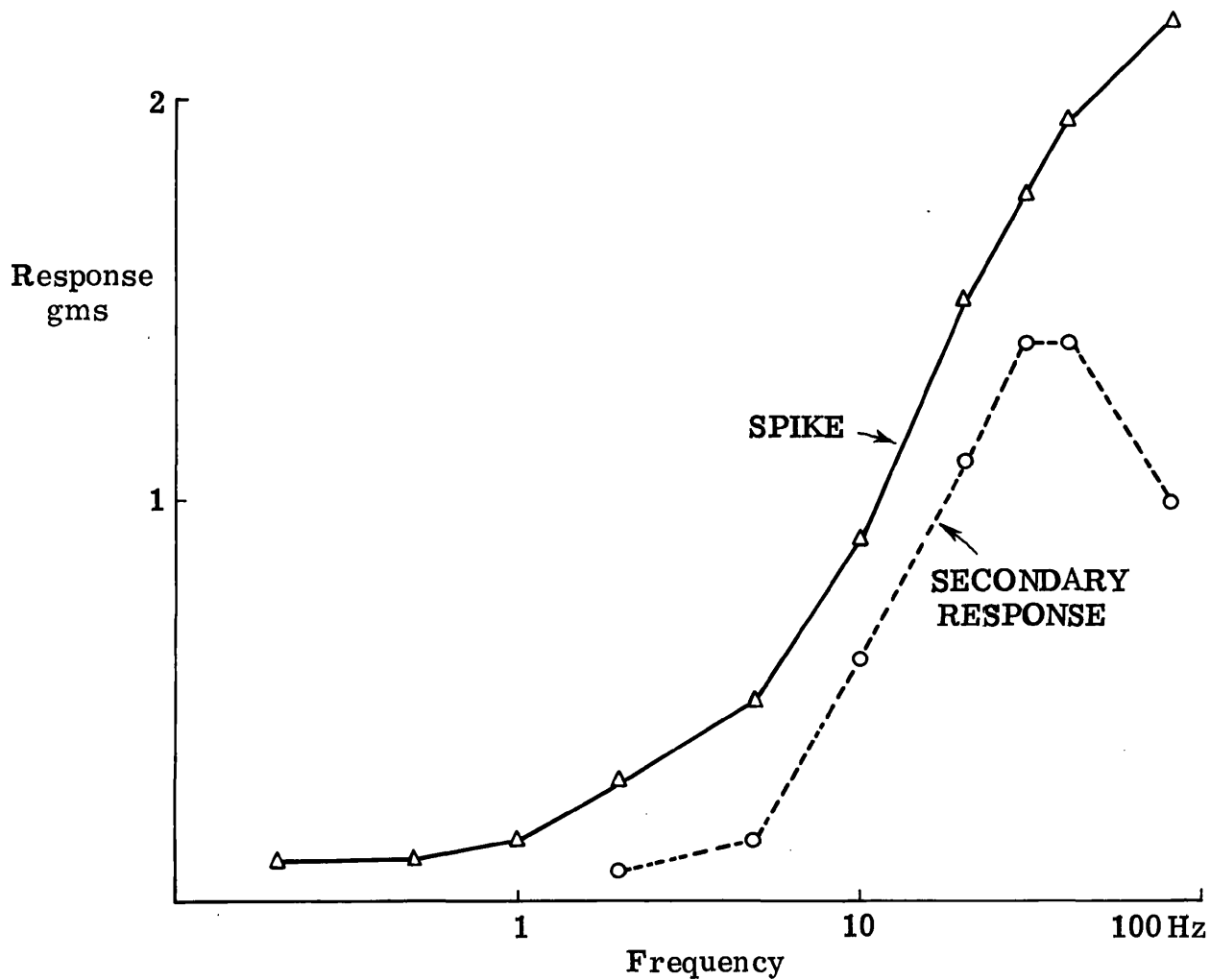
Stimulation with a 10 mm electrode at L 3. Longitudinal tension response in a single vas deferens to stimulation at the frequencies indicated for 10 sec. "SPIKE" is the initial rapid peak which is reached within 1.5 sec. "SECONDARY RESPONSE" is the slower peak reached after this initial response. At low frequencies the secondary response is small compared to the "spike", but as the frequency increases its relative size increases.

Spinal origin of the vas deferens response

Preliminary experiments, exploring the spinal canal, confirmed that the optimal position for the response in the vas deferens was L 2 - 3. Fig. 32 illustrates this and shows that this position is somewhat lower than the position for stimulation of preganglionic sympathetic nerves to the anococcygeus. If the extreme ends of these optimal positions were used, selective stimulation of either the anococcygeus at T 12 - 13 or vas deferens at L 3 - 4 was possible. If the electrode was placed at L 1 - 3, both muscles could be stimulated simultaneously.

Shape of vas deferens response

The isometric development of tension in the vas deferens during nerve stimulation showed a characteristic two phase response. An initial rapid "spike" contraction reached its maximum within 1.5 sec and then declined to be replaced by a slower smooth secondary contraction which reached its maximum after 8 - 10 sec (Fig. 33).

The relationship in the size of these two phases varied with frequency and with the duration of stimulation. Single pulses produced a rapid "spike" only. At low frequencies, the secondary response was small but as the frequency increased it became greater in proportion to the "spike" until at 30 Hz it was 80% of the "spike" (Fig. 34). The response declined immediately on cessation of stimulation. Continuous stimulation for up to 3 min at 30 Hz showed that the secondary response declined after 20 - 30 sec but remained at a lower plateau level for the remainder of the stimulation period (Fig. 17).

This two phase response in the vas deferens is in contrast to the single phase, smooth contraction of the anococcygeus. Had the "spike" been absent, the secondary response in the vas deferens would be, however, similar to the anococcygeus response (see Figs. 17 & 35).

In experiments on the vas deferens where perfusion pressure instead of longitudinal tension was used as a measure of the response, only a single phase of pressure increase was seen corresponding to the secondary component of the isometric longitudinal tension response. The maximum pressure was reached after 8 - 10 sec (Fig. 33). This may indicate an anatomical basis for the two phase tension response since longitudinal tension is likely to reflect activity in the outer longitudinal layer of smooth muscle in the vas, whereas the perfusion pressure is likely to reflect activity in the inner circular layer.

Another difference in the perfusion pressure response was that it showed a delayed response corresponding in time with the blood pressure and heart rate adrenal response when stimulating in the thoracic region, whereas the longitudinal tension showed no adrenal response (Fig. 32).

The absence of the "spike" in the perfusion response is not due to the inability of the pressure recording system to respond to rapid changes in lumen diameter since mechanical constriction of the vas with forceps can produce pressure increases at least as rapid as the "spike".

The longitudinal tension responses in the vas deferens to 10 sec periods of stimulation are reproducible if at least 3 min is left between periods. If less than 3 min is left, the "spike" component declines slightly. This decline is similar to the endogenous inhibition found in vitro and attributed by Swedin (1971 b) to release of prostaglandins within the tissue. To eliminate this additional factor, when investigating the effects of drugs on the response, at least 3 min was left between periods of stimulation.

Effects of drugs on the vas deferens response

With the pithing rod electrode at L 1 - 3, the autonomic outflows to both vas deferens and anococcygeus can be stimulated simultaneously. In order to compare the properties of the innervation of the two tissues, the effects of
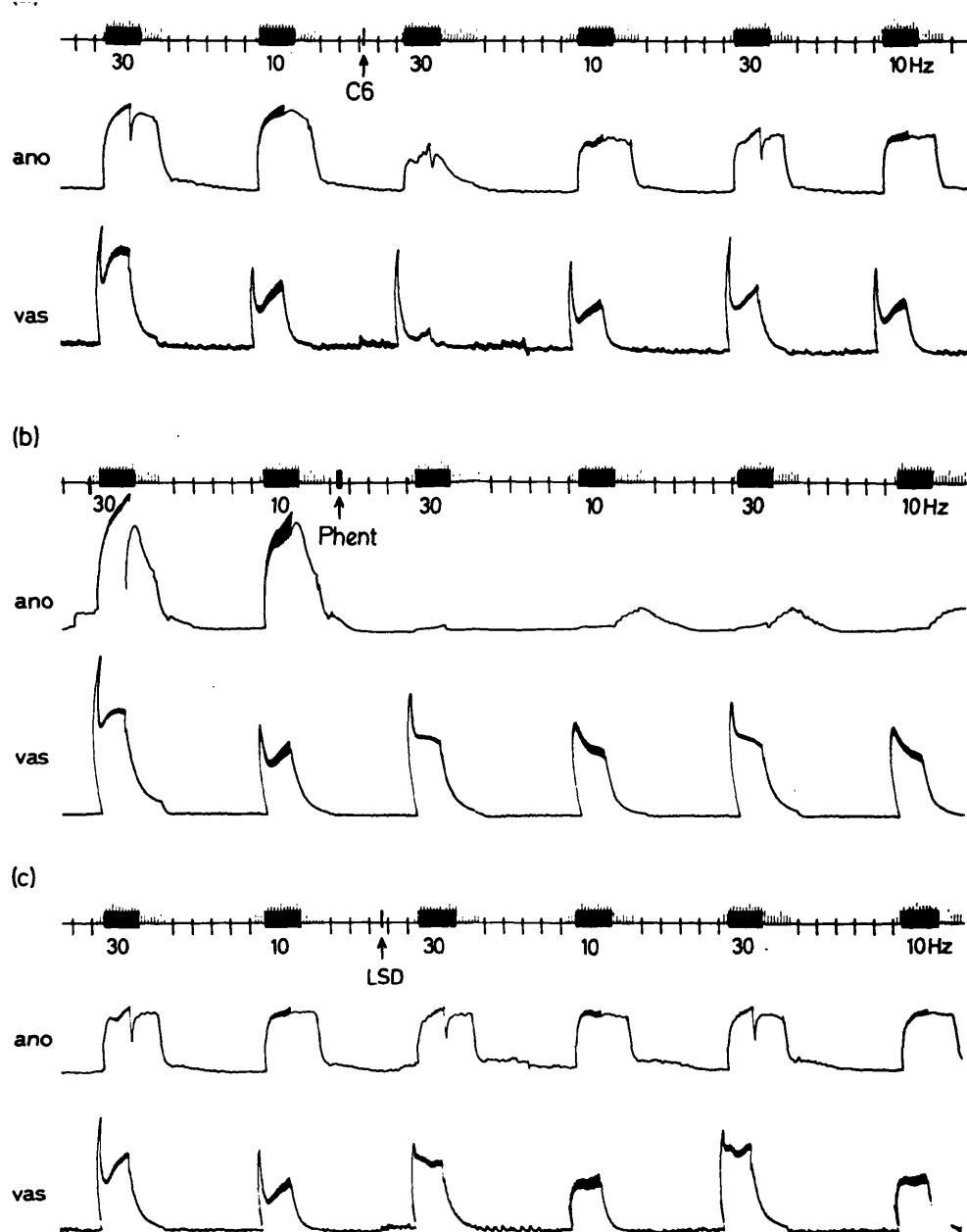
Fig. 35   Effects of blocking drugs on the responses of the
anococcygeus and vas deferens of the pithed rat.   Stimulation for
10 sec periods was applied alternately at 10 Hz and 30 Hz.

(a)   Hexamethonium (C6) 1 mg/kg inhibited the response in the
anococcygeus and inhibited preferentially the secondary response in
the vas deferens.   This inhibition was short - lived, the responses
in both tissues having recovered over 50% after 12 min.

(b)   Phentolamine (Phent) 4 mg/kg abolished the response in the
anococcygeus but modified the response of the vas deferens.

(c)   LSD 200 ug/kg produced a small motor response in the
anococcygeus but did not inhibit the response to nerve stimulation.
In the vas deferens, on the other hand, LSD preferentially inhibited
the "spike" phase of the response and potentiated the secondary
component.   This effect was more marked at 10 Hz.
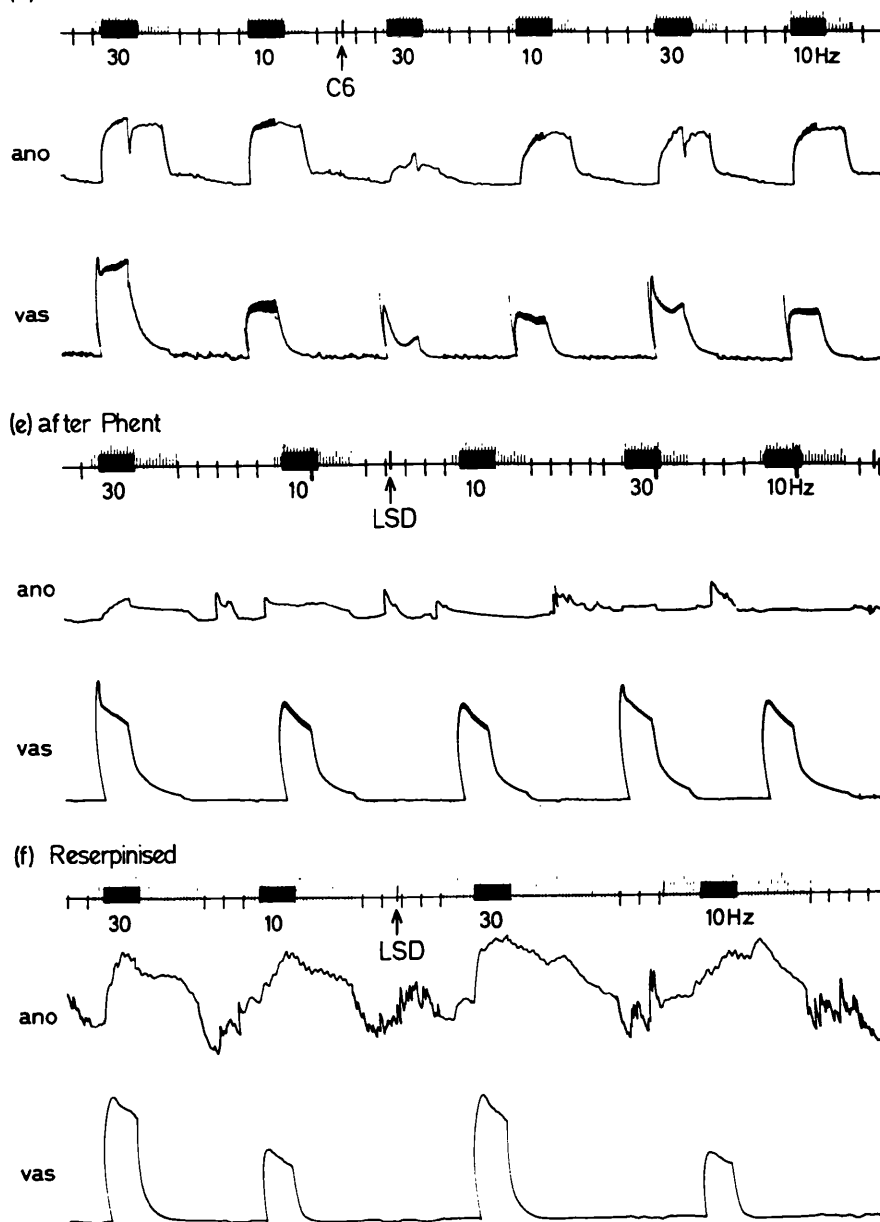
(d)   This trace follows on from (c).   After LSD 200 ug/kg, C6 inhibited
the response in the anococcygeus in a manner similar to its effect in the
absence of LSD, cf (a).   In the vas deferens, after LSD, C6 inhibited
the response remaining.   The net effect of LSD and C6 was to inhibit both
phases of the vas response as might be expected from the summation of the
effects in (a) plus (c).   As in (a) the effect of C6 was short - lived.

(e)   This trace follows on from (b).   After phentolamine 4 mg/kg, LSD
200 ug/kg had no effect on either the anococcygeus or vas deferens.

(f)   This trace was obtained from a rat pretreated 18 hrs before pithing
with reserpine 3 mg/kg.   The sensitivity of the recorder in this trace is
twice that in (a) - (e).   The responses in both anococcygeus and vas
deferens are reduced compared with controls and the vas response is mono -
phasic and similar to that obtained after phentolamine, cf (b).   After
reserpine, LSD 200 ug/kg had no effect on either tissue.   Time trace
upwards 1 sec, 5 sec; downwards 1 min.   Trace speed reduced between
stimulation periods.

ganglion blocking drugs and drugs which block or potentiate the action of
adrenergic nerves were studied. In addition, LSD was examined since it has
been shown to inhibit the response to field stimulation in rat and guinea-pig
vas deferens _in vitro_ (Ambache, Dunk, Verney & Zar, 1973). Except where
otherwise stated the vas deferens response referred to is the longitudinal
tension.

Hexamethonium (1 mg/kg) inhibited the responses in both anococcygeus and
vas deferens (Fig. 35 a). The secondary component of the vas deferens response
was inhibited more than the "spike". Two possible explanations for this
differential action on the vas deferens are either that the two phases are
produced by different pathways which have different sensitivities to ganglion
blocking agents or that partial ganglion blockade leads to a reduced frequency
of discharge in the postganglionic nerves which in turn produces a response in
which the "spike" is proportionately greater than the secondary response. In
favour of the former and against the latter argument is the fact that the
response to 30 Hz after hexamethonium has a larger "spike" but smaller
secondary response than the response to 10 Hz before hexamethonium. If
there are indeed two pathways, it is of interest that they appear to have a
common spinal origin since both phases are produced in similar proportions
at different spinal levels (Fig. 32).

A larger dose of hexamethonium (5 mg/kg) completely abolished the response in
both anococcygeus and vas deferens, indicating that both components of the
vas deferens response are produced by nerves having a ganglion synapse in
their pathway.

The effects of hexamethonium were short lasting. The effect of 1 mg/kg
had started to disappear after 5 min in both tissues. After 5 mg/kg, the
response in the vas deferens recovered particularly quickly, being back to its
full size within 15 - 30 min. After recovery, a second dose of 5 mg/kg
hexamethonium again produced a block but this was even more short lived

than before.

Reserpine (3 mg/kg) given intravenously, selectively inhibited the "spike" component of the vas deferens response without affecting the secondary component or the anococcygeus response. This effect developed within 5 min and so is unlikely to be related to any significant depletion of NA stores by reserpine.

In rats pretreated for 18 hr with reserpine (3 mg/kg, i.p.), at which time the NA content of both the vas deferens and anococcygeus had been depleted by at least 98% (i.e. the NA level was undetectable) a reduced, single phase response was obtained in the vas deferens to nerve stimulation. This response reached its maximum after 2 - 3 sec and then declined over the remainder of the 10 sec stimulation period to 50 - 80% of its maximum (Fig. 38 b). When the peak of this response was compared with the secondary response in control animals, it was only 60% of the latter (Fig. 39). Thus after reserpine pretreatment both components of the vas deferens response were reduced and the residual response was monophasic.

After reserpine pretreatment, the response to nerve stimulation in the anococcygeus was depressed compared with controls. Responses could not be produced to single pulses or low frequencies and even at high frequencies the response was slow to develop (Fig. 38 b) and had a reduced maximum (Fig. 39). When the response had developed, however, the raised tension could be maintained for up to 60 sec if stimulation was continued.

Reserpine has, therefore, a dual action on the vas deferens response; an acute effect to selectively inhibit the "spike" component and a delayed effect, related to the depletion of NA from the tissue, to reduce both phases of the response. On the anococcygeus, on the other hand, reserpine affects the response only when the NA content is depressed but even under these conditions the response is not abolished.

Guanethidine (10 mg/kg) completely abolished the responses to nerve
stimulation in both tissues. The anococcygeus response recovered after
1 - 1½ hr but the vas deferens response did not.

Phentolamine (4 mg/kg) completely abolished responses in the anococcygeus
but only changed the shape of the response in the vas deferens (Fig. 35 b).
This change in shape is open to various descriptions but one is to say that
the "spike" became prolonged and the secondary response became poorly
maintained. At 30 Hz, the height of both components was decreased but at
10 Hz the height of the two phases was little changed, only the shape being
changed. Alternatively it could be argued that the single phase response
after phentolamine is the secondary response which now starts earlier but is
poorly maintained.

In either case it is clear that phentolamine (4 mg/kg) does not block the
response in the vas deferens as it does the response in the anococcygeus and
no additional dose of phentolamine (up to 20 mg/kg) produces any further
change in response.

LSD - 25 (200 µg/kg) had no detectable effect on the motor response of the
anococcygeus (Fig. 27), but had a differential effect on the two phases of the
vas deferens response (Fig. 35 c). At 10 Hz, the "spike" was completely
abolished and at 30 Hz, was inhibited. The secondary response on the other
hand was slightly potentiated (Figs. 35 c & 37). Whatever the pathway
producing the "spike", it appears to be specifically inhibited by LSD, and
either as a result of this inhibition or due to some other property of LSD,
the secondary response is potentiated.

The differential effect of LSD is thus the opposite of that of hexamethonium.
When hexamethonium (1 mg/kg) was given after LSD (200 µg/kg) both phases of
the vas deferens response were inhibited as might be expected from the
differential effect of each drug on one phase (Fig. 35 d). The effect of

hexamethonium was short lived, the response returning to that found after LSD alone.

After the shape of the vas deferens response had been changed by the administration of phentolamine (4 mg/kg), LSD had little further effect (Fig. 35 e). If the dose of phentolamine was given after the LSD, however, the response returned to the shape it would have had after phentolamine alone. The effect of LSD is thus overridden by the effect of phentolamine, although it is not on this evidence possible to say that the effect of LSD is abolished by phentolamine since phentolamine itself exerts an effect on the response.

After pretreatment of the rat for 18 hr with reserpine (3 mg/kg), the response in the vas deferens was similar in shape to that found after phentolamine (Fig. 35 f). This response was not further altered by LSD or phentolamine.

There is, therefore, a similar change in the response of the vas deferens after $\propto$ - receptor blockade by phentolamine and after reduction of the NA content of the nerves by reserpine. The response remaining after both these treatments is resistant to the action of LSD. This suggests some role for $\propto$ - adrenergic receptors in the motor transmission process of the vas deferens which can be blocked by phentolamine or prevented by reduction of tissue NA content by reserpine (and therefore presumably reduction of NA release). Since LSD has no effect on the response remaining when this $\propto$ - receptor mechanism is absent, it is likely that LSD exerts its effect somewhere along the transmission process leading to the $\propto$ - response, possibly by facilitation of NA release or by an indirect sympathomimetic action, releasing NA from the nerve.

The perfusion pressure response in the vas deferens, which exhibits only a single smooth component, is unaffected by LSD (200 µg/kg) (Fig. 33). This provides pharmacological evidence that the two phases of the vas deferens
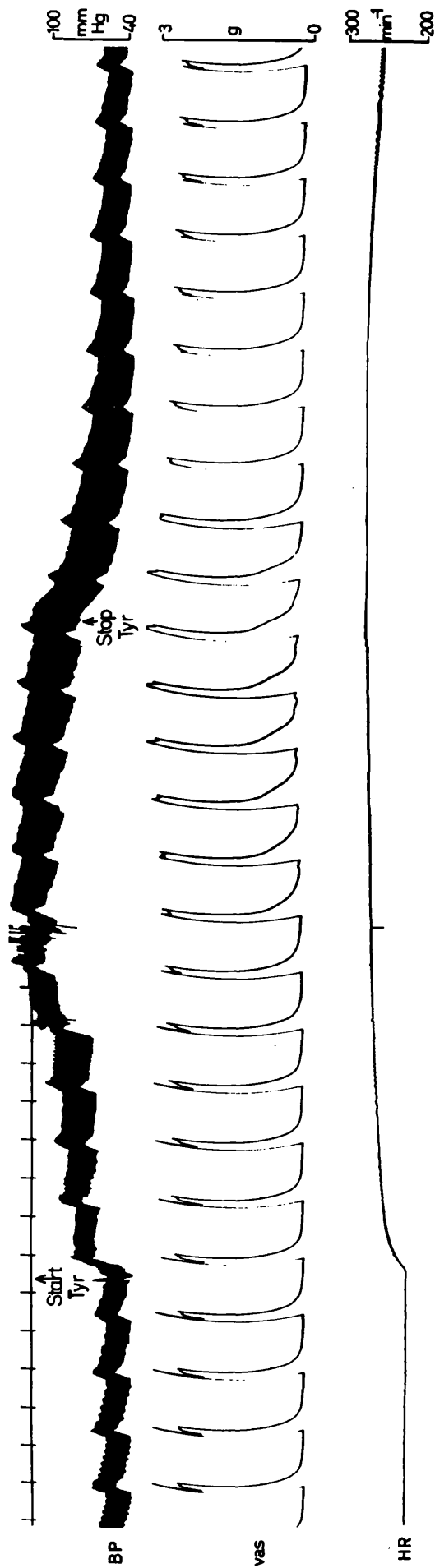
Fig. 36   Effect of a slow infusion of tyramine on the response to nerve stimulation of the vas deferens in the pithed rat.   Stimulation at L 1 - 3, 30 Hz, 10 sec every 90 sec.   Tyramine 40 ug/min first inhibited the "spike" phase of the response and then potentiated both phases.   On stopping the infusion, the process was reversed although the "spike" phase of the response never fully recovered.

Time 1 min.

(a)

BP

LSD

ano
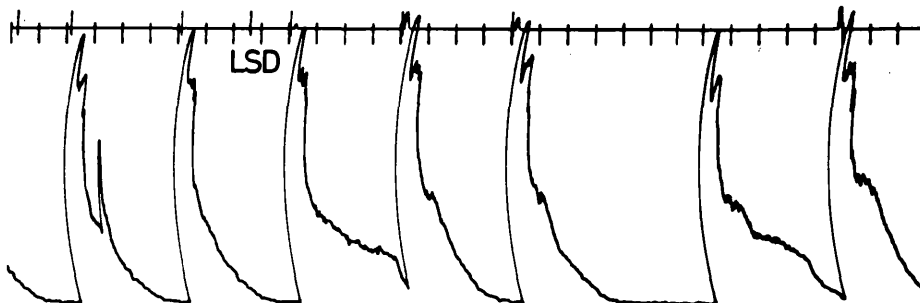
vas

HR

(b)

BP

Tyr

ano
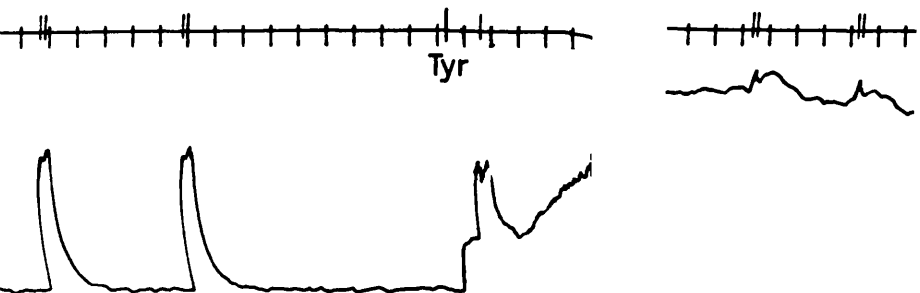
vas

HR

Fig. 37   Effects of LSD and tyramine on the responses
of the anococcygeus and vas deferens of the pithed rat.
Stimulation at L1 - 3 with a 10 mm electrode, 10 sec trains
at 30 Hz.

(a)   LSD 40 ug/kg had little effect on the height of the
anococcygeus response but slightly potentiated the duration.
On the vas deferens, LSD inhibited the "spike" and
potentiated the secondary response.

(b)   An intravenous infusion of tyramine (T) (40 ug/min)
raised the tone of the anococcygeus but did not prevent
motor responses to stimulation.   On the vas deferens,
tyramine, like LSD, inhibited the "spike" and potentiated
the secondary response.   Despite the similarity of the
effects of tyramine and LSD on the vas deferens, note
that the cardiovascular effects of LSD are far less
dramatic than those of tyramine.   Time 1 min.   The
gap in the lower trace occurred as a result of the
annual replacement of the trace paper.

response have a separate anatomical basis since the LSD - sensitive mechanism giving rise to the "spike" does not contribute significantly to the perfusion pressure.

Noradrenaline (0.2 - 4.0 µg/kg) produced motor responses in the anococcygeus which summated with the responses to nerve stimulation. In the vas deferens, NA produced small motor responses only in response to 4.0 µg/kg or more. Smaller doses of NA, e.g. 0.2 µg/kg, did not produce motor responses in the vas but reduced the size of the "spike" response without affecting the size of the secondary response (Fig. 26). Larger doses, e.g. 4.0 µg/kg, could also produce slight potentiation of both phases of the response before depression of the "spike".

Tyramine Single injections of tyramine (0.06 - 0.6 mg/kg), which produced motor responses in the anococcygeus summating with motor nerve responses, inhibited the "spike" response in the vas deferens by 10 - 50% without affecting the secondary response or raising tone (Fig. 26).

When tyramine was given by slow infusion (40 µg/min) in order to produce a high blood tyramine level, a maintained maximal contraction of the anococcygeus was produced, whereas in the vas deferens there was no contraction but a transient inhibition of the "spike" was found followed by potentiation of the secondary response (Fig. 36). During this potentiation, the shape of the response changed in such a way that only one phase remained. This response was larger than both phases of the pre - tyramine response but reached its maximum as rapidly as the "spike" and was maintained with only a slight decline throughout the 10 sec period. At this point, therefore, it was not possible to differentially assess the effects of the high dose of tyramine on each of the two phases.

This effect of tyramine; suppression of the "spike", potentiation of the secondary response; is similar to the effect of LSD (Fig. 37), but the

(a) Reser'pinised

BP

100 mm Hg
40

Tyr 50µg   Tyr 150µg   NA 1µg

ano

5g
0

vas

2g
0

HR

300 min⁻¹
200

(b)

10sec

Control   Reserpinised

ano

5g
0

5g
0

vas

2g
0

2g
0

Tyr   Tyr
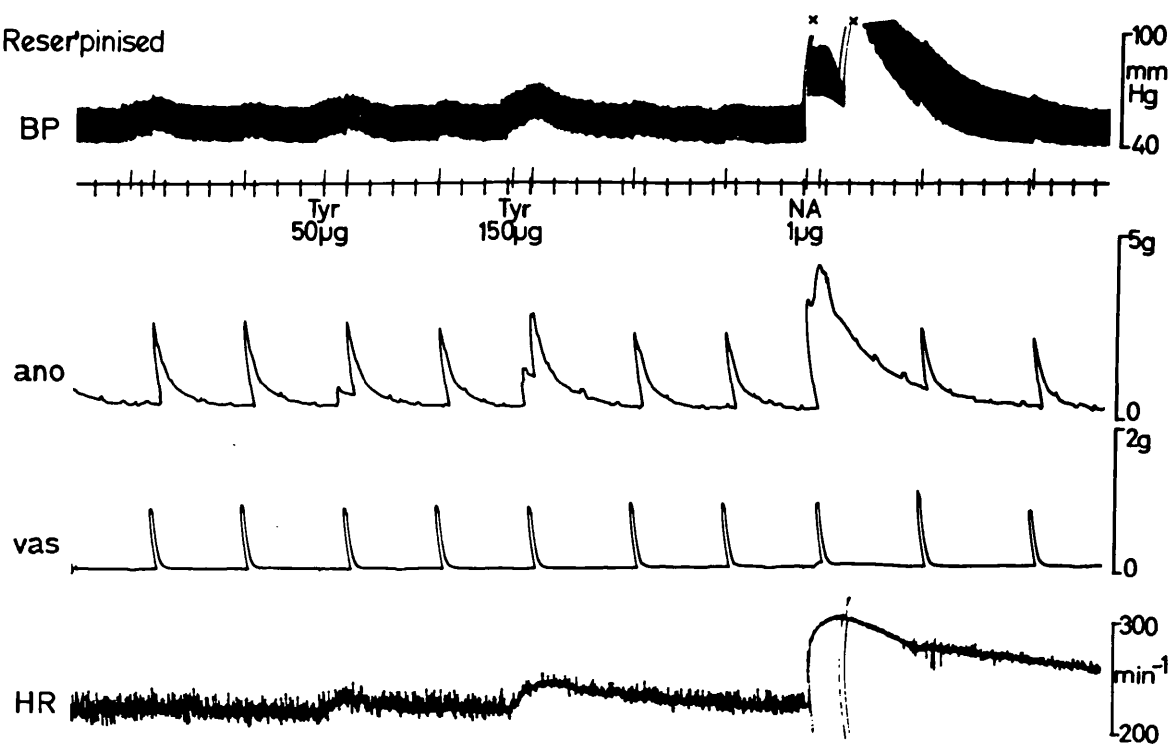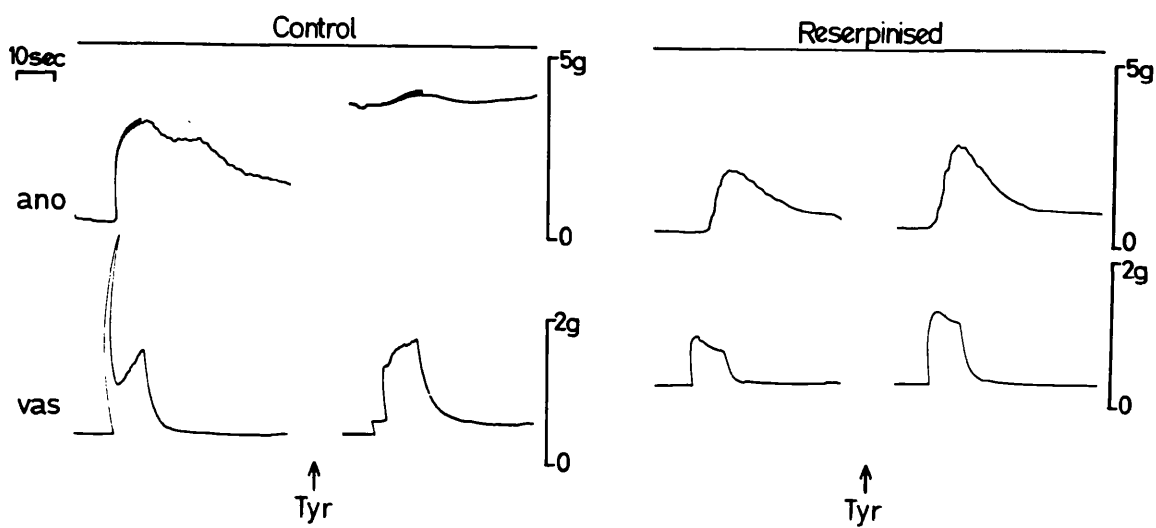
Fig. 38   Effect of reserpinisation on the effect of
tyramine on the pithed rat anococcygeus, vas deferens, blood
pressure and heart rate.

(a)   Pithed rat pretreated 18 hrs before pithing with
reserpine 3 mg/kg.   Stimulation at L 1 - 3, 30 Hz, 10 sec
every 4 min.   Tyramine produced very small motor responses
in the anococcygeus, no effect on the vas deferens response
and only small responses on B.P. and H.R.   NA, on the other
hand, produced responses on all 4 parameters, cf. control
rat in Fig. 26.   Time 1 min.   Note sensitivity of B.P.
trace was halved between crosses.

(b)   Comparison of the effect of a slow infusion of tyramine
(40 ug/min) on the responses to nerve stimulation in the
anococcygeus and vas deferens of the pithed rat in control
and reserpinised (18 hr, 3 mg/kg) pithed rats.   Stimulation
at L 1 - 3, 30 Hz, 10 sec.   In the control, the tone of
the anococcygeus is raised and the motor response to
stimulation is superimposed on this.   After reserpinisation,
tyramine produces no elevation of tone but the reduced response
to stimulation is slightly potentiated.   In the control vas
deferens, the "spike" is abolished by tyramine.   After
reserpinisation, the single phase response remaining in the vas
deferens was slightly potentiated by tyramine.

Fig.39   EFFECT OF RESERPINISATION ON THE RESPONSE TO STIMULATION OF
THE SYMPATHETIC OUTFLOW TO THE ANOCOCCYGEUS AND VAS DEFERENS
IN THE PITHED RAT.
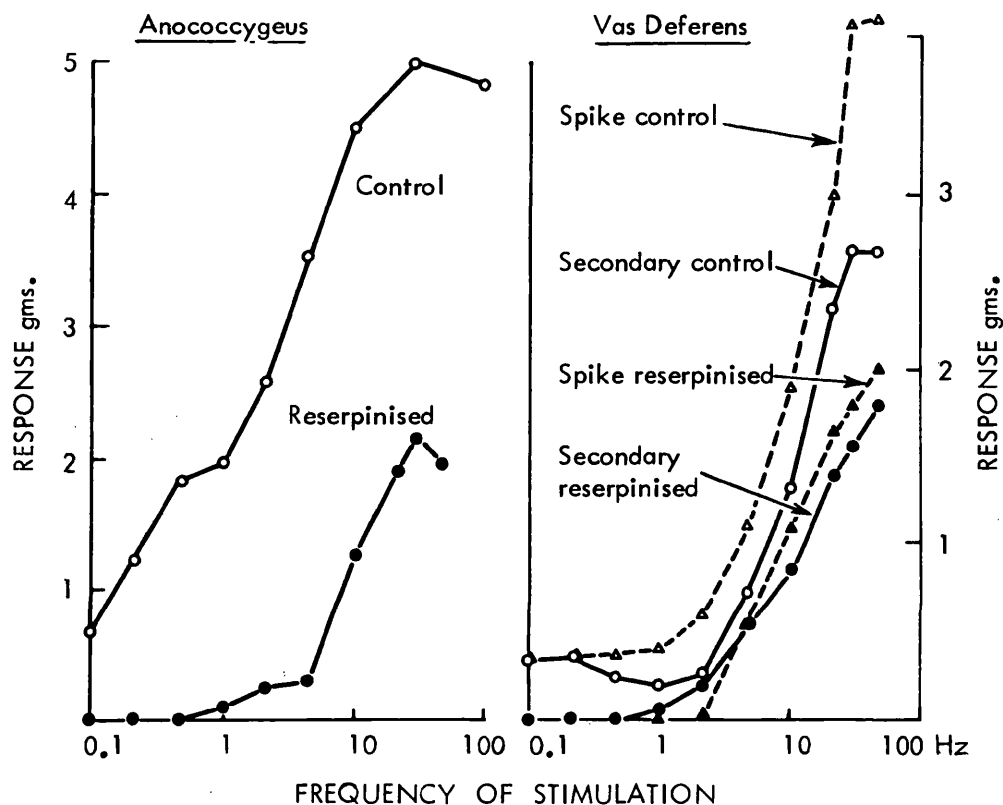
Responses of one anococcygeus muscle and one vas deferens to
stimulation at L 1 - 3 with a 10 mm electrode, 10 sec train,
1 msec pulse at frequencies indicated.   Both components of
the vas deferens response are shown.   Open symbols represent
the responses in control animals (n = 3).   Filled symbols
represent the responses in reserpinised (3 mg/kg, i.p., 18 hr)
animals (n = 3).

effect of tyramine unlike that of LSD is reversible (Fig. 36).

After reserpine (3 mg/kg, i.p., 24 hr) pretreatment, tyramine (0.2 - 0.6 mg/kg) produced weak motor responses on the anococcygeus which summated with the reduced motor nerve responses still present (Fig. 38). NA (4 µg/kg) produced contraction of the anococcygeus similar to controls. In the vas deferens, however, the diminished motor nerve response remaining after reserpine was unaffected by tyramine, although it could be potentiated by NA (4 µg/kg).

Reserpine pretreatment thus prevents the effects of indirect sympathomimetics (tyramine) but not direct sympathomimetics (NA) on the anococcygeus. By analogy, it is tempting to suggest that the effects of tyramine and LSD on the vas deferens, which are also absent after reserpine, are produced by an indirect sympathomimetic action.

Summary of motor responses in vas deferens compared with anococcygeus

In terms of classical autonomic pharmacology, these experiments suggest that the effects of drugs acting on the nerve - muscle junction in the anococcygeus are relatively straightforward compared with those in the vas deferens.

The anococcygeus shows a regular, frequency related, monophasic contraction to preganglionic sympathetic nerve stimulation which is abolished by interruption of the pathway at the ganglion, at the sympathetic terminal by neurone blockade and at the effector organ itself by α - adrenergic blockade, and is very much reduced after depletion of the tissue NA content by reserpine.

In the vas deferens, on the other hand, the motor response to nerve stimulation is complex and the pharmacological evidence does not conclusively prove that the motor transmission is adrenergic.

Part of the complex, biphasic nature of the longitudinal tension response in the vas arises from the anatomical structure of the organ as demonstrated by the much simpler monophasic response produced when perfusion pressure rather than longitudinal tension is recorded. This does not, however, fully explain the unconventional effects of drugs normally associated with modification of adrenergic transmission.

The responses in the vas were obtained from stimulation in the region L 1 - 3, an area normally associated with the sympathetic outflow to many tissues including the anococcygeus. That the responses so produced in the vas were due to stimulation of preganglionic sympathetic nerves appears to be verified by the blockade of the response by a large dose of a ganglion blocker or by the adrenergic neurone blocker guanethidine. This hypothesis is not, however, directly corroborated by the effects of the other drugs tested. The motor response is not directly blocked by phentolamine, as it is in the anococcygeus, but NA, tyramine and LSD all appear to modulate transmission via an α - receptor mechanism. This modulation appears to consist of inhibition of the "spike" and potentiation of the secondary response. Phentolamine cannot, therefore, block the motor response since it disinhibits the "spike" mechanism at the same time as inhibiting the secondary response.

On this evidence, therefore, the secondary response is modified in the manner expected of an adrenergic response, whereas the initial "spike" is unconventional apart from its abolition by hexamethonium and guanethidine.

Analyses which concentrate on the first component of the vas deferens response either by using the maximum amplitude or by giving so few stimuli that only the first component is elicited, naturally suggest that the motor transmission is non - adrenergic since it is resistant to phentolamine, inhibited by NA or tyramine and is still present after virtually complete depletion of the tissue NA content by reserpine. If, however, the shape of the response and

the above hypothesis on the role of α - receptors in this tissue are taken into account, it is clear that both reserpine and phentolamine produce a significant change in the pattern of response to nerve stimulation which implicates NA in the roles of both motor transmitter (in the secondary component at least) and modulator of the response (in the "spike" phase) possibly by feedback inhibition of its own release. The effect of reserpine is particularly interesting since an acute dose specifically inhibits the "spike", whereas after depletion of tissue NA a monophasic response is left, which from the above hypothesis, is a prolonged "spike" response. This acute inhibition could be due to initial release of NA from the nerve terminals by reserpine similar to the indirect sympathomimetic action postulated for tyramine and LSD. After depletion of NA by reserpine, no further release of NA by this indirect sympathomimetic action will occur, leaving the reserpine - resistant "spike" response disinhibited. Thus the main anomaly in the motor transmission process of the vas deferens emerges as the initial "spike" response which is resistant to phentolamine and to reserpine pretreatment. From these results there is no direct evidence that the transmitter giving rise to the latter response is or is not NA.

PART II – THE EFFECTS OF RESERPINE

ON THE RAT ANOCOCCYGEUS, VAS DEFERENS AND HEART

The adrenergic nerves of the rat vas deferens are depleted of their NA by
reserpine relatively more *slowly* ~~quickly~~ than those in other tissues such as the
heart (Sjostrand & Swedin, 1968).

Two possible explanations of this resistance are that either the rate of
depletion is dependent on the firing frequency in the nerves and therefore
reduced in the intermittently active genital organs or that the "short"
adrenergic neurones, which are found in the vas deferens and seminal vesicles
and have their cell bodies located close to the target organ, are less easily
depleted than the "long" adrenergic neurones found elsewhere.

With the pithed rat preparation it is possible to test the first hypothesis
since impulse traffic to tissues can be altered in two ways; the normal tonic
sympathetic discharge can be eliminated by pithing alone or traffic can be
increased by pithing followed by electrical stimulation of the sympathetic
outflow.

The second hypothesis can also be tested by simultaneous comparison of
depletion in the vas deferens and in the anococcygeus since the latter has the
anatomically conventional "long" adrenergic neurones as demonstrated in
Part I by the ability to stimulate postganglionic nerves to the tissue at a
distance of up to 40 mm from the tissue. Both these tissues were further
compared with the heart, a tissue known to be rapidly depleted of NA by
reserpine.

The dose dependence and time course of depletion in vivo in the tissues were
first determined and then the effect of stimulation of the nerves on the rate
of depletion was examined using pithed rats.

Dose µg/kg	ANOCOCCYGEUS			VAS DEFERENS			HEART		
	n	µg/g	% CON.	n	µg/g	% CON.	n	µg/g	% CON.
(a) CON.	5	2.56±0.48	100±19	5	15.04±0.86	100±6	3	1.31±0.05	100±4
50	3		54 15	3		53 7			
100	3		33 7	3		51 10			
200	5		9 2	5		20 7			
1000	1*		3	1		1			
(b) Time hr									
3	4		87 7	4		84 10	3		34 10
6	4		45 8	4		50 12	3		12 4
12	3		50 18	3		58 17			
24	5		9 2	5		20 7			
48	3		20 5	3		10 2			
Days									
21	3		19 4	3		49 4			
28	3		39 5	3		56 7			
35	3		113 17	3		95 6			

Effect of reserpine on the NA content of the rat anococcygeus, vas deferens and heart.   (a) Effect of various doses of reserpine administere[d] 24 hr before tissues taken for assay.

(b) Effect of a single dose of 200 µg/kg reserpine at various times after administration.

Table 2

	ANOCOCCYGEUS		VAS DEFERENS		HEART	
	n	Mean	n	Mean	n	Mean
Pithed (3 hr)	3	128 ± 8	3	95 ± 4	3	102 ± 5
Pithed + Stim.	3	101 ± 13	3	82 ± 6	4	104 ± 7
Reserp. (3 hr)	4	87 ± 6	4	84 ± 10	3	34 ± 10
Reserp. (6 hr)	4	45 ± 8	4	50 ± 12	3	12 ± 4
Reserp. + Pithed (1 hr)	2	69	2	72	2	32
Reserp. + Pithed (3 hr)	4	58 ± 14	4	64 ± 7	3	34 ± 9
Reserp. + Pithed + Stim.	4	18 ± 6	4	32 ± 5	3	12 ± 4

Effect of pithing, of nerve stimulation and of reserpine on the NA content
of the rat anococcygeus, vas deferens and heart.   The NA content is expressed
as a percentage of the mean values from untreated control rats for each tissue
(calculated as μg NA/g tissue).

Reserp. — Reserpine 200 μg/kg i.p. 3 hr before pithing or for time indicated.

Pithed — Tissues taken 3 hr after pithing or after time indicated.

Stim.   — Stimulation via pithing rod electrode at L1-3, 1 msec pulses at
30HZ, 10 sec trains every 1.5 min for 2 hr starting 1 hr after
pithing.

EFFECT OF RESERPINE ON NA CONTENT

24 hrs

NA CONTENT % CONTROL

VAS

ANO.

DOSE μg/kg
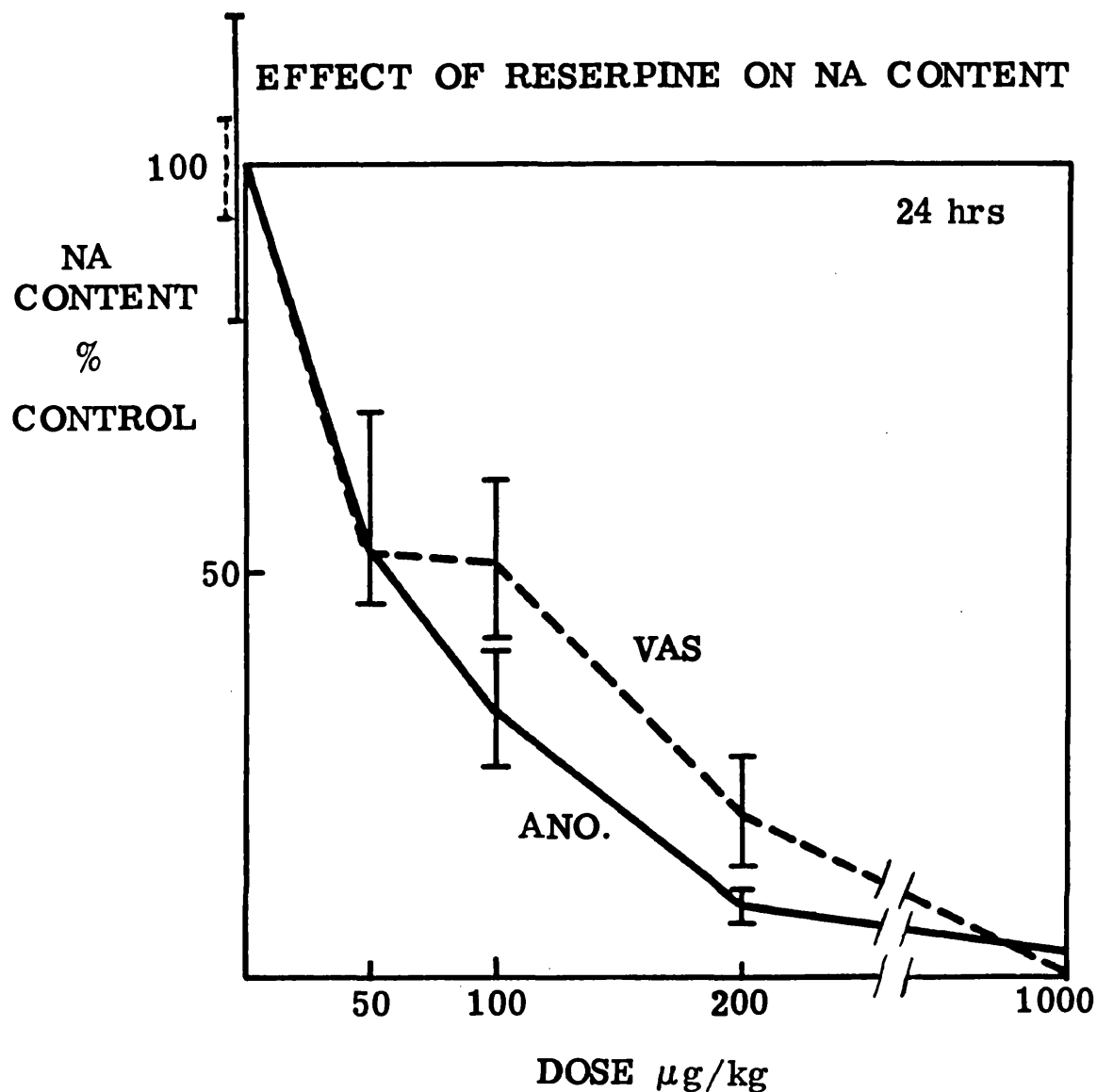
Fig. 40   Effect of reserpine in doses of 50, 100, 200 and 1000 μg/kg (i.p.) on the NA content of rat anococcygeus and vas deferens after 24 hr.   NA content is expressed as a percentage of the mean value from untreated controls for each tissue.   ———— anococcygeus;   — — —- vas deferens.   Solid I - bars represent S.E.M., dotted I - bars represent the S.E.M. for the untreated controls;   from left to right - anococcygeus, vas deferens.

EFFECT OF RESERPINE ON NA CONTENT

200μg/kg

NA CONTENT % CONTROL

VAS

HEART   ANO.

100

50

3   6   12   24

TIME Hrs.
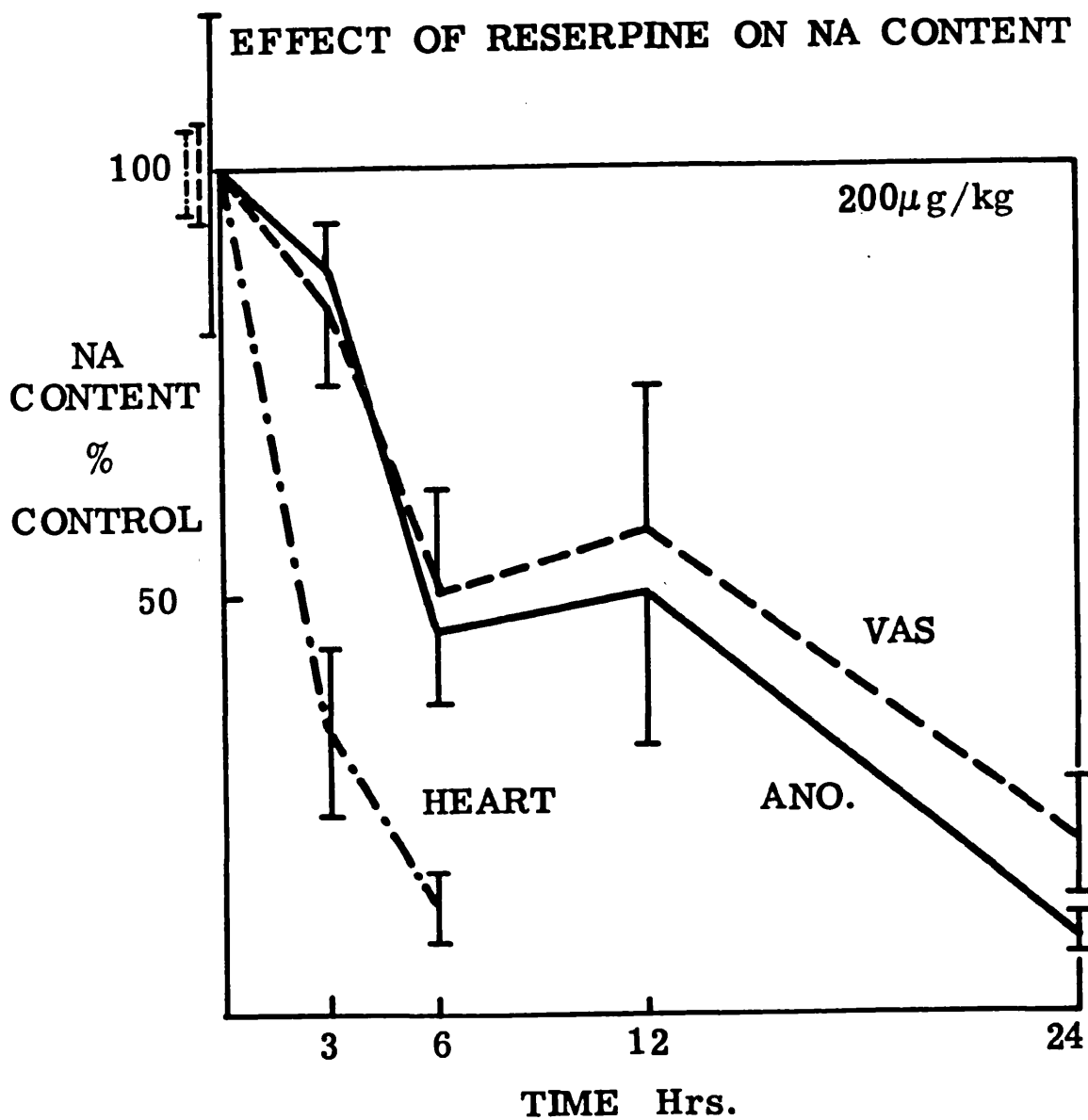
Fig. 41   Effect of reserpine 200 µg/kg (i.p.) after various times on the NA content of the rat anococcygeus, vas deferens and heart. NA content is expressed as a percentage of the mean value from untreated controls for each tissue.   ———— anococcygeus; ———— vas deferens;   —·—·—· heart. Solid I - bars represent S.E.M., dotted I - bars represent the S.E.M. for the untreated controls; from left to right - anococcygeus, vas deferens, heart.

Effect of reserpine dose on the tissue NA content

The normal level of NA in the three organs studied was - anococcygeus
2.56 ± 0.48 µg/g (n = 5), vas deferens 15.04 ± 0.86 µg/g (n = 5) and
heart 1.13 ± 0.05 µg/g (n = 3). The effect of various doses of reserpine on
the NA level in the anococcygeus muscle and vas deferens was measured at
24 hr and the results are shown in Table 1 and Fig. 40. Both tissues were
depleted in a similar dose dependent manner. At the highest dose level
(1000 µg/kg) there was almost complete depletion to 3% and 1% of normal for
the anococcygeus and vas deferens respectively. Reserpine 200 µg/kg
produced a sub - maximal depletion to 9 ± 2% (n = 3) for the anococcygeus
and 20 ± 7% (n = 3) for the vas deferens. There was no statistically
significant difference at any dose level between the percentage depletion in
NA of the two tissues.

Time course of depletion of NA by reserpine

At 24 hr a dose of 200 µg/kg of reserpine produced a large but sub - maximal
depletion and was therefore selected as a suitable dose which, in the
experiments on pithing and nerve stimulation, could show either enhanced or
decreased depletion. The time course of depletion by this dose was then
studied in the three tissues with the results shown in Table 1 and Fig. 41.
The anococcygeus and vas deferens were depleted of NA at a similar rate but the
heart was depleted much more rapidly. After 6 hr the NA content of the heart
was 12 ± 4% (n = 3), the anococcygeus 45 ± 8% (n = 7) and the vas deferens
50 ± 8% (n = 7) of their respective controls.

Since this large divergence in depletion existed over this initial 6 hr period,
the effects of pithing and nerve stimulation were examined over the period
from 3 to 6 hr after reserpine. In this way, direct comparison was possible
between depletion in vivo and that after the additional factors of pithing and
nerve stimulation had been added.

Fig.42   RECOVERY OF NA CONTENT OF RAT ANOCOCCYGEUS AND
         VAS DEFERENS AFTER RESERPINE PRE-TREATMENT.
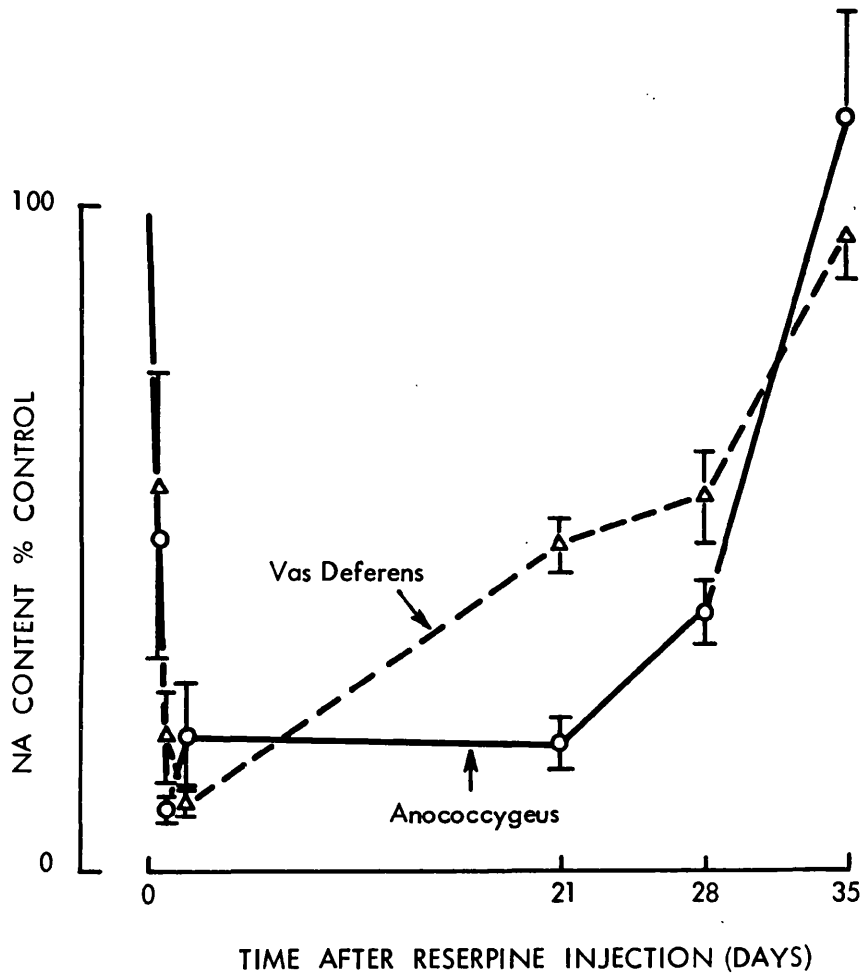
Rats reserpinised (200 ug/kg, i.p.) at time

zero.   Tissues taken at times indicated.

Recovery of tissue NA after reserpine

Since the adrenergic nerves of the vas deferens and anococcygeus were depleted of their NA contents at a similar rate by reserpine and this rate was slower than that found in other tissues, it was of interest to compare the rate of recovery of their NA contents after depletion by reserpine 200 μg/kg. The results are shown in Table 1 and Fig. 42.

48 hr after reserpine the NA content of the vas deferens had fallen further than at 24 hr to 10 ± 2% (n = 3) but the NA content of the anococcygeus showed a non - significant increase to 20 ± 5% (n = 3). 21 days after reserpine, however, the NA contents of the vas deferens and anococcygeus were 49 ± 4% (n = 3) and 19 ± 4% (n = 3) respectively. This difference between the two tissues was statistically significant, indicating a more rapid recovery in the vas deferens despite both tissues having been initially depleted to a similar extent. The recovery in the anococcygeus increased after 28 and 35 days and by 35 days after reserpine the NA content of both tissues was restored to normal levels.

Effects of pithing and of nerve stimulation in non - reserpinised animals

The effects of interrupting any spontaneous neuronal activity by pithing and of increasing neuronal activity by a 2 hr period of intermittent stimulation are shown in the first part of Fig. 43 and Table 2. Pithing alone produced a rise in the NA content of the anococcygeus with little change in the content of the vas deferens or heart. Nerve stimulation in the pithed animal produced a slight reduction in the NA level in the vas deferens with little change in the anococcygeus or heart. None of these changes was statistically significant.

ANOCOCCYGEUS

NA
CONTENT

μg/g

VAS

NA
CONTENT

μg/g

HEART

NA
CONTENT

μg/g

0   3   4   6 hrs
RESERPINE
PITHED
STIMULATED
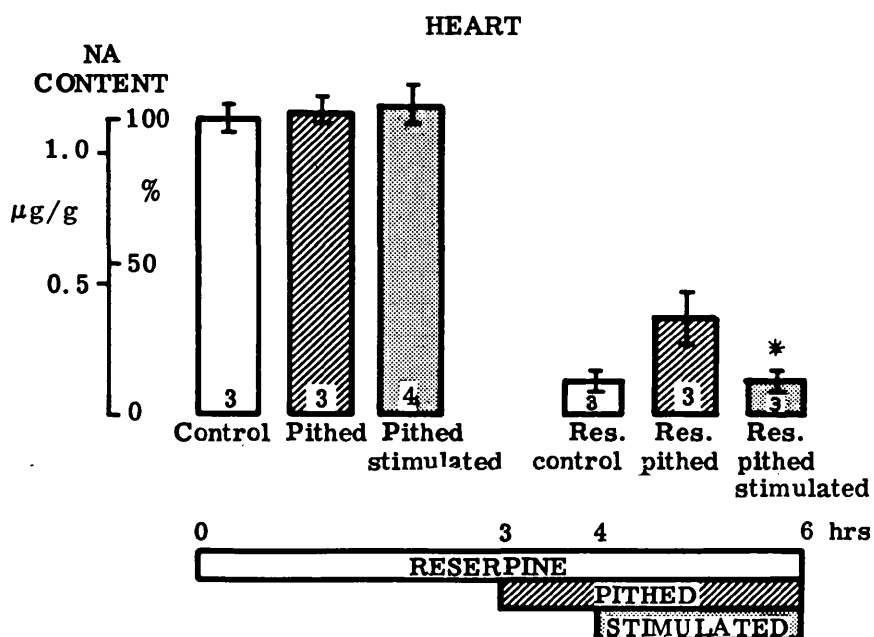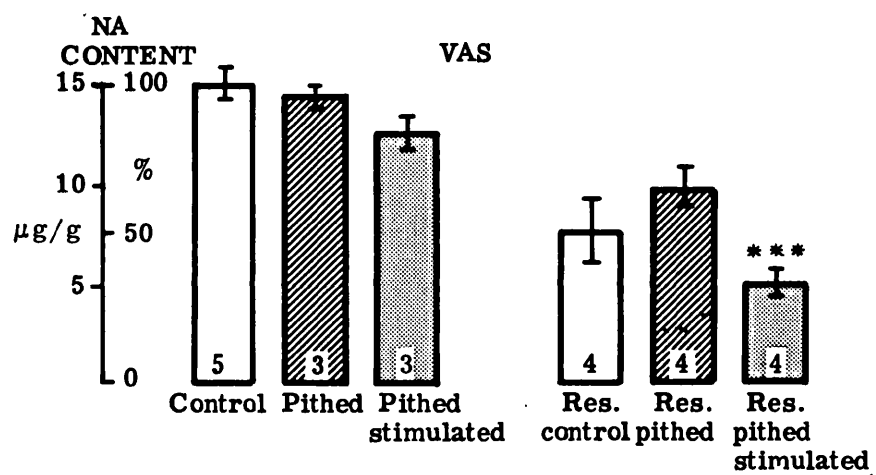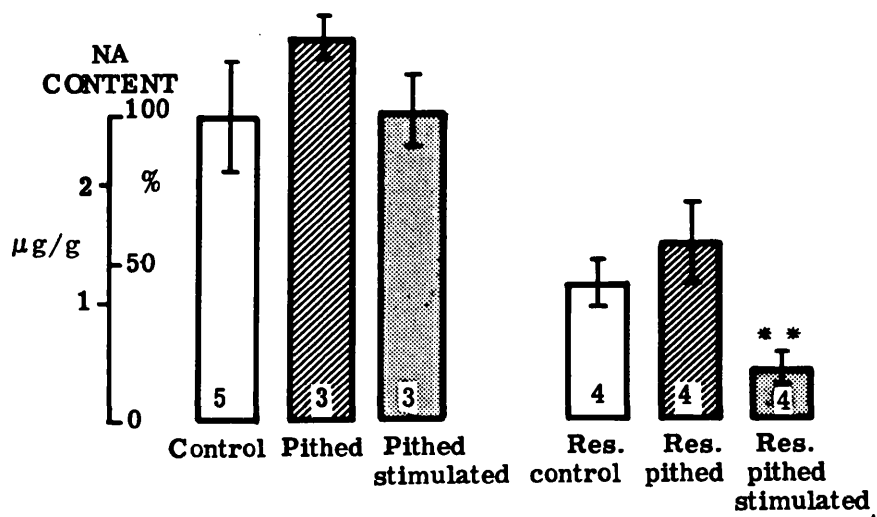
Fig.43. Effects of reserpine, pithing and lumbar sympathetic stimulation on the NA content of rat anococcygeus, vas deferens and heart.

Pithed — tissues taken 3 hr after rat was pithed.

Pithed stimulated — lumbar sympathetic outflow stimulated with 10 sec trains of pulses at 30Hz every 1.5 min for 2 hr starting 1 hr after pithing. Tissues taken 3 hr after pithing.

Res. Control — tissues taken 6 hr after reserpine 200 µg/kg (i.p.).

Res. pithed — as "pithed" but pretreated 3 hr before pithing with reserpine 200 µg/kg (i.p.), i.e. tissues taken 6 hr after reserpine.

Res. pithed stimulated — as "pithed stimulated" but pretreated 3 hr before pithing with reserpine 200 µg/kg (i.p.), i.e. tissues taken 6 hr after reserpine.

Numbers in columns indicate number of assays for each treatment.

Asterisks denote significant difference between the final two columns for each tissue.

* — $0.05 > P > 0.025$

** — $0.025 > P > 0.0125$

*** — $0.0125 > P > 0.01$

Horizontal bars along bottom indicate the time scale of the experiments.

NA CONTENT
% CONTROL

Anococcygeus

Vas Deferens

Heart

Effect of Reserpine alone

Effect of Pithing

Effect of Pithing +
stimulation of lumbar outflows
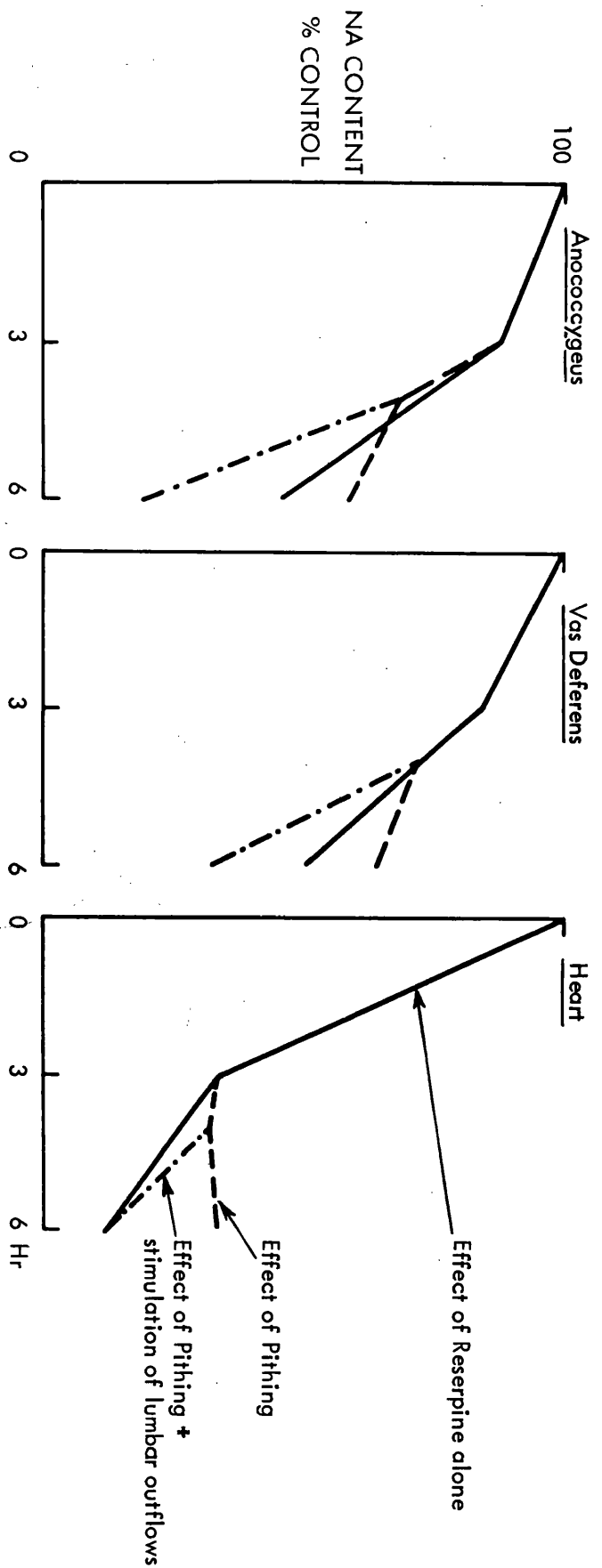
Fig. 44   Effect of pithing and of lumbar stimulation on the time course of depletion of tissue NA
content by reserpine (200μg/kg, i.p.). The values for NA content from table 2 are here
expressed on a time scale to illustrate the time course of the changes produced by the
various procedures.

Effects of pithing and of nerve stimulation in reserpinised animals

The effect of pithing on the NA content of the three organs in animals pretreated with reserpine depended on the time elapsing after pithing before the tissues were removed (Table 2 and Figs. 43 & 44). When only 1 hr had elapsed then the degree of depletion was essentially the same as would have been expected in animals treated with that dose of reserpine but unpithed. Where 3 hr had elapsed the degree of depletion in all three tissues was less than in unpithed animals, suggesting that the loss of normal neuronal activity slowed the NA depletion by reserpine.

Stimulation of the sympathetic outflows to the anococcygeus and vas deferens not only restored the normal rate of NA depletion by reserpine in unpithed animals but increased it. The increase in NA depletion in both anococcygeus and vas deferens between reserpinised animals which were pithed and which were pithed but had their sympathetic outflow stimulated was statistically significant (Fig. 43). It is this latter observation, the contrast in depletion between abolishing nerve impulses and reinforcing them by artificial nerve stimulation, which is the key observation in this study as can be seen in Figs. 43 & 44. Such a difference did not occur in the absence of reserpine so it can be concluded that the extent of neuronal activity affects the rate of depletion of tissue NA by reserpine and that this applies to tissues with either "long" or "short" neurones.

The stimulation of the lower lumbar outflow significantly increased the NA depletion in the heart compared with that in pithed but unstimulated animals. This observation was unexpected since, anatomically, stimulation in this region should not affect the cardiac sympathetic nerves and this is confirmed by the observed absence of cardio - acceleration on stimulation. It must, however, remain a possibility that some inadvertent stimulation could have occurred.

The extent of depletion in the heart in these reserpinised, pithed and stimulated rats was similar to, but not greater than, the depletion in reserpinised but unpithed rats after 6 hr, in contrast to the anococcygeus and vas deferens which were more depleted after nerve stimulation than they would have been after 6 hr. In addition, the significance level for the difference in the heart was less than that for the other two tissues (Fig. 43). None the less, this is a real effect for which I have no satisfactory explanation but which I hope does not affect the interpretation of the data from the other tissues.

The effects of reserpine on the response of the vas deferens

Since the tension response in both the anococcygeus and the vas deferens was recorded, it was possible to correlate the changes in NA content with the mechanical response.

The 10 sec trains of pulses at 30 Hz were sufficient to produce maximal contractions in both organs. The response in the anococcygeus was a smooth contraction, whereas the vas responded with the familiar two phase contraction, an initial quick twitch ("spike") reaching its maximum within 1.5 sec then declining to be followed by a slower, smooth secondary contraction which reached its maximum after 8 - 10 sec.

With stimulation repeated every 1.5 min, the response in the anococcygeus increased gradually over the first 15 min and then remained constant for the remainder of the 2 hr period. In the vas deferens the responses, particularly the initial rapid contraction, declined slightly.

In tissues first stimulated 4 hr after reserpine (200 µg/kg) there was no change in the response of the anococcygeus or the secondary response of the vas deferens and continued stimulation in the following 2 hr produced no greater tendency to fatigue than in non - reserpinised animals. The first, rapid

	Anococ.	Vas Def.	n
(a) control			4
(b) con. stim.	← stim →	← stim →	6
(c) reserp.			6
(d) reserp. stim.	← stim →	← stim →	13
(e) reserp. 18hr.			2
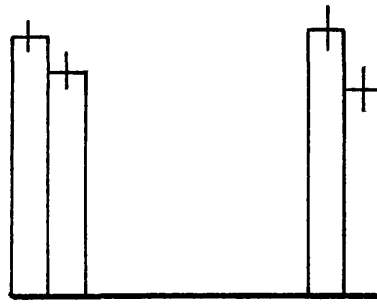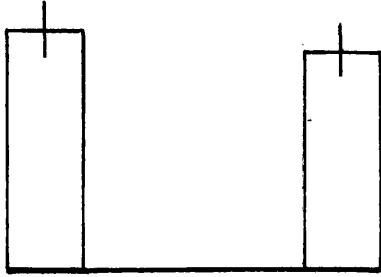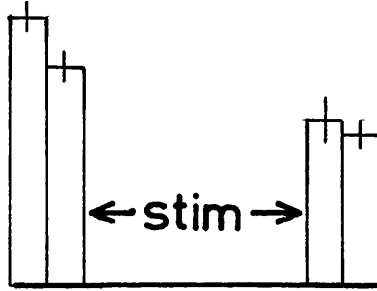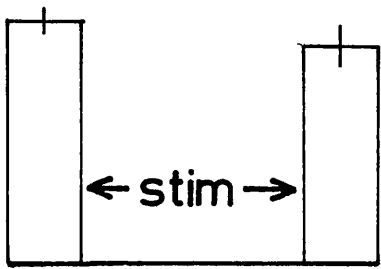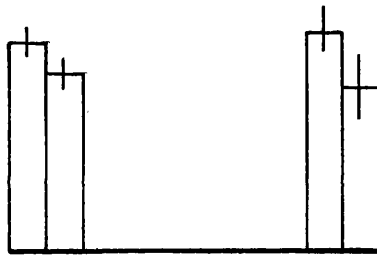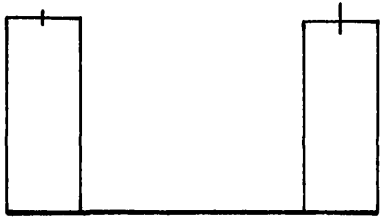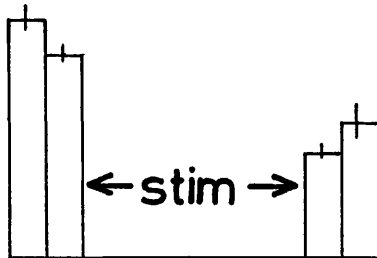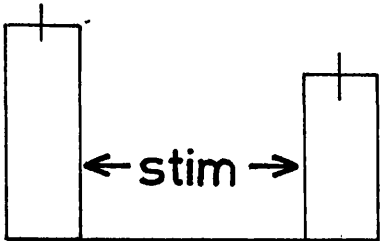
Fig. 45   Effect of reserpine pretreatment on the response to

spinal stimulation of the nerves to the anococcygeus and vas deferens

in the pithed rat.   Stimulation 30 Hz, 10 sec, 10 mm electrode at L 1 - 3.

In each experiment represented in (a) - (d) the response to the first

period of stimulation is shown followed by the response to a similar

period 2 hr later.   In (a) & (c) no other stimulation was delivered

between these two periods.   In (b) & (d) stimulation periods were

delivered every 90 sec throughout the 2 hr between the two responses

measured.   In (a) & (b) responses from control rats are shown, while

in(c) & (d) the corresponding responses are shown in rats pretreated

3 hr before pithing with reserpine 200 ug/kg.   The response of the

anococcygeus was simply the maximum tension developed by the

stimulation.   In the case of the vas deferens, however, both the

initial "spike" and the later secondary response were measured.   Where

the vas response did not show two clear cut phases as in the latter

responses in (d) and in (e), the "spike" was taken as the tension

after 1.5 sec stimulation, i.e. the normal peak of the "spike",

and the secondary response as the tension at 10 sec.   In (e) the

rats were pretreated 18 hr before pithing with reserpine 3 mg/kg

and only the first response is shown.   I - bars represent S.E.M.

"spike" component of the response to nerve stimulation was, however, influenced by reserpine in a characteristic manner. The response to the first period of nerve stimulation after reserpine, whatever time was allowed for the reserpine to act, contained a "spike" of normal amplitude but this quickly declined in subsequent stimulation periods until after 15 - 20 min it had quite disappeared at a time when the secondary component of the response was unaffected (Fig. 45).

There thus appears to be no correlation between NA content and mechanical response in either anococcygeus or vas deferens over the range of NA content produced in these experiments despite depletion of up to 82% for anococcygeus and 68% for vas deferens. Taken together with the results from Part I , which show that motor responses can still be obtained in both tissues when over 97% depletion has occurred, this indicates that only a very small proportion of the NA content is necessary in both these tissues for motor transmission to occur. The evanescence of the "spike" response in the vas deferens of rats pretreated with a moderate (200 μg/kg) dose of reserpine also correlates with the abolition of this response by a large (3000 μg/kg) acute dose. It is interesting that this effect of pretreatment does not fully develop until after several stimulation periods. This may indicate that there is some "pool" of transmitter responsible for the "spike" which is depleted by reserpine only after nerve stimulation or after a large dose, or that some other change occurs in the region of the neuro - muscular junction after reserpine which leads to the suppression of the mechanisms responsible for the "spike" and which only occurs after nerve stimulation or a large dose of reserpine, e.g. the build up in the area of some substance such as NA or prostaglandins which could act on either nerve or muscle to produce inhibition. This latter explanation would be in agreement with the α - mechanism postulated in Part I .

Summary of effects of reserpine on the rat anococcygeus, vas deferens & heart

It has been shown that the conventional "long" adrenergic neurones of the anococcygeus and the "short" adrenergic neurones of the vas deferens are depleted of NA by reserpine to a similar extent by various doses and at a similar rate by a moderate (200 µg/kg) dose, and that this rate is slow compared with that in the cardiac sympathetic nerves. In addition, it has been demonstrated that the rate of depletion in both anococcygeus and vas deferens is decreased by abolition of impulse traffic by pithing and increased by stimulation of the sympathetic outflow to the tissues. It is thus concluded that nerve impulse traffic may be an important factor in determining the rate of depletion of tissue NA by reserpine.

Further information arising from this study was that the NA content of the vas deferens recovered more quickly after reserpine treatment than did that of the anococcygeus and that no direct correlation was found between tissue NA content and the size of the response to motor nerve stimulation in either the anococcygeus or the vas deferens.

PART III - THE PROPERTIES OF THE CAT ANOCOCCYGEUS MUSCLES IN VITRO

AND A COMPARISON WITH THE RAT ANOCOCCYGEUS

The anococcygeus muscles in the cat were described as long ago as 1845 (Straus - Durckheim, 1845) but their physiological and pharmacological properties have never been examined. Since the pharmacological properties of the anococcygeus muscles have been examined in the rat alone, it was of interest to compare the properties of this tissue in the two species.

The necessity for this study was given further impetus just after it was started when a paper appeared by Garrett, Howard & Lansdale (1972), demonstrating histochemically the presence in the cat anococcygeus muscle not only of a dense adrenergic innervation similar to that found in the rat, but also of a diffuse distribution of acetylcholinesterase throughout the muscle. It was tempting to interpret this staining as evidence of a cholinergic innervation and to associate this with a possible inhibitory innervation.

The present experiments were, therefore, designed to determine whether the cat anococcygeus had a dual motor and inhibitory innervation similar to that in the rat muscle and if so whether either component could be cholinergic. In the course of this work several responses in the cat muscle were found to differ so radically from those in the rat that a simultaneous comparison was made on muscles from the two species and this developed into a pharmacological comparison of both differences and similarities in the properties of the two species.
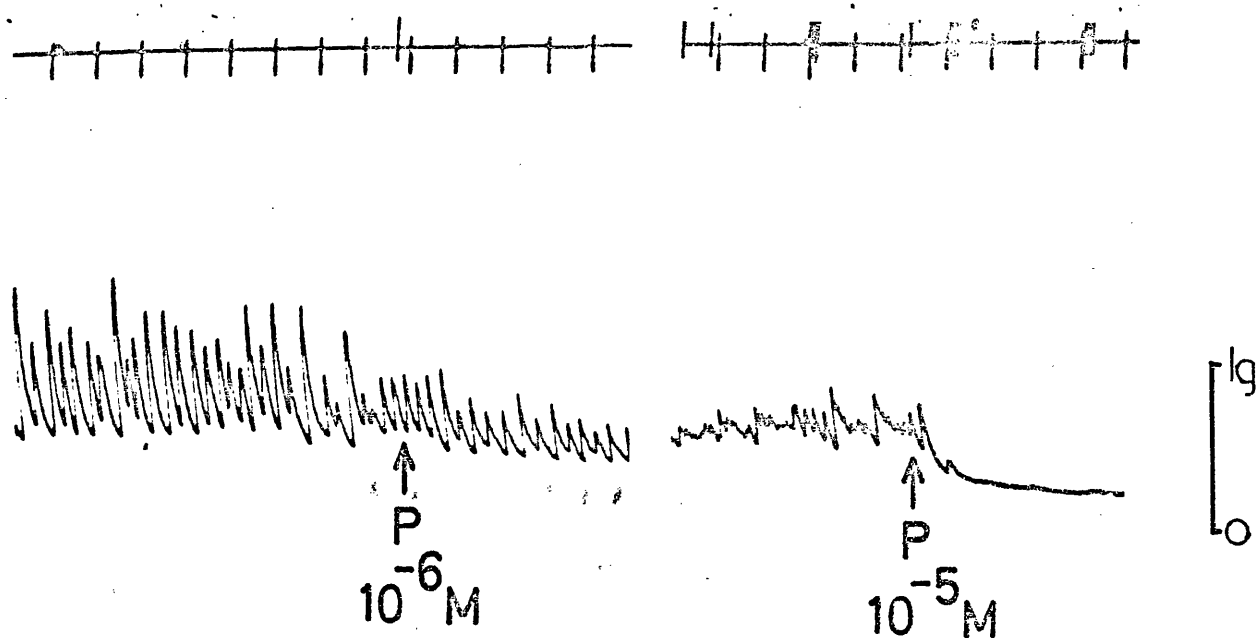
Fig. 46   The effect of papaverine on

the spontaneous activity and intrinsic tone

of the cat anococcygeus muscle.   Papaverine

(P) 10^{-6} M reduced spontaneous activity and

intrinsic tone.   Papaverine 10^{-5} M abolished

the remaining spontaneous activity and

intrinsic tone.   Time 1 min.

Spontaneous activity and intrinsic tone in the cat anococcygeus muscle

When rat anococcygeus muscles were stretched to give an initial tension of
1 g , they gradually relaxed over a period of 2 - 5 min to a resting tension
of 0.3 - 0.6 g , which was then maintained for up to 10 hr.   The muscles
showed no rhythmic activity and it was not possible to lower this resting
tension with papaverine or inhibitory nerve stimulation.   The resting tension
is, therefore, presumably sustained by connective tissue elements.
Stimulation of the motor nerves or the addition of agonist drugs causes
contraction in which the tension is then actively maintained by the smooth
muscle elements.

In the cat anococcygeus, similar stretching to give an initial tension of 1 g
was also followed by a relaxation in 5 min to a resting tension of 0.3 -
0.6 g but unlike that in the rat this resting tension involved an element of
active muscular contraction.   This was engendered in two ways.   First, in
31 out of 38 preparations, spontaneous rhythmic activity appeared with a
frequency of 1 - 6/min (mean 2.9 ± 0.3/min, n = 31) and amplitude range of
0.2 - 6.0 g (mean 1.1 ± 0.2 g, n = 31).   This rhythmic activity steadily
declined over the first 2 hr, a decline accelerated by regular field
stimulation of the preparation, and usually disappeared completely.   The
second evidence of a contribution of active muscle contraction to the resting
tension was the ability of papaverine (10^{-6} M - 10^{-5} M) to inhibit spontaneous
activity (Fig. 46) and lower the resting tension, and also the ability to
display, in appropriate circumstances, a similar reduction in resting tension
by stimulation of the inhibitory nerves.   This active component of the
resting tension will be referred to as "intrinsic tone" and was observed in
26 out of 30 preparations tested.

Intrinsic tone in the cat anococcygeus appeared to be myogenic in origin.   It
was unaltered by phentolamine (10^{-6} M) or atropine (10^{-6} M) and was found in

tissues stored in Krebs' solution at 4° C for up to 10 days, so long as a period of equilibration at 36° C was allowed before applying tension. Storage for longer than 10 days reduced but did not abolish intrinsic tone.

Effect of Agonist drugs

In the course of examining the effects of several putative transmitter or transmitter - like substances on the cat anococcygeus, several were found to contract the muscle including NA, adrenaline, 5HT and indirect sympathomimetics, and several to relax the muscle including acetylcholine, isoprenaline, prostaglandins, ATP and vasopressin.   Furthermore, several drugs such as 5HT which are normally considered as direct agonists appeared to have a large indirect component to their action.   This made it necessary to be able to distinguish between drugs acting directly on the smooth muscle and those acting indirectly by the release of the neuro - transmitter, NA, from the adrenergic nerves within the muscle.   An effective means of making this distinction is to sympathectomise the muscle using 6 - hydroxydopamine (6OHDA). In the case of the cat, it was not practicable to pretreat the animals with 6OHDA, which is the method normally employed (Malmfors & Thoenen, 1971).   It was, however, possible to produce a sympathectomy by exposure of the muscles to 6OHDA <u>in vitro</u> as has been demonstrated in the vas deferens by Wadsworth (1973).   Before considering the effects of the various agonist drugs, this 6OHDA treatment is described in the following section.

6OHDA in vitro treatment

The method employed was that of Wadsworth (1973) except that the high concentration of ascorbic acid was omitted from the incubation medium since all doses of ascorbic acid from 10^{-6} g/ml to 10^{-3} g/ml were found to produce contraction of both the rat and cat anococcygeus muscles.   The purpose of

saline

60HDA
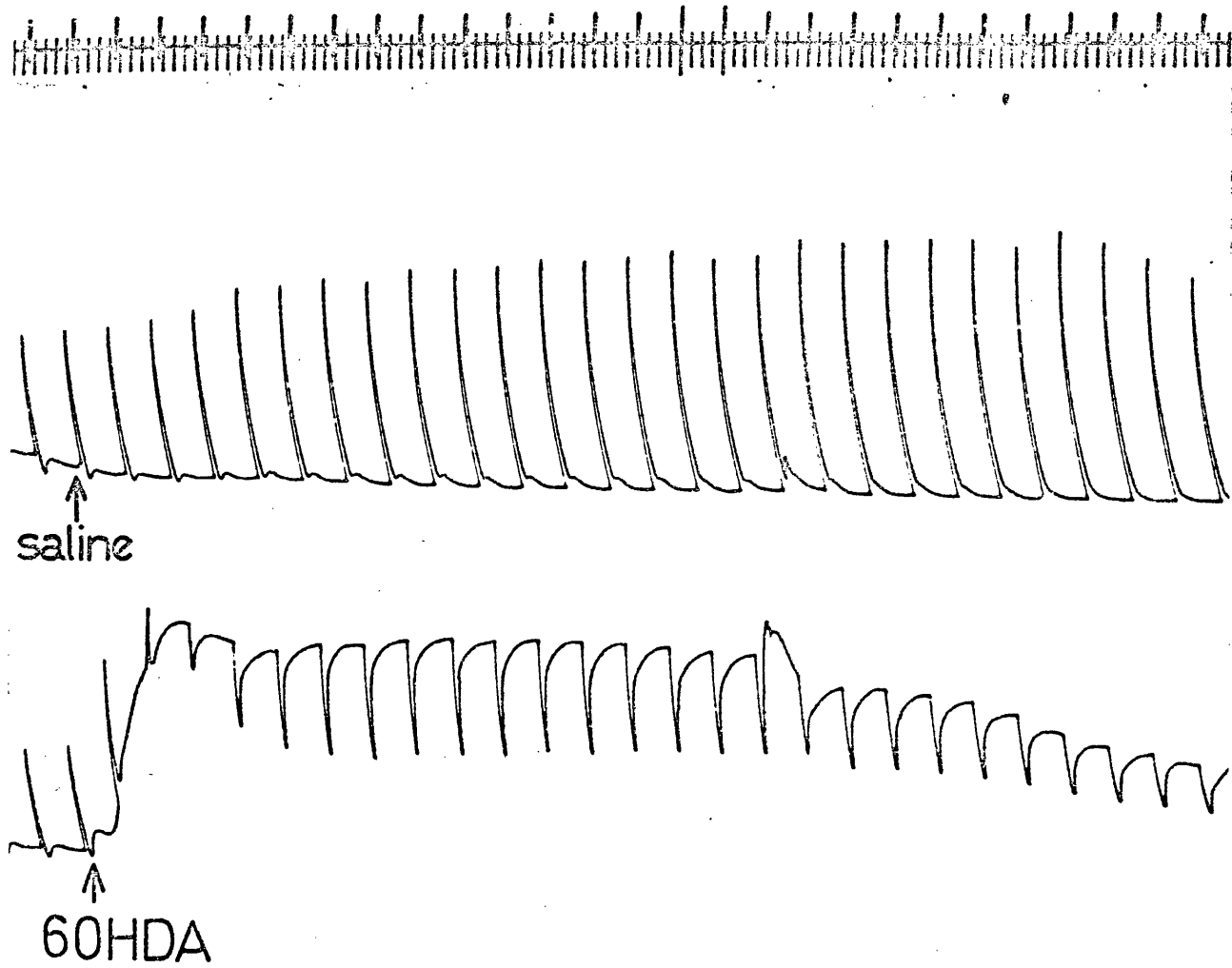
Fig. 47   Effect of 60HDA on the response to field

stimulation of the cat anococcygeus _in vitro_.   Stimulation

10 Hz, 1 msec pulses, for 10 sec every 4 min.   Upper trace –

untreated control muscle.   Lower trace –   60HDA added to

give a bath concentration of 250 μg / ml.   60HDA raised

the tone of the muscle and reversed the responses from motor

to inhibitory in a similar manner to guanethidine c.f. Fig. 67.

Time 1 min.

(a) Control   (b) 6OHDA   (c) 6OHDA PARGYL. NA
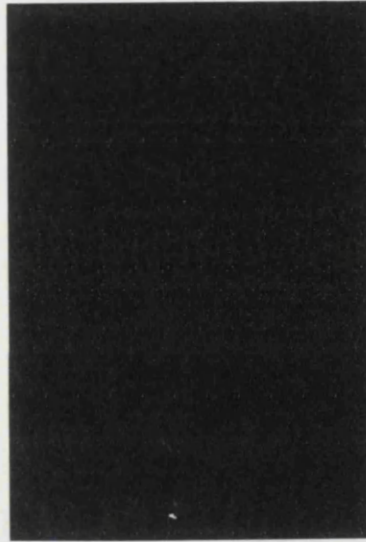
(d) Control   (e) RESERP.   (f) RESERP. PARGYL. NA
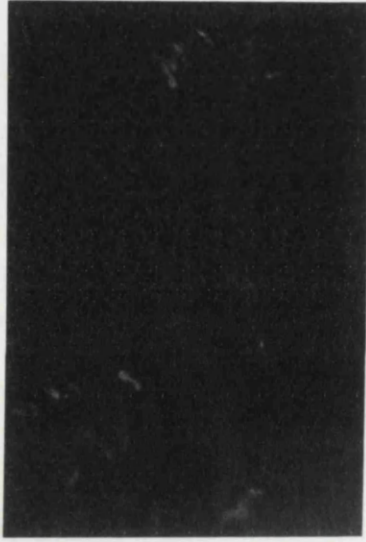
<u>Fig. 48</u>　　　　Effect of pretreatment with reserpine or

<u>in vitro</u> incubation with 6OHDA on the adrenergic nerves of

of the rat anococcygeus as shown by the fluorescence technique

of Falck & Hillarp.　Magnification　x 300.

(a)　Control muscle, transverse section.

(b)　Muscle incubated with 6OHDA 250 ug/ml for 2 hr then

washed for 2 hr.　Note absence of nerves but presence of intense

fluorescence within the smooth muscle cells.

(c)　Contralateral muscle from (b) treated similarly except

that following 6OHDA it was incubated with pargyline 5 x 10^{-5} g/ml

for 30 min in order to inhibit MAO and then incubated with NA

10^{-4} M.　As in (b), no nerves were visible.

(d)　Control muscle, transverse section.

(e)　Muscle from rat pretreated with reserpine 3 mg/kg, 18 hr.

No nerves are visible.

(f)　Contralateral muscle from (e) incubated with pargyline and

NA as (c).　Nerves become faintly visible once more.

This illustrates that whereas the reserpine treatment merely

depletes the NA from the nerves, the 6OHDA treatment destroys

the nerves making it impossible to revisualise them by filling

the terminals with exogenous NA.

including ascorbic acid was to prevent oxidation of 6OHDA, but omission was found to make no difference to the sympathectomy produced.

Tissues were suspended in the organ bath in the usual way and control responses to NA and to field stimulation obtained. Solid 6OHDA was then dissolved in 1 ml of Krebs' and immediately added to the bath to give a final concentration of 250 ug/ml. On addition of 6OHDA to the bath, the Krebs' immediately turned bright red (this colour change was not prevented by the prior addition of ascorbic acid to the Krebs'). Both rat and cat anococcygeus muscles immediately contracted on addition of 6OHDA to the bath and motor responses to field stimulation were reversed to inhibitory responses (Fig. 47). Maximal contraction of the muscles was maintained for 1 hr after which time the tension gradually waned, reaching 50% of maximum after 2 hr. If the 6OHDA was washed out after 1 hr, the rate of relaxation of the muscle was not significantly affected (Fig. 47). After 2 hr exposure to 6OHDA, the bath was washed out several times and the muscle left gradually to relax to the initial baseline. After a further 2 hr, the muscles were tested for their sensitivity to field stimulation and to agonist drugs.

At this time i.e. 4 hr after the beginning of exposure to 6OHDA, no adrenergic nerves could be detected in the tissue by the Falck technique (Fig. 48). These nerves did not reappear, in either species, when the tissues were subsequently exposed to a high concentration of NA (10^{-4} M) after inhibition of mono - amine oxidase by pargyline (5 x 10^{-5} g/ml, 30 min), a treatment which produced the reappearance of adrenergic nerve endings after depletion of the tissue NA content by reserpine (1 mg/kg, i.p., 24 hr) (Fig. 48). This suggests that the sympathectomy by 6OHDA treatment in vitro is produced by destruction of the nerve terminals or at least by depletion of neuronal NA coupled with complete blockade of the neuronal NA uptake mechanism.

Fig.49   RESPONSES OF CAT ANOCOCCYGEUS TO NA AND Adr. EFFECT OF PHENTOLAMINE
AND COMPARISON WITH THE RAT ANOCOCCYGEUS.
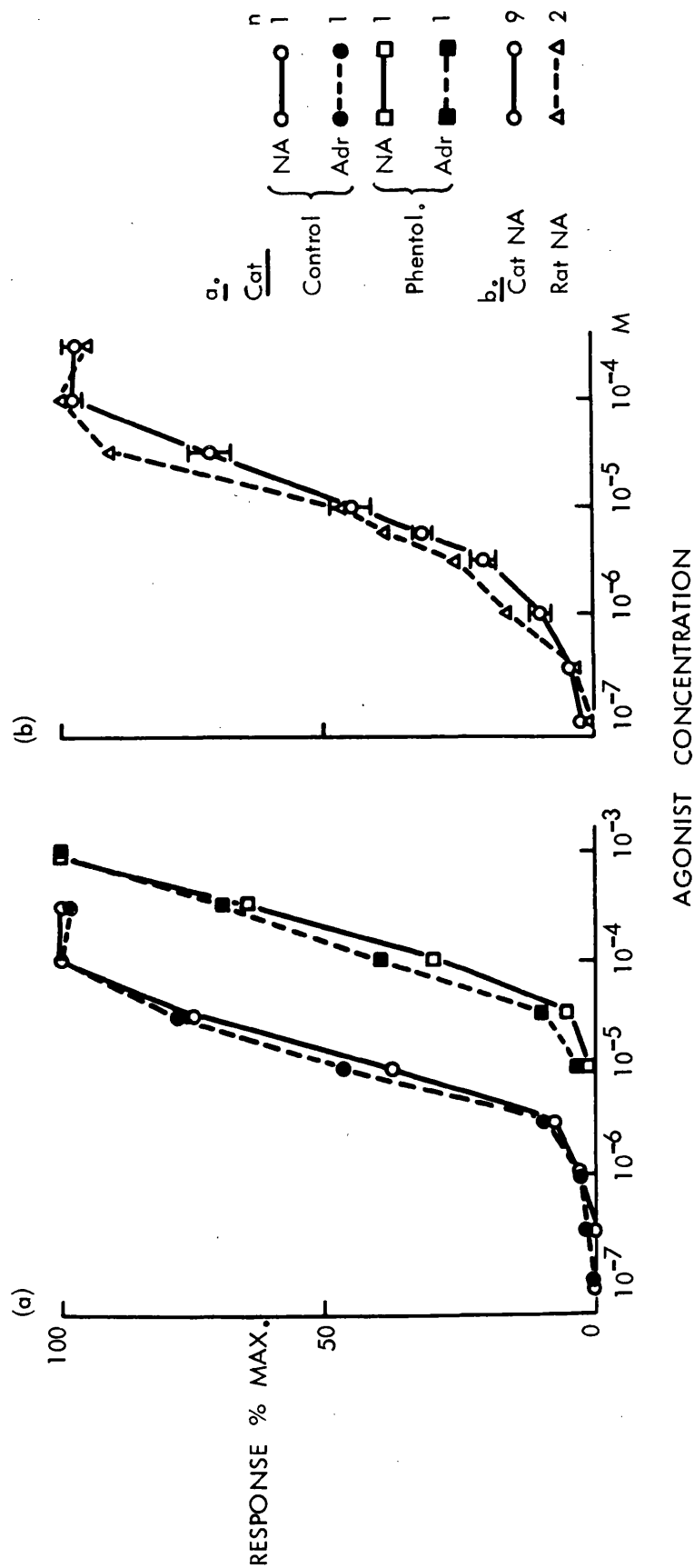
Cat and rat anococcygeus muscles recorded in vitro .

(a)   Cat.   Effect of phentolamine on responses to NA and Adr in a single muscle.

(b)   Comparison of effects of NA in cat and rat.
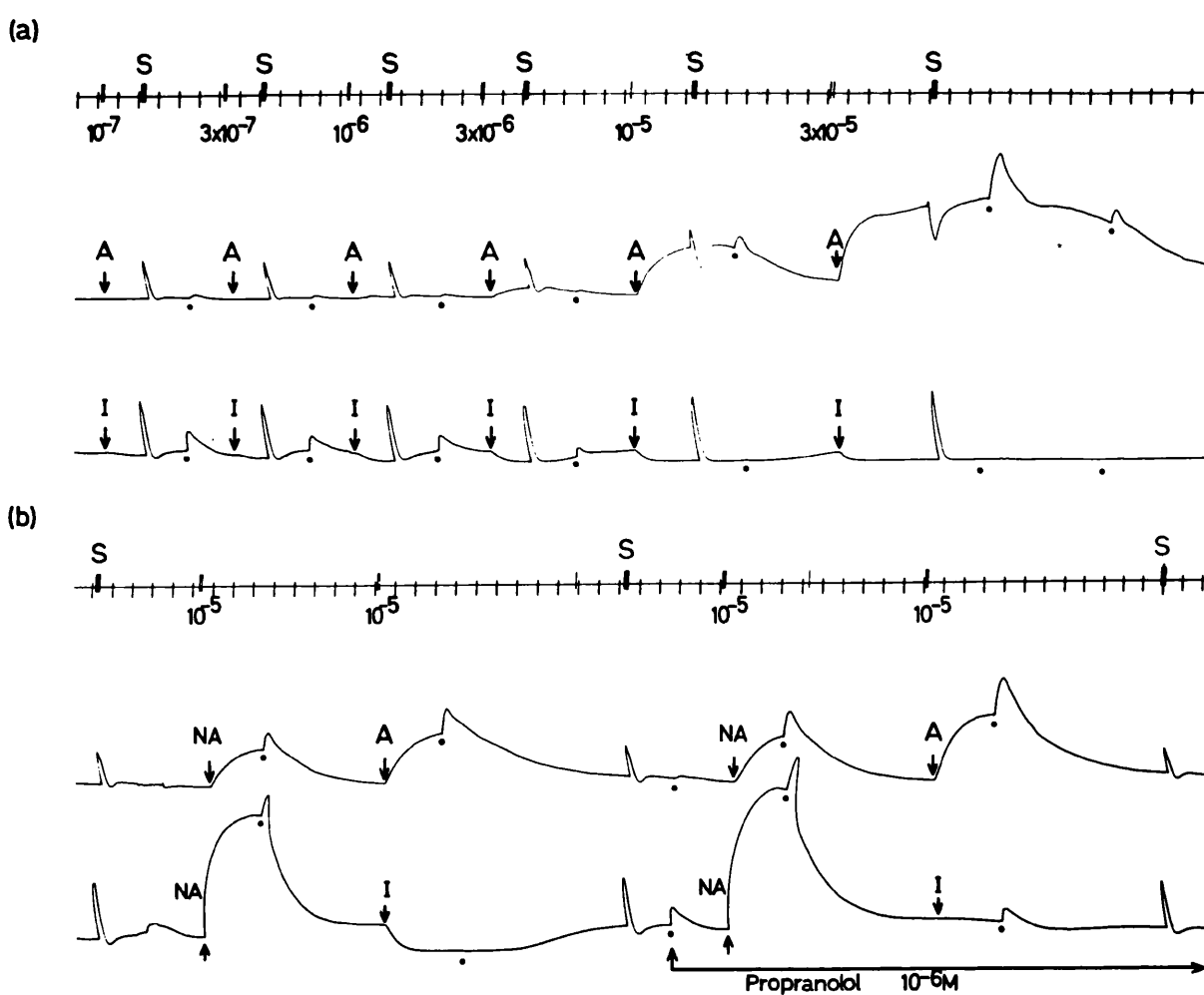
Fig. 50   Responses of the cat anococcygeus muscles <u>in vitro</u> to NA, adrenaline, (A), and isoprenaline (I), and the effect on these of propranolol.

(a)   Contralateral cat anococcygeus muscles.   Upper trace shows responses of one muscle to adrenaline, lower trace of the other muscle to isoprenaline, added simultaneously at the concentrations indicated below the time trace (10^{-7} M - 3×10^{-5} M).   The muscles were also field stimulated at 10 Hz for 10 sec periods at S.   In the upper trace, as adrenaline raised the tone of the muscle, the motor response to field stimulation showed a secondary inhibitory component.   In the lower trace, however, isoprenaline lowered the muscle tone and the motor response to field stimulation was unaffected.

(b)   A continuation of the trace in (a).   Control responses were obtained to 10^{-5} M doses of NA and A in the upper muscle and NA and I in the lower muscle.   These responses were then repeated in the presence of propranolol 10^{-6} M.   The responses to NA and A were potentiated and responses to I abolished by propranolol.   Again responses to field stimulation were obtained and these were unaffected by propranolol. Wash at dots.   Time 1 min.

After this sympathectomy, it was possible to demonstrate whether drugs were acting directly on the muscle or as indirect sympathomimetics, since the latter were no longer effective.

In the case of the rat it was possible to compare these in vitro effects of 6OHDA with the sympathectomy produced by pretreatment in vivo with the regimen; day 1 - 2 x 50 mg/kg i.p., day 4 - 2 x 100 mg/kg i.p., day 5 - tissues used.

The effects of 6OHDA on responses to field stimulation and to drugs are discussed in the appropriate sections.

Drugs causing contraction in both species

Direct sympathomimetics   Both NA and Adr produced dose - related contractions of the cat anococcygeus.   Their potency and the effect of antagonists were examined by constructing dose / response curves (Fig. 49).   Adr and NA were approximately equipotent (Fig. 49a).   Phentolamine (10^{-6} M) produced an equal and parallel shift to the right of both curves equal to a twelvefold increase in dose (Fig. 49a).   The sensitivity of the cat and rat anococcygeus to NA was almost identical (Fig. 49b).   Responses in the cat muscle to NA were unaffected by methysergide (2 x 10^{-7} M) (Fig. 51) or atropine (10^{-6} M).   Responses to Adr and to a lesser extent NA were potentiated by propranolol (10^{-6} M) (Fig. 50).   Isoprenaline did not produce contraction of the cat anococcygeus at any dose but at all doses tested from 10^{-7} M to 3 x 10^{-5} M produced relaxations which were abolished by propranolol (Fig. 50).

Responses in the cat anococcygeus to NA were potentiated by the presence of cocaine (3 x 10^{-5} M) in the organ bath.   The dose / response curve to NA was shifted to the left (Fig. 51) and responses were prolonged, returning to baseline more slowly after washout (Fig. 53).

Fig.51 RESPONSES TO DIRECT SYMPATHOMIMETICS OF THE CAT
ANOCOCCYGEUS. EFFECTS OF PHENTOLAMINE, COCAINE
AND METHYSERGIDE.
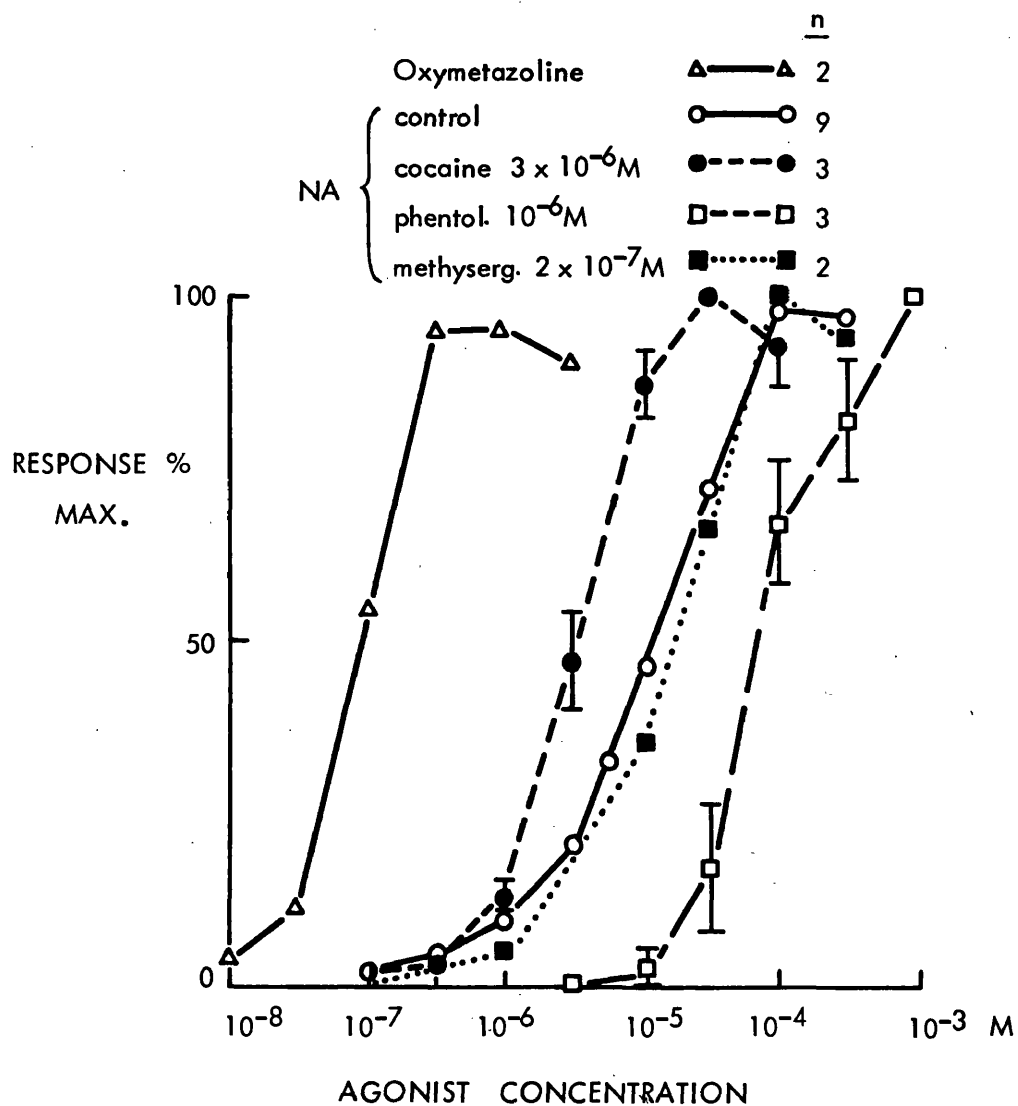
Cat anococcygeus recorded in vitro .

Fig.52 RESPONSES OF CAT AND RAT ANOCOCCYGEUS TO NA AND 5 HT.
EFFECT OF 6 OHDA TREATMENT
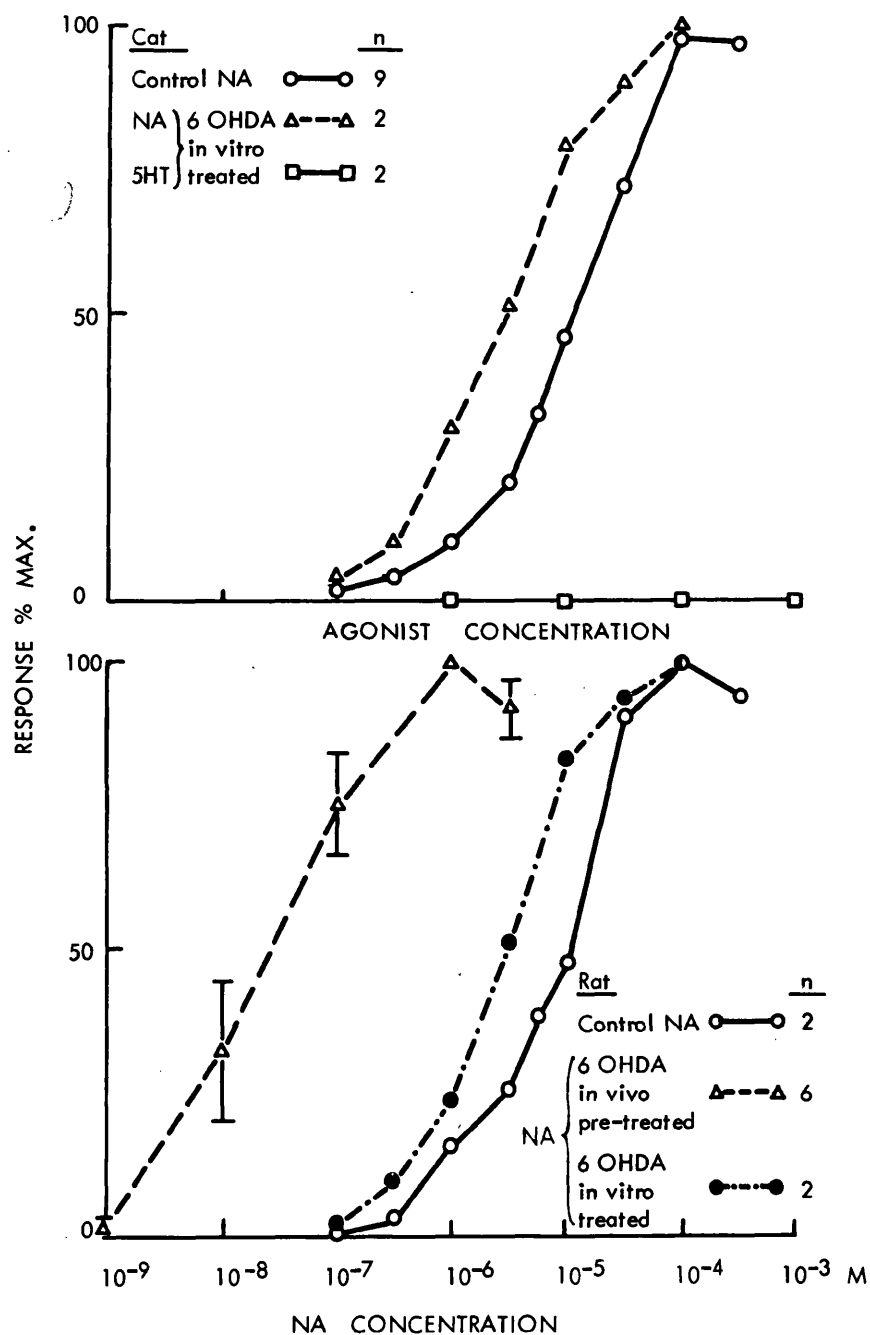
Cat and rat anococcygeus muscles recorded <u>in vitro</u> .

Upper graph - cat. Effect of 6OHDA <u>in vitro</u> treatment
on responses to NA and 5HT.

Lower graph - rat. Effect of 6OHDA <u>in vitro</u> and
<u>in vivo</u> treatment on responses to NA.

Fig.53 EFFECT OF 6 OHDA OR COCAINE ON THE RETURN OF TENSION
TO BASELINE AFTER WASH-OUT OF NA ON THE CAT ANOCOCCYGEUS.
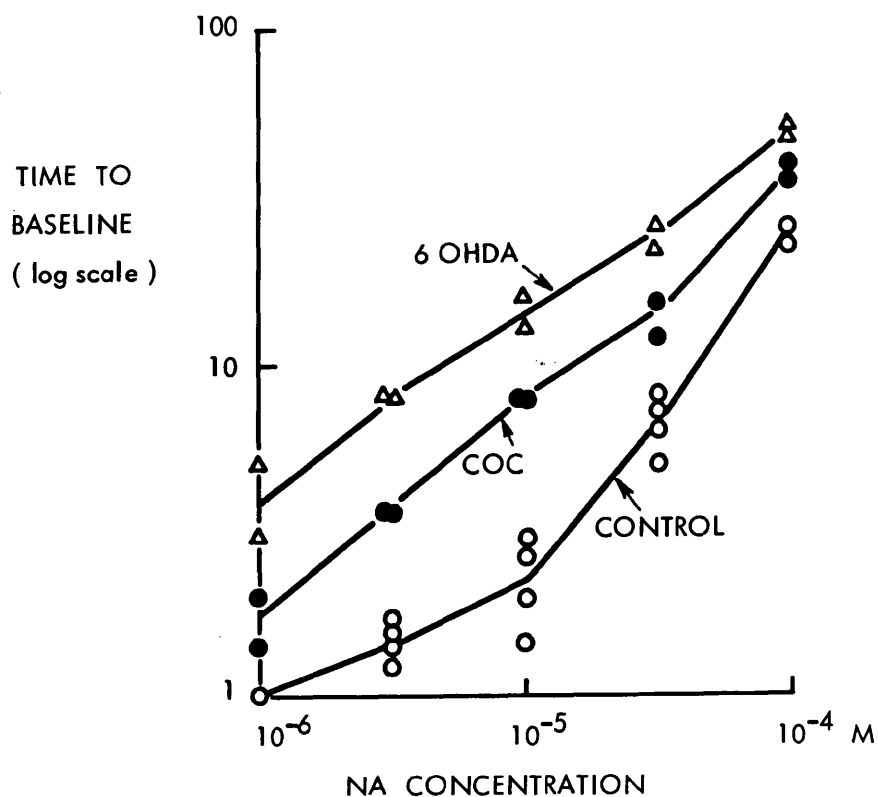
Cat anococcygeus muscles recorded in vitro.

Time elapsing between washout of NA and return to pre - NA

baseline is expressed for doses of NA indicated. Open

circles represent control muscles. Closed cirles

represent muscles given NA in the presence of cocaine

3×10^{-5} M . Open triangles represent muscles pretreated

in vitro with 6OHDA.

Fig.54   RESPONSES OF THE CAT AND RAT ANOCOCCYGEUS TO 5 HT
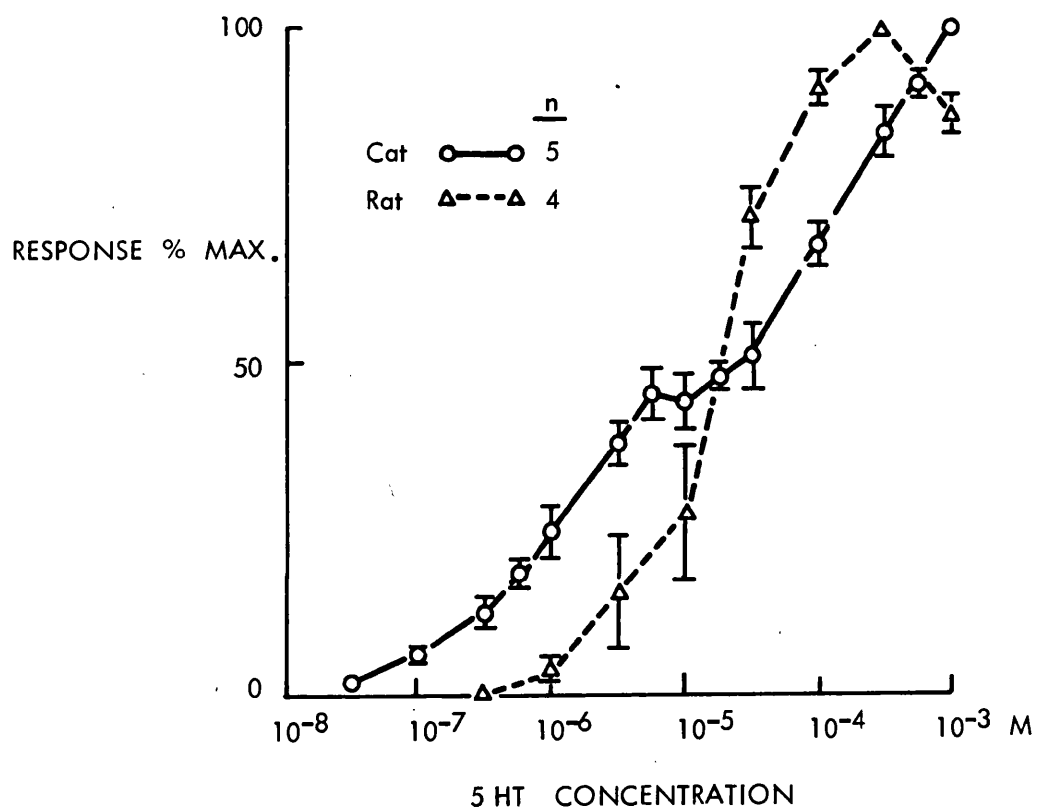
Anococcygeus muscles recorded in vitro .

After treatment of the muscle for 2 hr with 6OHDA (250 μg/ml), responses to NA were potentiated in a manner similar to that after cocaine. The dose / response curve to NA was shifted to the left (Fig. 52a) and the response was prolonged after NA washout (Fig. 53). This 6OHDA treatment in vitro produced a sympathectomy of the anococcygeus similar to that found after pretreatment in vivo but did not produce as great an increase in sensitivity to NA as the latter, as shown for the rat in Fig. 52b.

The cat anococcygeus was approximately x 200 more sensitive to oxymetazoline, an α- receptor agonist which is not taken up into adrenergic nerves (Mujic & Von Rossum, 1965), than to NA (Fig. 51). Together with the effects of cocaine and 6OHDA treatment, this suggests a role for neuronal uptake in determining the response to NA in this tissue.

The cat anococcygeus thus contracts in the presence of α - adrenergic agonists. This contraction is potentiated if the agonist is prevented from being taken up into adrenergic nerves and, in the case of agonists which also have β - adrenergic effects, is potentiated by blockade of β - receptors. These properties are similar to those of the rat anococcygeus except that in the rat no β - receptor effects were found (Gillespie, 1972).

5 - hydroxytryptamine (5HT) produced dose - related contractions of the cat anococcygeus. The cat anococcygeus was more sensitive to low doses of 5HT than was the rat anococcygeus (Fig. 54) and the log dose / response curve to 5HT of the former showed an inflection at 10^{-5} M. Beyond this inflection, the response to 5HT increased with increasing dose up to 10^{-3} M, which was the highest dose tested. Even the response to 10^{-3} M was, however, still lower than the maximum response to NA (Fig. 55) so it is likely that the theoretical maximum response to 5HT could not be produced until doses higher than 10^{-3} M.

Fig.56   RESPONSE OF CAT ANOCOCCYGEUS TO 5HT. EFFECTS OF PHENTOLAMINE
AND METHYSERGIDE.
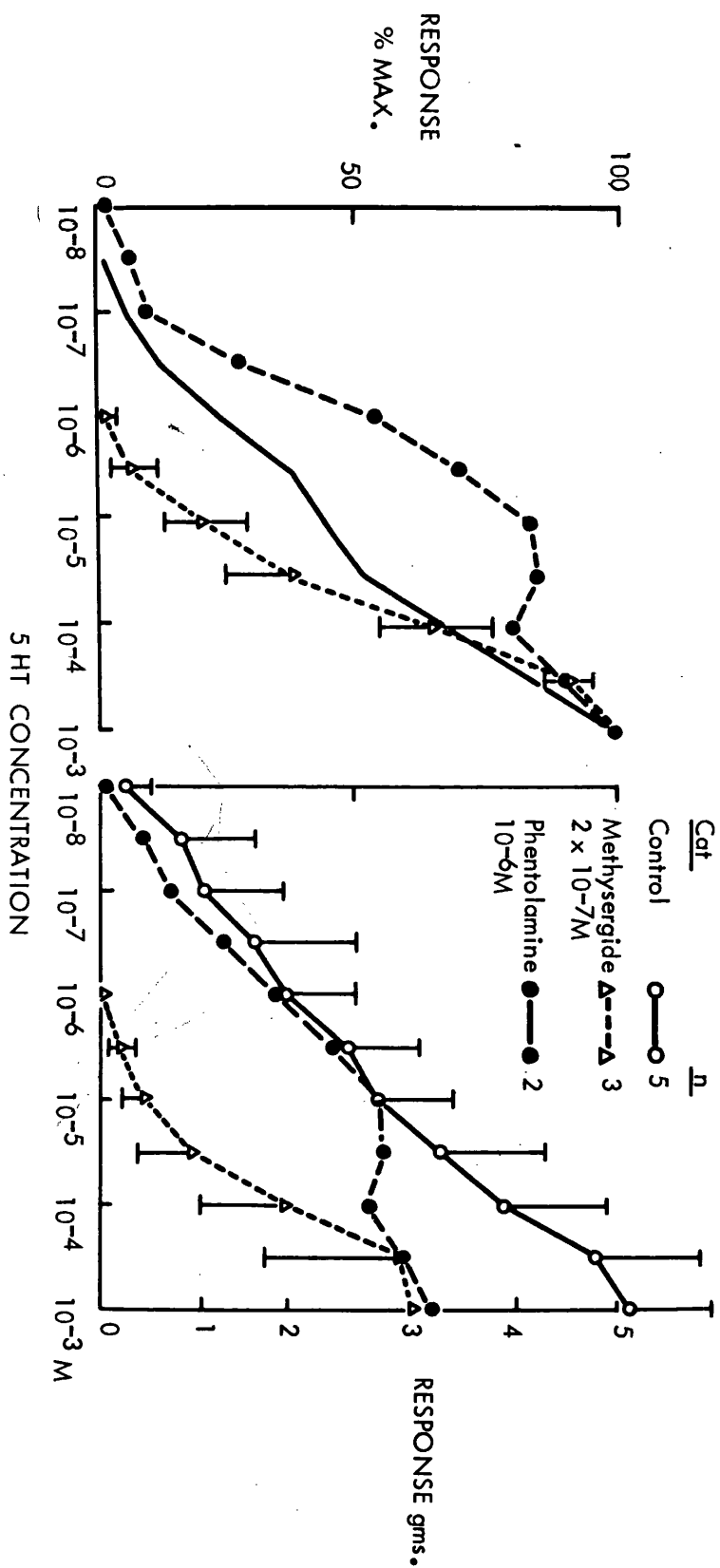
Cat anococcygeus muscles recorded in vitro .

Dose / response curve to 5HT.   Effect of phentolamine and methysergide.

Left   –   responses expressed as a percentage of the response obtained at 10^{-3} M.

Right   –   responses expressed in gms tension.
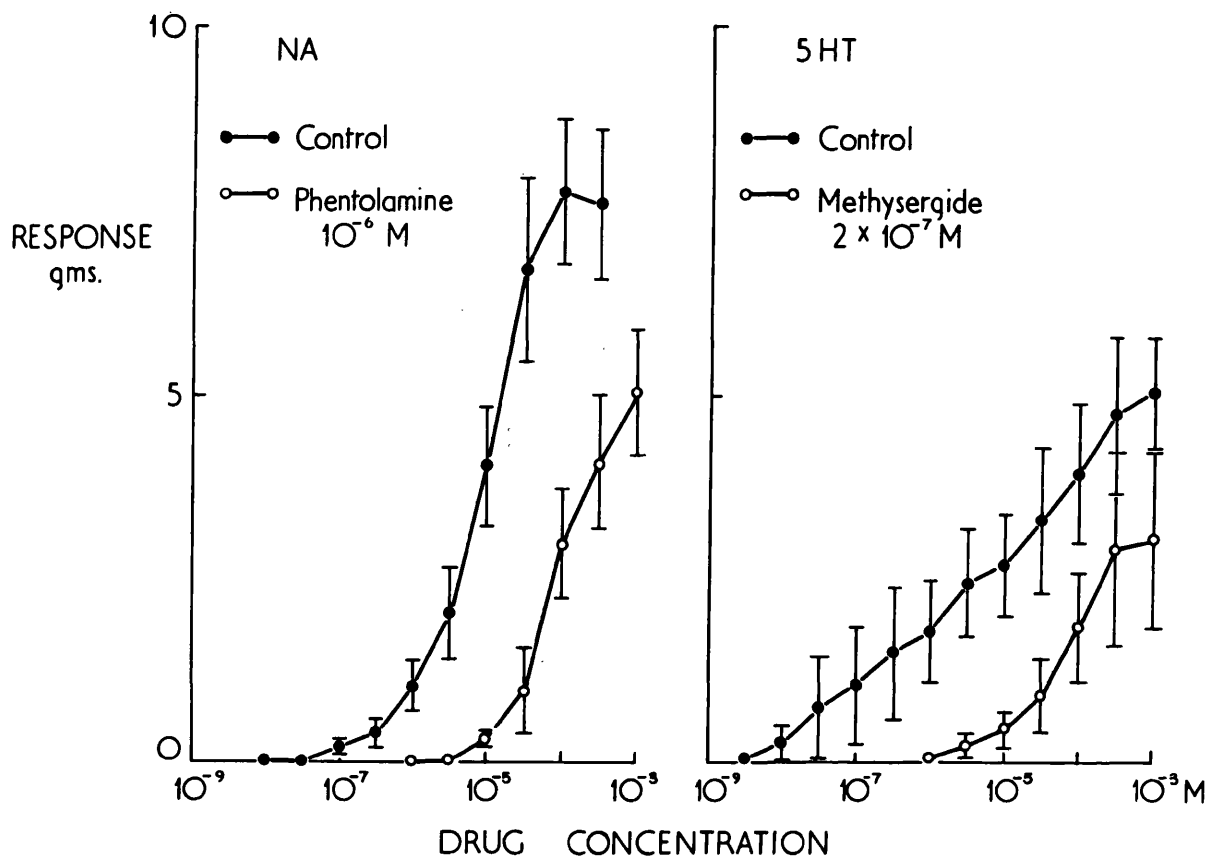
Fig. 55   Dose - response curves of the cat anococcygeus

to Noradrenaline (NA) and 5 - hydroxytryptamine (5 - HT) and the

effect of phentolamine 10^{-6} M and methysergide 2 x 10^{-7} M.

●————● Control.   ○————○ phentolamine or

methysergide.   Bars represent $\pm$ S.E.M.   Muscles from male and

female cats.

For construction of log dose / percentage maximum response curves, therefore, the response to 10^{-3} M was considered the maximum.

Analysis of the response to 5HT using antagonists proved more complex than in the case of NA. Methysergide (2×10^{-7} M), which did not affect sensitivity to NA (Fig. 51), increased the threshold to 5HT of the cat anococcygeus by a factor of 100 and depressed the size of contractions to higher doses (Figs. 55 & 56b). If this result is plotted as a log dose / percentage maximum response curve, however, the control and "inhibited" curves are not parallel (Fig. 56a). Three possible explanations for this lack of parallelism are (1) Block is non - competitive (2) The complex nature of the control curve represents the participation of 5HT at more than one site of action and this introduces complexity which makes analysis by blocking agents difficult (3) These log dose / percentage maximum response curves are not valid since the true maximum is not known.

Further evidence arises from the effect of phentolamine on the 5HT response. Phentolamine (10^{-6} M) did not affect the response of the cat anococcygeus to low doses of 5HT but inhibited the response to high doses (Fig. 56b). If an attempt is made to express this result as a log dose / percentage maximum response curve the result is again confusing (Fig. 56a). It is, however, clear that the response of the cat anococcygeus to 5HT is inhibited by both methysergide and phentolamine; at doses below 10^{-5} M by methysergide only and at doses above 10^{-5} M by both methysergide and phentolamine.

Since the response of the cat anococcygeus to 5HT has an inflection on the dose / response curve at 10^{-5} M and the contractions to doses on either side of 10^{-5} M show different susceptibility to antagonists, it was considered possible that the response might consist of two components; one to low doses and the other to high doses. Furthermore, since phentolamine inhibited part

Fig.57 RESPONSE OF RAT ANOCOCCYGEUS TO 5 HT. EFFECTS OF
6 OHDA TREATMENT, PHENTOLAMINE AND METHYSERGIDE
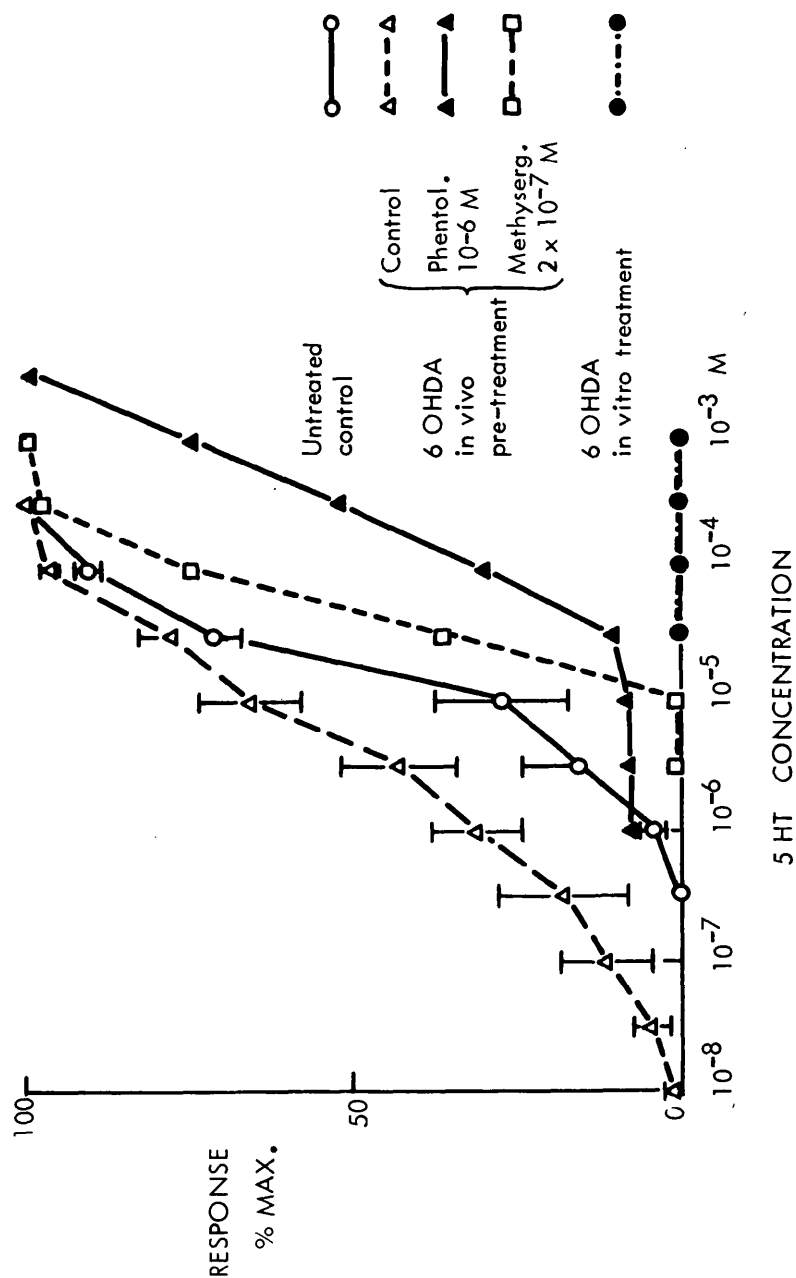
Rat anococcygeus muscles in vitro .

Dose / response curve to 5HT.

(a)

Control

10^{-6}M
5HT

3×10^{-6}M
5HT

10^{-5}M
5HT

1min

in vivo
6OHDA
treated

(b)

Phentolamine 10^{-6}M

3×10^{-4}M
5HT

3×10^{-4}M
5HT
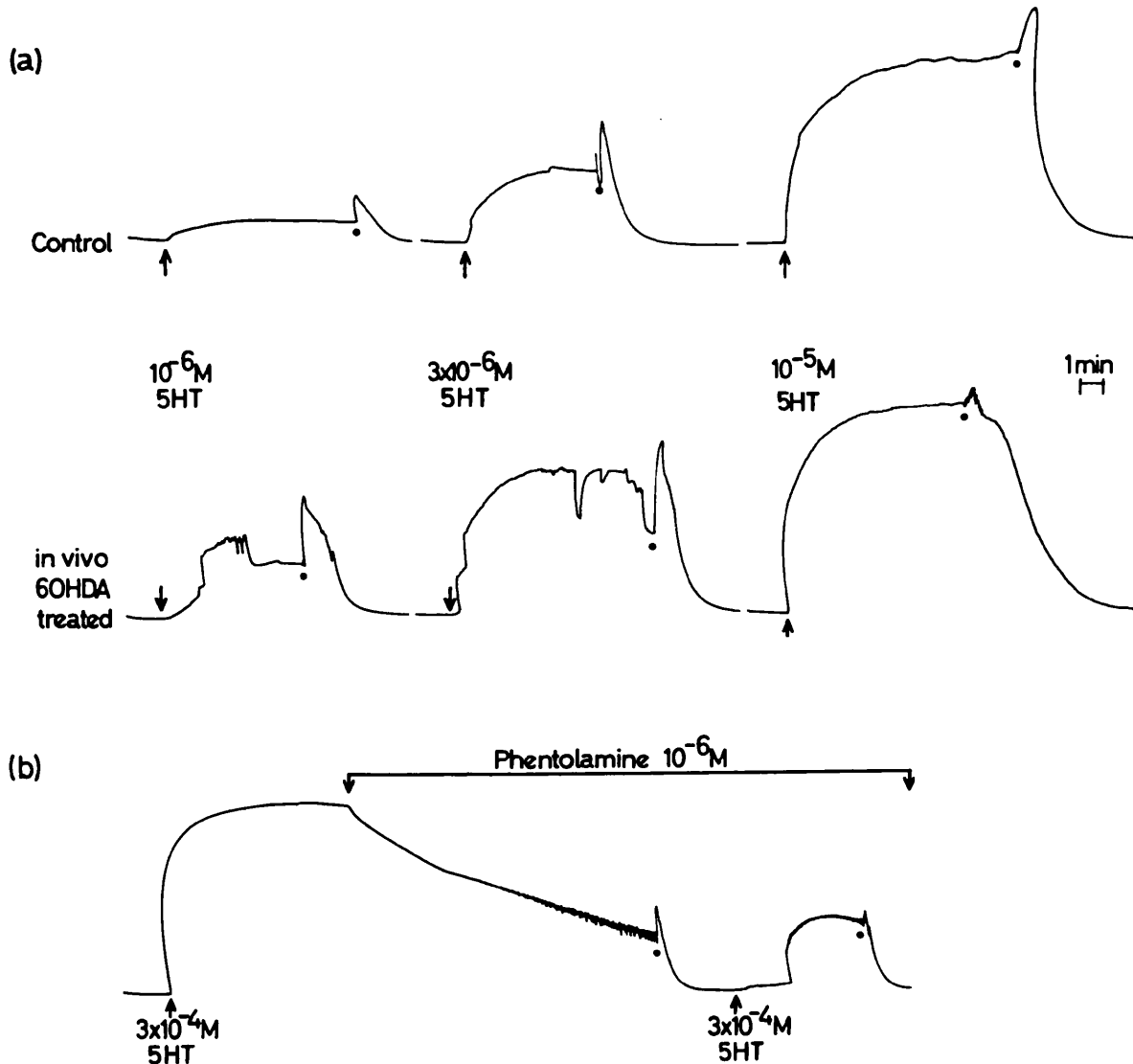
Fig. 58   Responses to 5HT of the rat anococcygeus muscles
in vitro.

(a)   The control responses to 5HT of a rat anococcygeus
muscle are compared with the responses to similar doses in a
muscle from a rat pretreated in vivo with 6OHDA.   The responses
in the control are smooth contractions which start immediately on
addition of 5 HT to the bath.   After 6OHDA treatment, the response
at low doses is slow to start, becoming faster but irregular.   As
the dose increases the contraction becomes faster.   Wash at dots.

(b)   Phentolamine 10^{-6} M added to the bath at the peak of a
contraction to 5HT 3 x 10^{-4} M reduces the 5HT tone.   In the
continued presence of phentolamine a subsequent dose of 5HT produced
a reduced response which developed in a manner similar to a low dose.

of the response, it was possible that 5HT was acting on α - receptors either directly or indirectly by release of NA from nerves. To test these two latter hypotheses, the effect of 5HT was examined on muscles which had been treated in vitro with 6OHDA.

Cat anococcygeus muscles which were exposed to 6OHDA (250 μg/ml) in vitro for 2 hr showed no response to 5HT at any dose up to 10^{-3} M, despite being more sensitive to NA than before 6OHDA (Fig. 52a). A similar result was found for rat anococcygeus exposed to 6OHDA in vitro for 2 hr (Fig. 57). This suggests that the effect of 5HT in both species is by an indirect sympathomimetic action. On the other hand, anococcygeus muscles from rats pretreated with 6OHDA showed a high sensitivity to 5HT (Fig. 57).

These responses to 5HT in the rat anococcygeus after in vivo 6OHDA pretreatment were, however, different in character from the responses in control tissues (Fig. 58a); in control tissues, low doses of 5HT produced small contractions which were sometimes irregular but reached a plateau within 3 min; after 6OHDA pretreatment, a delayed larger contraction appeared in addition to this control response - with increasing dose, the length of the delay diminished until at 10^{-5} M it had disappeared. (Occasionally a response such as this appeared to a low dose of 5HT in a control muscle.) Similar delayed responses were obtained in the rat anococcygeus treated with 6OHDA in vivo and subsequently tested with tyramine, guanethidine, LSD and cocaine (Fig. 61b) Untreated muscles from both species also showed these responses to low doses of LSD (Fig. 61a).

It may, therefore, be that 5HT does act as an indirect sympathomimetic as shown by the effect of sympathectomy in vitro with 6OHDA and that after in vivo 6OHDA treatment, indirect sympathomimetics, including 5HT, can still act due to a combination of incomplete sympathectomy and increased sensitivity to NA.

This hypothesis is supported by the effects of the blocking drugs phentolamine and methysergide which both inhibit the responses to 5HT after 6OHDA _in vivo_ pretreatment (Fig. 57). After phentolamine, in addition to the height of the response being reduced, the shape of the response to a high dose of 5HT is changed to that of a low dose (Fig. 58b).

The effect of 5HT on the cat anococcygeus is thus to produce contraction by an indirect sympathomimetic action since (a) 6OHDA treatment _in vitro_ abolishes the effect of 5HT but potentiates the effect of NA (b) methysergide inhibits responses to 5HT but not to NA (c) phentolamine inhibits responses to NA and 5HT. The apparent two - stage nature of the dose / response curve to 5HT cannot be fully explained.

These results suggest a mechanism of action for 5HT - 5HT releases NA from nerve varicosities by a methysergide - sensitive mechanism; NA diffuses across the synaptic gap to act on the smooth muscle membrane by a phentolamine - sensitive mechanism.

The apparent resistance to phentolamine of the response to low doses of 5HT could be explained if low doses of 5HT produce their action by releasing only sufficient NA to act on the muscle in the immediate vicinity of the nerves, but high doses can release sufficient NA to diffuse away to act on more distant receptor sites on the muscle and thus produce an additional component to the response. Since the response to NA added to the bath is more easily depressed by phentolamine than is the motor nerve response (Fig. 64), then by analogy the response to NA released from nerves but acting at distant sites might be more easily depressed than the response to a high concentration of NA in the synaptic region.

This hypothesis would also explain the complex two phase nature of the dose / response curve to 5HT; the first phase representing the effect of NA released
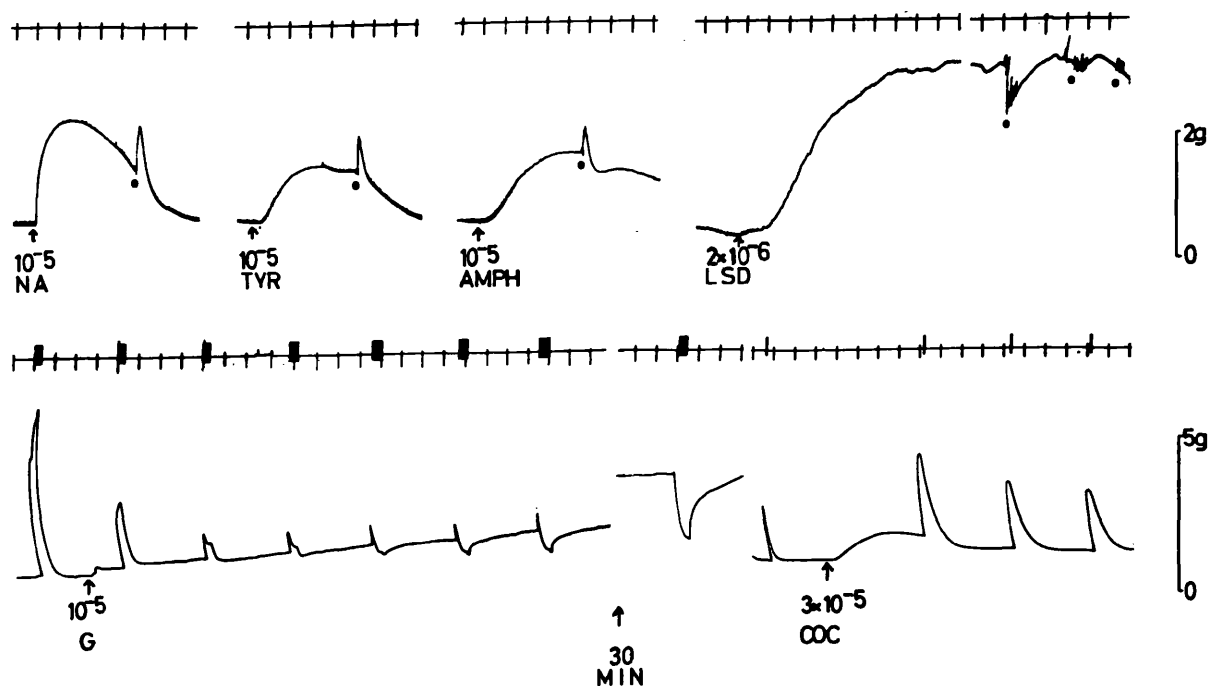
Fig. 59   The effect on the cat anococcygeus of a series of indirect sympathomimetics compared with the action of Noradrenaline (NA). Noradrenaline 10^{-5} M produced a rapid motor response. Tyramine (TYR) 10^{-5} M, dexamphetamine (AMPH) 10^{-5} M and lysergic acid diethylamide (LSD) 2×10^{-6} M produced responses slower in onset. Responses to LSD were persistent even after several washes. Wash at dots. Guanethidine (G) 10^{-5} M raised the tone slowly, reaching its maximum after 40 min and reversed the response to field stimulation (10 Hz, 20 sec) from motor to inhibitory. Cocaine (COC) 3×10^{-5} M produced a motor response and prolonged and potentiated the motor response to field stimulation (10 Hz, 1 sec). Time 1 min.

Fig.60 RESPONSES OF THE CAT ANOCOCCYGEUS TO INDIRECT SYMPATHOMIMETICS.
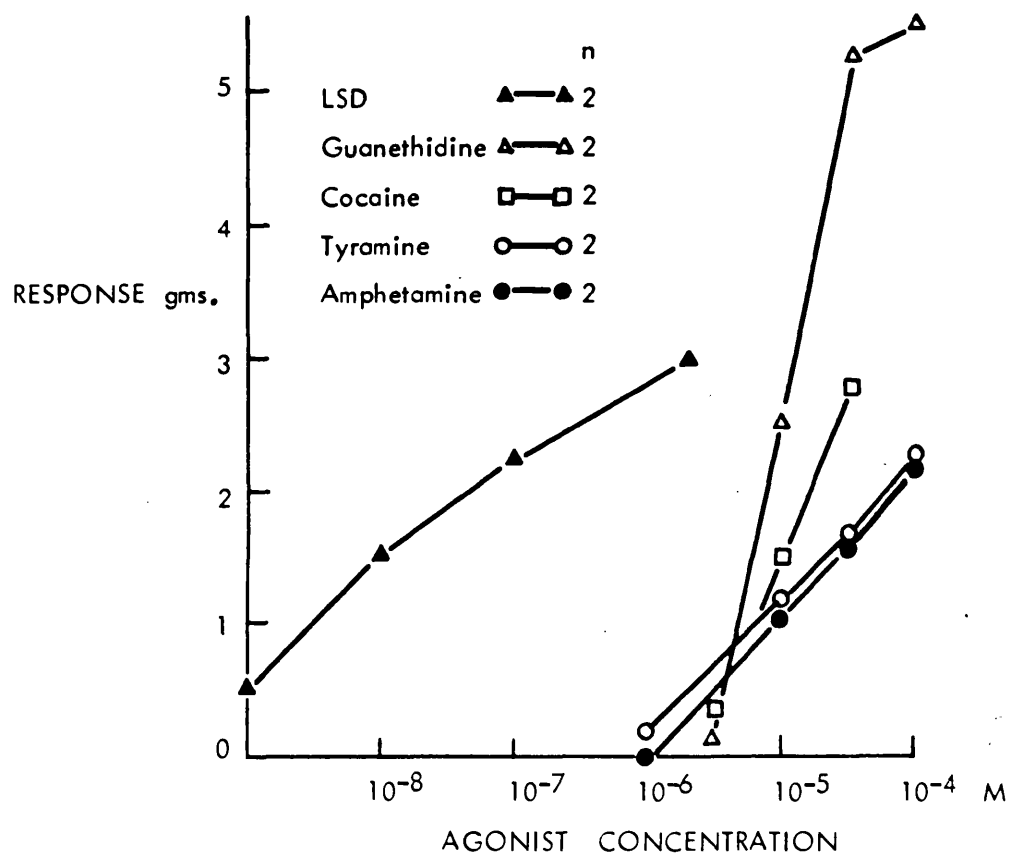
Cat anococcygeus muscles in vitro .

Note that these responses were obtained on

different muscles for each drug so that the

absolute tensions on their own do not reflect

the relative potency between agonists.

into the immediate vicinity of the nerves, this response being maximal by 10^{-5} M; and a second phase due to diffusion of NA to more distant regions of the muscle. The two phase contraction of the muscle to other indirect sympathomimetics under various conditions might also be accounted for in this system.

The rat anococcygeus is less sensitive than the cat muscle to the action of low doses of 5HT, but otherwise the response is essentially similar being due to the release of NA by 5HT. The _in vivo_ pretreatment with 6OHDA which was employed did not produce a sufficient sympathectomy to prevent the action of 5HT.

Indirect sympathomimetics   Tyramine, amphetamine, cocaine, guanethidine and LSD all produced contractions of the cat anococcygeus (Figs. 59 & 60) which were reduced or abolished by phentolamine (10^{-6} M). One distinguishing feature was the rate of development of the response. The contractions to tyramine, amphetamine and cocaine were complete within 5 min whereas guanethidine required up to 30 min to develop its full motor effects, even though its blocking effect on the adrenergic motor response developed rapidly and the final tension achieved was equal to that of the other drugs. LSD on the other hand often showed a two phase response at low doses; a slow gradual contraction, followed by a delayed more rapid contraction (Fig. 59). All five of these drugs, however, showed some delay before the response at low and moderate doses, in contrast to the immediate direct contraction produced by NA (Fig. 59). The length of this delay shortened with increase in dose till at high doses an immediate contraction was found. Even guanethidine at high doses produced a rapid contraction (Fig. 67). These drugs, therefore, at low doses showed to some degree the type of two phase response found to 5HT in the anococcygeus of the 6OHDA pretreated rat. LSD at the one extreme

(a)

CAT

Control

in vitro
6OHDA
treated

Tyr   LSD   Coc

RAT

Control

in vitro
6OHDA
treated

Tyr   LSD   Coc   NA

field
stim

(b)

RAT

in vivo
6OHDA
treated

Tyr   LSD   Coc   NA   Guan
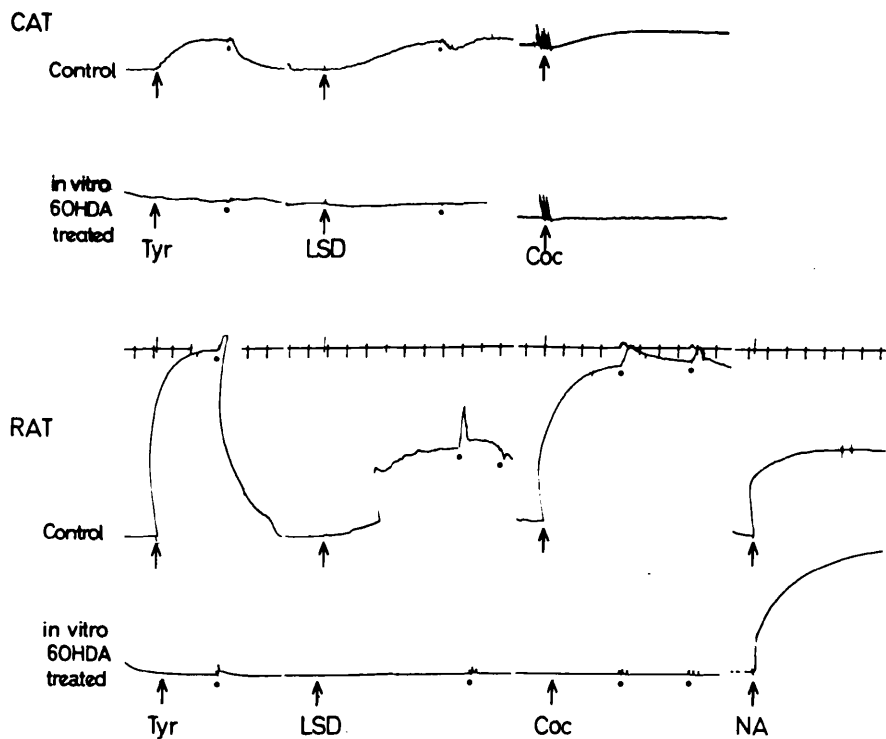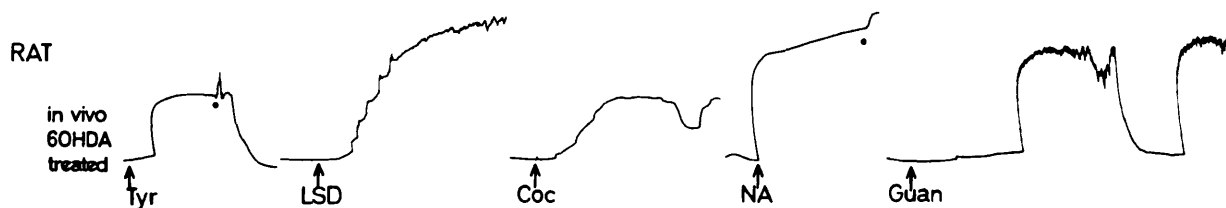
Fig. 61   Effect of 6OHDA treatment on drugs producing
sympathomimetic responses in the anococcygeus muscles in vitro.

(a)   6OHDA in vitro treatment.
Cat anococcygeus muscles. A control muscle is compared with the
contralateral muscle which has been incubated with 6OHDA 250 ug/ml
for 2 hrs. The control muscle shows responses to tyramine 10^{-5} M,
LSD 2 x 10^{-8} M, cocaine 10^{-5} M whereas the 6OHDA treated muscle
shows no response to any of these.
Rat anococcygeus muscles. A control muscle is compared with the
contralateral muscle which has been incubated with 6OHDA 250 ug/ml
for 2 hrs. The control muscle shows responses to tyramine 10^{-5} M,
LSD 2 x 10^{-9} M, cocaine 5 x 10^{-5} M and NA 10^{-7} M whereas the 6OHDA
treated muscle shows a response only to NA.   Wash at dots. Time 1 min.

(b)   6OHDA in vivo pretreatment.
Anococcygeus muscle from a rat pretreated with 6OHDA shows delayed
responses to tyramine 10^{-5} M, LSD 2 x 10^{-8} M, cocaine 5 x 10^{-5} M
and guanethidine 3 x 10^{-5} M but an immediate response to NA 10^{-5} M.
Compare this with the control muscle in (a).   When the tone was
raised with guanethidine, field stimulation at 10 Hz produced almost
complete abolition of the tone.   Wash at dots.   Time 1 min.

showed a slow contraction followed after a delay of up to several minutes by a rapid contraction, tyramine, amphetamine and cocaine showed a short period of slow contraction followed by a more rapid contraction and guanethidine showed only a slow, steady contraction.

After washout, the effects of these drugs were slow to disappear compared with the rapid return to baseline after washing out NA. The effect of LSD was particularly persistent, taking up to 45 min to disappear, but the effects of the others were also slow to wear off; in decreasing order of persistence - LSD, guanethidine, cocaine, amphetamine and tyramine.

In the rat anococcygeus, tyramine, amphetamine, guanethidine, LSD and cocaine all produced contraction in doses similar to those for the cat. Responses in the rat were in general rapid monophasic contractions apart from those to LSD and cocaine which showed an initial slow followed by a later more rapid contraction.

In both species, after 6OHDA (250 μg/ml, 2 hr) treatment _in vitro_, the responses to tyramine, guanethidine, LSD and cocaine were completely absent (Fig. 61a). This suggests that each of these drugs is acting by an indirect sympathomimetic action. In the anococcygeus muscles from rats pretreated _in vivo_ with 6OHDA, responses could be obtained to high doses of tyramine, guanethidine, cocaine and LSD. Unfortunately, dose / response curves were not obtained so the sensitivity change cannot be quantified. These responses had a characteristic shape; a slow gradual contraction followed by a more rapid contraction (Fig. 61b). This shape is similar to that of the control response to low doses of LSD and to the response to 5HT after 6OHDA _in vivo_ pretreatment. These responses to tyramine, guanethidine, cocaine, LSD and 5HT, remaining after 6OHDA _in vivo_ pretreatment, were abolished or reduced by phentolamine (10^{-6} M).

Fig.62 EFFECTS OF DRUGS WHICH RELAX THE ANOCOCCYGEUS
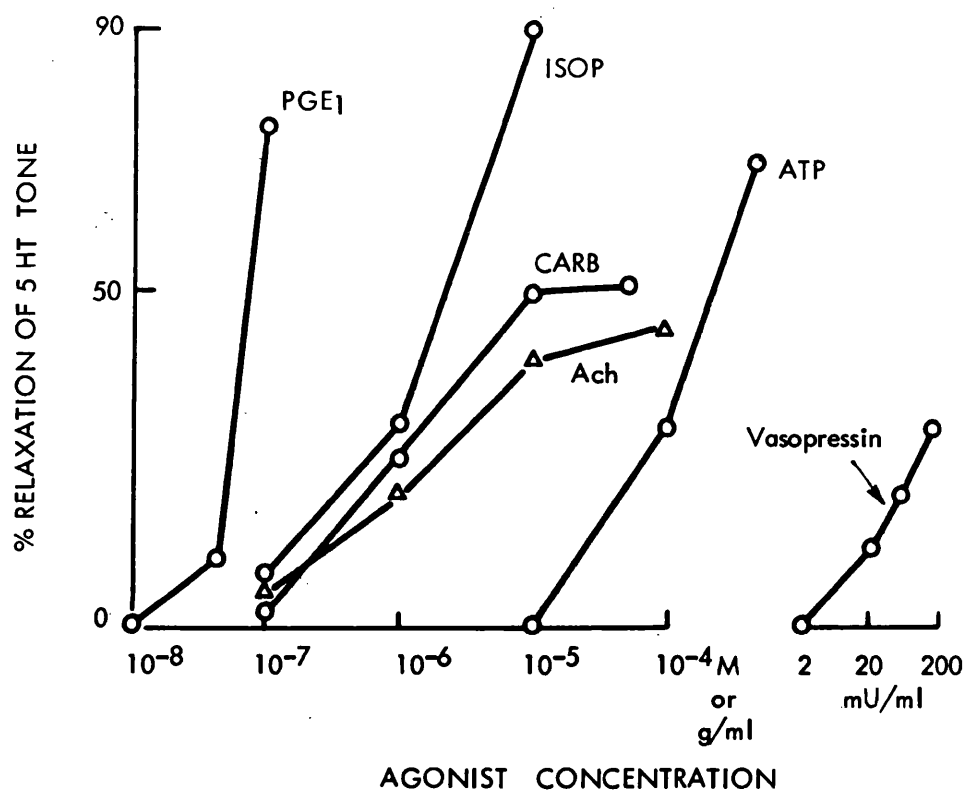
Cat anococcygeus muscles in vitro. Relaxation

by drugs of the tone produced by 5HT 10^{-5} M.

All concentrations expressed as molar except

vasopressin (m U) and PGE_1 (g / ml).

RAT

CAT

10^{-7} 10^{-6} 10^{-5} 10^{-6} 10^{-5} 10^{-4} 5×10^{-4} 10^{-7} 10^{-6} 10^{-5} 10^{-8} 5×10^{-8} 2g

0

2g

10^{-7} 10^{-6} 10^{-5} 5×10^{-5} 10^{-6} 10^{-5} 10^{-4} 5×10^{-4} 10^{-6} 10^{-5} 10^{-8} 5×10^{-8} 0

CARBACHOL   ATP   ISOPRENALINE   PGE$_1$
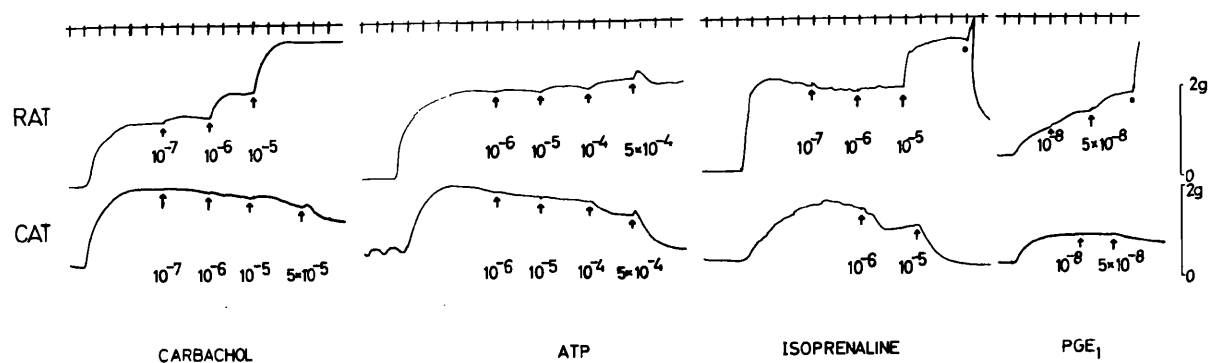
Fig. 63   Comparison of the effects of Carbachol, ATP,

Isoprenaline and PGE$_1$ on the rat and cat anococcygeus.   In

order to detect inhibitory responses, the tone of the muscles was

first raised with 5HT.   Since the cat is more sensitive to 5HT

the dose of 5HT given was 10^{-7} M compared with 10^{-5} M for rats.   In

the final rat trace Carbachol 10^{-6} M replaced 5HT for this

purpose.   All four substances contracted the rat muscle but

relaxed the cat muscle.   Time 1 min.   Concentrations shown are

molar for Carbachol, ATP and Isoprenaline, but g/ml for PGE$_1$.

It is thus concluded that tyramine, guanethidine, cocaine, LSD and 5HT all act
in the anococcygeus of both rat and cat as indirect sympathomimetics and that
the sympathectomy produced by _in vivo_ pretreatment of rats with 6OHDA was
insufficient to prevent this effect. The present experiments do not,
however, define the sites of action in the adrenergic nerve endings for this
indirect sympathomimetic effect. All six indirect sympathomimetics tested,
however, exhibited cross tachyphylaxis, suggesting a common mechanism of
action.

Histamine produced a small contraction of the cat anococcygeus muscle.
This response was, as in the rat, less than 5% of the maximum response
produced by NA, was seen only at concentrations of the drug of 10^{-4} M or
more, exhibited marked tachyphylaxis and was completely prevented by
mepyramine (10^{-6} M). Mepyramine (10^{-6} M) had no effect on the response to NA
in either species.

Drugs causing inhibition in the cat anococcygeus

A major difference between the pharmacology of the cat and rat anococcygeus
was the number of drugs causing inhibition in the cat. In the rat, no drug
capable of causing inhibition was found. In the cat, acetylcholine,
carbachol, isoprenaline, ATP, prostaglandins E_1, E_2 & $F_{2\alpha}$ and vasopressin,
all of which were motor in the rat, produced relaxation (Fig. 62). Because
of the presence of spontaneous tone, this inhibitory effect could be
demonstrated directly on the cat muscle, but the response was more dramatic if
tone was first raised by the addition of 5HT, tyramine or guanethidine. Fig. 63
contrasts this inhibitory effect on the cat anococcygeus with a simultaneous
motor action on the rat for carbachol, isoprenaline, ATP and PGE_1.
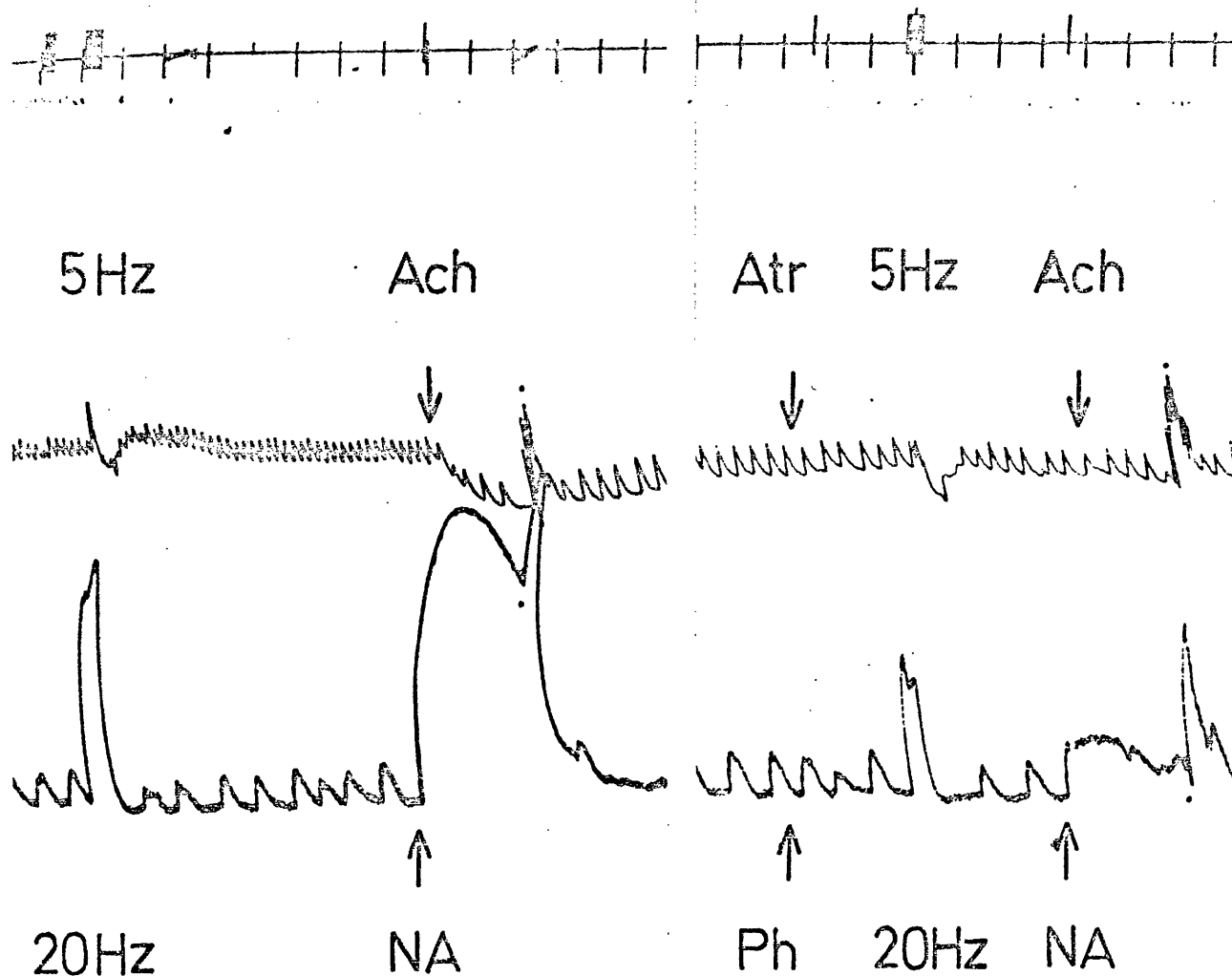
Fig. 64   Comparison of the effects of acetylcholine (Ach) and NA on contralateral cat anococcygeus muscles in vitro and the effects of atropine (Atr, 10^{-6} M) and phentolamine (Ph, 10^{-6} M). Responses to field stimulation are also shown for comparison. Upper muscle stimulated at 5 Hz for 20 sec at bars on time trace and shows a predominantly inhibitory response.   Lower muscle stimulated at 20 Hz for 20 sec shows a motor response.   The inhibitory nerve response, unlike the Ach response was unaffected by atropine whereas the motor nerve response, like the NA response, was inhibited by phentolamine.   Note, however, that the NA response is more inhibited than the motor nerve response.   Time 1 min.   Wash at dots.

RAT

CAT

10^{-6} 10^{-7}
5HT   ACH

10^{-6} 10^{-7}
5HT   ACH

NEO

10^{-7}
5HT

10^{-7} 10^{-6}
ACH   ACH

10^{-7}
5HT

10^{-7} 10^{-6}
ACH   ACH
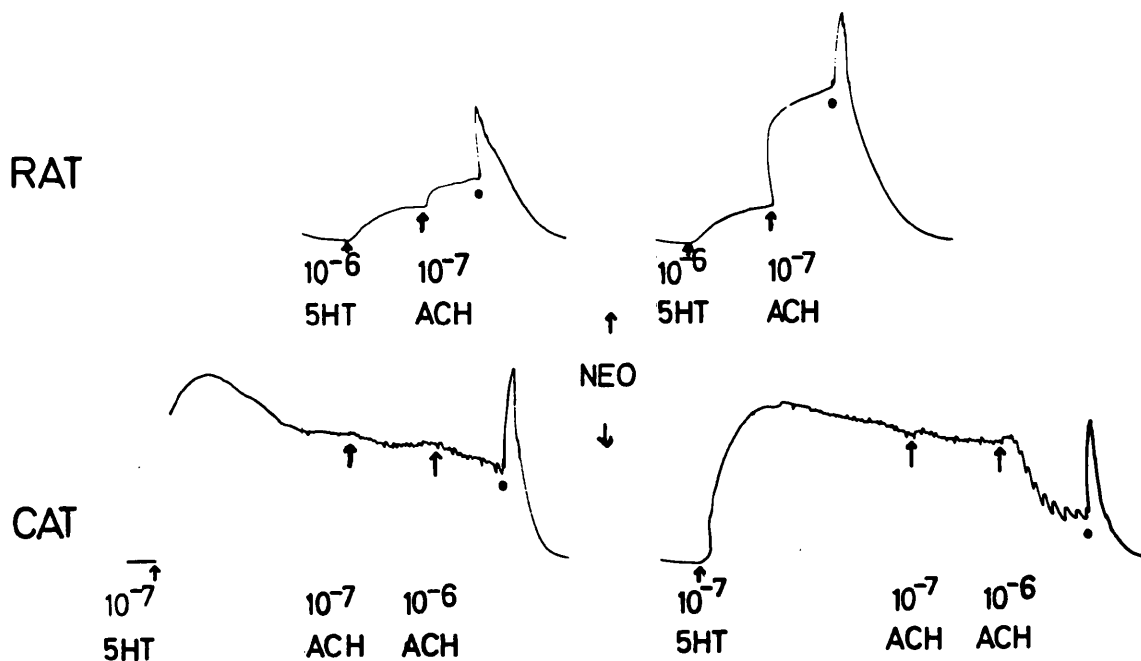
Fig. 65   Comparison of the effects of neostigmine (NEO) 10^{-7} M

on the response of the rat and cat anococcygeus to acetylcholine

(Ach).   The tone of the muscles was first raised with 5 -

hydroxytryptamine 10^{-6} M for rat, 10^{-7} M for cat.   Neostigmine

enhanced the motor response to acetylcholine 10^{-7} M in the rat

and the inhibitory response to acetylcholine 10^{-6} M in the cat

but did not affect the response to 5 - hydroxytryptamine in

either species.   Wash at dots.
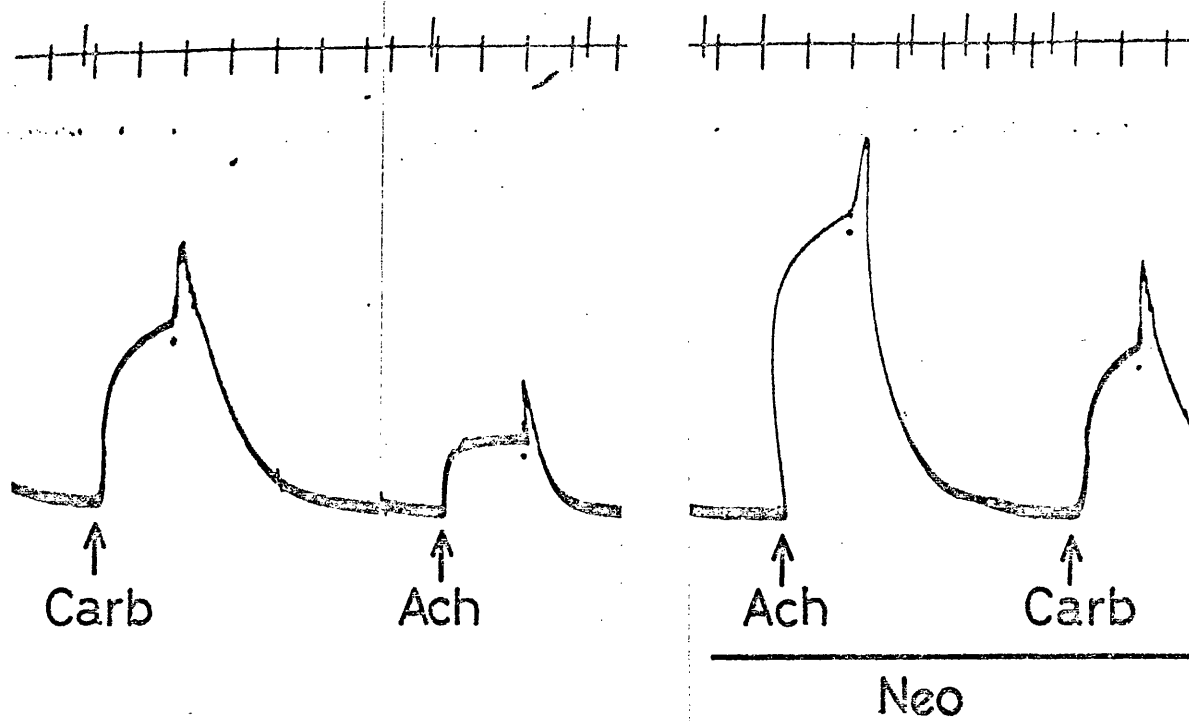
Fig. 66   Effect of neostigmine (Neo, 10^{-7} M)

on the responses to acetylcholine (Ach, 10^{-6} M)

and carbachol (Carb, 10^{-6} M) of the rat

anococcygeus in vitro.   The response to

acetylcholine but not to carbachol was potentiated.

Time 1 min.   Wash at dots.

<u>Muscarinic Agonists</u>   Acetylcholine and carbachol both produced relaxation of the cat anococcygeus.   This could be demonstrated in the presence of intrinsic tone alone (Fig. 64) or after raising the tone of the muscle with motor agonists (Figs. 63 & 65).   The relaxation produced by acetylcholine was potentiated by neostigmine (10^{-7} M) (Fig. 65) whereas the carbachol response was unaffected.   Atropine (10^{-6} M) abolished the relaxation produced by acetylcholine (Fig. 64) or by carbachol.

In the rat anococcygeus, acetylcholine and carbachol both produced contraction (Fig. 66).   This contraction by acetylcholine but not by carbachol was potentiated by neostigmine (10^{-6} M) (Figs. 65 & 66).   Motor responses to both acetylcholine and carbachol were abolished by atropine (10^{-6} M).   High doses of acetylcholine may produce small, atropine - sensitive contractions of the cat anococcygeus (Fig. 80).   These responses are small compared with the more powerful inhibitory effect of acetylcholine.

Muscarinic agonists thus produce the opposite effect on the tone of the anococcygeus in the cat or rat;   contraction in the rat, relaxation in the cat. Both these effects are abolished by atropine and the efficacy of acetylcholine to produce either response is potentiated by cholinesterase inhibition.

<u>Isoprenaline</u>   produced relaxation of the cat anococcygeus in the presence of intrinsic tone alone (Fig. 50) or after the tone of the muscle had been raised by agonist drugs (Fig. 63).   This relaxation by isoprenaline was abolished by propranolol (Fig. 63) but not by phentolamine (10^{-6} M), atropine (10^{-6} M) or hexamethonium (10^{-6} M) and so can be classified as a β - adrenergic effect.

The motor response produced in the cat anococcygeus by NA and adrenaline was potentiated by propranolol, presumably by inhibition of these same β - receptors. It thus appears that the cat anococcygeus possesses both α - and β - adrenergic receptors with opposing effects.

The rat anococcygeus was not relaxed by isoprenaline under any conditions. The only response produced by isoprenaline was contraction at high doses which was abolished by phentolamine (10^{-6} M). The rat, unlike the cat, anococcygeus thus appears to lack β - receptors.

Adenosine triphosphate (ATP)   at concentrations at and above 10^{-4} M relaxed the cat anococcygeus in the presence of intrinsic tone alone or after the tone had been raised by motor agonists (Figs. 62 & 63). In the presence of intrinsic tone alone, ATP produced a transient, small motor response at doses from 5×10^{-6} M to 5×10^{-4} M. This led to a biphasic response in the range 10^{-4} M - 5×10^{-4} M; transient contraction followed by relaxation.

In the rat anococcygeus, ATP produced contraction at all doses from 5×10^{-6} M to 5×10^{-4} M. In Fig. 63, the response to ATP 5×10^{-4} M appears to have a biphasic nature. The relaxation following the motor response is not, however, a true inhibitory response but rather a reflection of the diminishing response to a supra - maximal dose since the tone did not relax to the level from which the first dose of ATP produced contraction.

The effects of ATP on both species were unaffected by atropine (10^{-6} M), propranolol (10^{-6} M) or hexamethonium (10^{-6} M).

Prostaglandins E_1, E_2 and $F_{2\alpha}$ all produced relaxation of the cat anococcygeus if the tone was first raised by an agonist. PGE_1 and PGE_2 were approximately equipotent, being capable of relaxation 50 - 90% of the induced tone within 3 min at a concentration of 10^{-7} g/ml. $PGF_{2\alpha}$ produced a very weak effect at 10^{-7} g/ml and in one out of three experiments produced a small motor response at this concentration. The effects of PGE_1 are shown in Figs. 62 & 63.

G
10^{-6}

G
10^{-5}

G
3×10^{-5}

5g

0

5g

0

10^{-5}
G

10^{-4}
G
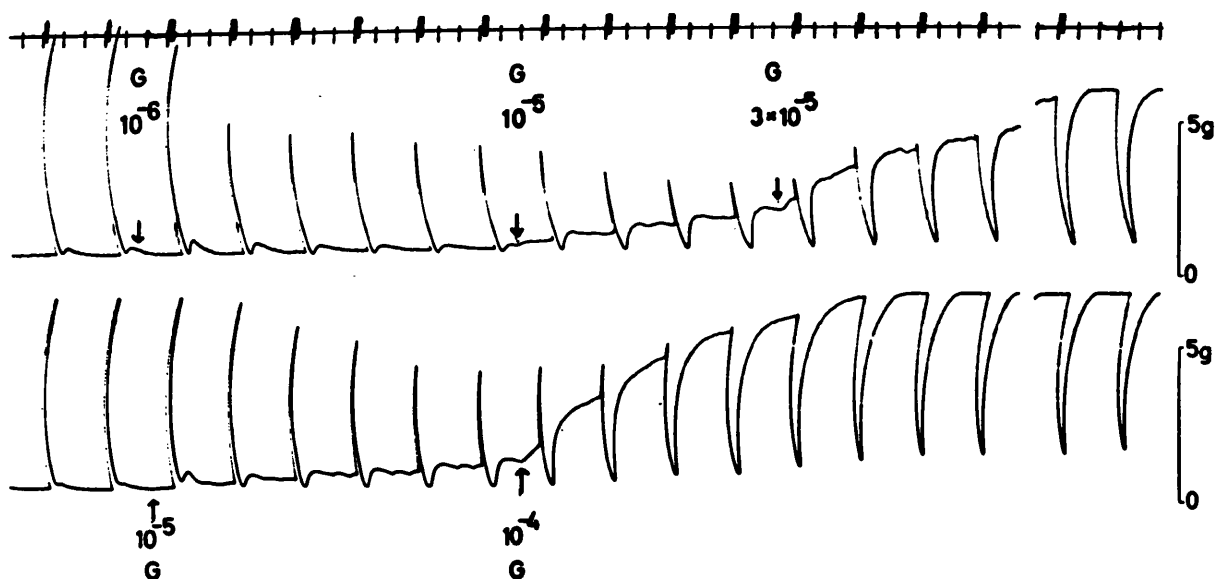
Fig. 67   The effect of Guanethidine on the response to field
stimulation in the cat anococcygeus muscle. In the upper trace,
Guanethidine (G) 10^{-6} M rapidly produced a 50% reduction in the motor
response to field stimulation but caused only a slight increase in
muscle tone. Guanethidine 10^{-5} M and 3 x 10^{-5} M further reduced the
motor response and increased the tone more rapidly thus uncovering a
large inhibitory response. The lower trace shows that a still larger
dose of Guanethidine 10^{-4} M will produce a faster increase in tone
and reversal of the response from motor to inhibitory. The
contractions on the lower trace were truncated by the limits of the
recorder, the first two motor responses on the left were 2g larger
than shown and at the right, the maximum tone produced by
Guanethidine 10^{-4} M was 1.5g larger than shown. Stimulation at
bars on time trace, 10 sec train at 10 Hz. Time 1 min.

In the rat anococcygeus, all three prostaglandins tested raised the tone of the muscle. The threshold varied from 3×10^{-9} g/ml to 10^{-8} g/ml. Maximal responses were always produced by 3×10^{-8} g/ml and were never larger than 50% of the maximal motor response to NA. In the rat anococcygeus, the three prostaglandins showed a spectrum of potency $E_2 > E_1 > F_{2\alpha}$.

The effects of prostaglandins on both rat and cat anococcygeus were unaffected by atropine (10^{-6} M), propranolol (10^{-6} M) or hexamethonium (10^{-6} M).

Vasopressin   produced transient motor responses in the cat anococcygeus at 6 - 200 m U / ml in the absence of induced tone. At 20 - 200 m U / ml these motor responses were followed by relaxation responses. When the tone of the muscle was raised with an agonist drug, no motor responses were found and inhibitory responses were produced by 20 - 200 m U / ml.

In the rat anococcygeus, vasopressin 2 - 200 m U / ml produced small motor responses. The maximum response was obtained at 20 m U / ml, larger doses producing smaller responses.

These responses to vasopressin in both species were unaffected by atropine (10^{-6} M).

Responses to Field Stimulation

Field stimulation of the rat anococcygeus produces motor responses due to its adrenergic innervation. When these motor nerves are blocked with guanethidine and the tone of the muscle raised, field stimulation then produces a powerful inhibitory response. Similar motor and inhibitory responses were found in the cat anococcygeus (Fig. 67).

Increasing doses of guanethidine gradually depressed the motor response to

30   20   10   5   2   1   0.5 Hz

RAT

CAT

5g

10g
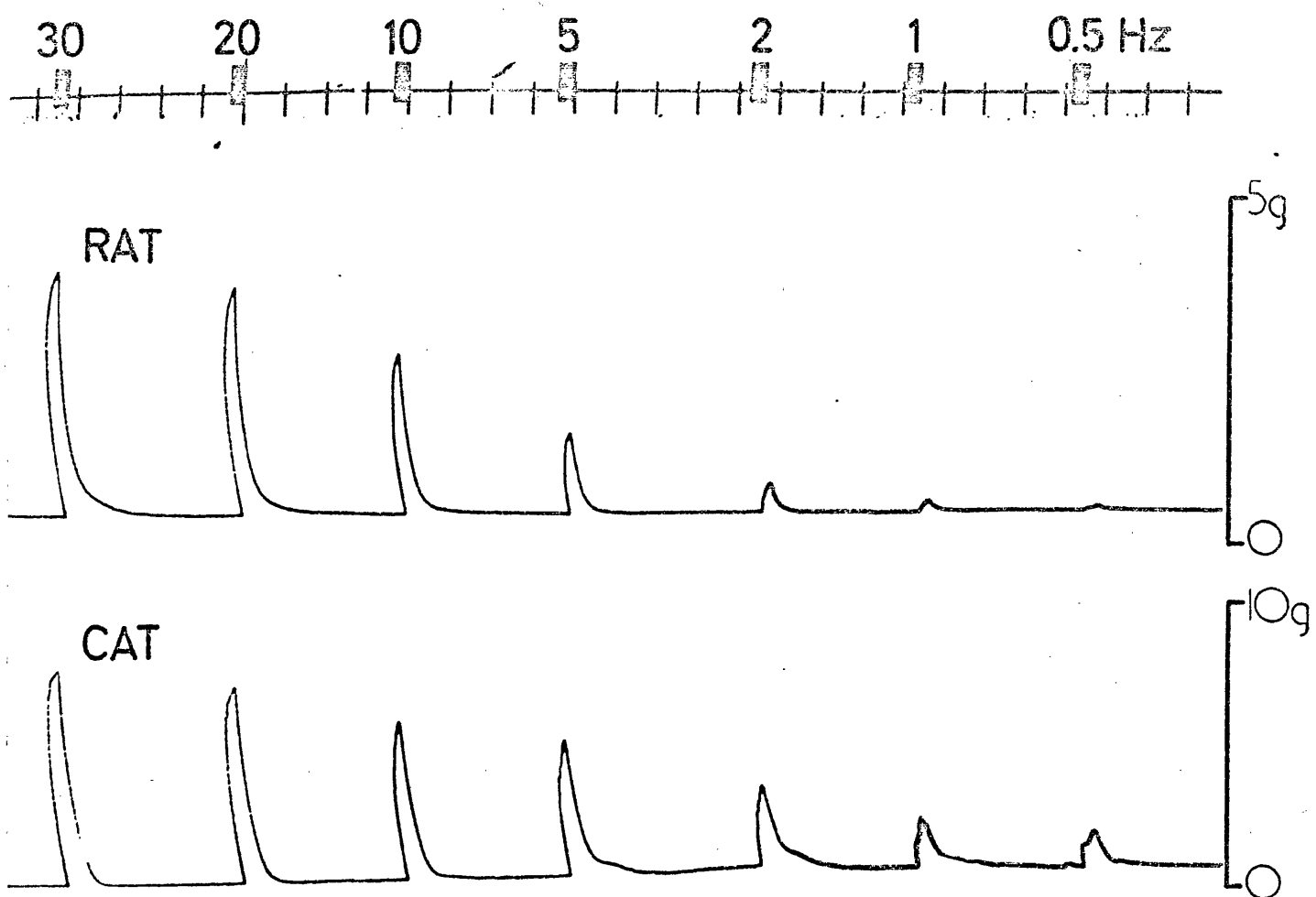
Fig. 68   Comparison of the responses in rat and cat anococcygeus muscles to field stimulation at varying frequencies. Stimulation at the frequencies indicated above the time trace for 10 sec. Note that this cat anococcygeus muscle showed pure motor responses in the absence of drugs in contrast to the complex responses obtained in other muscles, c.f. Fig. 70. Time 30 sec.

field stimulation. At 10^{-5} M and above, guanethidine also raised the tone of the muscle and at the same time uncovered an inhibitory response. At 3×10^{-5} M and above, guanethidine completely abolished the motor response, leaving a pure inhibitory response.

As in the rat, these inhibitory responses were abolished by tetrodotoxin $(3 \times 10^{-7}$ g/ml).

The nature of this dual innervation in the cat anococcygeus was studied by first examining the frequency characteristics of the response to field stimulation and the interaction between motor and inhibitory responses which this uncovered and then attempting to identify the transmitters involved by investigating (1) the effects of blocking drugs on both motor and inhibitory nerve responses compared with the corresponding effects of the blockers on putative transmitter substances i.e. can the transmitters be identified by being specifically blocked or mimicked?

(2) the effects of the putative transmitters on the motor and inhibitory nerve responses i.e. can the interaction between agonist drugs and the nerves provide evidence for the identity of the transmitters?

(3) the effects of cholinesterase inhibition on nerve responses and on the effects of acetylcholine i.e. does cholinesterase play a role in normal nerve activity and if not does it influence the effect of acetylcholine on nerve activity?

Responses to field stimulation in the absence of blocking drugs

In the rat anococcygeus, field stimulation with 1 msec pulses produced smooth contractions which were rapid in onset and declined rapidly on cessation of stimulation. Responses could be obtained to single pulses and summation of pulses occurred between 0.1 and 0.2 Hz. The maximum tension which could be developed increased with increasing frequency to reach a maximum at 20 - 30 Hz (Fig. 68). At all frequencies, contraction was maintained as long as
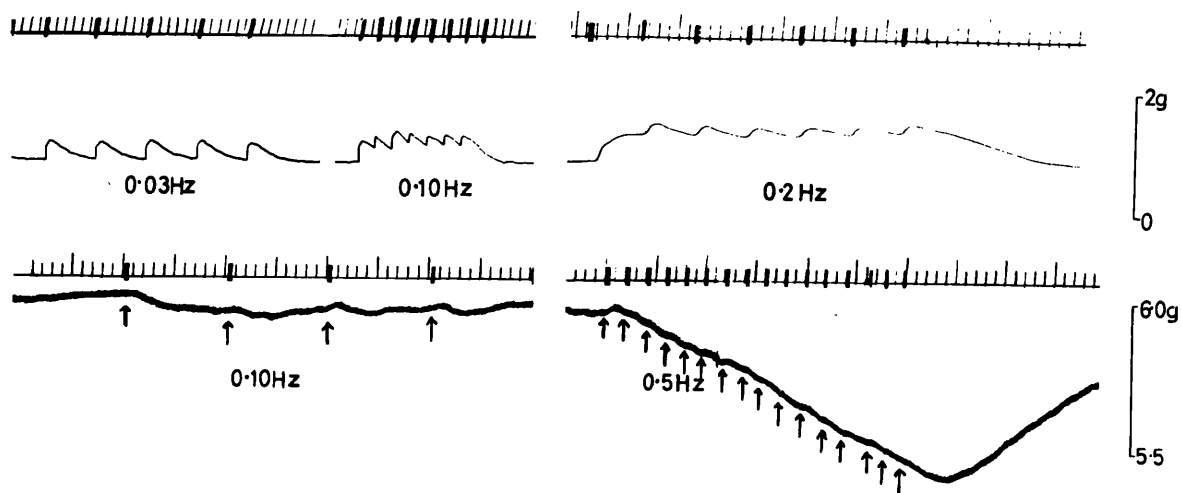
Fig. 69   Responses to stimulation at low frequencies of the cat anococcygeus in vitro. The upper trace shows motor responses in the absence of drugs. Mechanical summation of responses to the single pulses occurs between 0.03 and 0.10 Hz. The lower trace shows inhibitory responses obtained in the presence of adrenergic blockade and elevated tone produced by guanethidine 3 x 10^{-5} M. Single pulses produce inhibitory responses but these are small compared with the motor responses which are produced by single pulses. Summation of inhibitory responses occurs between 0.10 and 0.5 Hz, but at frequencies as low as 0.5 Hz the development of the response is slow. Time 1 sec.

Fig. 70   Complex responses of cat anococcygeus muscles to field stimulation in vitro.   Contralateral muscles from the same animal were stimulated for 1 min periods at the frequencies indicated above the time trace.   At 0.2 Hz, a pure motor response was found, but by 0.5 Hz a later inhibitory component was already appearing.   At 10 Hz on the upper trace and 20 Hz on the lower, the motor response re - established itself.   On cessation of stimulation, however, a further inhibitory response was found. Note that the muscle in the lower trace has more intrinsic tone than the other, enabling it to display inhibitory responses to better visual advantage.   In the upper trace, although the late inhibitory responses do bring the tension below the initial tone, the earlier inhibitory component appears only as an incisura on the motor response.   Time 1 min.

stimulation continued and declined on cessation to the original resting tension.

In the cat anococcygeus, the response to field stimulation was more complex. Some muscles showed responses similar to those found in the rat in which the contraction was well maintained during stimulation and on its cessation declined only to the original resting level (Fig. 68). In these muscles, single pulses produced responses proportionately larger than those in the rat and which summated mechanically at between 0.03 and 0.10 Hz (Fig. 69). The maximum tension which could be developed by continuous stimulation increased with increasing frequency up to 20 - 30 Hz (Fig. 81).

In most muscles, however, the response was more complex as shown in Fig. 70. Single pulses and low frequencies produced simple contractions and these were maintained for as long as stimulation continued. At frequencies at or above 0.5 Hz, however, the contraction was not maintained and declined after reaching a peak at 2 - 10 sec to a tension sometimes below the initial baseline. At frequencies at and above 10 Hz, if stimulation was prolonged, the contraction usually re - established itself and the decline in tension after the initial contraction either disappeared or remained as an incisura on the contraction. On cessation of stimulation, however, at all frequencies above 0.5 Hz, the tension normally declined to a level below the initial resting level.

This gave rise to responses which, if examined at any one frequency, might appear complex (e.g. Fig. 70, 10 Hz), but when examined over the range of frequencies are seen to follow a simple pattern as follows -

1) At frequencies below 0.5 Hz, pure motor responses were obtained.

2) At frequencies at and above 0.5 Hz, a secondary "inhibitory" response appeared which was slower in onset than the "motor" contraction response but in the range 0.5 - 10 Hz was capable of antagonising it.

3) At and above 10 Hz, during continuous stimulation, the motor response was capable of overcoming the inhibitory response.

Fig.71   COMPARISON OF FREQUENCY CHARACTERISTICS OF MOTOR AND
INHIBITORY RESPONSES IN THE CAT ANOCOCCYGEUS.

FREQUENCY OF STIMULATION
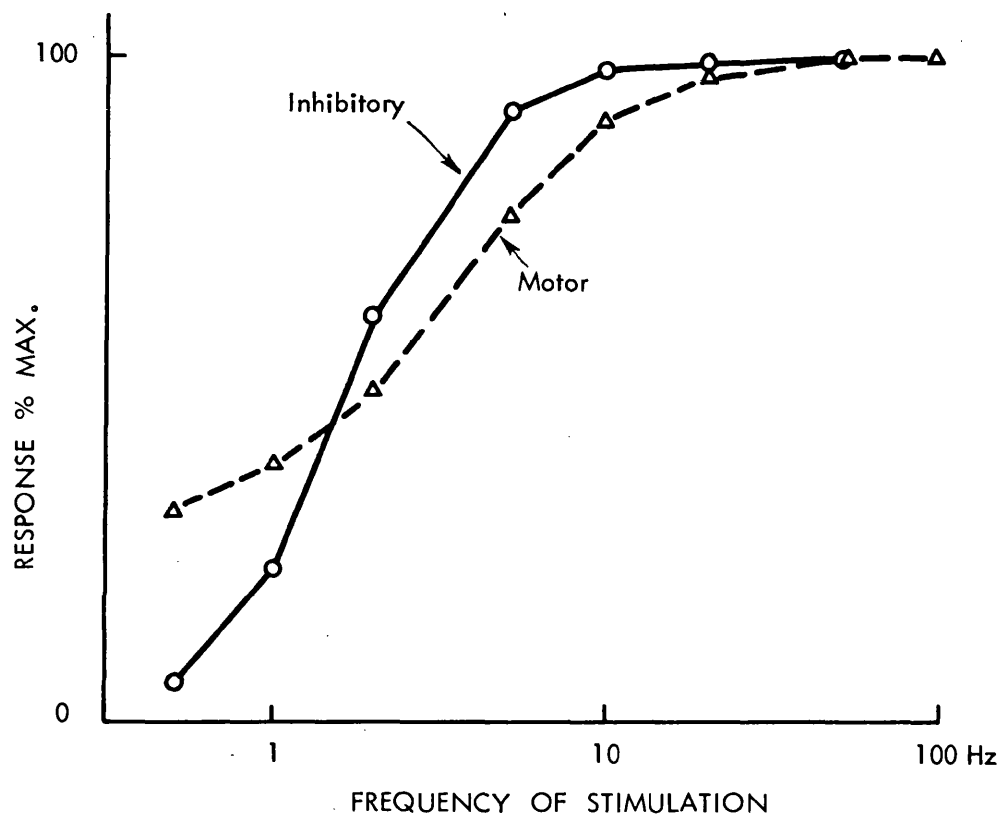
Cat anococcygeus muscles in vitro.   Responses to field stimulation.

Motor - maximum tension generated by continuous stimulation at
the frequencies indicated, in the absence of drugs.   Each response
expressed as a percentage of the response at 100 Hz.   (n = 4)

Inhibitory - maximum relaxation produced by continuous stimulation
at the frequencies indicated in the presence of tone generated by
guanethidine 3×10^{-5} M.   Each response expressed as a percentage
of the relaxation produced by 50 Hz.   (n = 4)
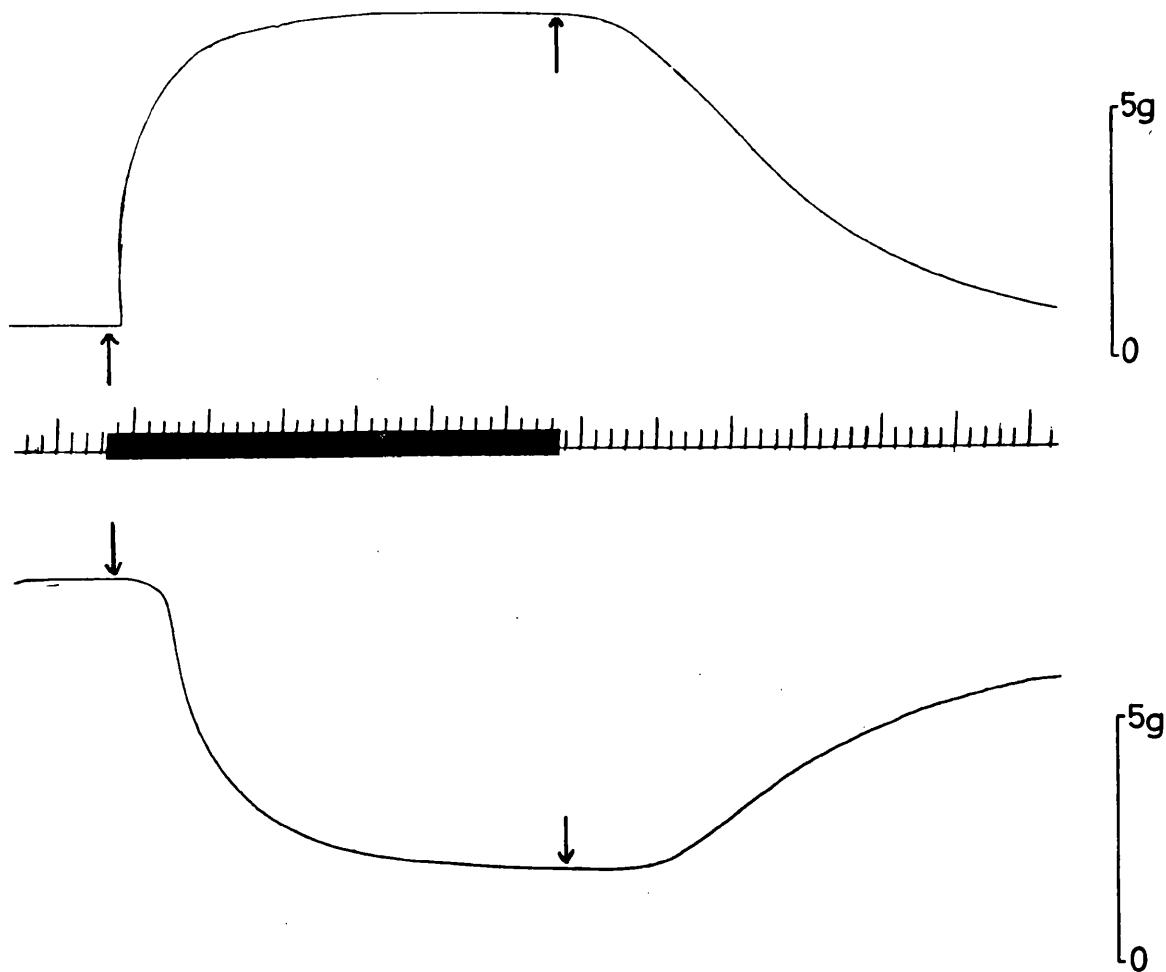
Fig. 72   Comparison of the time course of maximal motor and inhibitory responses in the cat anococcygeus muscle in _vitro_.   The upper trace · shows the response to field stimulation at 30 Hz for 30 sec in the absence of drugs.   After a short latent period, tension developed rapdily to reach a plateau within 15 sec.   Tension was then well maintained until stimulation ceased.   On cessation, tension declined to reach baseline within 35 sec.   In this muscle, no inhibitory component to the response developed in the absence of adrenergic blockade.   The lower trace shows the response of the same muscle to stimulation at 10 Hz for 30 sec in the presence of guanethidine 3×10^{-5}M. The inhibitory response started slowly after an initial short latent period but rapidly produced a steeper decline of tension.   This inhibition was well maintained and was still increasing on cessation of stimulation at 30 sec.   After a short delay, the tension began to recover slowly but had not reached its former level until 60 sec after the end of stimulation.   Time 1 sec, 5 sec.

4) On cessation of stimulation, at all frequencies above 0.5 Hz, the

    effects of the inhibitory response outlast those of the motor response

    and so produce a late relaxation.

This interpretation is borne out by an analysis of the interaction between

the two sets of responses as shown in the following section.

Interaction between motor and inhibitory nerves

Consideration of the frequency characteristics of the motor and of the

inhibitory responses shows agreement with the above interpretation of the

complex response.   Comparing the motor responses obtained to continuous

field stimulation in the absence of drugs with the inhibitory response

obtained to similar stimulation in the presence of guanethidine (3×10^{-5} M),

it is apparent that single pulses and low frequencies are more effective at

producing motor than inhibitory responses (Fig. 69) but that at intermediate

frequencies (2 - 10 Hz) the inhibitory response is more fully developed than

the motor response (Fig. 71).

The rate of development of the motor and inhibitory responses also agrees

with the above interpretation.   Fig. 72 compares a maximal motor response

in a cat anococcygeus which showed no signs of an inhibitory component in

the absence of guanethidine with a maximal inhibitory response in the same

muscle after guanethidine.   The latent period was short for both responses,

but the motor response showed a very rapid initial rate of development

whereas the inhibitory response had a slow initial rate of development which

gradually accelerated.   Similarly, after cessation of stimulation, the

motor response waned more rapidly than the inhibitory response.   This

correlates with the finding that when motor and inhibitory nerves are active

simultaneously the motor response may appear at first before the inhibitory

response, but since the inhibitory response takes longer to develop and lasts

↑
5HT
10^{-6}M

↑
5HT
10^{-5}M
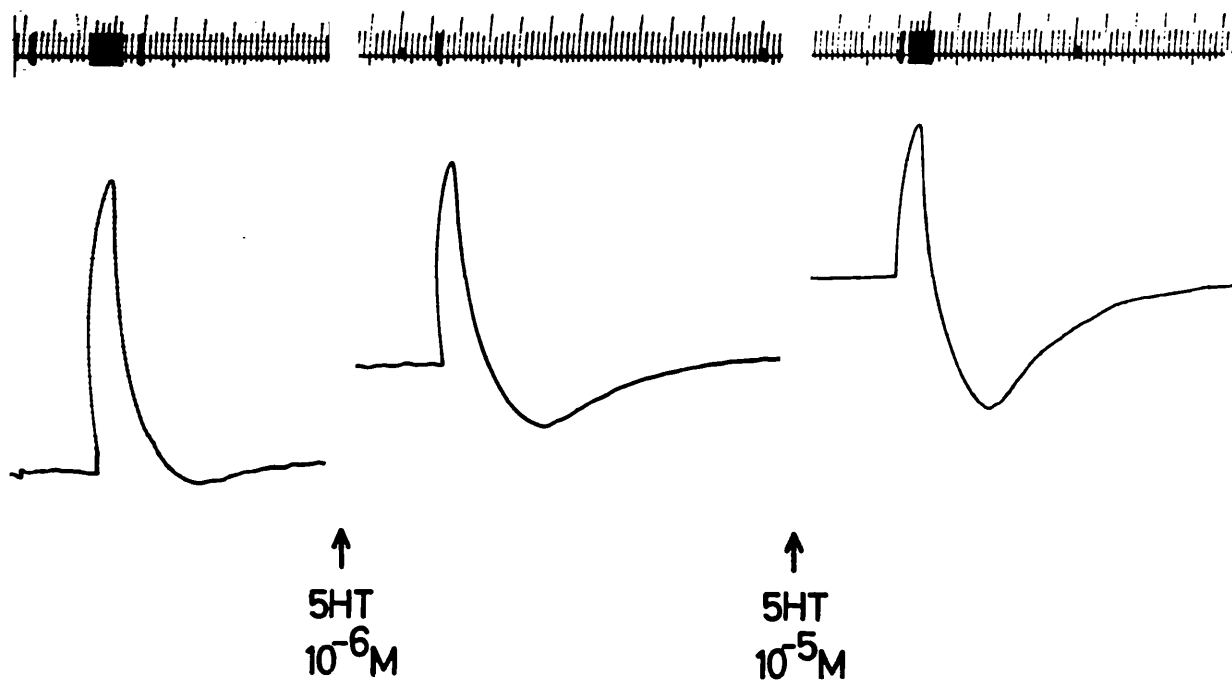
Fig. 73   Effect of raising muscle tone

on the response to field stimulation in a

cat anococcygeus muscle.   In the absence

of induced tone, the motor response to

stimulation at 5 Hz for 5 sec is not well

maintained and on cessation of stimulation.

a small overshoot of the resting baseline

occurs.   If the tone of the muscle is then

raised with 5HT, the depth of this overshoot

increases thus uncovering a previously hidden

inhibitory response.   Time 1 sec, 5 sec.

longer, it may reverse the motor response either during or after the stimulation period according to the frequency.

Two other experiments which analyse the response at 5 Hz (the frequency at which the inhibitory response is most evident) further confirm the above interpretation of the complex response.

Fig. 73 illustrates the first. When the tone of the muscle was raised by addition of 5HT to the bath, the inhibitory response following the motor response became more apparent and as the tone was raised further this inhibitory component produced a relaxation of tension to and beyond the initial resting baseline. This demonstrates that in the absence of adrenergic blocking drugs, both motor and inhibitory nerves are being stimulated and the mechanical response which is observed is the sum of their two opposing responses and will therefore vary according to the state of activity in the muscle. This biphasic response in the presence of 5HT can be further modified by guanethidine which will abolish the motor response leaving a pure inhibitory response, confirming the above interpretation.

This first experiment confirmed that when stimulating at the optimal frequency for the inhibitory nerves i.e. 5 Hz, after 3 - 4 sec the tone of the muscle comes under the dominant influence of the inhibitory nerves. The motor nerves, which are still being stimulated, are thus no longer capable of producing contraction. A second experiment was, therefore, designed to estimate the time course and magnitude of the inhibitory effect produced by transmitter liberated from the inhibitory nerves during a short optimal burst of stimulation. The response to a single pulse was used as a measure of the degree of inhibition at any given moment since a single pulse in the absence of other influences produces a relatively pure motor response.

This experiment is illustrated in Fig. 74. A single test stimulus was followed

(a)

sp   5Hz   sp

7

5

4

3

(b)

5Hz   0.5Hz
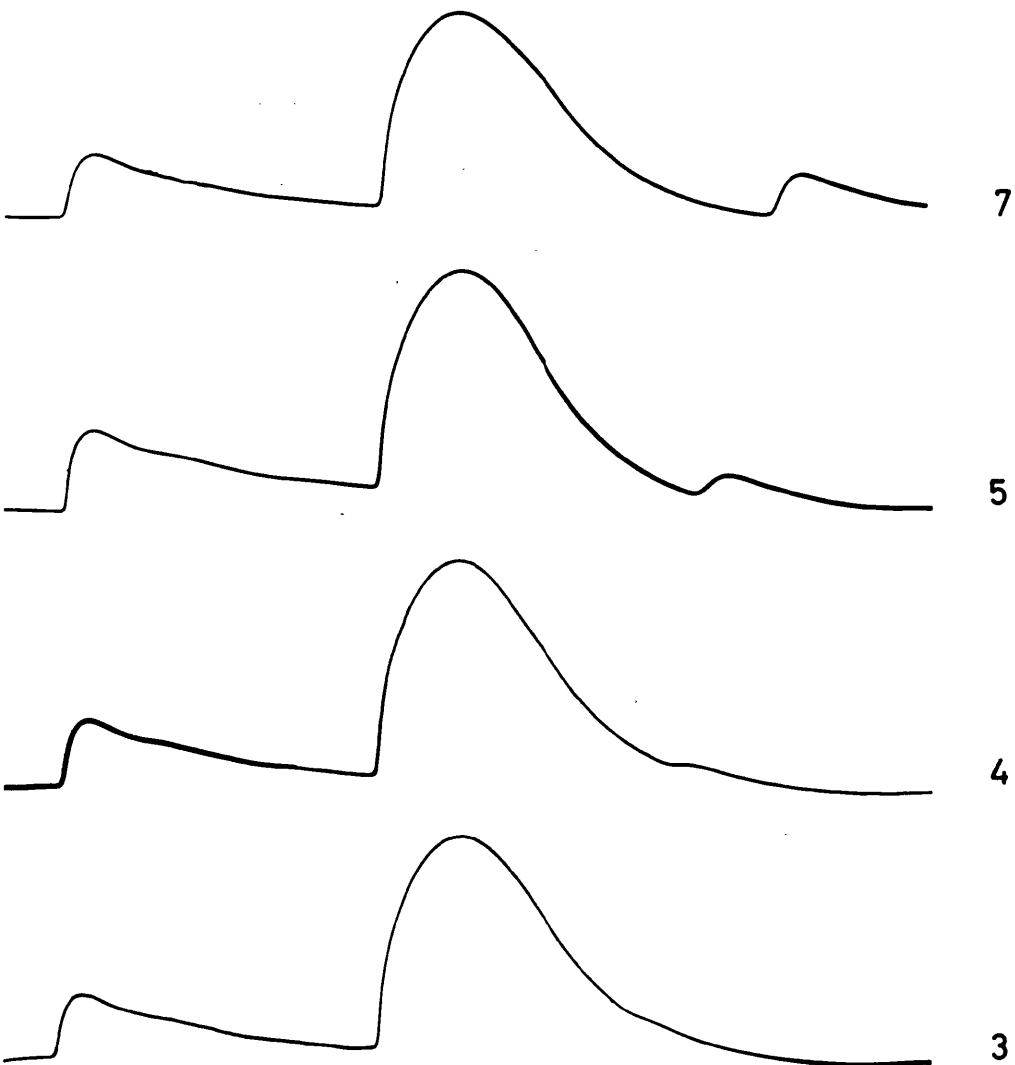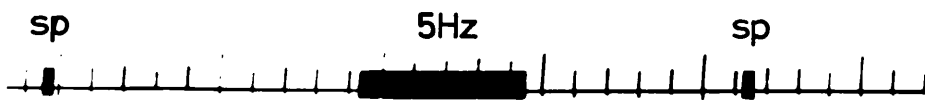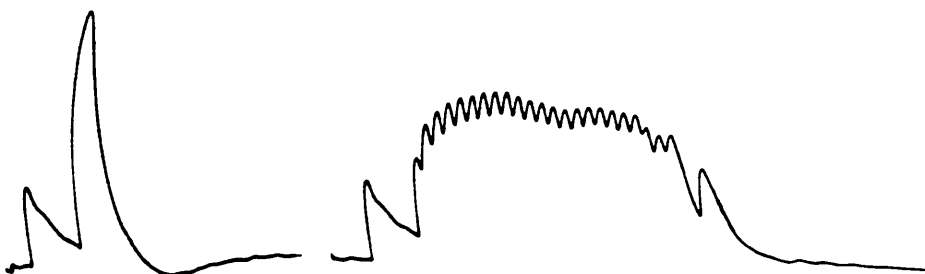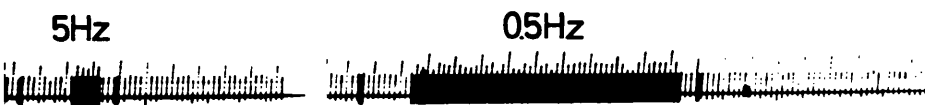
Fig. 74   (a)   Inhibition of the motor response to
a single pulse by a preceding train of pulses at 5 Hz in
a field stimulated cat anococcygeus muscle.   A single
pulse (sp) was followed after 10 sec by a train of 25
pulses at 5 Hz.   This train was then followed by a
further single pulse at varying intervals.   In this
illustration, these test pulses followed the train at
7, 5, 4 and 3 sec.   As the time of the single pulse
approached the train, the response diminished.   The
height of this response to the single pulse is plotted
as a percentage of the control response preceding the
train in Fig. 75.   This is the same muscle and
stimulation parameters (i.e. 5 Hz, 25 pulses) as are
employed in Fig. 73.   Time 1 sec, 5 sec.

(b)   The first panel shows the experiment in (a) with
the test single pulse following the train after 3 sec
on a slower trace speed.   The second panel shows a
similar experiment where the train at 5 ·Hz has been
replaced by one at 0.5 Hz.   At 0.5 Hz the motor response
is maintained at an oscillating level throughout the
stimulation period and when a single pulse is applied
3 sec after the end of the period, a response is
obtained which is as large as the control.   Time
1 sec, 5 sec.
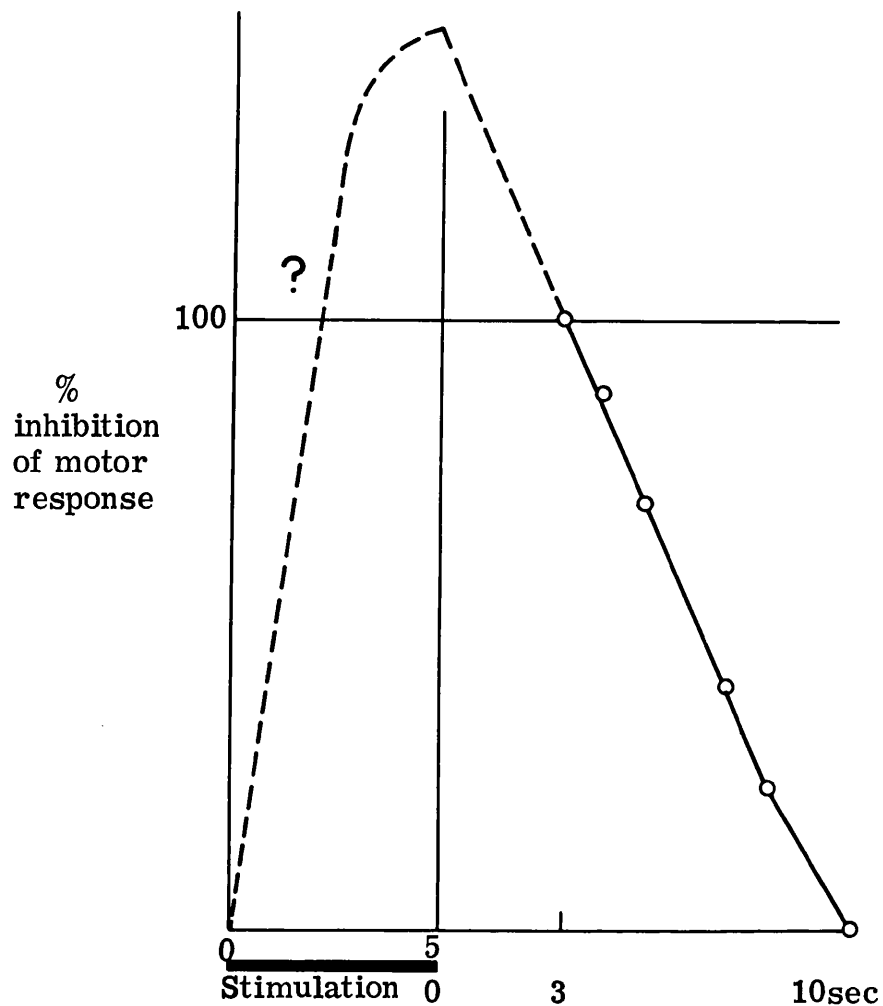
Fig. 75   Time course of the inhibitory

nerve response plotted from the recovery of

the motor response to a single pulse following

a train of 25 pulses at 5Hz i.e. experiment in

Fig. 74.   Dotted line plots hypothetical

development of the response.

after 10 sec by a train of 25 pulses at 5 Hz.   At varying times after this

train, the single test pulse was repeated.   The results are shown in Fig. 75.

10 sec after the train, a single pulse was capable of producing a motor

response similar to the control obtained before the train.   As the time of

the pulse approached nearer to the end of the train, inhibition first

appeared at 8 sec and the response diminished until at 3 sec it had almost

completely disappeared.   On its own, this finding might have been due to

post - tetanic fatigue of the contractile apparatus similar to the non-

specific post - tetanic inhibition described by Cantoni & Eastman (1946).

That this was not the case was shown by replacing the train of 25 pulses at

5 Hz with a 25 pulse train at 0.5 Hz which was capable of maintaining a raised

tone without signs of an inhibitory component.   Under these conditions, a

single pulse 3 sec after the train was capable of producing a motor response

as large as before (Fig. 74b).   Taken together with the responses obtained in

the absence of any drugs and in the presence of 5HT, this reinforces the idea

that in the cat anococcygeus the motor and inhibitory nerves can be activated

simultaneously by field stimulation and that at 5 Hz, after an initial motor

response, the inhibitory nerves dominate and their effects outlast those of

the motor nerves.

In the cat anococcygeus, it can thus be demonstrated in several ways that

both motor and inhibitory nerves are activated simultaneously by field

stimulation.   In the rat anococcygeus, the inhibitory component cannot be

easily demonstrated in the absence of a combination of adrenergic blocking

drugs and raised tone, although a small late inhibitory component can often

be elicited by field stimulation in vitro in the presence of raised tone

without adrenergic blockade, and in the pithed rat the inhibitory nerves can

be selectively stimulated in the absence of adrenergic blockade by stimulation

within the spinal canal (see Part I, Fig. 25b).
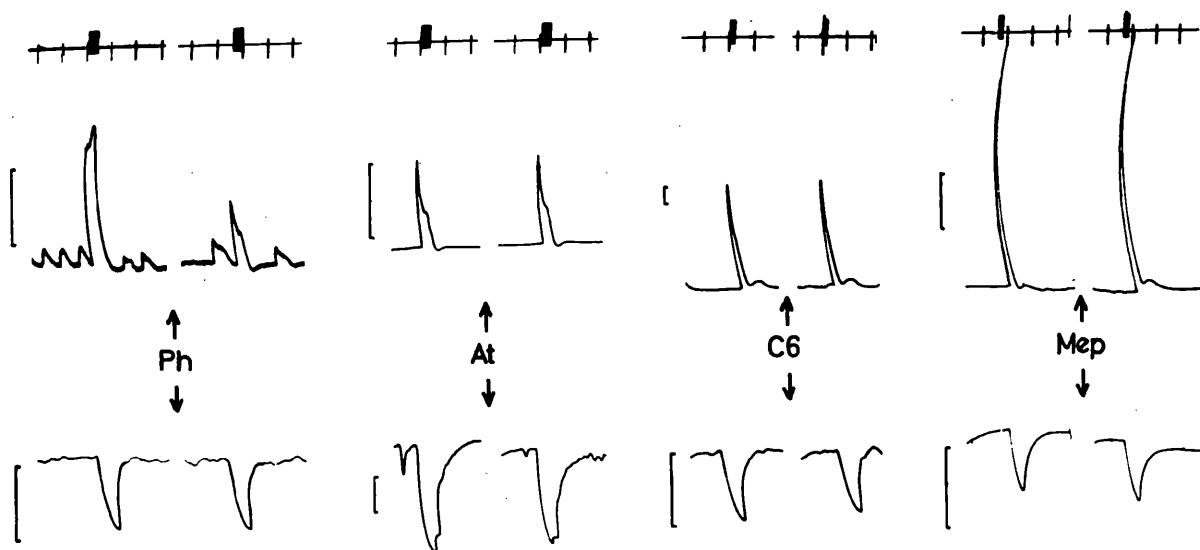
Fig. 76 The effect of Phentolamine (Ph) 10^{-6} M, atropine (At)
10^{-6} M, hexamethonium (C6) 10^{-5} M and mepyramine (Mep) 10^{-5} M
on the motor and inhibitory responses to field stimulation of
the cat anococcygeus. Each pair of responses (i.e. before and
after drug) was obtained on a different muscle. Motor responses
were obtained from stimulation at 10 Hz with the exception of
the first pair (30 Hz). Inhibitory responses produced in the
presence of Guanethidine 3 x 10^{-5} M with the exception of the
first pair of responses where the tone was raised by LSD 10^{-6} M.
Frequency of stimulation for inhibitory responses was 10 Hz with
the exception of the first pair (5 Hz). Phentolamine decreased
the size of the motor response but did not affect the inhibitory
response. Atropine, hexamethonium and mepyramine had no effect
on responses whether motor or inhibitory. Time 1 min.
Vertical calibrations 1 g.

The effects of blocking drugs on nerve responses

The effects of phentolamine, atropine, hexamethonium, pancuronium, methysergide, mepyramine and propranolol were tested on both motor and inhibitory responses in the cat anococcygeus. None of these drugs altered the inhibitory response and only phentolamine blocked the motor response (Fig. 76).

(In order to examine the effect of phentolamine on the inhibitory response, the motor nerves were first blocked with guanethidine and then the tone was raised with a supra - maximal dose of LSD which produced a powerful contraction resistant to the action of phentolamine. This situation allowed inhibitory responses to be seen while the tone of the muscle itself was unaffected by the blocker.)

Phentolamine thus blocks both motor nerves and NA suggesting that NA is the motor transmitter. This is supported by the histochemical demonstration of noradrenergic nerves in the muscle and by the abolition of the motor response after 6OHDA in vitro treatment. n.b. Phentolamine (10^{-6} M) does not completely abolish the motor response to stimulation at 30 Hz (Fig. 76). This dose does, however, abolish responses to lower frequencies and higher doses (10^{-5} M and above) completely suppress the motor response. Responses to NA are thus more easily inhibited by phentolamine than motor nerve responses (Fig. 64).

The inhibitory response, on the other hand, was unaffected by doses of atropine and propranolol which abolished responses to acetylcholine and isoprenaline respectively and so is unlikely to be either cholinergic or - adrenergic. The inhibitory transmitter thus remains unknown.

These blockers have also been tested on the rat anococcygeus with similar results. Motor nerves were blocked by phentolamine only; inhibitory nerves were not affected by any of these blockers.
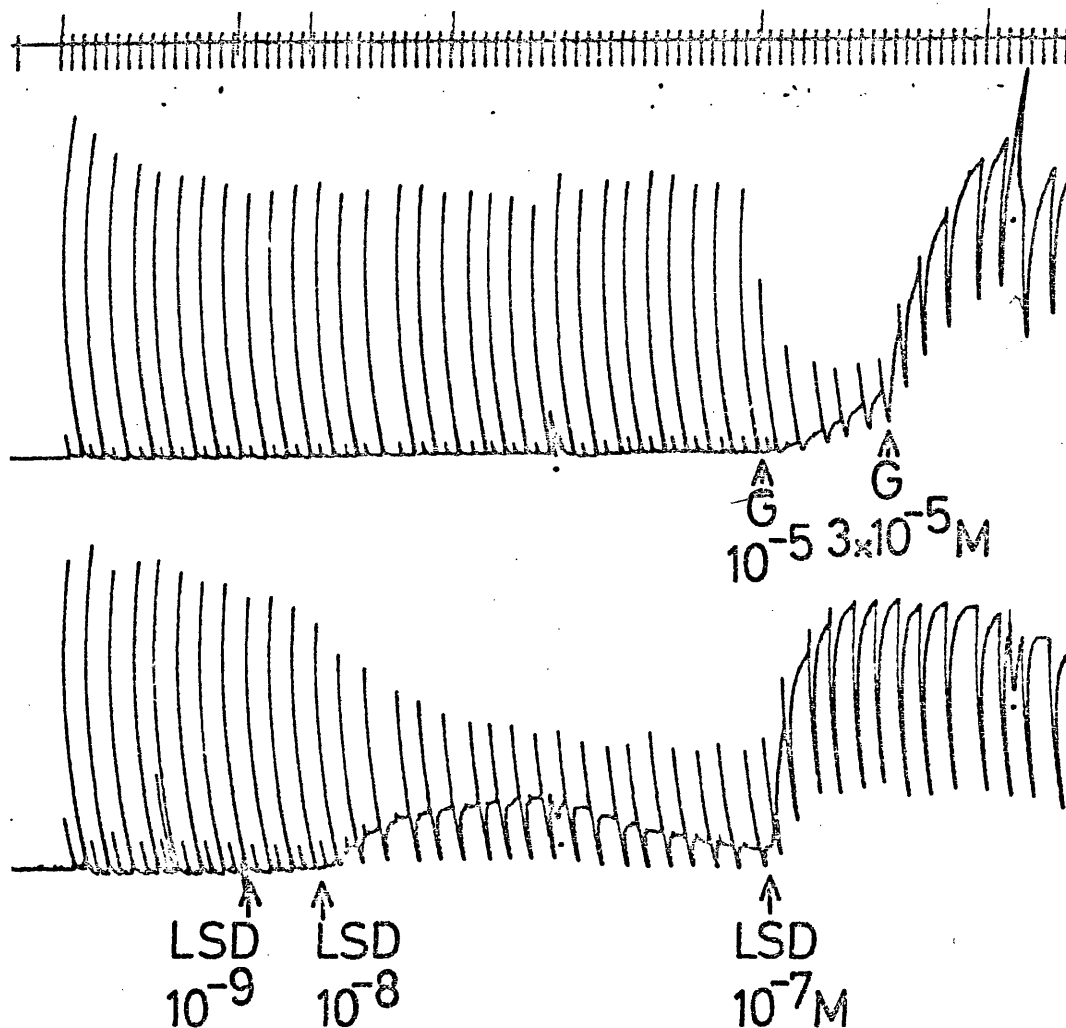
Fig. 77   Comparison of the effects of LSD
and guanethidine (G) on the motor response to
field stimulation in the cat anococcygeus muscles.
The contralateral muscles were stimulated alternately
with a single pulse and 30 pulses at 10 Hz.
Wash at dots.   Time 1 min.

Guanethidine also blocks the motor but not the inhibitory nerve responses in both species. Unlike phentolamine, however, guanethidine does not block the response to NA added to the bath and so is supposed to act by preventing the release of NA from the nerves i.e. neuronal blockade.

LSD is known to block the motor adrenergic response in the vas deferens (Ambache, Dunk, Verney & Zar, 1973). In a previous section, evidence has been given that LSD can act on the adrenergic nerves of the cat anococcygeus but as an indirect sympathomimetic. It was, therefore, of interest to determine whether LSD was acting as a pure indirect sympathomimetic or whether its action was close to that of guanethidine, which, in low doses, acts as a pure neuronal blocking agent and only at relatively high doses acts as an indirect sympathomimetic (Fig. 67). No evidence was found that LSD, in doses less than those required for an agonist action (10^{-9} M), reduced the motor response to field stimulation (Fig. 77). At doses producing a moderate agonist action (10^{-8} M), the motor response to field stimulation was diminished. At higher doses producing large contractions, the response to field stimulation was reversed to inhibition. This inhibition was as great as that usually seen after guanethidine (Figs. 67 & 77) and was not enhanced by further addition of guanethidine. In addition, these doses of LSD did not inhibit the response to NA 3 x 10^{-6} M so it is possible that LSD may be inhibiting the motor nerves by a neuronal blocking action similar to that of guanethidine.

The effects of agonist drugs on nerve responses

The effects of the substances which relax the cat anococcygeus on the responses to field stimulation of the nerves are interesting for several reasons. First, considered as putative transmitter substances, they might be expected to inhibit the responses to motor nerve stimulation and to summate with the responses to inhibitory nerve stimulation. In addition, whether or not a

(a) Control — Ach / CARB

(b) Neostig. — Ach / CARB
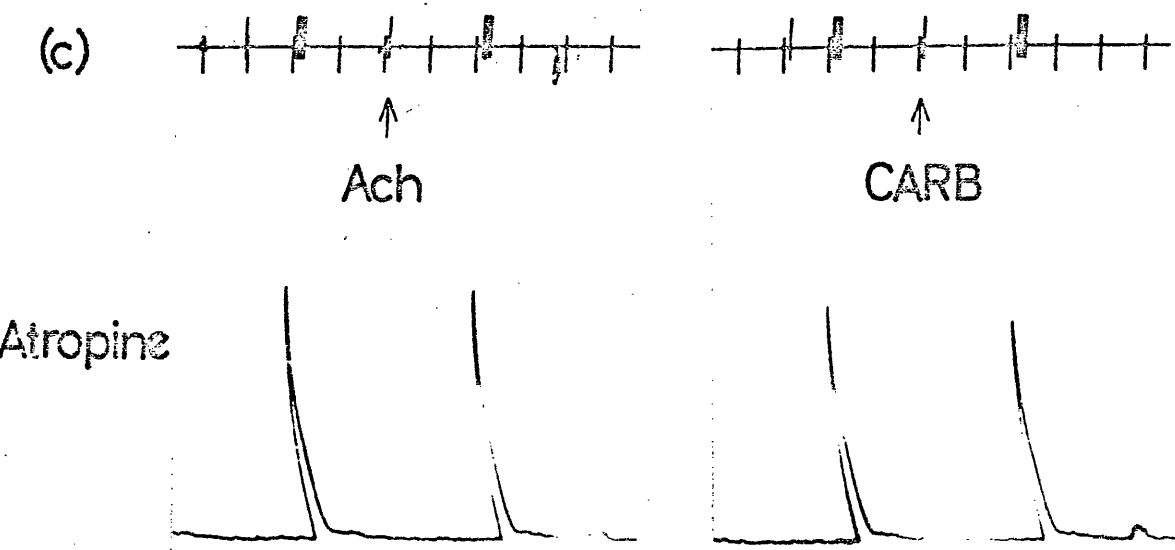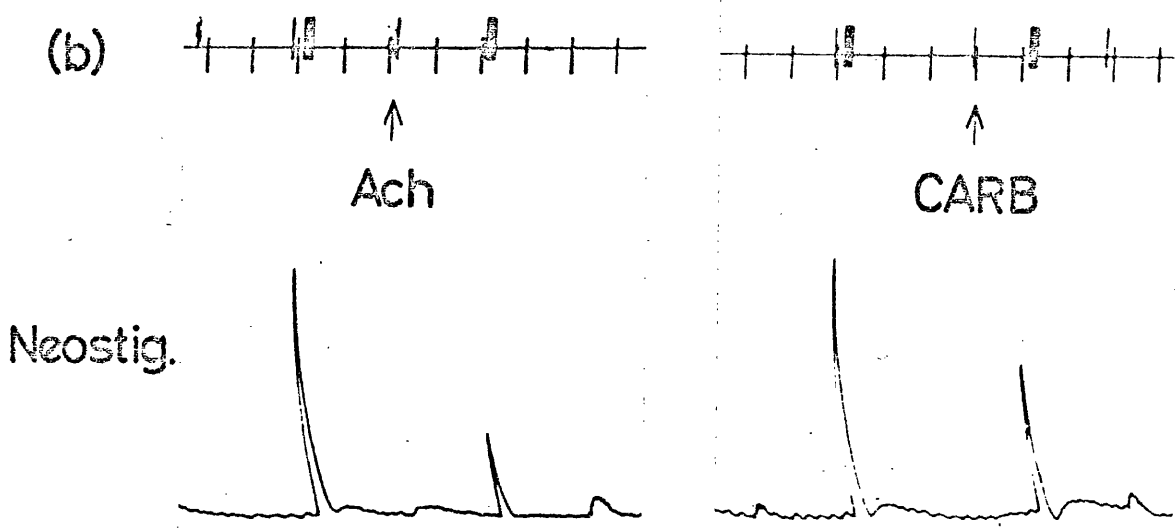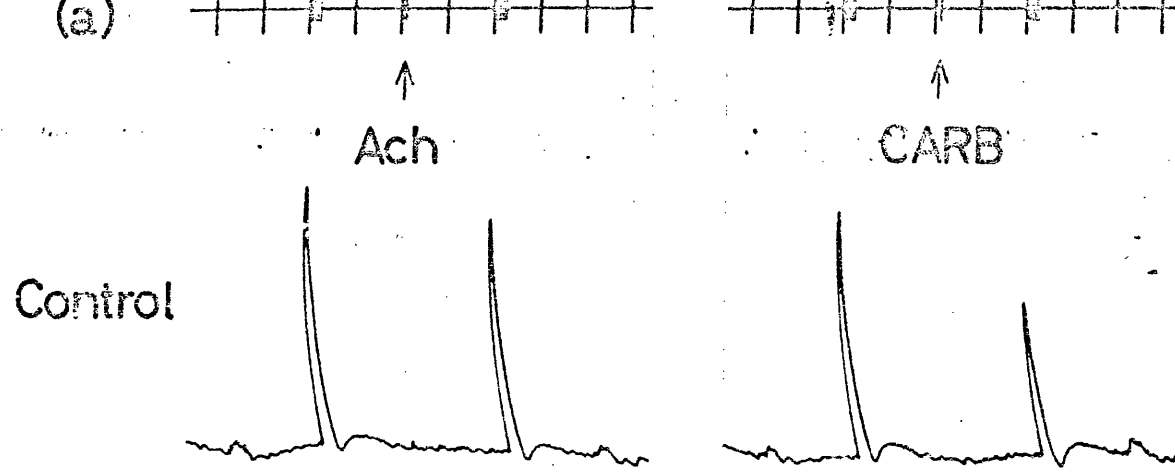
(c) Atropine — Ach / CARB

Fig. 78   Effects of acetylcholine (Ach, 10^{-5} M) and carbachol

(Carb, 10^{-6} M) on the motor response to field stimulation of the cat

anococcygeus muscle (10 Hz, 10 sec) in vitro.   (a)   control

(b)   in the presence of neostigmine (10^{-7} M)   (c)   in the presence

of atropine (10^{-6} M).   The effect of acetylcholine is potentiated

by neostigmine and abolished by atropine.   The effect of carbachol

is unaffected by neostigmine but abolished by atropine.   The motor

response to field stimulation is affected by neither neostigmine nor

atropine.   Time 1 min.

particular substance was the transmitter, its effects on nerve responses as opposed to those on intrinsic or drug - induced tone might be expected to provide more information on the type and location of pharmacological receptors present within the tissue.

Acetylcholine especially has been shown to have effects in several tissues not only directly on the muscle but also on sympathetic nerve terminals. These responses can vary considerably according to the dose and tissue employed. Thus, in the isolated perfused mesenteric artery, extremely low doses of acetylcholine (5×10^{-11} g/ml) can potentiate the response to sympathetic nerve stimulation while higher concentrations (5×10^{-9} g/ml) can inhibit the same response (Malik & Ling, 1969). On the other hand, in the isolated rabbit heart, concentrations of 10^{-9} - 10^{-6} g/ml have been shown to inhibit NA release by nerve stimulation while still higher doses (10^{-4} g/ml) released NA from the nerves (Löffelholz & Muscholl, 1969).

With these considerations in mind, the effects of the inhibitory substances on responses to field stimulation were examined, paying particular attention to the effects of acetylcholine over a wide dose range.

Muscarinic agonists   Acetylcholine and carbachol were found to have an inhibitory effect on the motor nerve response in the cat anococcygeus (Fig. 78). This inhibition was more effective at low than at high frequency of stimulation and, in the case of acetylcholine but not of carbachol, was potentiated by neostigmine (Fig. 79 a & b). The threshold for this inhibition was 10^{-8} M - 10^{-7} M. Acetylcholine had no apparent effect in the range 10^{-13} M - 10^{-8} M.

This inhibition of motor nerve responses, like the inhibitory response on muscle tone, did not occur in the rat anococcygeus. The sole effect of acetylcholine in the rat anococcygeus appeared to be to contract the muscle

Fig.79   EFFECT OF ACETYLCHOLINE ON THE MOTOR RESPONSES
TO FIELD STIMULATION AND TO NA ON THE CAT
ANOCOCCYGEUS.
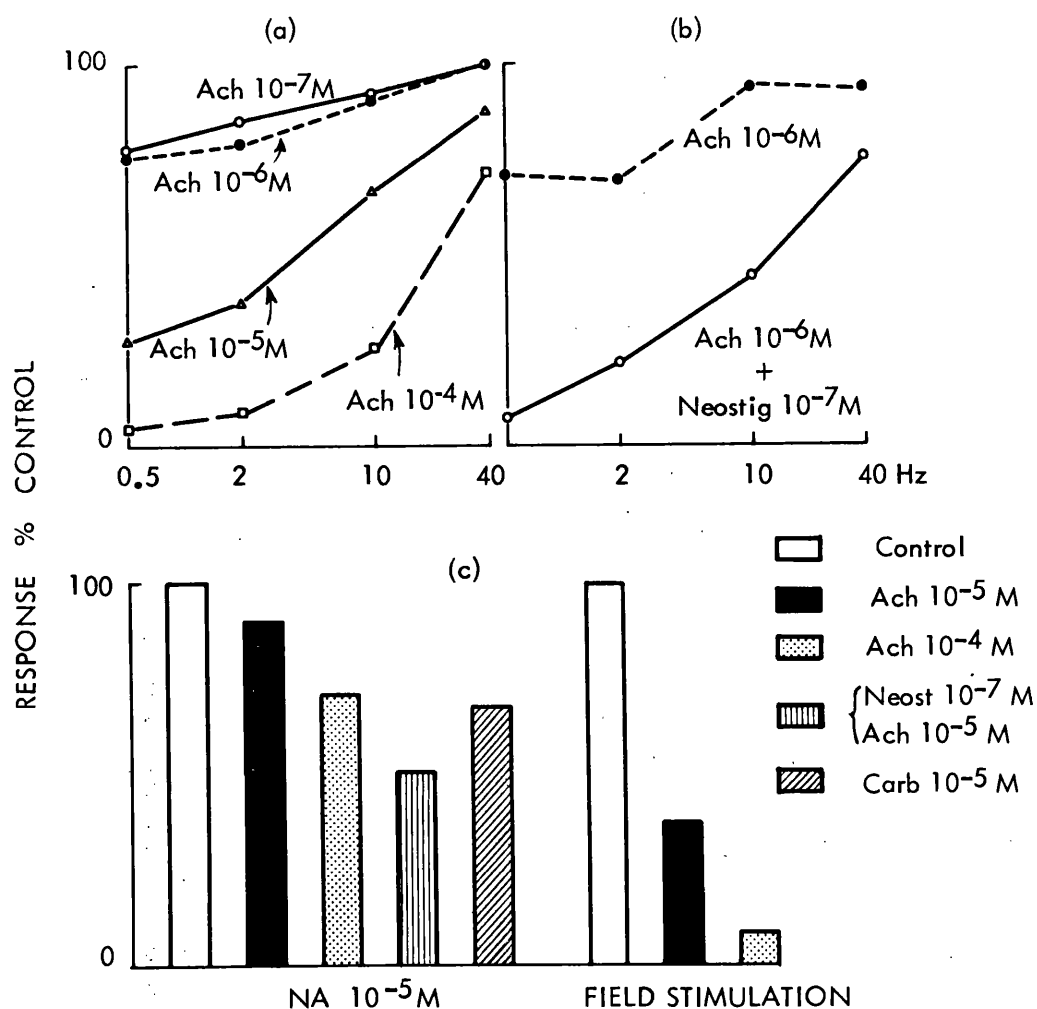
Cat anococcygeus in vitro.

(a)   Effect of Ach on the motor responses to field stimulation
(40 pulses) at the frequencies indicated.   (n = 4)

(b)   As (a).   Effect of neostigmine on the effect of Ach (n = 2)

(c)   Comparison of the effect of Ach on responses to NA 10^{-5} M
and to field stimulation at 2 Hz (40 pulses).   Control responses
to NA 10^{-5} M and to 40 pulses at 2 Hz were approximately equal.
Effects of carbachol and of neostigmine on the NA response are
also shown.   (n = 2)

and these contractions summated with the motor nerve responses. Fig. 80 contrasts the effects of acetylcholine in the cat and rat muscles and illustrates that at high doses acetylcholine can produce small contractions of the cat muscle.

Atropine (10^{-6} M) abolished the inhibition of motor nerve responses in the cat anococcygeus produced by acetylcholine or carbachol (Fig. 78) and also the small contractions produced by high doses of acetylcholine.

It is not possible to tell from these experiments whether acetylcholine is exerting its inhibitory effect on the release of NA from the motor nerves or by acting directly on the muscle. One test of this would be the effect of acetylcholine on the motor response produced by NA as shown in Fig. 79 c. The sub - maximal motor responses produced by NA 10^{-5} M were inhibited by the presence of acetylcholine 10^{-5} M or 10^{-4} M. This inhibition of the NA response by acetylcholine 10^{-5} M was only 10% but could be increased to 49% by the prior inhibition of cholinesterase with neostigmine 10^{-7} M. Carbachol 10^{-5} M produced an inhibition similar to that produced by acetylcholine 10^{-4} M. From this evidence, an inhibitory effect of acetylcholine on the cat anococcygeus muscle is likely to be exerted directly on the muscle.

It is not possible to make a direct comparison between the extent of the inhibitions by acetylcholine on the motor nerve response and on the NA response since the effects of acetylcholine have not been tested against the whole dose / response curve for NA, but only against a single sub - maximal dose. Comparison can, however, be made between the response to NA 10^{-5} M and the response to motor nerve stimulation at 2 Hz which produced motor responses of similar magnitude. The inhibition produced by acetylcholine 10^{-5} M or 10^{-4} M against the nerve response is greater than that against the NA response - Fig. 79 c. This suggests either that acetylcholine is acting at a site on

(a)

Rat

Control   Ach 10^{-6}M   Ach 10^{-4}M

Cat

(b)

Ach 10^{-7} Ach 10^{-6} Ach 10^{-5} Ach 10^{-4} Ach 3×10^{4} NA 10^{-6} NA 10^{-5}

Rat

Cat
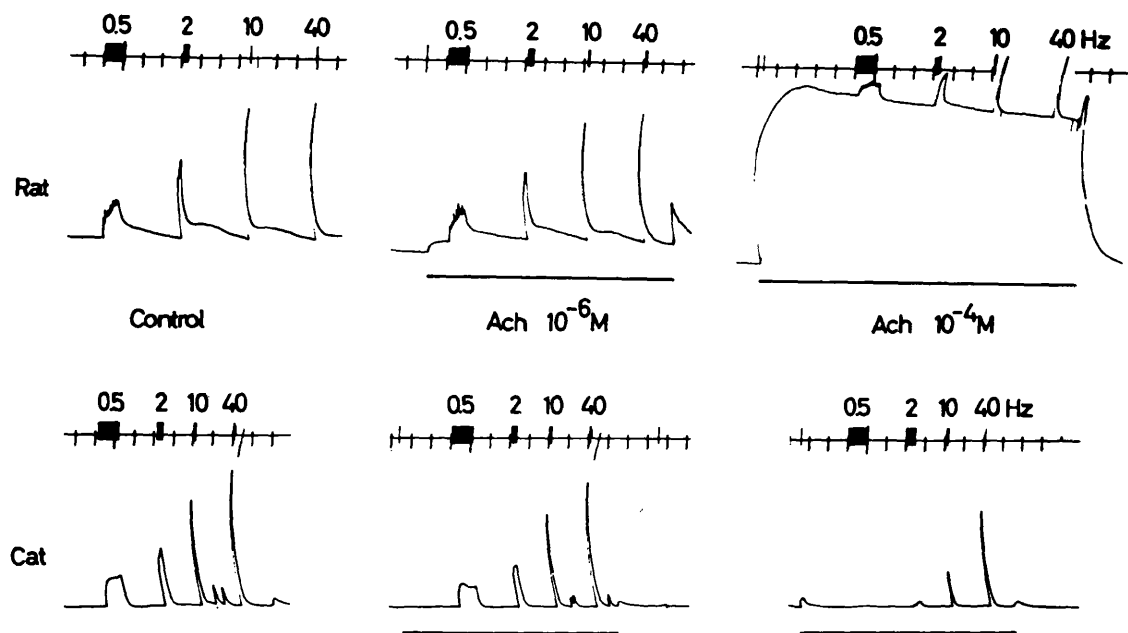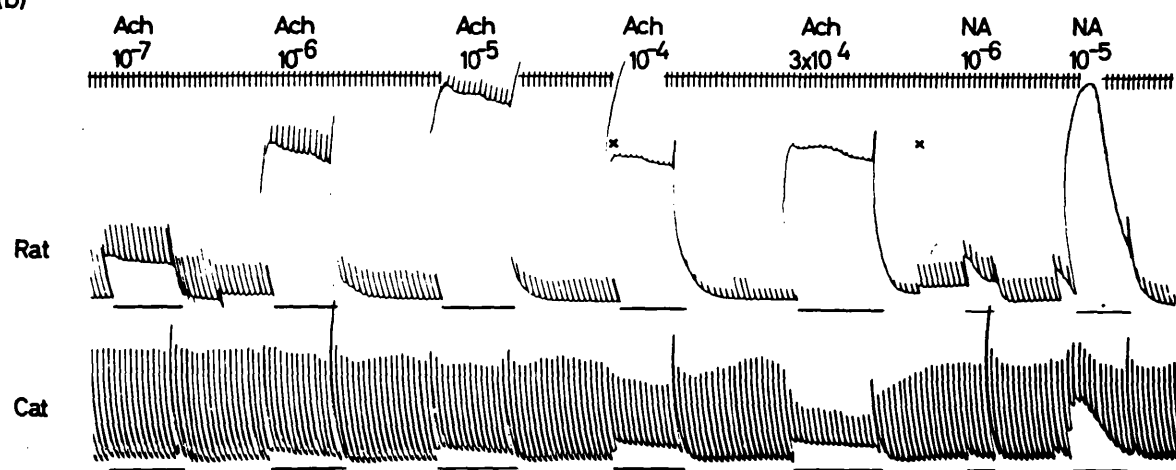
Fig. 80   Comparison of the effects of acetylcholine

(Ach) on the rat and cat anococcygeus.

(a)   Effects of Ach on the responses to field stimulation

in the two species.   Stimulation - 30 pulses at each

frequency.   In the rat, Ach raised the tone of the muscle

but did not affect the height of the motor responses to

field stimulation.   In the cat, on the other hand, Ach

inhibited the responses to field stimulation without

affecting the tone.   Time 1 min.

(b)   Effect of Ach on responses to single pulses in the

two species.   Stimulation 1 pulse every 1 min.   The

responses in the rat muscle are superimposed on the motor

response to Ach.   In the cat, however, the responses are

inhibited even in doses where the tone of the muscle is

raised.   Note the contrast with the effect of NA in the

cat.   When NA raises the tone, the responses are

superimposed on the raised tone.   This cat muscle

was cold stored for 24 hr at 4° C and as a result of this

shows greater motor responses to Ach than would a fresh

muscle.   Time 1 min.   Note that between the crosses

the sensitivity of the rat trace was halved.

the muscle which is more effective against nerve - released NA than against NA added to the bath e.g. acetylcholine and nerve - released NA act mainly in the region of the nerve - muscle junction but NA added to the bath acts generally over the surface of the muscle cells, or that the effect of acetylcholine against nerve responses is only partly by an action on the muscle and is perhaps also exerted on the nerve against release of NA.

The small contractions produced in the cat anococcygeus by high doses of acetylcholine were produced in the muscles from 3 out of 8 cats.   These responses were abolished by atropine but were not further investigated.   It is not, therefore, known whether they represent a direct or indirect effect of acetylcholine on the muscle but they are very small compared with the direct responses to similar doses on the rat (Fig. 80 b).

The effect of acetylcholine on the inhibitory nerve response in the presence of guanethidine was difficult to quantify since the tone of the muscle was altered by the acetylcholine itself.   Inhibitory nerve responses were superimposed on the acetylcholine - induced relaxation, but showed no obvious differences from responses which were obtained when the tone of the muscle was reduced to a similar level by reducing the concentration of guanethidine. Acetylcholine, therefore, produced no changes in the inhibitory nerve response which could not be accounted for by the change in muscle tone.

Other drugs which relax the cat anococcygeus   Apart from acetylcholine and carbachol, the drugs which produced relaxation of the cat anococcygeus did not have a significant effect on motor or inhibitory nerve responses in either species.

Isoprenaline had little effect on the motor nerve response at doses from 3×10^{-7} M to 3×10^{-5} M (Fig. 50) and had no effect on inhibitory responses.

In both species, ATP, vasopressin and prostaglandins all caused a slight

Fig.81   EFFECT OF 6 OHDA IN VIVO PRE-TREATMENT ON THE MOTOR NERVE RESPONSES OF THE RAT ANOCOCCYGEUS.
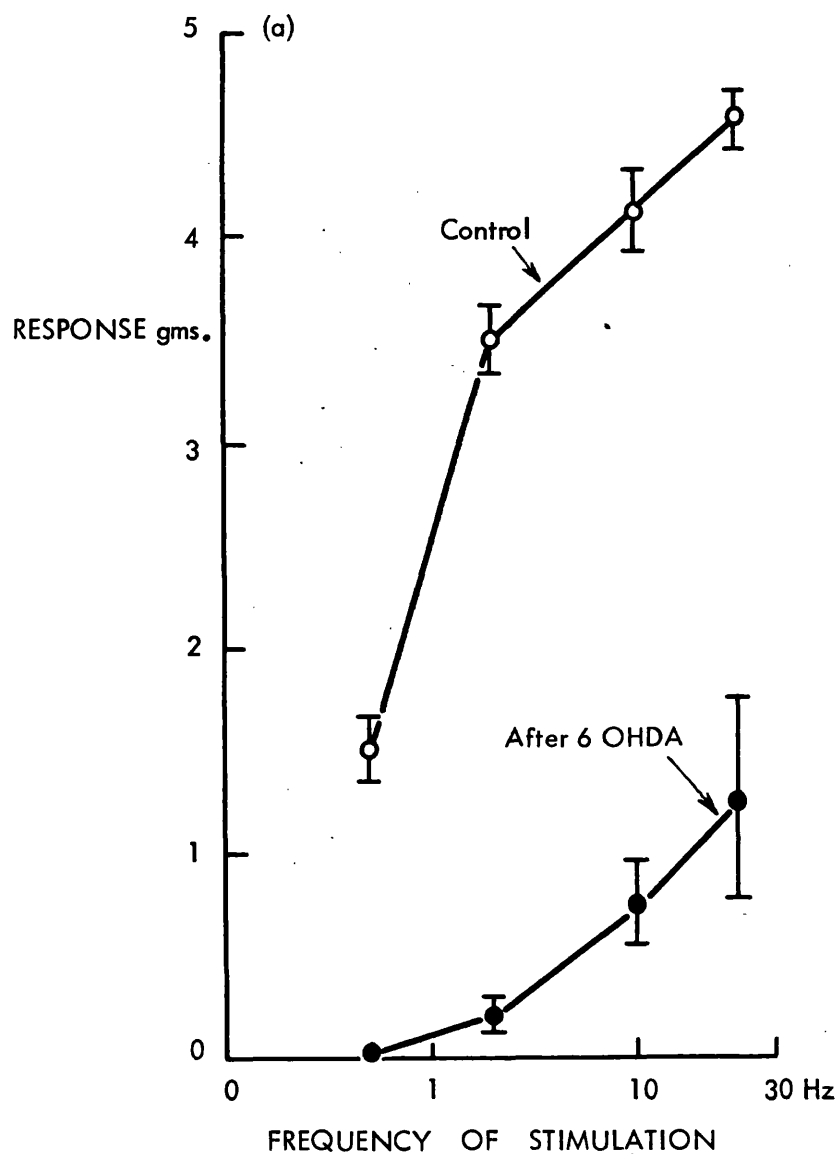
Rat anococcygeus muscles in vitro.

(a)   Responses expressed as maximum tension generated by continuous stimulation at each frequency.   Open circles - untreated controls.   Filled circles - muscles from 6OHDA pretreated rats.   (n = 6)

(b)   (Right hand page)   Comparison of the development of tension produced by field stimulation in tissues from control and 6OHDA treated rats.

(b)

0.5   2   10   30 Hz

CONTROL

5g

0

5g

0

0.5   2   10   30 Hz

6OHDA

5g

0

5g

0
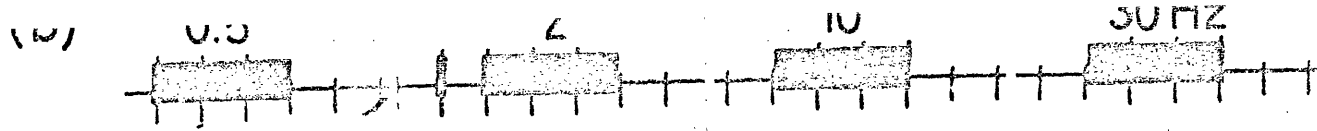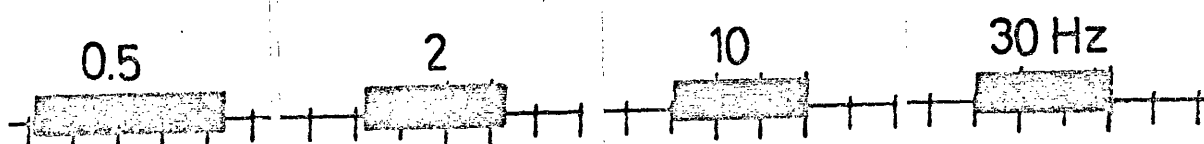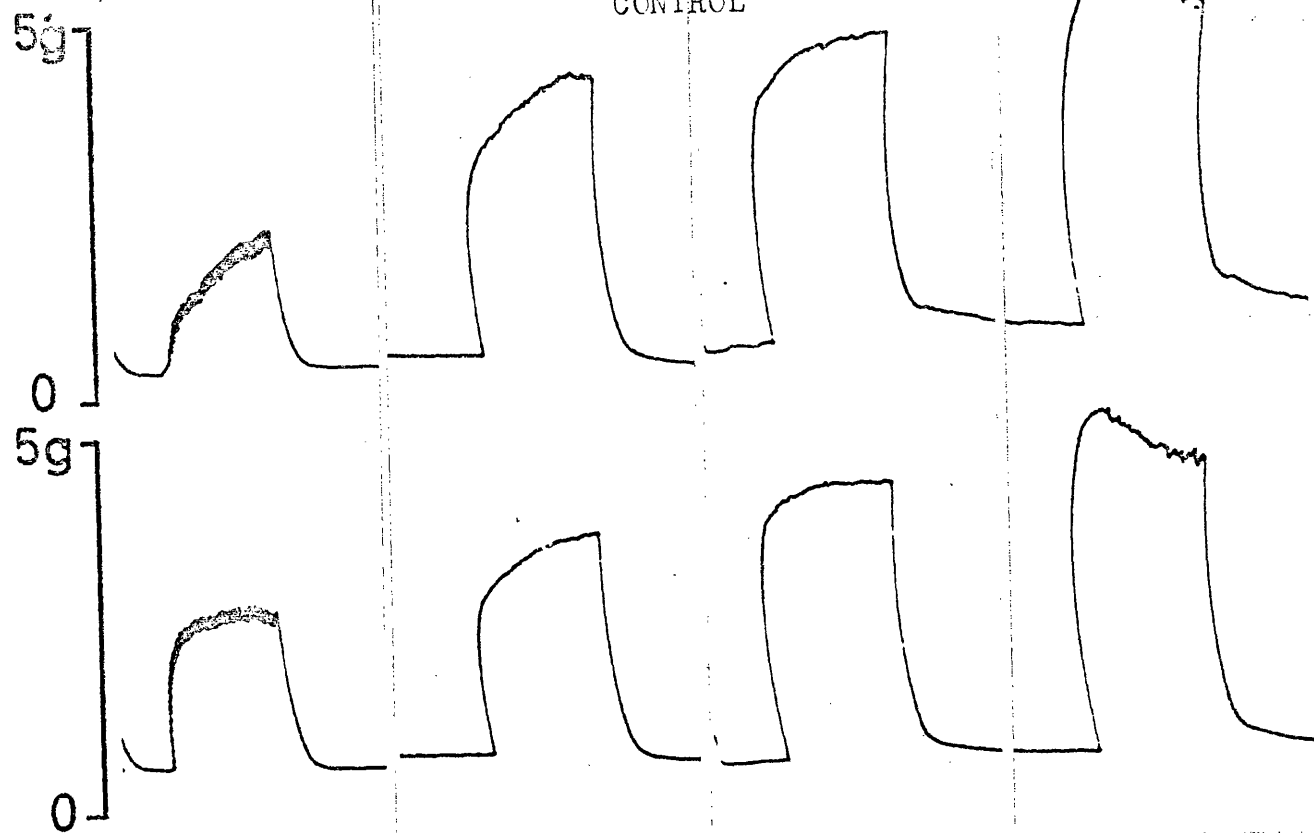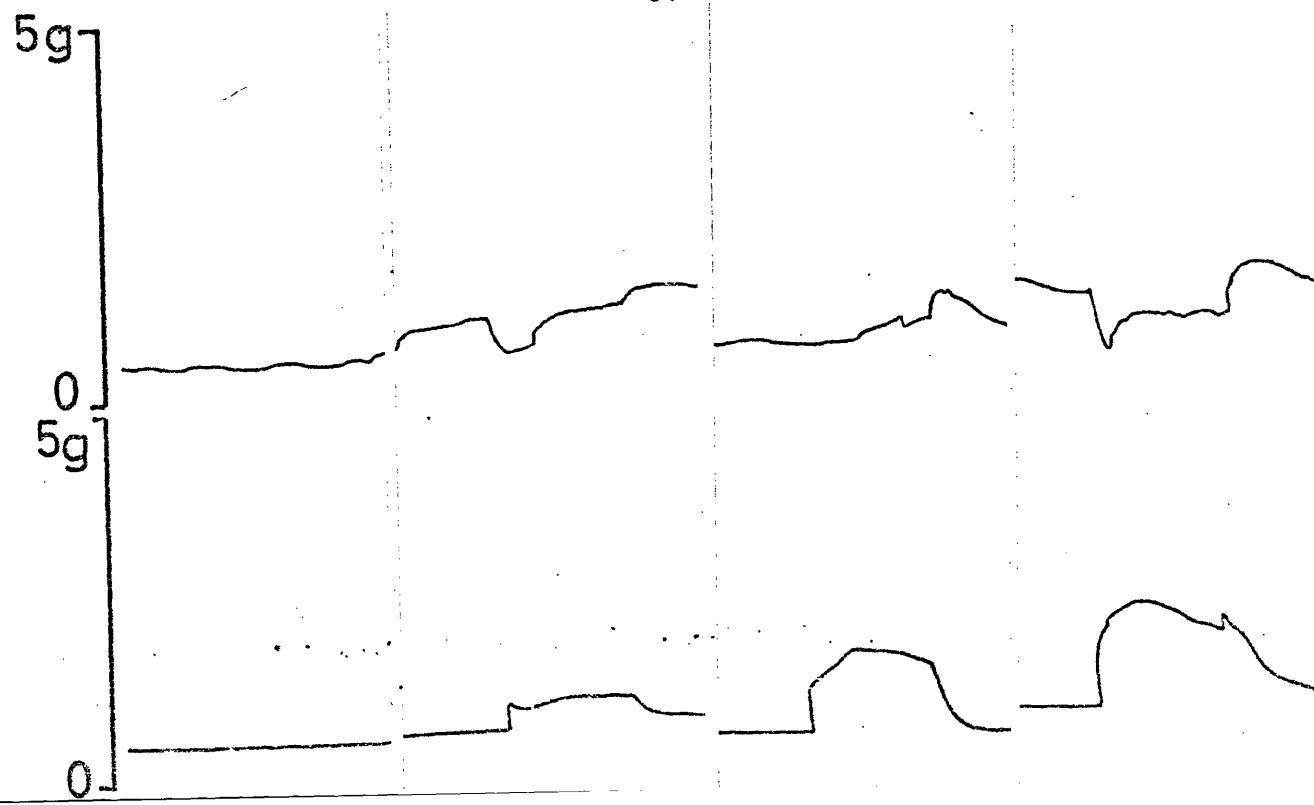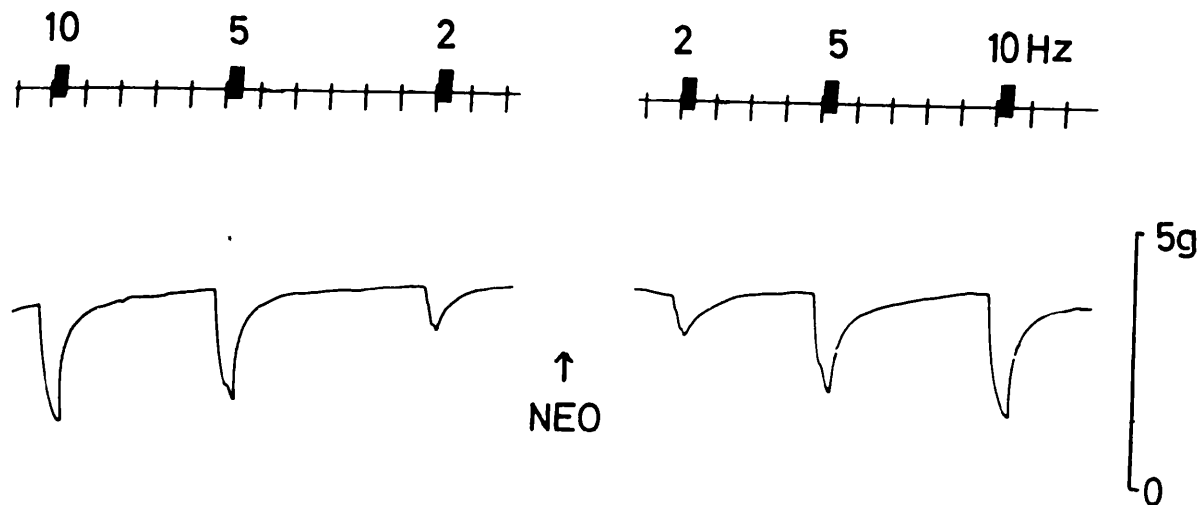
Fig. 82   Effect of neostigmine (NEO) 10^{-7} M on the inhibitory

response to field stimulation in the cat anococcygeus.   In

the presence of guanethidine 3 x 10^{-5} M, stimulation at the

frequencies indicated above the time trace produced inhibitory

responses.   Neostigmine had no effect on the size or

duration of responses at 2, 5, or 10 Hz.   Time 1 min.

depression of motor responses to field stimulation at the highest doses tested i.e. ATP 5 x 10^{-4} M; vasopressin 200 m U/ml; prostaglandins 10^{-7} g/ml; but produced no change in the size of the inhibitory nerve response.

There was, therefore, no evidence in either species of summation between any of the substances which were found to relax the cat anococcygeus and the inhibitory nerve response.

Effect of 6OHDA treatment on nerve responses

6OHDA added to the organ bath contracted the anococcygeus from both species and reversed motor nerve responses to inhibitory responses (Fig. 47). After washing out the 6OHDA, the motor response did not recover even up to 6 hr after washout. The inhibitory response, on the other hand, was produced by field stimulation in the presence of 6OHDA and after removal of 6OHDA inhibitory responses could be demonstrated when the tone was raised with NA. 6OHDA in vitro treatment thus appears to selectively destroy the motor nerves without affecting the inhibitory nerves.

In rats pretreated with 6OHDA in vivo, diminished motor nerve responses to field stimulation could be still be obtained (Fig. 81). Together with the evidence that indirect sympathomimetics can still produce contractions, this demonstrates that the sympathectomy produced by the 6OHDA in vivo pretreatment was incomplete.

Effect of neostigmine on nerve responses

Since the response to acetylcholine in the cat anococcygeus was potentiated by neostigmine (10^{-7} M) (Fig. 65) any role played by acetylcholine in the motor or inhibitory nerve transmission process might also be expected to be modified by cholinesterase inhibition. Neostigmine (10^{-7} M) had, however, no effect on the motor or inhibitory nerve responses in either species. This is shown for the cat motor nerves in Fig. 78 and the cat inhibitory nerves in Fig. 82.

Since the effects of acetylcholine in the anococcygeus of both species are potentiated by neostigmine and inhibited by atropine and the responses to stimulation of both inhibitory and motor nerves are unaffected by either neostigmine or atropine, it seems unlikely that acetylcholine has a significant role to play in the normal neuro - transmission processes of the anococcygeus muscles.

From the pharmacological evidence so far, it thus appears that the anococcygeus muscle of the cat contains a dual innervation similar to that in the rat.

A motor adrenergic innervation is present which can be activated by field electrical stimulation, whose responses can be mimicked by NA or by indirect sympathomimetics, which can be specifically blocked by the α - adrenergic blocker phentolamine or the adrenergic neurone blocking agent guanethidine and which can be functionally destroyed by the action of 6OHDA.

An inhibitory innervation exists which also can be activated by field stimulation, whose presence becomes most obvious when the muscle is contracted by motor agonists, for which no effective blocking drug has been found, which has only been abolished by tetrodotoxin and whose transmitter, therefore, remains unknown. Among the substances which can produce relaxation and therefore mimic inhibition, the effects of acetylcholine and isoprenaline can be specifically altered by drugs which do not affect the inhibitory nerve response. In addition, all these substances, which relax the cat anococcygeus, contract the rat anococcygeus.

The pharmacological properties of the innervation of the anococcygeus muscle are, therefore, similar in both species. It is, however, possible to demonstrate the presence of the inhibitory innervation in the cat muscle without resorting to the use of blocking drugs as is necessary in the rat.
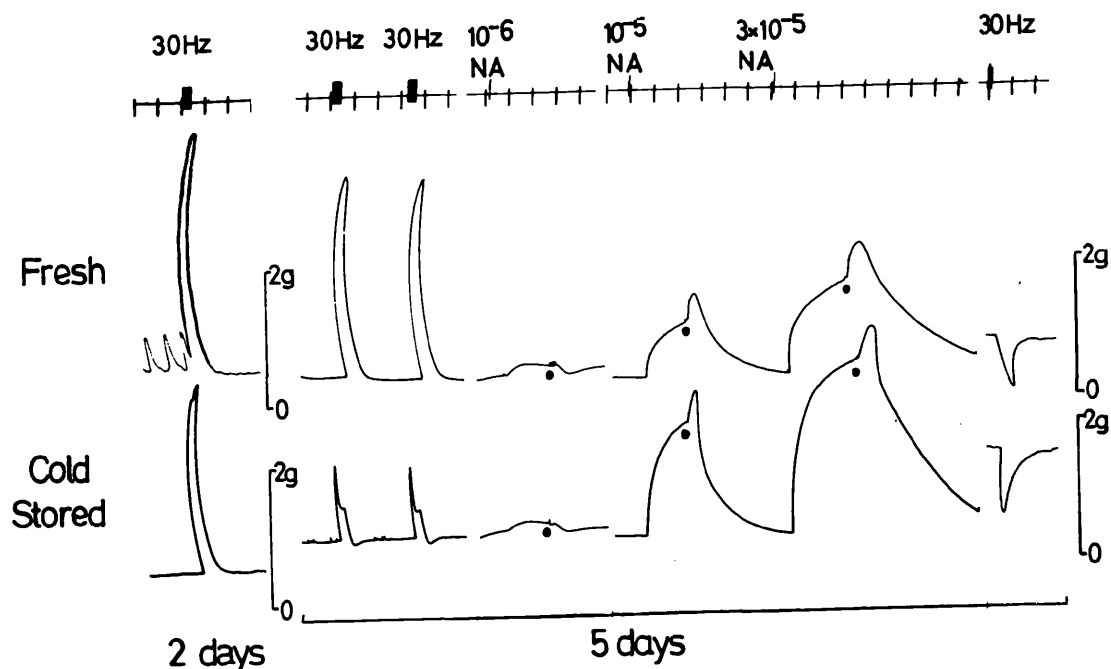
Fig. 83   The effect of cold storage on the responses to field
stimulation and to noradrenaline in the cat anococcygeus.   This
shows a simultaneous comparison of fresh muscles (upper trace) with
muscles stored for 2 or 5 days in Krebs' solution at 4° C (lower
trace).   In the first panel the motor response to field stimulation
of a 2 day stored muscle is compared with that from a fresh
preparation.   In the second panel the motor responses to field
stimulation and to noradrenaline, and the inhibitory response to
field stimulation after guanethidine 3×10^{-5} M (final panel) are
shown in fresh and 5 day cold stored muscles.   In this experiment
the motor response to field stimulation was reduced to 50%
compared with the fresh control, although the responses to
noradrenaline were larger.   There was no apparent change in the
ability of guanethidine to raise the tone of the muscle and
inhibitory responses were similar in 5 day stored and fresh
muscles.   Time 1 min.
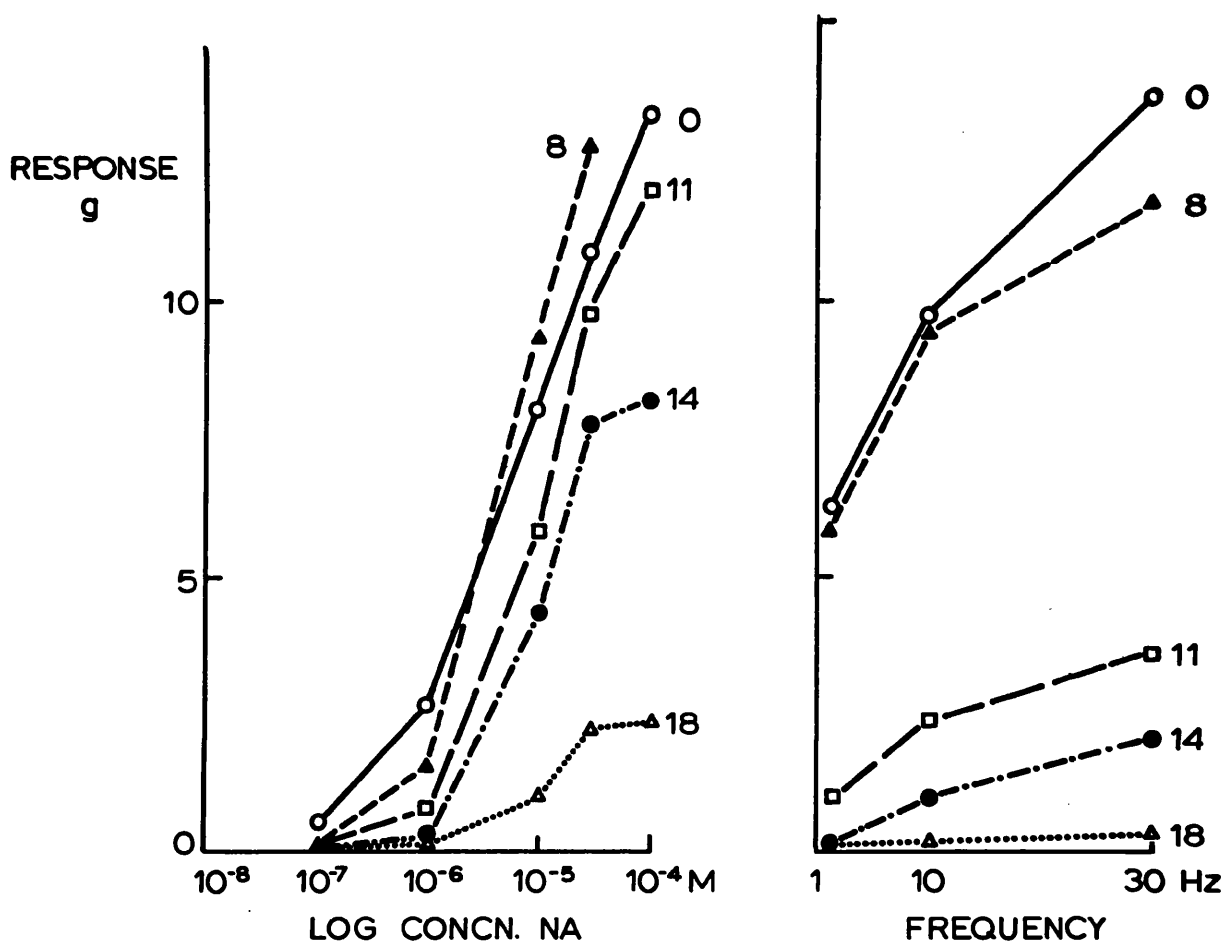
Fig. 84   Effect of cold storage on the response of a cat anococcygeus to NA and to field stimulation.   The mean of the responses in 2 fresh muscles (o————o 0) is compared with the responses of a muscle which was cold stored and intermittently tested between 8 - 18 days (▲— — —▲ 8, ◻— — —◻ 11, ●—·—·—● 14, △·······△ 18 days).   The response to NA declined steadily after the 8th day but survived longer than those to field stimulation.   The responses to field stimulation showed a sudden decrease between 8 and 11 days which may correspond to nerve degeneration.

Effects of cold storage on the cat anococcygeus

Since the anococcygeus muscles used were obtained from cats used in other experiments, some were stored in Krebs' solution at 4° C in a sealed beaker for a variable time before their responses were measured. Observations were, therefore, made on the effects of cold storage on the responses to field stimulation and to agonist drugs. The action of direct and indirect sympathomimetics and the responses to field stimulation proved remarkably resistant to cold storage. Up to 5 days there was little diminution of the motor response to either drug or nerve stimulation (Fig. 83).

In another experiment, the effect of NA and field stimulation was examined in the same muscle after 8, 11, 14 and 18 days cold storage (Fig. 84). At 8 days the responses to both NA and field stimulation were similar to those obtained with fresh tissues. After 8 days the responses to NA declined gradually but were still present at 18 days, the dose / response curve drawn as a percentage of the maximum remaining unchanged. The motor response to field stimulation, on the other hand, declined more rapidly, the greatest change occurring between 8 and 11 days. Motor responses to field stimulation were just detectable at 10 Hz and 30 Hz at 18 days.

The effect of cold storage on the inhibitory response was more difficult to examine since guanethidine was necessary to display inhibition and once given, further examination of the effect of cold storage on the motor response in that muscle was impossible. For this reason, the effect of cold storage on the inhibitory response was examined in the muscles from only 2 cats. In one cat, the response was measured in one muscle immediately after removal and in the contralateral muscle from the same animal, after 5 days cold storage. The muscles from the second cat were stored for 11 and 18 days respectively. The percentage inhibition of induced tone in the fresh muscle was 68%, comparable

(a) Control
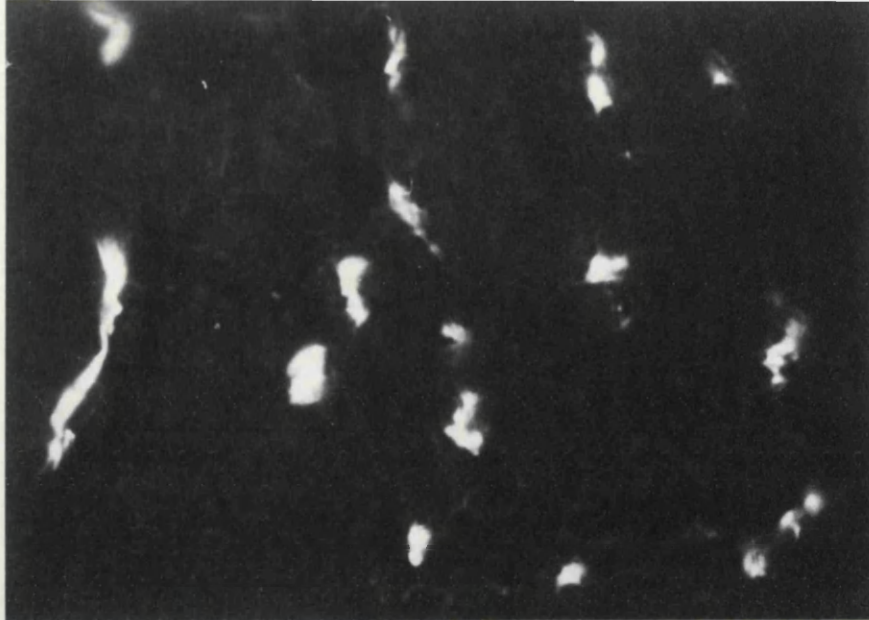
(b) 11 days
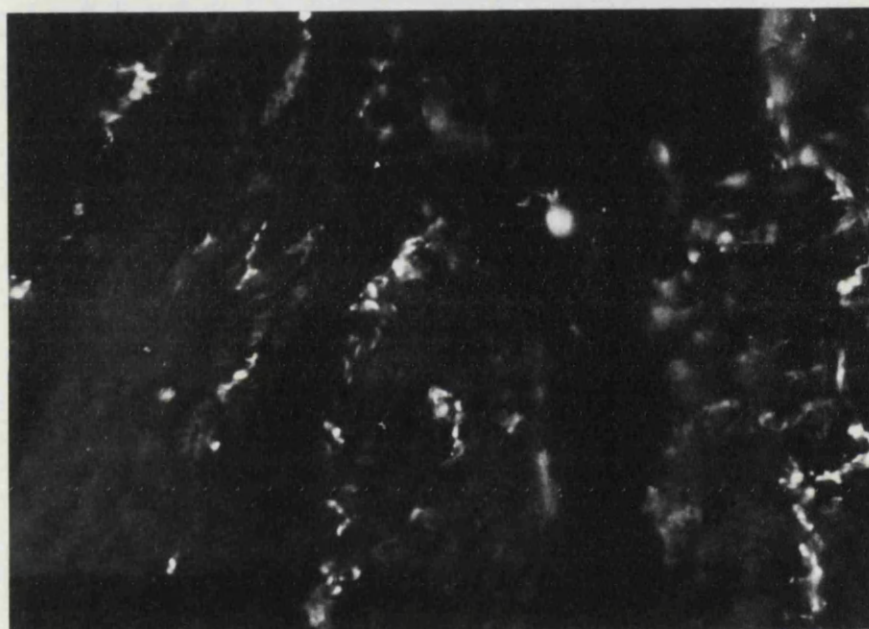
(c) 18 days

Fig. 85   Effect of cold storage on the adrenergic nerves
of the cat anococcygeus muscle as shown by the fluorescence
technique of Falck & Hillarp.

(a)   Control, transverse section.

(b)   After 11 days cold storage.   Note that varicosities
are less bright and have a more beaded appearance than the
control.

(c)   After 18 days cold storage no terminal varicosities
are visible.   The faint fluorescence at the bottom left
represents the remnants of an adrenergic nerve trunk.
Magnification x 1000.

to the average for all cats; at 5 days in the same cat the inhibition was 60%. In the other animal, at 11 days the maximum inhibition was 27% and at 18 days no response was obtained. The ability of guanethidine 3×10^{-5} M to raise the tone of the muscle was undiminished at 5 days, was reduced approximately 50% at 11 days and at 18 days only a small transient response was found.

Histochemical examination of a cat anococcygeus muscle after 5 days cold storage showed many fluorescent nerve terminals still visible. After 11 days, the terminals were less bright but still present and at 18 days they were just detectable (Fig. 85).

There was thus little change in the properties of the muscle over the 1 to 2 days for which muscles used in the experimental work were stored. The survival of the nerve and muscle elements of the cat anococcygeus after cold storage was considerably greater than in the corresponding muscles in the rat which do not respond to field stimulation or to NA after only 5 or 6 days (D. Templeton, personal communication). Species variation may, therefore, be as important as the properties of the particular tissue used in determining survival after cold storage.

DISCUSSION

The work on which this thesis is based falls conveniently into three related sections, the rat anococcygeus _in vivo_, the effects of nerve stimulation on NA depletion by reserpine and the cat anococcygeus _in vitro_. These are discussed in turn followed by a section linking them together.

RAT ANOCOCCYGEUS _IN VIVO_

The work of Gillespie (1972) made it clear that field stimulation of the rat anococcygeus could produce two separate and opposing nerve mediated responses, a motor response in the absence of drugs and an inhibitory response when the motor nerve response was blocked and the tone of the muscle raised. On histochemical and pharmacological grounds, it was shown that neither response was preganglionic and that the motor response was due to adrenergic neurones. The inhibitory response could, however, be neither blocked nor mimicked by conventional drugs and was, therefore, uncharacterised.

The possible mechanisms responsible for a dual response are several, e.g. (1). If only one type of fibre is concerned, the nature of the response is determined by (a) the "peripheral mechanism" (McCrea, McSwiney & Stopford, 1925) (b) Wedensky inhibition (e.g. Veach, 1925; Cannon, Raule & Schaefer, 1954) (c) variable effect of a single transmitter (e.g. Bozler, 1940; Burn & Robinson, 1951; Gardner & Kandel, 1972). (2) Two functionally different fibre groups exist, usually adrenergic and cholinergic, and these are organised either as (a) sympathetic and parasympathetic fibres with separate anatomical origin but which may come together peripherally to reach the target organ in a common nerve bundle (e.g. McSwiney & Robson, 1929; Finkleman, 1930) (b) fibres are of similar origin but sympathetic may be cholinergic (Dale & Feldberg, 1934) and parasympathetic may be adrenergic.

The experiments described, fortunately eliminate all of these explanations other than the existence of a double set of fibres with separate origins in the spinal cord and mediated by two different transmitters. The preganglionic fibres in the motor pathway originate in the lower thoracic and upper lumbar region of the spinal cord whereas the preganglionic fibres in the inhibitory pathway originate in the lower lumbar and upper sacral region. This demonstrates clearly that there are two separate nerve pathways giving rise to the two opposing responses and eliminates the possibilities of only one type of fibre being involved or of two different types of fibre with a similar origin. This has now been supported by the evidence that after a sympathectomy by 60HDA, the motor response to field stimulation is greatly reduced but the inhibitory response remains unchanged (Gillespie & Gibson, 1973).

The nature of the motor fibres is clear. Anatomically, preganglionic fibres arising from the thoracico - lumbar outflow belong almost exclusively to the sympathetic nervous system (Mitchell, 1953; Pick, 1970) so the classification as sympathetic of the motor nerves is justified on anatomical grounds alone as well as being supported by histochemical and pharmacological evidence of their adrenergic nature.

The nature of the inhibitory fibres is more interesting. On the basis of their spinal origin, they could have been either autonomic efferents or possibly, posterior root afferents equivalent to the posterior root vaso - dilators (Bayliss, 1902; Lewis & Marvin, 1927; Wybauw, 1936; Holton & Perry, 1951). If they are autonomic they are not cholinergic as their spinal origin (similar to sacral parasympathetic) might suggest since they are not blocked by atropine. The blockade of the inhibitory responses by hexamethonium, on the other hand, suggests that they are autonomic efferent

fibres. If they are not cholinergic, then they might have been purinergic but their spinal position does not commend the inhibitory nerves to classification within the purinergic system either. In his review of this system, Burnstock (1972) cites only one tentative, unpublished example of purinergic nerves under sacral parasympathetic control. Central control of purinergic nerves, where it occurs at all, appears to be via the vagus.

The possibility of antidromic stimulation of posterior root afferent fibres was also eliminated by the use of ganglion blockers. This possibility is excluded if a ganglion is shown to be in the pathway since afferent nerves lack a peripheral synapse, and even if they had one, it would be unlikely to transmit impulses in the antidromic direction.

The demonstration of ganglion blockade again became useful when it was found that the motor nerves could be stimulated postganglionically with the spinal electrode in a position which partially overlapped with the origin of the inhibitory nerves. Since the inhibitory region extends one segment further rostrally to L 5 , enabling selective stimulation of the inhibitory nerves alone, as demonstrated in the presence of tyramine, ganglion blockade was not a necessary condition for the demonstration that the motor and inhibitory pathways were separate. It served, however, as a further adjunct.

This ability to stimulate the motor nerves postganglionically was, in itself, interesting since in previous investigations of this method of stimulating the spinal outflows, it had never been possible to stimulate postganglionic fibres (Gillespie, MacLaren & Pollock, 1970) and it is believed that the usual location of such stimulation is the point at which the ventral roots leave the vertebral canal. One explanation for the present exception could have been the position of the anococcygeus directly in front of the vertebral column,

enabling stimulation of the nerves within the muscle. This was, however, excluded by the radiographic demonstration that the distance between the electrode and the muscle was at least 20 mm and up to 40 mm.

The most likely explanation is that some anatomical feature brings the postganglionic sympathetic nerve which supplies the anococcygeus close to the vertebral column in the upper sacral region, bringing it into close apposition with the spinal electrode in this position and producing favourable conditions for stimulation. The precise anatomical feature which makes this possible is not known but it has been shown that postganglionic fibres of the genitofemoral nerve, which supplies the anococcygeus, can take an unusual route which might make it particularly susceptible to stimulation by a spinal electrode (Pick, 1970). The postganglionic sympathetic fibres leave the sympathetic ganglion by the usual route via the gray ramus to the spinal nerve but then may leave the spinal nerve again and fuse with the sympathetic trunk at a more caudal level. This feature has been reported only in the rhesus monkey (Zuckerman, 1938; Sheehan &Pick, 1943) and in man (Pick, 1970) but if it occurs in the rat, it might explain why   (1)   preganglionic fibres can be stimulated in the upper lumbar region, in the usual way, (2)   no fibres to the anococcygeus can be stimulated in the central lumbar region since the preganglionic outflow to the tissue ceases at L 3 and (3)   postganglionic fibres can be stimulated in the lower lumbar and upper sacral region where the postganglionic nerve again comes near to the vertebral column. This hypothesis is, however, not wholly satisfactory since it brings the postganglionic nerve only as near to the vertebral column as the sympathetic trunk, and we have no reason to suppose that the spinal electrode can normally stimulate this structure. This may, nevertheless, point to the type of feature involved.

Another possible factor bearing on the apparent uniqueness of this ability to stimulate the anococcygeus postganglionically might be the location of the ganglia. In many of the previous investigations carried out using the pithing rod as the electrode, the responses measured have been due to either parasympathetic nerves with their ganglia located close to the target organ e.g. sacral parasympathetic to the bladder or colon (Gillespie, MacLaren & Pollock, 1970), or to sympathetic nerves with ganglia located at sites distant from the sympathetic chain e.g. thoracico -lumbar outflow to the vas deferens via the hypogastric nerve with the ganglia located along the peripheral end of the nerve, to the heart via the stellate ganglia, to the nictitating membrane via the superior cervical ganglion and although not strictly a ganglion synapse, to the adrenal medulla (Gillespie & Muir, 1967 a; Gillespie, MacLaren & Pollock, 1970; Gillespie, MacLaren, Marshall, Muir & Pollock, 1970; MacLaren & Pollock, unpublished). In the anococcygeus, on the other hand, the sympathetic motor nerves originate from the genito - femoral nerve which has paravertebral ganglia located in the sympathetic chain. It is, therefore, possible that the anococcygeus is one of the few tissues we have examined in the pithed rat which has a "classical" sympathetic innervation, the other main exception being the thoracico - lumbar vasomotor outflow, and that if other such pathways were examined, postganglionic stimulation might be possible there also.

Whatever the reason for this facility for pre - or post - ganglionic nerve stimulation, it does provide a simple preparation in which both pre - and post - ganglionic nerve stimulation can be selectively achieved and the responses of the end organ measured _in situ_ with a minimum of dissection. This might provide a convenient _in vivo_ means of assessing drugs acting on ganglia, especially since the motor pathway to the anococcygeus appears to be relatively susceptible to ganglion blockade compared with other pathways

such as the motor nerves to the vas deferens or the inhibitory pathway to the anococcygeus itself.

The reason for this differential susceptibility of ganglia to blockade in various pathways is not clear from the present experiments. The motor pathway to the vas deferens, the inhibitory pathway to the anococcygeus and the nerve - mediated depressor responses seen when blood pressure was raised all showed comparatively rapid recovery from the effects of ganglion blockers when compared with the motor pathway to the anococcygeus. In addition, the inhibitory pathway to the anococcygeus exhibited a form of desensitisation whereby an initial low dose of hexamethonium prevented blockade by a subsequent larger dose.

Differential action between tissues in terms of both time scale and the extent of blockade is not surprising since at their introduction as ganglion blockers by Paton & Zaimis (1951), it was noticed that the methonium salts had quantitatively different actions on different pathways and that the relative efficacy on different pathways varied between different members of the series. It has subsequently become accepted that differences do exist in the susceptibilities of various autonomic ganglia to ganglion blocking agents and indeed that they may exist between cell groups within a given ganglion (Volle & Koelle, 1965). It has further been suggested that morphological differences between ganglia may produce uneven distribution of blocking agents and that this may be a primary reason for the different sensitivities to blockade (Shaw, MacCallum, Dewhurst & Mainland, 1951; Volle, 1962). In the present investigation, the paravertebral ganglia of the anococcygeus motor pathway and those of the vasopressor nerves were more susceptible to blockade than those elsewhere but there is no justification in the literature for a simple correlation between the anatomical nature of the ganglion and the effects of blocking agents; indeed the difference between the susceptibilities of cells

within a given ganglion make this clear. A further possibility which was at first considered for the relative resistance of the ganglia in the anococcygeus motor pathway was that after blockade of the hexamethonium - sensitive transmission process in the ganglia, an atropine - sensitive muscarinic transmission process might have taken over (Steinberg & Hilton, 1966). Atropine, however, produced no further ganglion blockade when the pathway had recovered from or was desensitised to hexamethonium. Muscarinic transmission does not, therefore, appear to be responsible for the resistance to conventional ganglion blockade.

The anococcygeus responded to stimulation of the adrenals on a time scale similar to that of the heart rate and blood pressure. Stimulation of the thoracic outflow to the adrenals with a 300 pulse train at 30 Hz produced a response in the anococcygeus similar to that of 1 ug NA in a 250 g rat, this response being 70% of the response to maximal motor nerve stimulation. This high in situ sensitivity to catecholamines of either endogenous or exogenous origin is similar to the response of the nictitating membrane (Rosenblueth & Bard, 1932) but different from that of the vas deferens which contracts to neither adrenal stimulation nor injected catecholamines. In the vas deferens, only a small response in the perfusion pressure was recorded following adrenal stimulation. Since all three tissues, anococcygeus, vas deferens, and nictitating membrane, receive a motor adrenergic innervation and are contracted in vitro by catecholamines, this differential effect of circulating catecholamines may be produced by several factors which are common to the anococcygeus and nictitating membrane but different for the vas deferens. Differences in sensitivity to drugs are usually explained by differences in the access of the drug to its site of action or to differences in the sensitivity of the tissue itself to the drug.

Access of catecholamines to their site of action has been invoked as a factor determining the response to catecholamines in two ways.

In the anococcygeus, vas deferens (Birmingham, 1970; Greenberg & Long, 1971) and nictitating membrane (Trendelenburg & Weiner, 1962), blockade of the neuronal NA uptake mechanism or complete removal of adrenergic nerves by sympathectomy produces increased sensitivity to NA. It has been suggested that in the vas deferens, the highly efficient neuronal uptake mechanism may be responsible for the comparatively low sensitivity to NA (see Birmingham, 1970). There is, however, no evidence that blockade of this mechanism leads to any greater increase in sensitivity of the vas than of the other two tissues (Birmingham, 1970; Tsai, Denholm & McGrath, 1968; Gibson & Gillespie, 1973), which suggests that this is not the primary reason for the difference in sensitivity.

Also under the heading of access, it has been suggested that the low sensitivity of the vas to several drugs may be due to a relatively low rate of perfusion of blood through the tissue (Kopin, Gordon & Horst, 1965; Sjostrand & Swedin, 1968). This might be expected to lead to a lower net drug concentration at the site of action and therefore a smaller response for a given concentration, but this hypothesis does not hold true for all drugs e.g. guanethidine and reserpine both exert their blocking action on the motor nerves within 2 min, and judging by the lack of fatigue after vigorous, repetitive stimulation of the sympathetic outflow of the tissue over a 2 hr period, the tissue is very adequately perfused for maintenance of its metabolic activity.

The most convincing explanation for the differences in sensitivity between these tissues is the sensitivity of the muscle cells themselves to the drug. This may be related to the cytological arrangement of the muscle and to the physiological role of the muscle cells in the various tissues. In the vas

deferens, the synaptic cleft between nerve and muscle is relatively close
(Burnstock, 1970) and each cell receives at least one synapse (Richardson, 1962).
Conditions are thus favourable for synaptic transmission in this narrow cleft
where high concentrations of NA may be built up by release from nerves.   Over
the surface of the cell as a whole, however, sensitivity to NA is insufficient
for either exogenously administered NA or catecholamines from the adrenals to
act.   In the anococcygeus (Gillespie, personal communication) and in the
nictitating membrane (Esterhuizen, Graham, Lever & Spriggs, 1968), however, the
synaptic gap is larger i.e. 1000 - 3000 Å cf 200 Å for vas deferens, and from
both the _in vitro_ and _in vivo_ evidence, sensitivity of the cells as a whole
to catecholamines is greater.   Thus in the latter tissues, the nerve and muscle
are organised in such a way that the transmitter may act over a wider area than
in the vas, perhaps that from one varicosity acting on several cells, and also
so that blood catecholamines may produce an effect on the tissue.   In the vas
deferens, on the other hand, the organisation favours specific nerve transmission
via discrete connections and prevents contraction of the muscle by blood
catecholamines.

This situation thus favours a simple contraction of the anococcygeus and
nictitating membrane by either sympathetic nerve or adrenal activity.   Since
both tissues consist of sheets of smooth muscle and the function of the
nictitating membrane and probably also of the anococcygeus is to contract under
conditions of sympathetic stimulation, then their cytological organisation,
which manifests itself in the present experiments by influencing the
pharmacological sensitivity to NA, is seen to be necessary for their function.
In the vas deferens, on the other hand, the muscle is organised in a complex
manner with inner circular and outer longitudinal layers.   The function of this
muscle is not simply to shorten, but to propel the seminal fluid, in one

direction, during the process of emission. It is thus likely that specific control of each muscle cell, or at least of small bundles, is necessary in order that the contractions of the various components are integrated to produce this response. Since sexual activity takes place only under specific circumstances, it would be undesirable if adrenal activity, whenever it occurred, produced emission. In addition, the greater sensitivity to circulating catecholamines of the circular compared with the longitudinal muscle, suggests some further physiological correlate. The structure and pharmacological properties of the vas deferens are thus less surprising if its function is taken into account. So far as I can determine from the literature, very little is known of the precise mechanism of muscle contraction leading to emission (see Bell, 1972) despite the popularity of the vas deferens for pharmacological investigation.

The cardiovascular responses in these experiments corresponded with the findings in the original experiments of Gillespie, MacLaren & Pollock, (1970), with the additional uncovering of a nerve - mediated depressor response.

The origin of the splanchnic nerve fibres to the adrenal medulla overlaps with the direct sympathetic outflow to blood vessels. For this reason it is not possible to elicit a pressor response from adrenal stimulation without a superimposed response from stimulation of the sympathetic nerves to the arterioles. Simultaneous experimental release and assay of adrenal catecholamines in the one animal is therefore not possible utilising the pressor response. Since the sympathetic outflow to the anococcygeus arises some considerable distance from the fibres to the adrenal medulla and since the anococcygeus shows a high sensitivity to circulating adrenaline, it is possible to use it as an assay organ for adrenal output.

In the presence of raised blood pressure, an unexpected depressor response was produced by stimulation in the lower lumbar and upper sacral regions. This response was abolished by ganglion blockade so is therefore not due to antidromic stimulation of afferent nerves, and was unaffected by atropine. This effect of atropine eliminates the possibility of cholinergic vasodilator nerves.

Dusting and Rand (1972) have demonstrated depressor responses to stimulation of the entire autonomic outflow after artificially raising the blood pressure with NA. In their experiments, no neuro - muscular blocker was adminstered to prevent skeletal muscle twitching. By sequential administration of blockers, they demonstrated that a large part of their complex depressor responses was due to (1) excitation of motoneurones with consequent (a) functional hyperaemia in skeletal muscle, producing delayed depressor responses (b) overflow of acetylcholine from cholinergic nerves to blood vessels (2) β - effects of adrenaline released from the adrenal medulla (3) an extra component which they could not block and therefore did not characterise n.b. this response was resistant to among other things, hexamethonium. None of these factors are likely to be relevant to the present findings. Functional hyperaemia is not a factor since pancuronium was administered. Overflow of acetylcholine seems an unlikely cause since atropine did not block the response and, due to the selective method of stimulation, the adrenals are not a factor. The final uncharacterised part of their response is also unlikely to be related to the present response since their electrode ended at L 5 or L 6 which is just at the rostral end of the origin of the present depressor nerves and finally their response, unlike the present one, was unaffected by hexamethonium.

There thus appears to be a sacral preganglionic outflow in the rat with an

origin similar to that of the inhibitory nerves to the anococcygeus and which produces depressor responses by a non - adrenergic, non - cholinergic mechanism. These depressor responses, in absolute terms, are relatively small but, if as is likely, the vascular bed affected is limited to a particular area, then the effect in that bed would be substantial. No such responses have previously been reported in the pithed rat, but Hughes & Vane (1967) have demonstrated a non - adrenergic, non - cholinergic inhibitory nerve response in the isolated portal vein which might be analogous to the peripheral component of this response.

The similarity of this depressor response in the pithed rat to the inhibitory response in the anococcygeus is quite clear. Both share a similar spinal origin, have ganglia with a similar sensitivity to hexamethonium and are neither adrenergic nor cholinergic. It may, therefore, be tentatively postulated that they belong to related systems.

In recent years radical new theories on the nature of transmission in the vas deferens have been advanced. Ambache & Zar (1971) have postulated that the motor transmitter is not NA but some unknown chemical. NA is, of course, present in large quantities in the vas deferens and, furthermore, is undoubtedly present in nerves. Ambache and co - workers postulate that NA and the adrenergic nerves are inhibitory. This theory is based mainly on four observations on the isolated guinea - pig and rat vas deferens -

(1) The undoubted low sensitivity of the vas to the motor effect of NA (Ambache & Zar, 1970).

(2) The inability of α- receptor blockers such as phentolamine or NA depletion by reserpine to abolish the response to motor nerve stimulation (Ambache & Zar, 1971).

(3) The ability of NA, tyramine or cocaine to inhibit the motor response

to nerve stimulation (Ambache & Zar, 1970; Ambache, Dunk, Verney & Zar, 1972).

(4) The ability of LSD to block the motor response to nerve stimulation (Ambache, Dunk, Verney & Zar, 1973).

The only evidence which they found to be against their theory was that guanethidine and bretylium completely abolished the motor nerve response. /

This was, however, explained as a non - specific action of guanethidine and bretylium since they have been shown to be capable of paralysing cholinergic transmission in the phrenic nerve - diaphragm preparation (Dixit, Gulati & Gokhale, 1961).

A major factor which must be borne in mind when comparing the work of Ambache and co - workers with that of other authors is that they employ only short pulse trains, preferably 15 pulses or less, which they use because they consider this to be as many pulses as are likely to occur physiologically although they do not quote the basis for this assumption. As a result of this, they are observing only the first phase of the biphasic response seen in the present experiments or in those of Swedin (1971). They also show that inhibition by NA and LSD is more effective on short than on long trains (Ambache & Zar, 1971; Ambache, Dunk, Verney & Zar, 1973). This first part of the response is, of course, the part which is most unconventional in its response to drugs affecting adrenergic systems. This confinement to the study of a short train of pulses will, therefore, put an undue bias against adrenergic motor transmission.

Another hypothesis which seeks to explain the motor transmission mechanism but taking into account the two phases of the response was put forward by Swedin (1971). From studies on the rat and guinea - pig isolated vas deferens, he concluded that both phases were due to adrenergic nerves but that the basis of the two phases was that the NA released from the nerves acted at two

different sites of action on the muscle. In this hypothesis, NA released into the synaptic cleft produces the fast "spike" response. This same NA then diffuses to more distant sites on the muscle to produce the slower secondary response. It is difficult to see, since nerve stimulation continues, why the initial response should wane before the secondary response appears, and this is not explained in his argument.

These two theories of Ambache et al and of Swedin are obviously mutually antagonistic although the main conflicts arise from differences of experimental approach. The present experiments, however, offered experimental advantages in investigating this problem since the anococcygeus has a similar motor innervation arising from a similar spinal level but with a sensitivity to both agonists and antagonists which corresponds to a conventional adrenergic innervation. It therefore serves as an excellent control on which to study simultaneously the effects of drugs on the vas deferens. In this way, a comparative study could be made of the similarities and differences which this motor innervation in the vas deferens has to the motor adrenergic nerves in another smooth muscle tissue.

In the present study, the effects of drugs on the motor response to stimulation of the sympathetic outflow to the vas are discussed in relation to the biphasic nature of the response. Three main factors bear on any interpretation of this response - (1) The two phases appear to have a common spinal origin (2) They are differentially affected by several drugs which act at either the ganglion or the nerve - muscle junction (3) Only one component of the response appears to be present in the circular muscle. With these points in mind, it is concluded from the present experiments that there are two anatomically and pharmacologically distinct components to the motor innervation of the vas although these have a common spinal origin.

This leads to the development of yet another hypothesis concerning the nature of the motor innervation in the vas deferens. Each component of the response, in this hypothesis, is considered to be due to a separate nerve pathway.

The first, which produces the initial "spike" response, acts mainly on the longitudinal muscle of the vas, is less susceptible to ganglion blockade than the second pathway and is inhibited by an α- adrenergic mechanism which normally terminates the response by negative feed - back. This α- inhibition can be reinforced by exogenously applied NA or, most effectively, by endogenous release of NA by the indirect sympathomimetic action of tyramine or LSD and can be blocked by phentolamine or by pretreatment with reserpine, both of which produce prolongation of the "spike". Apart from guanethidine and the chronic effects of reserpine treatment, drugs normally associated with modification of adrenergic responses do not affect this response in the way that they affect, for instance, the anococcygeus response or the secondary phase of the vas's own response.

The second component in the pathway produces the secondary response, which occurs in both the circular and the longitudinal layers, is more susceptible to ganglion blockade than the "spike" and is potentiated by an α- adrenergic mechanism. This secondary response is thus potentiated by exogenous NA or by the indirect sympathomimetic action of tyramine or LSD and is inhibited by phentolamine or by reserpine pretreatment. The effects of all the drugs tested on this part of the response were similar to those on the anococcygeus and are as might be expected in a conventional adrenergic system.

This interpretation stands in partial disagreement with the explanations of motor transmission in the vas given by Ambache et al and by Swedin but is basically an attempt to resolve some of the problems inherent in both these

theories in order to form a more cohesive working hypothesis.

The experimental findings which led to this hypothesis are not in conflict with those of Ambache and co - workers except in some points of interpretation. Their view that NA, tyramine and LSD are all inhibitory to the motor nerves is confirmed, provided that it is acknowledged that only the first component of the response is so affected.

A major difference arises, however, when the effects of reserpine are considered. Ambache, Dunk, Verney and Zar, (1972), concluded that tyramine and cocaine exerted their inhibitory effect by an indirect sympathomimetic action but that LSD did not produce its action through this means since reserpine pretreatment did not prevent its effect (Ambache, Dunk, Verney & Zar, 1973). Since it is also part of their hypothesis that transmission is non - adrenergic since reserpine pretreatment does not abolish it, a large part of their argument is seen to rest on the ability of reserpine to completely prevent both adrenergic transmission and indirect sympathomimetic effects. From the present work on the anococcygeus and other work on other tissues (e.g. Sedvall & Thorson, 1965; Anden & Henning, 1966; Lee, 1967) it is clear that even depletion of NA which is virtually complete as measured by biochemical estimation of NA content, may be insufficient to cause a complete blockade of adrenergic transmission. If this is the case in the vas deferens also, as seems likely (see Swedin, 1971), then the sympathectomy produced by reserpine pretreatment is insufficient to prevent the action of indirect sympathomimetics as well as adrenergic transmission. To counter this, Ambache, Dunk, Verney & Zar (1973), claim that the failure of tyramine to inhibit motor responses after reserpine treatment is a measure of the failure of indirect sympathomimetics under these circumstances. The present experiments in vitro with both rat and cat anococcygeus demonstrated that in this tissue at least, LSD is a more powerful and persistent indirect

sympathomimetic than tyramine and so its effects might be expected to be more difficult to suppress. In support of adrenergic transmission, and relevant to this point, Wadsworth (1973) has demonstrated that if a complete sympathectomy is produced in the rat vas deferens by _in vitro_ 6OHDA treatment, then no motor nerve responses can be obtained. This suggests that the reserpinisation employed by Ambache, Dunk, Verney & Zar (1973) is indeed insufficient to produce complete sympathectomy.

In addition to this, both the present experiments and those of Ambache, Killick, Srinivasan & Zar (1973) have demonstrated that LSD also inhibits the motor response in the anococcygeus which certainly is adrenergic. This weakens the argument that inhibition by LSD points to a non - adrenergic mechanism. More interesting, and possibly more relevant, is the clear similarity between the effect of LSD and that of guanethidine, both of which block the adrenergic response in the anococcygeus as well as releasing NA from the nerves. Since guanethidine is the one drug whose action in the vas is consistent with an adrenergic innervation, LSD should also perhaps be interpreted as reinforcing the adrenergic nature of the initial component in the response. Whether the mechanism of action is similar for LSD and guanethidine, the net result of NA release and neurone blockade is different in the vas and anococcygeus. Since the anococcygeus is highly sensitive to the motor action of endogenously released NA, it would be expected that indirect sympathomimetics would cause contraction whereas the vas with a low sensitivity to the motor action of endogenously released NA could be expected not to show the indirect sympathomimetic effects. If the present hypothesis is true, then the feed - back inhibition of NA release in the vas deferens produced by an α - receptor action via indirect release of NA should be prevented by phentolamine. Direct neurone blockade of the guanethidine type, on the other hand, presumably would not.

From this it is concluded that there is insufficient evidence available to

justify the rejection of NA as motor transmitter in the vas, even for the ubiquitous initial component.

Many of the results reported by Swedin (1971) suggest that motor transmission in the vas is adrenergic and corroborate with the present results e.g. similar results with reserpine pretreatment and guanethidine, and a preferential inhibition of the first phase by PGE_2 is demonstrated which suggests a further pharmacological difference between the two phases.

Swedin's hypothesis that the two phases are produced by NA acting at two separate sites of action, while fitting his own experimental findings, is difficult to adapt to the present results. These demonstrate a differential action of ganglion blockers and a difference in the response of circular and longitudinal muscle. Ambache, Dunk, Verney & Zar (1972) raise a related objection to Swedin's hypothesis. This is the apparent contradiction whereby PGE_2 is claimed to inhibit the "spike" phase by inhibition of NA release and yet the secondary phase, which is also supposed to be due to this same NA, is unaffected. These objections are resolved if the two hypotheses are combined so that the two phases are due to different nerves, both of which are adrenergic but only one of which is susceptible to PGE_2. This latter explanation is the view of the present author.

A further objection to Swedin's hypothesis is raised by Ambache, Dunk, Verney & Zar (1972) which might also apply to the present hypothesis. This is based on their own findings that the motor effect of tyramine is abolished by α - blockers which they claim negates the existence of α - receptors resistant to α - blockers, a concept postulated by several authors (see Holman, 1970) and which is necessary in Swedin's system. This objection is not entirely sound since it is quite likely from the system suggested by Swedin that both the high concentration of NA and its localisation to the

synaptic cleft is responsible for the resistance to phentolamine. It is

unlikely that tyramine will release NA in this localised fashion or at the

same initial rate as does nerve stimulation and so it is hardly surprising

that it is more susceptible to blockade.

It is hoped that the present hypothesis ties together the work of both

Ambache et al and Swedin to provide a more cohesive working hypothesis of

motor transmission in the vas deferens and that it does not simply lead to

further confusion. It brings together the major contribution of the work of

Ambache et al which is that the motor nerves of the vas deferens are

unconventional by the standard of other known adrenergic systems and that

sympathomimetic agents are inhibitory to these nerves and the contribution of

Swedin which is that there are two phases to the response and that both bear

the characteristics of adrenergic responses. It is further suggested that

many of the experimental anomalies arising from these properties may be

explained by the presence of two sets of nerves. No new concepts have been

introduced in forming this further hypothesis except that LSD should be

considered as both a neurone blocking agent and an indirect sympathomimetic

and that the two components of the vas deferens response are both adrenergic

but elicited from two different muscle groups, the initial fast "spike" from

the longitudinal and the slow response from both longitudinal and circular

layers. The properties of the adrenergic nerves innervating these two regions

also appear to differ in that those serving the longitudinal muscle are

influenced by prostaglandins. Even the suggestion that differences exist

between the two muscle layers has been demonstrated by Donald (1969). She

showed that splitting the vas longitudinally led to a decrease in the

longitudinal tension response to nerve or drug stimulation which could only be

explained if the circular muscle was contributing a significant part of the

longitudinal tension in the unsplit vas. She further demonstrated considerable

qualitative differences between the split and unsplit vas in response to agonist drugs which suggested different properties for the longitudinal and circular layers. The only other concept which is employed in forming the present hypothesis is that of endogenous inhibition of NA release by an α - receptor mechanism. This is now a well established phenomenon in the vas deferens (see Stjarne, 1973).

In summary, it is hoped that this work with the anococcygeus of the pithed rat has demonstrated that the anococcygeus muscle receives two anatomically separate nerve pathways, one motor and one inhibitory, which originate from different levels of the spinal cord, producing opposing responses on the muscle, that both belong to the autonomic nervous system as indicated by the presence of ganglia, that the transmitter in the motor nerves is noradrenaline, that the transmission in the inhibitory nerves is unlikely to be either cholinergic or purinergic and that both sets of nerves have properties which are qualitatively similar to those in the cardiovascular system. In addition, comparison of the effects of drugs on the motor innervation of the anococcygeus and vas deferens demonstrated that transmission in the anococcygeus is relatively straightforward and may help to clarify some of the problems involved in the understanding of the more complex motor transmission in the vas.

EFFECTS OF NERVE STIMULATION ON NA DEPLETION BY RESERPINE

Fluorescence histochemistry shows the anococcygeus to have a dense adrenergic innervation distributed throughout the muscle. The appearance of this innervation is similar to but less dense than that of the vas deferens. In

addition, preliminary experiments using the microspectrofluorometric technique of Caspersson, Hillarp & Ritzen (1966), which identifies the substance giving rise to fluorescence after formaldehyde condensation according to its emission spectrum, have indicated that the fluorophore in both tissues bears the characteristics of NA. This is confirmed by the biochemical measurement of NA content, which shows the anococcygeus to contain 2.56 μg NA / g tissue as opposed to 15.56 μg / g in the vas. Although less than in the vas, this NA content in the anococcygeus is nevertheless fairly high compared with other smooth muscle tissues e.g. rat gastro - intestinal tract, 0.22 - 0.98 μg / g; cat nictitating membrane, 2.4 - 4.3 μg / g; rabbit blood vessels, 0.24 - 0.90 μg / g (for further comparisons see Holzbauer & Sharman, 1972). This similarity combined with the fact that the adrenergic innervation is motor in both tissues provides grounds for making comparisons between the properties of the nerves in the anococcygeus and vas deferens as demonstrated in Part I. One major difference between the two tissues, however, is that the ganglia on the sympathetic pathway to the vas deferens occur along the hypogastric nerve, near the tissue itself, giving rise to the so - called "short" adrenergic neurones, whereas the corresponding ganglia in the sympathetic pathway to the anococcygeus are located in the paravertebral ganglia, giving rise to the conventional "long" adrenergic neurones (see Part I). It has been suggested that this property of the vas neurones might be responsible for the tissue's relative resistance to depletion by reserpine.

It was thus interesting that reserpine should deplete both the anococcygeus and vas deferens of NA to a similar extent with different doses and at a similar rate with a fixed, moderate (200 μg / kg) dose. The basis for the belief that resistance to NA depletion in the vas by reserpine was due to the short adrenergic neurones, was that tissues such as the vas deferens and seminal

vesicles were more resistant to depletion than tissues receiving long
adrenergic neurones (see Sjostrand & Swedin, 1968). The discovery of a
tissue with long adrenergic neurones which is depleted in a manner similar to
that of the vas but different from that of other tissues thus makes this less
likely. That both the anococcygeus and vas are depleted more slowly than the
heart, confirms that a similar "resistance" to reserpine exists in the
anococcygeus and vas deferens.

If it is not the length of the neurone that is the significant factor, what
then is the common factor in the vas and anococcygeus giving rise to this
resistance? Comparisons carried out so far show that the two tissues recover
at a different rate from the effects of reserpine, their nerves show major
differences in response to many drugs (see Part I) and the cytological
organisation of the tissues as shown by electron microscopy is quite different
(Gillespie, personal communication). There are thus, on pharmacological
and morphological grounds, many differences as well as similarities between
the two tissues.

Another factor suggested by Sjostrand & Swedin, that low resting impulse traffic
to the vas deferens could be responsible for resistance to depletion, could be
tested with the pithed rat. Since comparison between the three tissues showed
that the major difference between depletion in the vas and anococcygeus was in
the rate of depletion rather than the final level reached (see also Sjostrand
& Swedin, 1968), then impulse traffic seemed a possibility. It is known that
there is a continuous discharge in the sympathetic nerves to the heart (Bronk,
Ferguson, Margaria & Solandt, 1936) and that this will be reflexly increased
as reserpine lowers systemic blood pressure by its action on the adrenergic
nerves to the blood vessels (Iggo & Vogt, 1960). No information is as yet
available on the presence of spontaneous activity in the nerves to the

anococcygeus nor am I aware of such information in the literature for the nerves to the vas deferens although the function of the vessel makes this unlikely. None the less, the observations that pithing the animals reduces the depletion produced by reserpine in all three tissues and that nerve stimulation enhances the depletion in the anococcygeus and vas deferens, support the idea that a major factor determining the extent of depletion is the amount of neuronal activity.

It is not possible to say from the present experiments whether nerve activity could fully explain the difference between the heart and the other two tissues. If it does, then one would expect the degree of depletion to be similar in animals pithed and then given reserpine. These experiments have not been carried out since it is difficult consistently to keep pithed rats in good condition for the necessary length of time. In the experiments where reserpinised rats were pithed, reserpine had three hours to act before the abolition of impulse flow by pithing and this could explain the greater depletion in the heart (66%) than in the anococcygeus (42%) and vas deferens (36%) at 6 hr.

These observations confirm and extend previous evidence that reduction of nerve impulse traffic by nerve section or drugs blocking ganglia will reduce the depletion of NA by reserpine in various tissues (Holzbauer & Vogt, 1956; Karki, Paasonen & Vanhakartano, 1959; Mirkin, 1961; Hertting, Potter & Axelrod, 1962; Benmiloud & Euler, 1963). This is in disagreement with the experiments reported by Sjostrand & Swedin (1968) who sectioned rat hypogastric nerve and found that the depletion in the vas deferens was unaltered. The explanation may lie in the different conditions. In the vas deferens, where impulse traffic is low, it is unlikely that a big effect will be seen by abolition alone. Furthermore, these authors used a smaller

dose (50 µg/kg) of reserpine given subcutaneously so that the rate of depletion was less. Since the effect of abolishing impulse traffic to the vas in the present experiments is small, it might be non - existent at smaller doses. In the present experiments the role of nervous activity in depletion was exaggerated by contrasting the depletion when nerve impulses were abolished with that when activity was reinforced by artificial nerve stimulation.

The act of pithing a rat leads to considerable autonomic discharge, presumably as a result of destruction of the spinal cord. This discharge continues for 10 - 15 min and is manifested by the initially high blood pressure and heart rate characteristic of the pithed rat and also by a contraction seen on pithing in the anococcygeus if its tension is recorded in the anaesthetised rat prior to pithing. These blood pressure and anococcygeus responses are absent if the animal is pretreated with phentolamine and are presumably due to a combination of sympathetic nerve discharge to the target organs themselves and to the adrenal medulla. In the present experiments, where reserpine pretreatment is employed, this discharge, if severe enough, resulted in an increased depletion of NA. A comparison of the NA contents of the tissues 1 hr after pithing i.e. 4 hrs after reserpine, with the contents 4 hrs after reserpine in unpithed animals showed no significant difference. If there had been no discharge, the rate of depletion during this 1 hr period might have been expected to fall. This discharge produced by pithing thus slightly reduces the effectiveness of pithing as a means of reducing the net impulse flow in the subsequent 3 hr period. Nevertheless, since it is a small effect, this does not interfere with the comparison of the NA content after pithing alone with that after pithing plus sympathetic stimulation. This is illustrated in Fig. 44.

In any study of the effect of nerve stimulation on NA content, the effect of homeostatic mechanisms on the maintenance of normal NA levels must be taken into account. For example, in the isolated guinea - pig vas deferens, during hypogastric nerve stimulation, the uptake and retention of radioactive NA from the surrounding fluid was increased (Bhagat & Zeidman, 1970) and the formation of NA from tyrosine was also increased (Thoa, Johnson, Kopin & Weiner, 1971). Any change produced in NA content is thus the net result of competing homeostatic mechanisms tending to maintain normal amine levels and nerve stimulation depleting these levels. The outcome can thus only be adequately understood if each parameter can either be measured and a balance sheet drawn up or else controlled by pharmacological or other means. In the present study on the effects of reserpine, the homeostatic factors cannot maintain the NA content at normal levels since the NA made available by any increased synthesis or reuptake cannot be retained by the storage granules since it is here that reserpine exerts its action. Instead of being made available for replenishment of the stores, any such NA would thus be exposed to metabolism by intracellular mono - amine oxidase (see Kopin & Gordon, 1962).

The effects on NA content of the tissues of pithing the animals and / or stimulating the sympathetic outflows in the absence of reserpine were not statistically significant probably due to the small number of examples used for each comparison. This provided a valid basis for comparison with the statistically significant differences which did exist after reserpine pretreat-ment, but unfortunately precluded a proper assessment of the effects of pithing and stimulating per se. As a basis for further comparison, it is, however, interesting to compare the non - significant changes in the mean values which did occur with analogous changes found in other tissues. Thus the effect of pithing might be compared to that of decentralisation. Decentralisation of the cat spleen led to an increase in the NA content of the

organ (Brown, Dearnaley & Geffen, 1967) while comparable decentralisation of

the guinea - pig vas deferens produced no such change (Blakeley, Dearnaley

& Harrison, 1970). In the present experiments pithing produced a non -

significant rise in the mean NA content of the anococcygeus but no change at

all in the vas deferens or heart. Moderate stimulation of adrenergic nerves

does not normally decrease the NA content of tissues (see Euler & Hellner -

Bjorkman, 1955; Blakeley, Dearnaley & Harrison, 1970) although exceptions

to this have been reported e.g. cat spleen (Dearnaley & Geffen, 1966). On

the other hand, more vigorous stimulation can produce depletion e.g. guinea -

pig vas deferens (Chang & Chang, 1965; Roth, Stjarne & Euler, 1967). In

the present experiments, stimulation of the sympathetic outflows to the two

tissues produced a non - significant fall in the content of the vas deferens

with no change in the anococcygeus. Clearly any change in content after

changing the amount of nerve activity will depend on the net result of release,

metabolism, reuptake and resynthesis as mentioned above. Even with the

complete abolition of sympathetic activity or vigorous stimulation employed,

the present results are for the vas deferens and heart in accord with the

previous literature and demonstrate that the anococcygeus, while possibly

increasing its NA content on resting, can maintain its NA stores in the face

of prolonged and repetitive stimulation. It would be interesting to know if

this small effect is due to the efficiency of the compensating homeostatic

mechanisms so that NA synthesis or turnover is enhanced.

The faster rate of recovery of NA contents after reserpine pretreatment in

the vas deferens compared with the anococcygeus is interesting in view of the

similarity in the rate and extent of depletion in the two tissues. It is

also interesting that the rate of recovery in both tissues after this 200 μg/kg

dose was slower than the recovery following 10 mg/kg in rat gastrocnemius

muscle, sub - maxillary gland and heart found by Haggendal & Dahlstrom (1971).

These authors demonstrated that recovery of the NA content of tissues after reserpine is dependent on and can be explained solely by the axonal down - transport of newly formed amine granules. Implicit in their argument is that after the almost total depletion of NA resulting in complete loss of adrenergic function, there is a supranormal down - transport of new granules leading to almost full recovery of NA content after three weeks, a fall at four to five weeks as these "extra" new granules reached the end of their life - span and then finally complete recovery to control levels at six weeks. It is thus interesting that in the present experiments, where the initial depletion was not sufficient to abolish sympathetic function, the initial rate of recovery should be slower than that found after a larger depletion. If the initial stimulus which produces the increased formation and transport of new granules is dependent on a negative feed - back loop resulting from the loss of nerve function, then this would explain this apparently contradictory slower recovery after a smaller dose. Recovery in the present experiments, therefore, might depend on the arrival of newly formed granules at the normal rate. It has been suggested by Dahlstrom (1970) that the stimulus for the increased formation and transport of granules might be a reflex increase in sympathetic activity after reserpine as was demonstrated by Iggo & Vogt (1960). The consequent increased bombardment of the postganglionic neurone cell body might then result in an increased protein synthesis similar to that shown by Hyden (1960) resulting in increased formation of new granules and the accompanying enzymes required for catecholamine synthesis, such as dopamine β - oxidase and tyrosine hydroxylase (Thoenen, Mueller & Axelrod, 1969; Axelrod, Mueller & Thoenen, 1970). In the present experiments such an increased nerve activity would be unlikely, at least as a consequence of impairment of transmission, and thus the stimulus to rapid recovery would be absent. Another factor which might be of interest but has not been examined

here would be whether the postulated increase in protein synthesis in the cell body would occur in all tissues whose transmission was prevented by a large dose of reserpine. In a tissue which was not part of a homeostatic reflex loop this phenomenon might be absent. Into this category would fall the vas deferens and possibly also the anococcygeus if it plays a role in defaecation. This point could be answered by examining the rate of recovery of the tissues' NA content after a large dose of reserpine.

The reason for the slower rate of recovery of NA content in the anococcygeus than in vas deferens is not known. On the basis of the "normal" rate of down - transport of new granules (72 - 240 mm/day, Dahlstrom & Haggendal, 1966, 1967), a 50 mm difference in axone length on its own would be unlikely to result in a significant difference in the rate of arrival at the terminals. This would tend to eliminate the length of axone as a direct contributory factor.

The effects of reserpine on the mechanical responses due to sympathetic nerve stimulation of the anococcygeus and vas deferens further illustrate a dual action of the drug on the response of the latter organ. These effects of reserpine were observed in three different situations. First, in a series of experiments where reserpine 200 µg/kg i.p. was administered three hours prior to pithing, the responses of the anococcygeus were as found in untreated controls but in the vas, although the response to the first period of stimulation was normal, in subsequent periods, the "spike" phase rapidly diminished. Secondly, when a large dose of reserpine 3 mg/kg i.p. was given 18 hours prior to pithing in order to produce a large depletion of NA content, the response of the anococcygeus was slower to develop and reduced in size compared with controls while in the vas, although both phases were reduced in size, the "spike" was prolonged and the secondary response was absent. In

the third situation, when reserpine 3 mg/kg was given intravenously to the pithed rat, the response in the anococcygeus was unaffected but the "spike" in the vas was acutely inhibited, leaving only the secondary response. The anococcygeus response was thus affected in the manner expected of a motor adrenergically innervated tissue, being unaffected by acute doses and reduced in size only after considerable depletion of the catecholamine stores. In the vas deferens, on the other hand, there were two different effects. The "spike" was susceptible to acute large doses of reserpine and was also reduced 4 hrs after a small dose if stimulation was repeated.

This effect is analogous to that reported by von Euler (1969, 1970) in the isolated guinea - pig vas deferens _in vitro_. He found that reserpine added to the bath rapidly reduced the responses to either field or hypogastric nerve stimulation. Relating this to the effect of reserpine on inhibition of NA release from isolated granules (Euler & Lishajko, 1963; Euler, 1966 a & b), he concluded that this acute effect of reserpine could be due to reduction of NA release by nerve stimulation resulting from a reduction in the transfer of NA from the storage granules to the actual neuronal release sites. In agreement with this concept, the first stimulus response after addition of reserpine was normal, whereafter the contractions declined gradually at a rate depending on the dose. In these experiments the stimul - ation period lasted only 5 sec so that according to the time scale (shape) of the two phase response in the isolated guinea - pig vas deferens reported by Swedin (1971), only the initial "spike" phase of the response was being examined. This effect is thus clearly analogous to the inhibition of the "spike" in the present experiments by an acute large dose or 4 hrs after a small dose of reserpine. In addition, the gradual disappearance of the "spike" with subsequent stimulation periods is similar to his findings.

Although the effect appears to be similar in every respect, the present experiments do, however, throw some doubt on von Euler's explanation. The first difficulty is that this effect appears to be absent in the anococcygeus. It is true that a similar acute inhibition of adrenergic responses by reserpine has been demonstrated on the isolated colon (Gillespie & Mackenna, 1961) and on the rabbit ear artery (Day & Owen, 1963) but the evidence was not strong, the former authors acknowledging that the effect could have been due to a solute effect and in the latter case the effect was so small that the authors did not comment on it in the text. It thus appears that this acute effect of reserpine may be relatively specific for the "spike" phase of the vas deferens response in a manner analogous to the similar actions of PGE_1 (Swedin, 1971) or LSD (Ambache, Dunk, Verney & Zar, 1973).

This similarity raised two possibilities which cannot be satisfactorily resolved by either von Euler's or the present experiments, viz. either (1) LSD and perhaps also PGE_1 act in a manner similar to reserpine in producing their effects i.e. by reducing NA release from granules by the nerve impulse, or (2) reserpine acts in a manner similar to that postulated by the present author for LSD, producing inhibition of the "spike" by acute release of NA.

The first of these possibilities is difficult to explain in view of the fact that only the "spike" phase is altered unless it is accepted that there are two different adrenergic motor nerve pathways and only one of them is affected. Furthermore, 18 hrs after reserpine 3 mg/kg, a "spike" can be obtained and so the initial inhibition of the "spike" appears to have disappeared at this time. LSD has, however, no effect at this time which together with the prevention of its effect by phentolamine is the reason for postulating that

it has an indirect sympathomimetic action. To answer this point adequately, however, a systematic study of the time scale of the development of the reserpine effects would be required. In addition, if LSD does act by an indirect sympathomimetic action, the present experiments leave open the possibility that the NA so released might act at the same site postulated by von Euler for reserpine.

The second possibility, that reserpine produces its acute effect by an indirect sympathomimetic effect resulting in feedback inhibition of NA release by nerve impulses, deserves consideration in view of the similarity of this effect of reserpine with those of LSD and tyramine. It is well documented that reserpine can produce gross sympathomimetic effects on administration (see Trendelenburg, 1972) as do other drugs which block sympathetic activity such as guanethidine. Part of this activity is due to release of adrenal catecholamines and to increased activity in the peripheral nerves produced by central effects (see Carlsson, 1966) and much of the NA released by reserpine in sympathetic nerve terminals is undoubtedly degraded by mono - amine oxidase (Kopin & Gordon, 1962). It must, however, remain a possibility that in some tissues part of the NA might reach the extracellular space to produce effects. The vas deferens might be particularly suited to this due to the small size of the synaptic gap, and if the action under consideration was in any case a presynaptic inhibitory action then this might be quite likely. The present evidence neither confirms nor denies either of these possibilities. The effect of phentolamine on the effect of reserpine on the isolated guinea - pig vas deferens might, however, strengthen either case according to the results.

Whatever the mechanism of action, the acute depression of the motor nerve response in the guinea - pig vas deferens shown by von Euler (1969) has been demonstrated in the rat _in vivo_ and an alternative explanation offered which

is not necessarily mutually exclusive of that of von Euler (1969, 1970).

There was no direct relationship between the motor nerve response and NA content in either anococcygeus or vas deferens. At up to 80% depletion, no correlation existed between response and NA content, and even after extensive depletion of 97 - 99%, reduced motor responses could still be obtained in both tissues. This is similar to previous findings in the vas deferens (Ambache & Zar, 1971; Swedin, 1971) and in other tissues (Sedvall & Thorson, 1965; Anden & Henning, 1966; Lee, 1967).

These results thus suggest a dual effect of reserpine on the response to motor stimulation in the rat vas deferens, analogous to that suggested by von Euler (1970). Large doses acutely inhibit transmitter release with a particular effect on the initial component of the response and unrelated to depletion of the main store of transmitter. Chronically, depression involved both components of the response and is probably related to transmitter store depletion. In contrast, only the latter effect is seen on the anococcygeus.

In summary, results have been presented which suggest that nerve impulse traffic may be an important factor in determining the rate of depletion of NA by reserpine and in the vas deferens may explain the apparent resistance to depletion. Furthermore, a dual effect of reserpine on the motor response of the vas deferens has been demonstrated.

CAT ANOCOCCYGEUS

The cat anococcygeus has been shown to be a smooth muscle receiving a dual innervation similar to that of the corresponding tissue in the rat. In the cat, however, the muscle displays some experimental differences in its responses to field stimulation and to drugs compared with that of the rat.

Similarities between the tissues can be summarised as -

(1)   Both species have a dense adrenergic innervation as demonstrated by fluorescence histochemistry and by their NA content.   This adrenergic innervation is motor as demonstrated _in vitro_ by the effects of adrenergic blocking drugs on the motor responses to field stimulation.

(2)   Both species also have an inhibitory innervation which can best be demonstrated _in vitro_ when the adrenergic nerves are blocked and the tone of the muscle raised.   The identity of the transmitter involved is unknown since the response can be blocked only by tetrodotoxin and not by any receptor blocking drugs.   The pharmacological evidence suggests, however, that this transmission is neither adrenergic nor cholinergic.

(3)   In both species the muscles are contracted by drugs acting as direct sympathomimetics viz. NA, Adr, oxymetazoline, and by drugs acting as indirect sympathomimetics viz. tyramine, amphetamine, guanethidine, cocaine, LSD and possibly also 5HT.

On the other hand, differences between the muscles in the two species are -

(1)   The cat but not the rat muscle exhibits spontaneous activity and intrinsic tone _in vitro_.   Partly as a result of this, inhibitory responses to field stimulation can be demonstrated on the cat muscle without either blocking the adrenergic nerves or raising the tone.

(2)   No agonist drugs have so far been found which relax the rat anococcygeus.

The cat muscle, however, is relaxed by several drugs all of which contract the rat muscle viz. acetylcholine, carbachol, isoprenaline, ATP, prostaglandins E_1, E_2 and F_2 and vasopressin. In addition to relaxing the muscle itself, acetylcholine also inhibits responses to adrenergic nerve stimulation in the cat anococcygeus.

The implications of this comparison are clear. The dual innervation found in the rat anococcygeus with motor adrenergic and inhibitory "unknown" transmission has a direct counterpart in another species. In this further species the effects of stimulation of the motor adrenergic nerves and of drugs which mimic their effects are similar, confirming both the classical effects on this tissue of NA and tyramine, which produce respectively direct and indirect sympathomimetic responses of the muscle and also the somewhat less expected actions of guanethidine, cocaine, LSD and 5HT, all of which produce indirect sympathomimetic responses. The ability to observe the effects of the inhibitory nerves in the cat muscle in the absence of the complicating effects of drugs strengthened the case for their being an entity independent of the adrenergic nerves and permitted the interaction and thus the interrelationship between motor and inhibitory nerves to be examined. Since several possible candidates for the role of inhibitory transmitter produced opposite actions in the two species and specific blocking or potentiating drugs could affect their responses without affecting the inhibitory nerve responses, the candidature of these substances was considerably weakened. Acetylcholine, for example, which must rank as the first candidate as transmitter in any nerves "physiologically" opposed to adrenergic nerves, produced opposite responses in the two species not only on the muscle but also by its effect on the adrenergic nerves. This not only made it an unlikely inhibitory transmitter in this tissue but also raised questions as to the general significance of any effects of acetylcholine on this tissue.

Many of these points require consideration in closer detail.

Even without advance knowledge of the existence of a dual innervation in the rat anococcygeus and the information on the separate anatomical origins of the motor and inhibitory nerves, the responses of the cat anococcygeus to field stimulation _in vitro_ would have themselves provided convincing evidence of two sets of nerves in that tissue. The experiments illustrating the time course of development of the dual response to field stimulation, combined with the frequency characteristics of the two sets of nerves, allay the possibilities of the inhibitory response being due to some transformation in the effects of the motor nerves such as might be explained by "Wedensky Inhibition" or some change in the "peripheral mechanism". The complex, compound responses produced by field stimulation in the absence of drugs are seen to be due to enhancement of either motor or inhibitory components of the response by selection of the appropriate frequency and duration of stimulation. Thus at very low frequencies and again at high frequencies the motor nerves dominate, while at intermediate frequencies the inhibitory nerves dominate. The time course for development of both motor and inhibitory responses also fits in with the observed compound responses. Thus contraction from motor nerve stimulation develops rapidly while inhibition from inhibitory nerve stimulation, although having a similarly short latent period, develops more slowly. On cessation of stimulation, the motor response wanes more rapidly than the inhibitory.

This leads to compound responses in which the relative sizes of the various components vary with frequency but which in general consist of (1) an initial motor response followed by (2) an inhibitory component (3) a period when either response may dominate and (4) on cessation of stimulation, a further inhibitory component may be found. These findings would be

difficult to justify in terms of a varying output of transmitter from a single nerve type (Wedensky Inhibition) or by variations in the properties of the nerve - muscle complex (peripheral mechanism). Furthermore, the initial resting tone in the muscle is purely myogenic in origin as demonstrated by the relaxation produced by papaverine but not by phentolamine. The inhibitory component of the response to field stimulation can reduce this tension in the muscle to below the resting baseline, demonstrating that a direct inhibitory mechanism exists and not simply a variation in the extent of motor stimulation.

In addition to demonstrating the separate natures of the two sets of nerves, these experiments on field stimulation also have a bearing on their physiological function. With separate stimulation of the two sets of nerves and appropriate selection of frequency, muscle activity could be made to range over the entire spectrum from almost pure inhibition through various intermediate states of activity to a maximum contraction. Thus mechanisms exist which under physiological conditions could produce a rapid and fine control of the muscle tone. No direct light is, however, thrown by this onto the function of the muscle since the possibilities for this control appear so flexible. Thus the muscle might be normally held contracted under the influence of a low frequency tonic discharge in the motor nerves and might be rapidly relaxed during some intermittent function by stimulation of the inhibitory nerves. This hypothesis would, however, be opposed by a corollary of the results of the experiments on the effect of reserpine (see Part II). These experiments suggest that a possible reason for the slow rate of depletion of NA from the anococcygeus is that this tissue receives only a low tonic impulse traffic in its motor nerves. If this is the case, the above hypothesis would be unlikely. Conversely, the muscle may normally be relaxed and play some intermittent role by a combination of motor and / or inhibitory responses. These two possibilities appear to be

the most likely simply on the grounds of economy, since it is difficult to comprehend why the muscle would be normally under the tonic influence of either the inhibitory nerves alone or a combination of motor and inhibitory.

Information on the time course of the inhibitory response was also gained from these experiments on field stimulation. Previous information on this had relied on the rate of recovery of tone after inhibition and was thus a measure of the rate at which inhibition was overcome by the presence of a motor agonist. It was, however, possible to obtain a more direct measure of inhibitory effect by observing the influence of a burst of stimulation on the motor response to a single stimulus. This demonstrated that the motor response to a single pulse was still completely abolished 3 secs after a burst of stimulation at 5 Hz, the optimum frequency for the inhibitory response, and that this response had not recovered until 8 - 10 sec after cessation of stimulation. This indicates that the influence of the inhibitory nerves on the muscle lasts a shorter time than might be imagined from the recovery of drug - induced motor tone after inhibitory nerve stimulation. This is illustrated by a comparison of Figs. 73 & 74 which were obtained with similar stimulation parameters on the same muscle. When field stimulation was applied in the presence of 5HT - induced tone, the peak of the relaxation of muscle tone was reached 10 -15 sec after the end of the stimulation period, (Fig. 73). Similar stimulation in the absence of induced tone and using a test stimulus as a measure of the inhibitory effect, showed recovery after 8 - 10 sec (Fig. 74). Several factors could explain this temporal difference in the maximal effects. (a) The inhibitory transmitter may affect both the muscle and the motor nerve and the effect on the muscle may outlast that on the nerve. (b) The time when the tension is at a minimum may not reflect the point of maximum action of the inhibitory transmitter on

the muscle. A more accurate indication of the effect of the inhibitory transmitter might be the rate of change of tone. This would reach a maximum much earlier and correspond more closely with the time course of suppression of the motor nerve response.

The fairly rapid decline of the inhibitory effect suggests that some inactivation process exists for the transmitter. In the absence of any information on the nature of the transmitter it is pointless to speculate whether this consists of enzymic breakdown and / or active reuptake into some site as exists for cholinergic and adrenergic systems.

Drug analysis of the motor and inhibitory responses confirmed the conclusions from these early experiments. Receptor and neurone blocking agents confirmed the adrenergic nature of the motor nerves. The "unknown" nature of the inhibitory nerves was demonstrated by a series of essentially negative results. Neither muscarinic, nicotinic, β - adrenergic, 5 - hydroxy - tryptaminergic or histaminergic blocking agents affected the response.

Hexamethonium had no effect on either response, demonstrating that only the postganglionic neurones are present in each case. This was confirmed histochemically by the lack of any ganglia in or around the muscles. The interpretation of responses in this tissue is thus simpler than in gastro - intestinal smooth muscle where the additional presence of Auerbach's and Meissner's plexuses leads to difficulties. The adrenergic nerves in the anococcygeus appear, on pharmacological evidence alone, to directly innervate the smooth muscle and do not act as has been suggested in the gastro - intestinal tract via a further neurone (Norberg, 1967). This is confirmed by the E.M. studies on the rat anococcygeus (Gillespie & Lullman, prepared for publication) which show the terminal varicose nerve fibres distributed

throughout the muscle in direct relation with muscle fibres. Within the muscle the inhibitory nerves also appear to be solely postganglionic. This stands in contrast to most cholinergic (Burnstock, 1969) or purinergic (Burnstock, 1972) systems which normally have their cell bodies in or close to the tissue innervated. While there is as yet no direct proof of a preganglionic component to the inhibitory pathway to the cat anococcygeus, the presence of such a ganglion in the rat makes this a reasonable assumption. The E.M. evidence so far does not conclusively demonstrate separate adrenergic and non - adrenergic nerves in the rat anococcygeus, but nerves containing agranular vesicles are present throughout the tissue and may represent the inhibitory nerves (Gillespie & Lullman, prepared for publication).

The effects of agonist as well as antagonist drugs also serve to reinforce the adrenergic and "unknown" natures of the motor and inhibitory nerves.

The cat anococcygeus shows a sensitivity to NA similar to that of the rat muscle and these responses are specifically blocked by phentolamine in concentrations which also block the motor nerve response. Indirect sympathomimetics such as tyramine also contract the muscle and are blocked by phentolamine. These α - receptor responses are similar to those in the rat. A difference between the species appears when β - receptor effects are considered. The β - blocker, propranolol, has no effect on the motor response to NA in the rat, but in the cat it potentiates motor responses to NA and Adr. The inhibitory β - adrenergic effect which this uncovers can be seen directly by the effect of isoprenaline. In the cat, isoprenaline relaxes both intrinsic tone and that induced by an agonist drug. This inhibitory effect is abolished by propranolol. The cat anococcygeus thus possesses inhibitory β - adrenergic receptors in addition to motor α - receptors. These β - receptors are activated by NA and Adr as well as by

isoprenaline, although this action of NA and Adr is normally masked by their predominant motor α- effect.

The site of these β- receptors is not known but they do not appear to play an important role in the motor transmission process since the motor response to field stimulation was unaffected by propranolol. Possible reasons for this could be -

(1) β - receptors occur over the surface of the muscle as a whole but not specifically near the nerve - muscle junction. In this case they would be more likely to be affected by exogenously administered NA than by nerve released NA.

(2) If β - receptors do occur in the region of the nerve - muscle junction, the β - response to nerve stimulation might be relatively resistant to blockade by propranolol in a manner analogous to the relative resistance of the motor nerve response to phentolamine.

No β - receptors appear to be present in the rat anococcygeus as shown by the lack of effect of isoprenaline whose only action is to produce direct α - effects in high concentrations. This lack of β - effects in the rat tends to eliminate β - adrenergic transmission as a possible candidate for the inhibitory nerve response, an argument which will also be applied to cholinergic, purinergic and prostaglandin - mediated transmission. The most convincing evidence, however, that β - receptors are not involved in the inhibitory pathway is the observation that propranolol has no effect on inhibitory responses in concentrations which abolish the effects of isoprenaline.

Even if isoprenaline is not the inhibitory transmitter, it would be expected to summate with or potentiate the inhibitory response. This was not observed. If both inhibitory transmitter and agonist acted by a similar mechanism such

as might be produced by hyperpolarisation via a change in permeability to a specific ion, then some potentiation might have been expected. The lack of such an effect suggests a difference in the mechanism which can only be resolved by electrophysiological means. The present experiments, with specific blocking drugs can only provide evidence that different receptors are involved. Whether the next step activated by receptor occupation is common can only be properly answered by electrophysiological techniques.

Acetycholine had to be considered as a possible inhibitory transmitter in the cat anococcygeus since it produced an inhibitory response and since Howard & Garrett (1973) had found acetylcholinesterase in the tissue and postulated that cholinergic nerves might be present. The evidence in Part I of the present study that the inhibitory nerves originate in the upper sacral region of the spinal cord (the origin of parasympathetic fibres) supports this though the motor effect of acetylcholine in the rat anococcygeus led to its rejection in that species as a possible inhibitory transmitter. This possibility was, however, re - opened by the finding that acetylcholine relaxed the cat anococcygeus. Since the intrinsic, as well as the drug - induced, tone could be relaxed this was a direct effect on the muscle. Carbachol produced a similar effect to acetylcholine, the effect of acetylcholine was potentiated by neostigmine and the effects of both acetylcholine and carbachol were blocked by atropine. Neither atropine nor neostigmine had, however, any effect on the inhibitory nerve response, nor did acetylcholine potentiate the inhibitory nerve response. For these reasons together with completely opposite effects of acetylcholine on the rat anococcygeus, cholinergic transmission was again rejected as a possible inhibitory nerve process in the cat anococcygeus.

On the rat muscle the muscarinic receptors appear to play a directly opposing

role, producing a motor response but still potentiated by neostigmine and
blocked by atropine. This opens the question as to the role of both
acetylcholine and cholinesterase in this tissue. The presence in the cat
anococcygeus of cholinesterase, demonstrated by Howard & Garrett (1973), was
confirmed by the effect of neostigmine. In the rat, however, attempts to
demonstrate cholinesterase met with no success (Gillespie, 1972) leading to
the conclusion that no cholinesterase was present. The specific potentiation
of acetylcholine by neostigmine in the present experiments contradicts this
latter conclusion, but it is possible that the reaction conditions employed,
despite producing a dense staining of motor end plates in rat diaphragm, were
not so vigorous as those employed by Howard & Garrett (1973). This
treatment might, therefore, have been insufficient to detect small quantities
of cholinesterase present, especially if this was diffusely distributed
throughout the muscle.

Assuming then that acetylcholinesterase is present in the anococcygeus of both
species, what function if any is it playing? The lack of any effect of either
atropine or neostigmine on the motor or inhibitory nerves in either species
rules out the suggestion of Howard & Garrett (1973) that cholinergic nerves
may be present in the muscle, unless some further nerves are present which
subserve neither a motor nor inhibitory function and whose effects cannot be
detected by field stimulation of the muscle with 0.1 - 10 msec pulses. In
addition, this same evidence makes it unlikely that in this tissue a
cholinergic link exists in the adrenergic nerves as suggested by Burn & Rand
(1959) or that acetylcholine plays a significant role in the activation of the
axone as suggested by Nachmanson (1966).

The effects of acetylcholine on the nerve responses to field stimulation shed
no further light on the role of cholinesterase. In the rat, acetylcholine
appears to have no effect on the motor response to field stimulation, other

than can be explained by simple summation of the direct responses on the muscle of the motor transmitter and of acetylcholine. Cholinesterase inhibition simply potentiates the motor effect on the muscle of acetylcholine. Inhibitory nerve responses are similarly unaffected by acetylcholine and in fact after 6OHDA <u>in vivo</u> treatment carbachol or acetylcholine provide a convenient means of raising muscle tone in order to observe inhibitory nerve responses (Gibson & Gillespie, 1973). In the cat, on the other hand, acetylcholine inhibits motor nerve responses, an effect potentiated by cholinesterase inhibition and blocked by atropine. This might be expected from the inhibitory effect of acetylcholine on the muscle, but other agonists which relax the cat anococcygeus such as isoprenaline, ATP, prostaglandins and vasopressin are not effective against motor nerve responses except in very high doses, in contrast to acetylcholine and carbachol which inhibit motor nerve responses in doses as low as those which relax the muscle. This points to a possible presynaptic effect of acetylcholine on motor nerves which is borne out by comparison of the effects of acetylcholine against NA and against motor nerves. Acetylcholine inhibits motor nerve responses to a greater extent than NA responses of a similar magnitude. This stands in contrast to the effects of the specific α- receptor blocker phentolamine which was more effective in a similar situation against NA. A presynaptic inhibition of NA release by acetylcholine is thus suggested, similar to that demonstrated on the rabbit heart by Loffelholz & Muscholl (1969). Another possibility which is not ruled out by these experiments is, however, that the muscarinic receptors might be concentrated in the region of the nerve - muscle junction resulting in a greater effectiveness against nerve as opposed to exogenously administered NA. This question would be resolved by transmitter overflow studies.

The inhibitory nerve response was unaffected by acetylcholine. As

mentioned previously, potentiation between the inhibitory nerve and a drug which lowers the tension is difficult to judge. There was, however, no overt evidence of such potentiation or of reduction of the inhibitory nerve response. The inhibitory effect of acetylcholine on the motor response in the cat in doses of 10^{-8} M - 10^{-4} M was thus the only effect of acetylcholine found on either set of nerves in either species. No potentiation of adrenergic responses was observed at lower doses (down to 10^{-13} M) as reported by Malik & Ling (1969) for the perfused mesenteric artery.

A further action of acetylcholine on the cat anococcygeus which has not been properly examined but which deserves mention was the small motor response at high doses. This response may be significant in two separate respects. Firstly, it may be produced by release of NA from the motor nerves. Such an effect was found by Loffelholz & Muscholl (1969) in doses higher than those required to produce inhibition of NA release and would be similar to the well - known sympathomimetic effects of acetylcholine in high concentrations (see Kosterlitz & Lees, 1972). This pattern may thus be repeated in the anococcygeus. Loffelholz & Muscholl found that the inhibitory effect was muscarinic, while the release of NA was nicotinic. In the present experiments both responses were blocked by atropine, but no attempt was made to analyse the specificity of blockade of this motor action of acetylcholine. In a second respect this small response may be related to the phenomenon of reversal of acetylcholine responses reported by Burn & Robinson (1951). These motor responses did not occur in every experiment and it was noted that they were more regularly present in muscles cold stored overnight. This can be seen from Fig. 80 b. There may thus be some evidence of reversal of acetylcholine effects produced by isolation of the tissue in physiological saline. Whether this would consist of reversal of the effect of stimulating receptors on the muscle or be due to development of the indirect release of NA, remains an open question.

Experiments with acetylcholine thus suggest that neither acetylcholine nor cholinesterase play a significant role in the nerve transmission process of the anococcygeus muscle but that exogenously applied acetylcholine can exert effects on the tissue and these effects are potentiated by cholinesterase inhibition. In the rat the only effect of acetylcholine is to produce contraction by a muscarinic action on the muscle. In the cat acetylcholine produces relaxation by a direct muscarinic action on the muscle and also inhibits motor nerve responses by a presynaptic mechanism. A universal physiological role for acetylcholine or acetylcholinesterase cannot, therefore, be suggested in this tissue. It is, however, possible that since there is no evidence for the presence of endogenous activity of acetylcholine itself in the anococcygeus, the acetylcholinesterase may be present either solely as a safety factor to prevent the action of acetylcholine from exogenous sources or to regulate some function of acetylcholine in the tissue which has not yet been suggested. This explanation is on a similar basis to those put forward for the various effects of acetylcholine on adrenergic transmission in other tissues (e.g. Malik & Ling, 1969; Löffelholz & Muscholl, 1969). Since the effects of acetylcholine are opposite in the two tissues, it becomes even more difficult to encompass them into a universal role in adrenergic transmission as suggested by Burn & Rand (1959) and again more recently by von Euler (1970).

The effects of ATP on the cat anococcygeus were examined in order to test whether the inhibitory innervation was purinergic. Burnstock (1972) has suggested that a great number of formerly uncharacterised inhibitory mechanisms may be due to nerves having as their transmitter ATP or a related nucleotide. In addition, he tentatively postulated that the non - adrenergic, non - cholinergic inhibitory system demonstrated by Gillespie (1971) in the rat anococcygeus might be related to this system. While Gillespie (1972)

has refuted this by demonstrating that ATP produces only motor responses in the rat anococcygeus, it was felt necessary to re - examine the possibility in the cat. As in the case of isoprenaline and acetylcholine, ATP produced relaxation of the cat anococcygeus, in contrast to its motor action in the rat. Considering the cat anococcygeus on its own, this response suggests ATP as a candidate for inhibitory transmitter. No attempt has as yet been made to obtain further pharmacological evidence to settle this point such as might be achieved by examining the action of the adenosine uptake blocking drug dipyridamole (see Burnstock, 1972). On the basis that the effect of ATP is motor in the rat muscle, it is at present felt that ATP is unlikely to be the inhibitory transmitter.

Another factor concerning the possible involvement of purinergic transmission in this tissue is that purinergic nerves have so far been demonstrated only in tissues where the adrenergic nerves are inhibitory. The only examples quoted by Burnstock (1972) of mammalian systems which receive a motor adrenergic innervation as well as a postulated purinergic inhibitory innervation are the monkey internal anal sphincter (Rayner, 1969), the cat internal anal sphincter (Garrett, Howard & Lansdale, 1971) and the rat anococcygeus (Gillespie, 1971). It is tempting to suggest that in each of these cases the inhibitory nerves involved are the inhibitory nerves of the anococcygeus or of a closely related but functionally similar tissue. This would in no way detract from the validity of purinergic nerves in tissues where adrenergic nerves are inhibitory, but the similarity in the anatomical location of the only examples of non - adrenergic, non - cholinergic nerves in systems where the adrenergic nerves are motor seems a great coincidence and suggests a system of non - adrenergic, non - cholinergic, non - purinergic innervation in such tissues.

Prostaglandins have been implicated as modulators of adrenergic transmission in several tissues (Horton, 1973) or even as inhibitory transmitters (Campbell,

1970). Like acetylcholine, isoprenaline, ATP and vasopressin, the prostaglandins E_1, E_2 and $F_{2\alpha}$ all contract the rat anococcygeus and relax the cat anococcygeus. Even at high doses the contraction of the rat muscle is small compared to the contraction produced by other motor agonists such as NA and only a slight inhibition of the motor response to field stimulation was produced at the highest dose tested. In the cat, the inhibitory response was produced against either intrinsic or drug - induced tone. As in the rat, these responses were not as large as those produced by other agonists such as isoprenaline, although it is possible that they might have been if larger doses had been given. Only a small inhibition of motor responses to field stimulation was found at high doses and no apparent effect on inhibitory responses was found. As with the other inhibitory agonists, no summation occurred between prostaglandin - induced relaxation and inhibitory nerve responses. Although no further attempt was made to study the pharmacology of prostaglandins on this tissue by manoevres such as inhibition of prostaglandin synthesis, it was concluded that the inhibitory transmitter was not a prostaglandin of the type tested here since they exerted an opposing action on the muscles from the two species and since no summation occurred with the inhibitory nerve response. Since prostaglandins are active in this tissue, albeit in fairly high concentrations, a role in modulation of adrenergic nerve responses, such as is found in guinea - pig vas deferens (Euler & Hedqvist, 1969) and in other tissues (see Horton, 1973), cannot be completely ruled out.

Vasopressin also produced motor responses on the rat and inhibitory responses on the cat anococcygeus. There is no suggestion that vasopressin might represent the inhibitory transmitter. It was included in the study simply as another example of a substance which may either relax or stimulate smooth muscle (e.g. Woo & Somlyo, 1967; Gilmore & Vane, 1970; Mackay & McKirdy, 1972). The fact that it produces opposite responses in the two species merely

adds vasopressin to the list of substances tested producing such responses. It is suggested that each of these substances may act on a similar receptor in each species, but that the net effect on the muscle of occupation of this "receptor" is different in each species. Presumably the receptor involved is different for each substance. This can be proved at least to the extent that acetylcholine and carbachol act on muscarinic receptors, isoprenaline on β - adrenergic receptors and the others, ATP, prostaglandins and vasopressin, on receptors which are neither of these. The present evidence provides grounds for speculation that these receptors are all different again from the receptors for the inhibitory nerve transmitter and that the mechanism activated by this latter receptor is different from that of the others. It would thus be of interest to direct an electrophysiological study towards determining the two unknown factors here, viz. (1) what is the mechanism or mechanisms set in action by acetylcholine etc. which leads to contraction of the rat and relaxation of the cat anococcygeus, is this the same basic mechanism e.g. change in permeability to some ion, in both species, and what is the difference between the two species which results in a net opposite response? (2) what is the difference between this mechanism in (1) and that set in motion by the inhibitory transmitter, which leads to an inhibitory response in both species? If these factors could be elucidated then characterisation of the inhibitory nerves would be a further step closer.

A striking feature of the anococcygeus in both species is the ease with which a wide range of drugs may produce sympathomimetic effects i.e. contractions which were reduced or prevented by phentolamine. In order to determine whether these effects were direct effects on the α - receptors of the smooth muscle or indirect effects produced by release of NA from the motor nerves, chemical sympathectomy using 6OHDA was employed. The methods available for differentiating between direct and indirect sympathomimetic effects have

recently been reviewed by Trendellenburg (1972) who enumerates three methods, viz. (1) the use of cocaine which will block the neuronal uptake process producing block of indirect and potentiation of direct actions (2) denervation, which will similarly prevent indirect effects by destruction of the nerves (3) reserpine pretreatment which will remove the NA which is available for release by indirect agents. In the present circumstances, cocaine could not be usefully employed since it was itself a powerful sympathomimetic, surgical denervation was not possible in the anococcygeus since the surgical problems of this have not yet been solved and reserpine treatment was considered unsuitable since it had been shown to produce depletion of NA stores which was insufficient to prevent adrenergic nerve responses. The alternative but related method of producing sympathectomy using 6OHDA was therefore chosen. Pretreatment of rats _in vivo_ with 6OHDA had been shown to produce a sympathectomy in the anococcygeus as demonstrated histochemically by the absence of adrenergic nerves and by the abolition of motor responses to field stimulation (Gibson & Gillespie, 1973). In addition, after this treatment the sympathomimetic effect of guanethidine was absent. This treatment was thus suitable for the rat. Treatment of a sufficient number of cats with 6OHDA was financially impracticable so in this case the muscles were incubated _in vitro_ with 6OHDA, a method previously demonstrated to produce an effective chemical sympathectomy in a variety of tissues (e.g. Sachs, 1971; Wadsworth, 1973). This _in vitro_ sympathectomy was carried out on rat as well as cat anococcygeus, so that in this species the two treatments could be compared.

Chemical sympathectomy either _in vivo_ or _in vitro_ greatly reduced or completely abolished the responses to tyramine and potentiated the responses to NA in both species. This suggested that a destruction of the adrenergic nerves had occurred resulting in both the inability of the indirect sympathomimetics to

produce responses and the potentiation of the direct sympathomimetics by removal of the neuronal uptake process. Of the test drugs, the responses of guanethidine, LSD and cocaine were either abolished or greatly reduced while 5HT produced a paradoxical result. In vitro 6OHDA treatment in both species resulted in abolition of the 5HT response, suggesting a wholly indirect mechanism of action, while 6OHDA in vivo treatment of rats produced a sensitivity to 5HT greater than before.

A clear - cut result was thus obtained with guanethidine, LSD and cocaine whose responses were reduced to a similar extent to those of tyramine and could thus be considered to produce their action entirely by release of NA. It was possible that the residual responses remaining to these drugs after 6OHDA could be produced by either direct or indirect actions, but several factors suggest that this is a residual ~~direct~~ INDIRECT action on the muscle. Thus the size of these responses varied between groups of rats which were treated from different batches of 6OHDA and this was reflected in the size of the motor responses which could be obtained to field stimulation. This suggests that the 6OHDA which was obtained from various sources may have been of varying purity. After in vitro 6OHDA treatment, residual responses to these indirect agents were uncommon. For these reasons a wholly indirect action is suggested for tyramine, guanethidine, LSD and cocaine, although a direct component cannot be ruled out completely. Tyramine, guanethidine and cocaine have all been shown in the past to possess both indirect and direct effects on smooth muscle tissues (see Trendelenburg, 1972). As Trendelenburg (1972) has pointed out " each sympathomimetic amine should be regarded as potentially able to exert both direct and indirect effects. Its overall effects in any given organ or system depend on the affinity of the amine to the receptor, to the uptake mechanism, on its ability to impair the re - uptake of the released transmitter as well as on the factors which influence the concentration of the amines at the receptors (such as the morphology of the synaptic region).". These same factors are

also likely to apply to non - amine sympathomimetics and it appears that the conditions existing in the anococcygeus are particularly conducive to indirect action.

The factors producing this susceptibility to indirect actions are not yet clear. The nerve - muscle distance is no smaller than in other tissues so this is unlikely to be a significant factor. Other morphological factors, an efficient neuronal uptake mechanism or a particular susceptibility to displacement of the granular NA may all be relevant.

LSD has surprisingly never been directly linked with sympathomimetic actions although several factors suggest the possibility. Thus (1) LSD has been shown to inhibit NA uptake in isolated storage granules (Euler, 1970), a property it shares with tyramine (Burgen & Iversen, 1965) (2) other ergot derivatives have been shown to possess indirect sympathomimetic actions e.g. ergotamine (Salzmann, Pacha, Taeschler & Weidmann, 1968) (3) the isolated cat nictitating membrane which is similar to the anococcygeus in being contracted by cocaine and 5HT as well as NA, is also contracted by LSD, ergotamine and dihydro - ergotamine (Thomson, 1958). It was not, therefore, surprising that LSD should exert an indirect sympathomimetic action on the anococcygeus. A further connection is that Gant & Dyer (1971) have shown that LSD and 5HT can both produce contractions of isolated strips of human umbilical vein and umbilical artery which are abolished by phenoxybenzamine. Since these preparations are nerve - free, an indirect sympathomimetic action seems at first sight unlikely. It has, however, been suggested by Gulati & Kelkar (1971) that tyramine may act on human umbilical artery by liberation of extraneuronal NA. It might, therefore, be possible that LSD and possibly also 5HT might act in this way. It would be rash to suggest that this indirect sympathomimetic property of LSD in the anococcygeus might have any significance for its CNS hallucinogenic action, but other peripheral properties such as inhibition of

5HT receptors have in the past led to much psychopharmacological speculation (see Mantegazzini, 1966).

Another property of LSD in the anococcygeus was an adrenergic neuronal blocking action. This property has been recently reported also by Ambache, Killick, Srinivasan & Zar (1973) for the rat ancococcygeus. They related this to the similar blocking action of LSD on the vas deferens of the guinea - pig and rat and demonstrated that this inhibitory action was more effective against short than long pulse trains. The present experiments demonstrate that LSD can inhibit adrenergic responses in the rat and cat anococcygeus but show no difference in inhibition of the response to pulse trains of different lengths. In addition, this inhibition could take up to 1 hr to reach its maximum effect and was produced by doses of LSD which raised tone. Earlier in this thesis in discussing the effect of LSD on the vas deferens of the pithed rat, it was suggested that since the inhibition produced in this tissue was restricted to the first phase of the motor response, it resembled the action of tyramine. This was related to the work of Ambache, Dunk, Verney & Zar (1972, 1973) and it was suggested that the reason for the inhibition found in their experiments being more effective against short than against long pulse trains was that only the first part of the two phase response was affected. When LSD was then shown to produce an indirect sympathomimetic effect on the anococcygeus, this lent further weight to this hypothesis. Ambache, Killick, Srinivasan & Zar (1973), however, demonstrated that this relatively greater inhibition of short pulse trains also existed in the anococcygeus and thus suggested that LSD was capable of producing a presynaptic neurone blocking action of the adrenergic nerves in both the anococcygeus and vas deferens and that this was somehow more effective against the first few impulses. In the present experiments, the inhibition of motor responses by LSD in the anococcygeus could not be shown to be different at any particular length of train.

It is thus felt by the present author that while LSD undoubtedly has a presynaptic blocking action on the anococcygeus, this is not necessarily either a simple effect or directly analogous to its effect on the vas deferens. In interpreting these effects and making comparisons, several points must be borne in mind -

(1) There is only one component to the motor response of the anococcygeus and this is inhibited by LSD. There are two components to the response of the vas deferens but only one of those is inhibited by LSD.

(2) In addition to producing inhibition of the motor nerves, LSD produces an indirect sympathomimetic action in the anococcygeus. This may be reflected in the potentiation of the secondary phase of the response in the vas deferens.

(3) In the anococcygeus the effect of LSD resembles that of guanethidine with the exception that with guanethidine the threshold for the neurone blocking action is lower than for sympathomimetic action, whereas with LSD inhibition does not occur until sympathomimetic doses have been reached. In the vas deferens, guanethidine inhibits both phases of the response but LSD only the first phase.

(4) In the rat vas deferens there is evidence (see Part II) that the two phases of the motor response may be due to two anatomically and pharmacologically distinct sets of nerves. The first phase, which is resistant to some methods of blocking adrenergic nerves e.g. reserpinisation or α - blockers, is blocked by LSD, but the latter phase, which is susceptible to these adrenergic blocking manoevres, is resistant to, and may be potentiated by, LSD.

With these factors in mind, it is suggested that LSD and guanethidine each possess the dual properties of adrenergic neurone blockade and indirect release of NA from adrenergic nerves. When the additional possibility of α - receptor inhibition of NA release by an indirect sympathomimetic is

added to this, the interpretation of the effect of either drug on a given system must take into account the net effect of these multiple factors. In this way all the effects of both LSD and guanethidine on the anococcygeus and vas deferens can be accounted for except the inability of LSD to block the secondary phase in the vas deferens. This latter exception may, however, be related to differences in morphology or metabolism of the nerves giving rise to this response. The lack of references in the literature to either sympathomimetic or adrenergic blocking actions of LSD in other tissues would support this. The reasons for this differential susceptibility to LSD of adrenergic nerves in different tissues remains to be elucidated.

The paradoxical effect of 6OHDA treatment on the response to 5HT was reflected in the effects of specific blocking drugs. The initial observation that the response to 5HT of the rat anococcygeus was blocked by phentolamine suggested that 5HT might act either directly on α - receptors or indirectly by release of NA from nerves. It was subsequently found that the cat anococcygeus was more sensitive to 5HT than the rat muscle, that phentolamine did not block at all concentrations and that the dose / response curve in the cat had a definite inflection at 10^{-5} M. Moreover, methysergide caused a marked inhibition of responses to low doses of 5HT, whereas phentolamine inhibited only responses to high doses. This then suggested that perhaps low doses exerted a direct effect on methysergide - sensitive 5HT - receptors and high doses exerted, in addition, an indirect effect which by acting via NA was therefore phentolamine - sensitive. A similar hypothesis to this has recently been put forward by Pluchino (1972) for the cat nictitating membrane. He showed that the dose / response curve to 5HT in this latter tissue also had an inflection and demonstrated that whereas the response to high doses was (a) blocked by phentolamine, cocaine or reserpinisation (b) potentiated by blockade of MAO (c) showed cross - tachyphylaxis to tyramine, the response to

low doses was unaffected by these procedures. His conclusion from this was thus that the response to high doses was an indirect sympathomimetic action, whereas that to low doses was a direct action on 5HT receptors. Unfortunately, he did not report on the action of methysergide which in the present study abolished the response to low doses. Nevertheless, he suggested, on the negative evidence of lack of blockade by phentolamine that this direct effect was exerted on specific 5HT receptors and not on adrenergic α - receptors.

With this background in mind, it was hoped that after sympathectomy by 6OHDA, direct and indirect components in the anococcygeus could be resolved and if direct components existed, they could be classified as 5HT or α - receptors by using specific blockers. After 6OHDA __in vitro__ treatment a clear answer to these questions appeared to be presented. In both species, 5HT produced no responses whatsoever, in contrast to NA whose responses were potentiated. On this basis it appeared that 5HT acted wholly as an indirect sympathomimetic in both species and that methysergide might then inhibit the responses at all concentrations of 5HT by presynaptic inhibition of the release of NA by 5HT. The only weakness in this hypothesis was that phentolamine inhibited only the responses to high doses which would be surprising if the whole response was due to liberation of NA. This could be rationalised by suggesting that the response to low doses might be due to an effect in the immediate vicinity of the nerves and that the high dose of NA built up in this region might be relatively resistant to phentolamine in much the same way that NA released by nerve impulses is. This argument also might explain the inflection on the dose / response curve to 5HT in the cat anococcygeus. Thus if the maximal effect of occupation of receptors by NA in the immediate vicinity of the nerves was reached at a lower concentration than the threshold for the effect on more distant receptors, then an inflection on the curve might be expected. The lack of such an effect coupled with the lower sensitivity to 5HT in the rat anococcygeus might suggest some morphological difference in this tissue. This

type of argument of differential effects on morphological grounds is
analogous to those of de la Lande & Waterson (1968), Trendelenburg, Draskoczy
& Pluchino (1969), Urquilla, Stitzel & Fleming (1970).

When muscles from 60HDA _in vivo_ treated rats were tested, however, a
conflicting result was obtained.   In this case the muscles were sensitive to
5HT, although the development of this response was much slower than in controls.
This response was inhibited by either phentolamine or methysergide.   If the
final maximum tension developed by each dose was used for the construction of
dose / response curves, these 60HDA sympathectomised tissues appeared to be
even more sensitive to 5HT than were untreated controls.   The difference in
rate of development of the response did, however, suggest that these responses
were qualitatively very different from those obtained in controls.   In the
case of the other indirect sympathomimetics tested, viz. tyramine,
guanethidine, LSD and cocaine, similar slow - onset responses were obtained in
some 60HDA _in vivo_ sympathectomised muscles.   The ability of these drugs to
produce these responses appeared to be inversely related to the degree of
sympathectomy, so for this reason it is assumed that these responses are due to
residual indirect effects.   Since 60HDA destroys nerve terminals before nerve
trunks (Malmfors, 1971), it is also possible that part of these residual
responses arises from NA released from axones rather than from terminal
varicosities.   This is partly substantiated by the presence of occasional
fluorescent axone profiles in the histochemical picture of these tissues.   For
this reason it is suggested that the residual response to 5HT in these 60HDA
in vivo treated tissues is due entirely to the release of NA remaining in
nerves following the incomplete sympathectomy produced by this treatment.   It
is further suggested that the apparent increase in the magnitude of the response
to 5HT under these conditions might be due to the abolition of factors
normally operating to limit the effectiveness of 5HT but which are largely

removed by the 6OHDA treatment, e.g. the destruction of the majority of the terminal varicosities will result in the loss of the neuronal uptake of 5HT and subsequent destruction by intra - neuronal MAO.

On the basis of these experiments, the final hypothesis so far reached for the action of 5HT in the anococcygeus is that its action is entirely indirect. This is based both on the actions of phentolamine and methysergide and crucially on the sympathectomy produced by in vitro 6OHDA treatment. The results of in vivo instead of in vitro 6OHDA treatment might have led to the conflicting conclusion that the effects of 5HT are both direct and indirect and that the direct component may be potentiated by destruction of the varicosities leading to an increased amount of 5HT available for direct action. It must, therefore, be stated that although this latter hypothesis has been discarded on the basis that the sympathectomy so produced is incomplete, there are drawbacks to the use of 6OHDA in vitro.

6OHDA may itself produce blockade of receptors. It has been shown to block α - adrenergic receptors (Haeusler, 1971) and muscarinic receptors (Furness, 1971). This effect may be due to 6OHDA itself or to oxidation products (Malmfors & Thoenen, 1971). To eliminate this effect the organ bath was thoroughly washed throughout the 2 hr period following 6OHDA treatment. Despite this precaution, the sensitivity of the rat anococcygeus to NA after this treatment was less than that after in vivo 6OHDA treatment. This could have been due to depression of the α - receptors or alternatively due to fatigue of the muscle since the 6OHDA treatment produced a prolonged maximal motor response. At the dose of 6OHDA used in these experiments, this response is likely to be due partially to the indirect sympathomimetic action of 6OHDA and partially to the release of NA following disruption of the nerve varicosities (Sachs, 1971; Trendelenburg & Wagner, 1971). The former effect is suggested by the rapid onset of the contraction and the latter by the slow

decline of the response after washout. This prolonged contraction of the muscles could thus have led to fatigue and thus to a limitation on sensitivity to NA but still does not eliminate the possibility that either α - receptors or hypothetical 5HT receptors on the muscle may have been depressed. The possibility that 5HT may act on either its own specific receptors or on α - adrenergic receptors has been suggested by Innes (1962) who demonstrated by receptor - protection experiments that 5HT has affinity for - receptors in the cat nictitating membrane.

A further factor which necessitates caution in these experiments is the effect of ascorbic acid. In several recent studies on the _in vitro_ effects of 6OHDA, ascorbic acid has been included in the medium (e.g. Sachs, 1971; Wadsworth, 1973) ostensibly to prevent oxidation of the 6OHDA along the lines that NA is so protected (see Hamberger, 1967). In the present study, ascorbic acid in the concentrations employed by these authors (6×10^{-3} M) produced a maximal contraction of the anococcygeus muscles from both species, and even in a 1,000 time dilution, still produced motor responses. It is not yet known whether these responses are due to a direct effect of ascorbic acid on the muscle or due to release of NA. Whatever the action, a direct pH effect is unlikely since the buffering capacity of the Krebs' solution prevented significant change especially at the lower concentrations. Effects of ascorbic acid on smooth muscle tissues have long been noted (e.g. Eliasson, Euler & Stjarne, 1955; Gillespie & Mackenna, 1961) so it should be included in physiological bathing media only with caution. In the present experiments, the inclusion of ascorbic acid was found to make no difference whatsoever to the chemical sympathectomy produced. This may be partly due to the unnecessarily large dose of 6OHDA employed, which was utilised simply because it had been shown to be successful by previous authors, or alternatively due to the lack of any effect of ascorbic acid on oxidation of 6OHDA.

With all these provisos in mind, it is thus concluded that 5HT acts as an

indirect sympathomimetic in the anococcygeus muscle, the cat muscle being more

sensitive than the rat. A direct effect of 5HT on the muscle cannot, however,

be completely eliminated. Further studies using the more conventional means)

of differentiating between direct and indirect effects are, therefore,

necessary.

The final aspect of the cat anococcygeus preparation which was examined was

the effects of cold storage. This was necessary since the experiments were

carried out on tissues which might be cold stored up to a maximum of 2 days.

The necessary control studies on the effect of cold storage showed that little

change in nerve or drug responses occurred over this short period.

Less expected was the ability of these nerves, cut off from their ganglion cell

body, to survive and continue functioning after 11 days, as shown by the

histochemical demonstration of adrenergic nerve terminals together with the

responses to field stimulation and to the indirect sympathomimetic action of

guanethidine. Stimulation of postganglionic adrenergic nerves fails to

produce responses after 2 days in mouse, rat and rabbit ileum, 3 days in

guinea - pig colon (Hölman & Hughes, 1965) and 7 days in guinea - pig taenia

caecum (Hattori et al, 1972). Adrenergic nerves were not demonstrable

histochemically after 5 days cold storage in guinea - pig caecum (Hattori et

al, 1972) or rabbit aortic strips (Shibata et al, 1971). The capacity of

the anococcygeus muscle to respond to NA after the nerves had ceased to

function is in agreement with the usual finding that in cold stored tissues

the smooth muscle cells remain viable longer than the nerves. Thus

intestinal smooth muscle from various species remains responsive to

acetylcholine for several days after the cholinergic nerves have ceased to

function (Ambache, 1946) and in guinea - pig taenia caecum, phenylephrine still

relaxes the muscle after 18 days cold storage (Fukuda & Shibata, 1972) despite the complete absence of nerve responses after 5 days (Hattori et al, 1972).

It was not possible, however, to differentiate between motor and inhibitory nerves in the cat anococcygeus by their ability to survive cold storage. In previous studies where cold storage of a tissue has been employed successfully to differentiate between the components of a multiple innervation, the different anatomical arrangement of the nerves has been a major factor contributing to the preferential survival of one type, e.g. one group of fibres separated from their cell body (Holman & Hughes, 1965; Hattori et al, 1972). In the isolated cat anococcygeus muscle, however, as in the rat (Gillespie, 1972), both motor and inhibitory nerves consist purely of postganglionic fibres. There is, therefore, no reason to expect differential survival. Despite the long survival after cold storage of the nerve and muscle elements of the cat anococcygeus, species variation may be at least as important as the properties of the particular tissue used since the rat anococcygeus muscle does not respond to either field stimulation or NA after only 5 or 6 days (D. Templeton, personal communication).

All these experiments with the cat anococcygeus muscles have thus demonstrated that the dual innervation first found in the rat anococcygeus exists and has similar properties in the corresponding tissue in the cat. They confirm that the motor nerves are adrenergic and demonstrate the basic pharmacological properties of the nerves and muscle which vary slightly from those of the rat. They do not lead any further towards a positive identification of the inhibitory nerve transmitter but demonstrate the interaction between the motor and inhibitory nerves and provide evidence that the inhibitory nerves are not cholinergic and are unlikely to be purinergic.

GENERAL DISCUSSION OF ANOCOCCYGEUS

The separate but related aspects of the anococcygeus muscle which have been investigated come together to answer several questions concerning the nature of the muscle and its innervation. Thus it has been clearly demonstrated that the dual innervation exists in two species, that the motor nerves are adrenergic and that the two sets of nerves may interact with each other. The main unswered question is the identity of the inhibitory transmitter, although several candidates have been eliminated. The physiological function of the muscle has not been determined although sufficient evidence has been accumulated concerning the properties of the nerves to permit tentative suggestions.

The separate nature of the two sets of nerves has been demonstrated in three ways -

(1) The anatomical separation of the two sets of nerves was achieved by the experiments with the pithed rat which demonstrated the separate anatomical origins of the motor and inhibitory nerves. Either motor or inhibitory outflows or both could be stimulated selectively by appropriate selection of the electrode position. Pure motor or inhibitory responses or responses including components from both could thus be obtained. These experiments also demonstrated that both sets of nerves possessed a ganglion synapse and could thus be classified as part of the autonomic nervous system, but since hexamethonium had no effect on the responses to field stimulation _in vitro_, these ganglia were shown to lie somewhere between the spinal cord and the tissue and not in the tissue itself. In the case of the motor nerves, the ganglia were further shown to lie at least 40 mm from the tissue.

(2) The contrasting "physiological" properties of the two sets of nerves were demonstrated both _in vivo_ and _in vitro_ by their different characteristics

with regard to frequency and the development and decline of the responses.
This led in turn to an understanding of the complex responses which were
produced by the simultaneous stimulation of both sets of nerves by field
stimulation in vitro . This further clarified also the reasons for the
dominance of either effect according to the tone of the muscle. One
separation which could not be made, however, was that of stimulating one or
the other nerves preferentially by varying the pulse width. Motor and
inhibitory nerves have a similar rheobase and chronaxie.

(3) The pharmacological separation of the two sets of nerves was achieved
by the effects of blocking drugs either in vivo or in vitro and by the effect
of reserpine or of 6OHDA pretreatment.

The evidence for the adrenergic nature of the motor nerves is also considerable.

(1) The adrenergic blocking manoevres mentioned above not only separate the
two sets of nerves but clearly demonstrate the adrenergic nature of the motor
nerves. This is further confirmed by the effects of direct or indirect
sympathomimetics and by the effects on these of various pharmacological
manoevres. A corollary of this was that the anococcygeus is particularly
susceptible to indirect sympathomimetic effects, showing responses, which were
due to the release of NA, to guanethidine, LSD and cocaine as well as to the
more usual tyramine and amphetamine. A pharmacological comparison with the
motor nerves of the vas deferens also demonstrated the classical adrenergic
nature of the anococcygeus motor nerves.

(2) The histochemical evidence derived from the Falck technique shows a
wide spread distribution of fluorescent terminal varicosities throughout the
tissue in both species. Microspectrofluorometric analysis confirms that this
fluorescence is due to the presence of NA.

(3) Assay of the catecholamine content shows that the tissue from both
species contains a relatively high NA content and no detectable Adr.

Reserpine treatment produces depletion of NA, this depletion is accelerated by stimulation of the motor outflow and ultimately depression of the motor response results from NA depletion. This is also reflected in the decline in the number and brightness of terminal varicosities visible with the Falck technique. Similar results apply to the motor nerves of the vas deferens although the effect of reserpine treatment on its motor response is more complex.

(4) The thoracico - lumbar origin of the preganglionic motor nerves corresponds with what might be expected of the sympathetic nerves to a tissue in the pelvic region.

(5) Further evidence of the presence and properties of adrenergic nerves in the anococcygeus is currently being accumulated which demonstrates that

(a) the E.M. picture of the tissue shows nerves which have granular vesicles after 5OHDA treatment (Gillespie, personal communication) (b) evidence from intracellular recording of the membrane potential of the smooth muscle demonstrates depolarisation produced by field stimulation of the muscle. This depolarisation is abolished by phentolamine (Gillespie, Creed & Muir,1973).

The identity of the inhibitory transmitter remains unknown, but the evidence suggests that it is not any of the more obvious candidates.

(1) That they are not cholinergic is demonstrated by (a) the conflicting effects of acetylcholine on the tissue from the two species tested (b) the lack of effect on the inhibitory nerve response of atropine and neostigmine which respectively block and potentiate the response to acetylcholine. This leaves open the question of the function of acetylcholinesterase in the tissue, which appears to be involved in neither motor nor inhibitory transmission.

(2) Several factors also suggest that transmission is not purinergic.

(a) ATP has opposite effects in the two species. (b) The inhibitory

response which is produced by ATP in the cat anococcygeus is only produced by extremely high concentrations of 10^{-4} M or more. (c) The spinal origin of the preganglionic inhibitory fibres together with the presence of the ganglion between the spinal cord and the tissue but not in or near the tissue would be uncharacteristic for the type of purinergic nerves postulated by Burnstock (1972). Where purinergic nerves come under central control, this appears to be via the vagus and synaptic connections with the postganglionic nerves are usually within the tissue. (d) Purinergic inhibitory nerves occur in tissues whose adrenergic innervation is exclusively inhibitory. The only tentative exceptions to this are tissues related to the anococcygeus.

(3) - adrenergic transmission can also be excluded on the basis of (a) inhibitory responses occur in the presence of guanethidine both <u>in vivo</u> and <u>in vitro</u> (b) - receptors are absent in the rat (c) in the cat, where inhibitory effects do occur, these are abolished by propranolol whereas the inhibitory nerve responses are not.

(4) Prostaglandins are eliminated as inhibitory transmitter candidates since they too have opposing responses in the two species.

(5) The possibility of antidromic stimulation of afferent fibres, which is always a possibility with an unknown response in an isolated tissue, is eliminated by the presence of a ganglion on the inhibitory pathway.

(6) On the positive side, although the activity of the inhibitory nerves can at present only be visualised by the change in tone of the muscle or by inhibition of motor nerve responses, attempts are being made to obtain electrophysiological evidence of their effects and also to detect the inhibitor by bioassay. In the rat anococcygeus field stimulation in the presence of guanethidine has not so far produced evidence of any definite change in membrane potential or conductance, but preliminary experiments with the rabbit anococcygeus suggest that the inhibitory nerves may produce

hyperpolarisation of the membrane (K. Creed, personal communication). In addition, preliminary experiments employing one rat anococcygeus as the assay tissue to detect overflow of transmitter from another rat anococcygeus, suggest that the inhibitory transmitter can be so detected. Further bioassay experiments may thus shed more. light on the nature of the inhibitory transmitter.

All this provides evidence on the structure and capacity to function of the anococcygeus and its dual innervation. An aspect that remains unclear, however, is its actual physiological function. From the anatomical position of the muscles, "wrapped around" the terminal colon and fixing it to the pelvic floor at the coccygeal vertebrae two opposing functions spring to mind. The first is that the muscles have a sphincteric function and the other that they may play a role in the defecation reflex, to assist defecation. The motor nature of the adrenergic nerves suggests a sphincteric function, since the sympathetic nervous system is normally associated with closing sphincters and relaxing the other areas of the gastro - intestinal tract. Since the motor nerves are capable of generating a considerable motor response even at very low frequencies either in vivo or in vitro, this would facilitate a sphinteric function. Inhibitory nerves might then play a role in the relaxation of the muscle during defecation. This is an attractive hypothesis but is in conflict with the slow rate of depletion in the tissue after reserpine, which suggests a low tonic discharge in the motor nerves. It is, however, possible that since such a low discharge frequency is necessary to maintain tone, this might be insufficient to produce a rapid depletion. The most likely alternative to a maintained tone via the motor nerves would be a normally relaxed state of the muscle with an intermittent role to be played by both sets of nerves. Such a role might also play a part in defecation and would in this case be consistent with the reserpine

experiments.   Which of these hypotheses proves to be the most likely could be
resolved in two ways.   First the effects on the animal and on the tissue
itself of cutting in turn each of the nerves to the muscle should be
examined and secondly the tonic discharge rate, if any, in these nerves should
be measured and examined in decerebrate or anaesthetised animals.   In this
way, the physiological function of the anococcygeus may become clear.

The anococcygeus muscle has thus been investigated in two species, the rat and
the cat, and experiments performed both in vivo and in vitro in order to
examine the nature of this muscle and its dual innervation.   This has
enabled the relationship of structure and function in this tissue to be
examined.   In addition to answering several questions as to the nature of this
tissue and the effects of drugs on this, this study has also presented
several more questions on the subject of both the nature of this tissue and
on the effects of drugs in general.

ACKNOWLEDGEMENTS

SUMMARY

(1)   The subject of this thesis was the investigation of the dual innervation of the anococcygeus muscle.   The rat anococcygeus had previously been shown in vitro to have a motor adrenergic innervation and also an inhibitory nerve response whose transmitter was unknown.

(2)   The object of the present study was threefold -

(a)   To determine whether this inhibitory response was due to a nerve pathway distinct from the motor innervation and with a separate spinal origin and if so whether this pathway had a ganglion synapse and so could be considered as part of the autonomic nervous system.

(b)   To compare the pharmacological properties of the anococcygeus muscle and vas deferens and to determine whether the rate of depletion of noradrenaline by reserpine in these two tissues was affected by nerve stimulation.

(c)   To compare the properties of the anococcygeus muscles from the cat and the rat in order to find whether the dual innervation found in the rat was represented in this further species and if so whether this comparison would throw any more light on the nature of the inhibitory response.

(3)   Using a pithed rat preparation permitting selective stimulation of the autonomic spinal outflows, it was shown that the inhibitory pathway to the anococcygeus muscles arose from the spinal canal, that it was interrupted by a ganglion synapse and that the spinal origin of its pre-ganglionic nerves was L 5 - S 2 as opposed to T 10 - L 3 for the preganglionic nerves in the motor pathway.

(4)   Using this same preparation, the pharmacological properties of the motor nerves to the anococcygeus were examined in situ and compared with those of the vas deferens.   This comparison demonstrated that the pharmacological properties of the anococcygeus motor innervation were those

of a classical adrenergic innervation whereas the vas deferens showed responses which were in themselves complex and showed unconventional responses to drugs.   A hypothesis is suggested to explain this unconventional nature of the vas deferens response.

(5)   An analysis of the dose dependence and time course of the depletion of tissue noradrenaline by reserpine showed that the rat anococcygeus and vas deferens were depleted to a similar extent and at a similar rate and that this was slower than that found in the heart.   Increase in sympathetic nerve activity by spinal stimulation in pithed rats significantly increased the noradrenaline depletion in both anococcygeus and vas deferens.   From this it is suggested that nerve impulse traffic may be an important factor in determining the rate of depletion of noradrenaline by reserpine and in the vas deferens may explain the apparent resistance to depletion.

(6)   The cat anococcygeus muscle was investigated _in vitro_ and shown to possess a dual innervation similar to that in the rat.   Due to the presence of intrinsic tone, both motor and inhibitory nerve responses could be demonstrated in the absence of blocking drugs and their interaction studied. The pharmacological properties of the cat anococcygeus were similar to those of the rat except that several substances relaxed the cat muscle which contracted the rat including acetylcholine, isoprenaline, prostaglandins and ATP.   These substances were therefore assessed as possible inhibitory transmitters but further analysis with blocking drugs suggested that the relaxations produced by these drugs were different from that produced by the inhibitory nerves.   The inhibitory effect of acetylcholine on the cat muscle was particularly interesting since it inhibited motor nerve responses as well as relaxing the muscle.   Several substances not normally associated with release of noradrenaline from nerves, including guanethidine, cocaine

LSD and 5HT produced indirect sympathomimetic effects in both species.

(7)   It is concluded that the anococcygeus muscle receives a dual innervation consisting of a motor adrenergic pathway originating from the lower thoracic and upper lumbar cord and a separate inhibitory pathway with its preganglionic fibres originating from the lower lumbar and upper sacral region of the vertebral column.   This dual innervation is found in both the rat and cat anococcygeus but in neither species does the inhibitory pathway appear to be adrenergic, cholinergic or purinergic and the transmitter remains unknown.

REFERENCES

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Aberg G. & Eranko O. (1967). Localisation of noradrenaline and acetylcholinesterase in the taenia of the guinea - pig caecum. Acta physiol. scand. 69 383 - 384.

Ambache N. (1946). Interaction of drugs and the effect of cooling on the isolated mammalian tissue. J. Physiol. 104 266 - 287.

Ambache N., Dunk L.P., Verney J. & Zar M. Aboo (1972). Inhibition of postganglionic motor transmission in vas deferens by indirectly acting sympathomimetic drugs. J. Physiol. 227 433 - 456.

Ambache N., Dunk L.P., Verney J. & Zar M. Aboo (1973). An inhibition of postganglionic motor transmission in the mammmalian vas deferens by D - Lysergic acid diethylamide. J. Physiol. 231 251 - 270.

Ambache N., Killick S.W., Srinivasan V. & Zar M. Aboo (1973). Presynaptic inhibition of adrenergic motor transmission in rat anococcygeus muscle by D - Lysergic acid diethylamide (LSD). J. Physiol. 233 35 - 37 P.

Ambache N. & Zar M. Aboo (1970). Motor transmission in the vas deferens; the inhibitory action of noradrenaline. Br. J. Pharmac. 40 556P - 558P.

Ambache N. & Zar M. Aboo (1971). Evidence against adrenergic motor transmission in the guinea - pig vas deferens. J. Physiol. 216 359 - 389.

Anden N.-E. & Henning M. (1966). Adrenergic nerve function, noradrenaline level and noradrenaline uptake in cat nictitating membrane after reserpine treatment. Acta physiol. scand. 67 498 - 504.

Axelrod J., Mueller R.A. & Thoenen H. (1970). Neuronal and hormonal control of tyrosine hydroxylase and phenylethanolamine N - methyl transferase. pp. 212 - 219 In "New aspects of storage and release mechanisms of catecholamines." ed. H.J. Schumann & G. Kroneberg: Springer - Verlag; Berlin, Heidelberg, New York, 1970.

Bayliss W.M. (1902). Further researches on antidromic nerve impulses. J. Physiol. 28 276 - 299.

Bell C. (1972). Autonimic nervous control of reproduction: Circulatory and other factors. Pharmac. Rev. 24 657 - 736.

Benmiloud M. & Euler U.S. von (1963). Effects of Bretylium, reserpine, guanethidine and sympathetic denervation on the noradrenaline content of the rat submaxillary gland. Acta physiol. scand. 59 34 - 42

Bentley G.A. & Sabine J.R. (1963). The effects of ganglion - blocking and postganglionic sympatholytic drugs on preparations of the guinea - pig vas deferens. Br. J. Pharmac. 21 190 - 201.

Bertler A. (1961) Effect of reserpine on the storage of catechol amines in brain and other tissues. Acta physiol. scand. 51 75 - 83.

Bertler A., Carlsson A. & Rosengren E. (1958). A method for the fluorimetric determination of adrenaline and noradrenaline in tissues. Acta physiol. scand. 44 273 - 292.

Bevan J.A. & Verity M.A. (1967). Sympathetic nerve - free vascular muscle. J. Pharmac. exp. Ther. 157 117 - 124.

Bhagat B. & Zeidman H. (1970). Increased retention of norepinephrine $-^3$H in vas deferens during nerve stimulation. Am. J. Physiol. 219 691 - 696.

Birmingham A.T. (1970). Sympathetic denervation of the smooth muscle of the vas deferens. J. Physiol. 206 645 - 661.

Birmingham A.T. & Wilson A.B. (1963). Preganglionic and postganglionic stimulation of the guinea - pig isolated vas deferens preparation. Brit. J. Pharmac. 21 569 - 580.

Bjorklund A., Ehinger B. & Falck B. (1968). A method for differentiating dopamine from noradrenaline in tissue sections by microspectro - fluorometry. J. Histochem. Cytochem. 16 263 - 270.

Blakeley A.G.H., Dearnaley D.P. & Harrison V. (1970). The noradrenaline content of the vas deferens of the guinea - pig. Proc. R. Soc. B 174 491 - 502.

Bozler E. (1940). An analysis of the excitatory and inhibitory effects of sympathetic nerve impulses and adrenaline on visceral smooth muscle. Am. J. Physiol. 130 627 - 634.

Bronk D.W., Ferguson L.K., Margaria R. & Solandt D.Y. (1936). The activity of the cardiac sympathetic centres. Am. J. Physiol. 117 237 - 249.

Brown G.L., Dearnaley D.P. & Geffen L.B. (1967). Noradrenaline storage and release in the decentralized spleen. Proc. R. Soc. B 168 48 - 56.

Bulbring E. & Burn J.H. (1935). The sympathetic dilator fibres in the muscles of the cat and dog. J. Physiol. 83 483 - 501.

Bulbring E. & Gershon M.D. (1967). 5HT participation in the vagal inhibitory innervation of the stomach. J. Physiol. 192 823 - 846.

Burn J.H. & Rand M.J. (1959). Sympathetic postganglionic mechanism. Nature, Lond. 164 163 - 165.

Burn J.H. & Rand M.J. (1960). The relation of circulating noradrenaline to the effect of sympathetic stimulation. J. Physiol. 150 295 - 305.

Burn J.H. & Robinson J. (1951). Reversal of the vascular responses to acetylcholine and adrenaline. Br. J. Pharmac. 6 110 - 119.

Burnstock G. (1969). Evolution of the autonomic innervation of visceral and cardiovascular systems in vertebrates. Pharmac. Rev. 21 247 - 324.

Burnstock G. (1970). Structure of smooth muscle and its innervation.
pp. 1 - 69 In "Smooth muscle" ed. E. Bulbring, A.F. Brading,
A.W. Jones & T. Tomita: Edward Arnold; London, 1970.

Burnstock G. (1971). Neural nomenclature. Nature, Lond. 229 282 - 283.

Burnstock G. (1972). Purinergic nerves. Pharmac. Rev. 24 509 - 581.

Burgen A.S.V. & Iversen L.L. (1965). The inhibition of noradrenaline
uptake by sympathomimetic amines in the rat isolated heart.
Br. J. Pharmac. 25 34 - 49.

Campbell G. (1970). Autonomic nervous supply to effector tissues. pp. 451 -
495. Chapter 15. In "Smooth Muscle" ed. E. Bulbring, A.F. Brading,
A.W. Jones & T. Tomita: Edward Arnold; London, 1970.

Cannon W.B. (1939). The wisdom of the body, Norton, New York, 1939.

Cannon P., Raule W. & Schaefer H. (1954). Zur Physiologie eines
sympathischen Ganglions und zur Frage der vasodilatoren und des
sympathischen Tonus. Pflugers Arch. ges Physiol. 260 116 - 136.

Carlsson A. (1966). Drugs which block the storage of 5-hydroxytryptamine
and related amines. pp. 527 - 592 In "Handbook of experimental
pharmacology" Vol. XIX "5-hydroxytryptamine and related
indolealkylamines" ed. V. Erspamer: Springer - Verlag; Berlin,
Heidelberg, New York, 1966.

Carlsson A., Rosengren E., Bertler A. & Nilsson J. (1961). Effect of
reserpine on the metabolism of catechol amines pp. 363 - 372
In "Psychotropic drugs" ed. S. Garattini & V. Ghetti: Elsevier;
Amsterdam, 1957.

Carlsson A., Falck B., Hillarp N.A. & Thieme G. (1961). A new
histochemical method for visualisation of tissue catechol amines.
Med. exp. 4 123 - 124.

Caspersson T., Hillarp N.A. & Ritzen M. (1966). Fluorescence
microspectrophotometry of cellular catecholamines and 5-hydroxytrypt -
amine. Expl Cell Res. 42 415 - 428.

Chang C.C. & Chang J.C. (1965). A change in the subcellular distribution
of noradrenaline in the rat isolated vas deferens effected by nerve
stimulation. Br. J. Pharmac. 25 758 - 762.

Cliff G.S. (1968). Drug induced rhythmicity in smooth muscle. J. Pharm.
Pharmac. 20 579 - 580.

Corrodi H. & Hillarp N.A. (1963). Fluoreszenzmethoden zur histochemischen
Sichtbarmachung von Monoaminen (1) Identifizierung der
fluoreszierenden Produkte aus Modellversuchung mit 6,7Dimethoxy -
isochinolinderivaten und Formaldehyd. Helv. chim. Acta 46 2425 -
2430.

Corrodi H. & Hillarp N.A. (1964). Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen (2) Identifizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd. Helv. chim. Acta 47 911 - 918.

Corrodi H., Hillarp N.A. & Jonsson G. (1964). Fluorescence methods for the histochemical demonstration of monoamines. (3) Sodium borohydride reduction of the fluorescent compounds as a specificity test. J. Histochem. Cytochem. 12 582 - 586.

Corrodi H. & Jonsson G. (1965 a). Fluorescence methods for the histochemical demonstration of monoamines (4) Histochemical differentiation between dopamine and noradrenaline in models. J. Histochem. Cytochem. 13 484 - 487.

Corrodi H. & Jonsson G. (1965 b). Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen (5) Identifierung des fluoreszierenden Produktes aus modellversuchen mit 5-Methoxytryptamin und formaldehyd. Acta histochem. 22 247 - 258.

Corrodi H. & Jonsson G. (1966). Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen (6) Identifierung der fluoreszierenden Produkte aus m-Hydroxyphenylathylaminen und Formaldehyd. Helv. chim. Acta 49 798 - 806.

Dahlstrom A. (1970). The effects of drugs on axonal transport of amine storage granules. pp. 20 - 36 In "New aspects of storage and release mechanisms of catecholamines" ed. H.J. Schumann & G. Kroneberg: Springer - Verlag; Berlin, Heidelberg, New York, 1970

Dahlstrom A. & Haggendal J. (1966). Studies on the transport and life - span of amine storage granules in a peripheral adrenergic neurone system. Acta physiol. scand. 67 278 - 288.

Dale H.H. (1965). Adventures in Physiology, The Wellcome Trust: London, 1965.

Dale H.H. & Feldberg W. (1934). The chemical transmission of secretory impulses to the sweat glands of the cat. J. Physiol. 82 121 - 128.

Daniel E.E. & Wolowyck M. (1966). The contractile response of the uterus to cocaine. Can. J. Physiol. & Pharmacol. 44 721 - 730.

Day M.D. & Owen D.A.A? (1963). The interaction between angiotensin and sympathetic vasoconstriction in the isolated artery of the rabbit ear. J. Pharm. Pharmac. 15 221 - 224.

Dearnaley D.P. & Geffen L.B. (1966). Effect of nerve stimulation on the noradrenaline content of the spleen. Proc. R. Soc. B 166 303 - 315.

De La Lande I.S. & Waterson J.G. (1968). The action of tyramine on the rabbit ear artery. Br. J. Pharmac. 34 8 - 18.

Dixit B.N., Gulati O.D. & Gokhale S.D. (1961). Action of bretylium and guanethidine at the neuromuscular junction. Br. J. Pharmac. 17 372 - 379.

Donald F.M.S. (1969). The effects of nerve stimulation, agonists and antagonists on the vas deferens of the guinea - pig. B.Sc. Honours Thesis, University of Glasgow, 1969.

Dusting G.J. & Rand M.J. (1972). Depressor responses to spinal stimulation in the pithed rat. Br. J. Pharmac. 45 218 - 228.

Dyer D.C. & Gant D.W. (1973). Vasoconstriction produced by hallucinogens on isolated human and sheep umbilical vasculature. J. Pharmac. exp. Ther. 184 366 - 375.

Ehrlen I. (1948). Fluorimetric determination of adrenaline II. Farm. Revy 47 242 - 250.

Eranko O. (1952). On the histochemistry of the adrenal medulla of the rat, with special reference to acid phosphatase. Acta anat. 16 suppl. 17.

Eranko O. (1955). Distribution of fluorescing islets, adrenaline and noradrenaline in the adrenal medulla of the hamster. Acta endocr. Copenh. 18 174 - 179.

Esterhuizen A.C., Graham J.D.P., Lever J.D. & Spriggs T.L.B. (1968). Catecholamine and acetylcholinesterase distribution in relation to noradrenaline release. An enzyme histochemical and autoradiographic study on the innervation of the cat nictitating membrane. Br. J. Pharmac. 32 46 - 56.

Euler U.S. von (1966 a). Release and uptake of noradrenaline in adrenergic nerve granules. Acta physiol. scand. 67 430 - 440.

Euler U.S. von (1966 b). 20 years of noradrenaline. Pharmac. Rev. 18 29 - 38.

Euler U.S. von (1969). Acute neuromuscular transmission failure in vas deferens after reserpine. Acta physiol scand. 76 255 - 256.

Euler U.S. von (1970). Effect of some metabolic factors and drugs on uptake and release of catecholamines in vitro and in vivo. pp. 144 - 158 In "New aspects of storage and release mechanisms of catecholamines" ed. H.J. Schumann, G. Kroneberg: Springer - Verlag; Berlin, Heidelberg, New York, 1970.

Euler U.S. von (1972). Synthesis, Uptake and Storage of Catecholamines in Adrenergic Nerves. pp. 186 - 230 Ch. 6 In "Handbook of experimental pharmacology" Vol XXXIII "Catecholamines"; Springer - Verlag; Berlin, Heidelberg, New York, 1972.

Euler U.S. von & Gaddum J.H. (1931). Pseudomotor contractures after degeneration of the facial nerve. J. Physiol. 73 54 - 56.

Euler U.S. von & Hellner - Bjorkman S. (1955). Effect of increased adrenergic nerve activity on the content of noradrenaline and adrenaline in cat organs. Acta physiol. scand. 33 suppl. 118, 17 - 20.

Euler U.S. von & Hedqvist P. (1969). Inhibitory action of prostaglandins E₁ & E₂ on the neuromuscular transmission in the guinea - pig vas deferens. Acta physiol. scand. 77 510 - 512.

Euler U.S. von & Lishajko F. (1959). The estimation of catecholamines in urine. Acta physiol. scand. 45 122 - 132.

Euler U.S. von & Lishajko F. (1961). Improved technique for the fluorometric estimation of catecholamines. Acat physiol scand. 51 348 - 355.

Euler U.S. von & Lishajko F. (1963). Effect of reserpine on the uptake of catecholamines in isolated nerve storage granules. Int. J. Neuropharmac. 2 127 - 134.

Euler U.S. von & Lishajko F. (1966). A specific kind of noradrenaline granules in the vesicular gland and the vas deferens of the bull. Life Sci. 5 687 - 691.

Falck B. (1962). Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. Acta physiol. scand. suppl. 197.

Falck B., Hillarp N.A., Thieme G. & Torp A. (1962). Fluorescence of catecholamines and related compounds condensed with formaldehyde. J. Histochem. Cytochem. 10 348 - 354.

Falck B., Owman Ch. & Sjostrand N.O. (1965). Peripherally located adrenergic neurons innervating the vas deferens and the seminal vesicle of the guinea pig. Experientia (Basel) 21 98 - 103.

Falck B. & Torp A. (1962 a). A fluorescence method for histochemical demonstration of noradrenaline in the adrenal medulla. Med. exp. 5 429 - 432.

Falck B. & Torp A. (1962 b). A new evidence for the localisation of noradrenaline in adrenergic nerve terminals. Med. exp. 6 169 - 172.

Ferry C.B. (1963). The innervation of the vas deferens of the guinea - pig. J. Physiol. 166 16 P.

Ferry C.B. (1966). Cholinergic link hypothesis in adrenergic neuroeffector transmission. Physiol. Rev. 46 420 - 456.

Ferry C.B. (1967). The innervation of the vas deferens of the guinea - pig. J. Physiol. 192 463 - 478.

Finkleman B. (1930). On the nature of inhibition in the intestine. J. Physiol. 70 145 - 157.

Fukuda H. & Shibata S. (1972). The effect of cold storage on the inhibitory action of isoprenaline, phenylephrine and nicotine on the mechanical and membranal activities of guinea - pig caecum. Br. J. Pharmac. 46 438 - 448.

Furchgott R.F. (1955). The pharmacology of vascular smooth muscle. Pharmac. Rev. 7 183 - 265.

Furness J.B. (1971). Some actions of 6-hydroxydopamine which affect autonomic neuromuscular transmission. pp. 205 - 214 In "6-hydroxydopamine and catecholamine neurons" ed. T. Malmfors & H. Thoenen: North-Holland; Amsterdam, London, 1971.

Furness J.B. & Burnstock G. (1969). A comparitive study of spike potentials in response to nerve stimulation in the vas deferens of the mouse, rat and guinea - pig. Comp. Biochem. Physiol. 31 337 - 345.

Gabella G. & Costa M. (1968). Le fibre adrenergiche nel canale alimentare. G. Accad. Med. Torino 130 1 -

Gaddum J.H. & Hameed K.A. (1954). Drugs which antagonise 5HT. Br. J. Pharmac. 9 240 - 248.

Gaddum J.H. & Picarelli G. (1957). Two kinds of tryptamine receptors. Br. J. Pharmac. 12 323 - 328.

Gant D.W. & Dyer D.C. (1971). d-Lysergic acid diethylamide (LSD-25); a constrictor of human umbilical vein. Life Sci. 10 235 - 240.

Garrett J.R., Howard E.R. & Lansdale J.M. (1972). Myenteric nerves in the hind gut of the cat. J. Physiol. 226 103P - 104P.

Gaskell W.H. (1916). The involuntary nervous system. London, Longmans, 1916.

Gibson A. & Gillespie J.S. (1973). The effect of immunosympathectomy and of 6-hydroxydopamine on the responses of the rat anococcygeus to nerve stimulation and to some drugs. Br. J. Pharmac. 47 261 - 267.

Gillespie J.S. (1955). The extrinsic and intrinsic innervation of the colon. Ph.D. Thesis, Glasgow University, 1955.

Gillespie J.S. (1972). The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. Br. J. Pharmac. 45 404 - 416.

Gillespie J.S., Creed K.E. & Muir T.C. (1973). Electrical changes underlying excitation and inhibition in intestinal and related smooth muscle. Phil. Trans. R. Soc. Lond. B 265 95 - 106.

Gillespie J.S., Hamilton D.N.H. & Hosie R.J.A. (1970). The extraneuronal uptake and localization of noradrenaline in the cat spleen and the effect on this of some drugs, of cold and of denervation.; J. Physiol. 206 563 - 590.

Gillespie J.S. & Kirpekar S.M. (1966). The histological localisation of noradrenaline in the cat spleen. J. Physiol. 187 69 - 79.

Gillespie J.S. & Mackenna B.R. (1961). The inhibitory action of the sympathetic nerves of the smooth muscle of the rabbit gut, its reversal by reserpine and restoration by catecholamines and by DOPA. J. Physiol. 156 17 - 34.

Gillespie J.S., MacLaren A., Marshall R.W. & Pollock D. (1970). Segmental stimulation of spinal autonomic outflows in the pithed cat and rat. J. Physiol. 211 11 - 12 P.

Gillespie J.S., MacLaren A. & Pollock D. (1970). A method of
stimulating different segments of the autonomic outflow from the
spinal column to various organs in the pithed cat and rat. Br. J.
Pharmac. 40 257 - 267.

Gillespie J.S. & Maxwell J.D. (1971). Adrenergic innervation of
sphincteric and nonsphincteric smooth muscle in the rat intestine.
J. Histochem. Cytochem. 19 676 - 681.

Gillespie J.S. & Muir T.C. (1967) a. A method of stimulating the
complete sympathetic outflow from the spinal cord to blood vessels
in the pithed rat. Br. J. Pharmac. 30 78 - 87.

Gillespie J.S. & Muir T.C. (1967) b. The origin of the decline in the
vasopressor response to infused noradrenaline in the pithed rat.
Br. J. Pharmac. 30 88 - 98.

Gillespie J.S. & Muir T.C. (1967) c. The effect of infusing noradrenaline
or of stimulating the sympathetic nerves on the blood pressure
response to indirect sympathomimetics. Br. J. Pharmac. 30 99 - 107.

Gilmore N.J. & Vane J.R. (1970). A sensitive and specific assay for
vasopressin in the circulating blood. Br. J. Pharmac. 38 633 - 652.

Graham J.D.P., Al Katib H. & Spriggs T.L.B. (1968). The isolated
hypogastric nerve - vas deferens preparation of the rat. Br. J.
Pharmac. 32 34 - 45.

Greenberg S. & Long J.P. (1971). The effects of cocaine, norepinephrine
and ionic stimulants on the isolated superfused rat vas deferens.
J. Pharmac. exp. Ther. 177 136 - 145.

Gulati O.S. & Kelkar V.V. (1971). Effect of tyramine on human umbilical
artery in vitro. Br. J. Pharmac. 42 155 - 158.

Haeusler H. (1971). Short and long term effects of 6-hydroxydopamine on
peripheral organs. pp. 193 - 204 In "6-hydroxydopamine and
catecholamine neurons" ed T. Malmfors & H. Thoenen: North -
Holland; Amsterdam, London, 1971.

Haggendal J. (1966). Newer developments in catecholamine assay. Pharmac.
Rev. 18 325 - 329.

Haggendal J. & Dahlstrom A. (1971). The recovery of noradrenaline in
adrenergic nerve terminals of the rat after reserpine treatment.
J. Pharm. Pharmac. 23 81 - 89.

Hamberger B. (1967). Reserpine - resistant uptake of catecholamines in
isolated tissues of the rat. Acta physiol. scand. Suppl. 295.

Hamberger B., Levi-Montalcini R., Norberg K.A. & Sjoqvist F. (1965).
Monoamines in immunosympathectomized rats. Int. J. Neuropharmac.
4 91 - 95.

Hattori K., Kurahashi K., Mori J. & Shibata S. (1972). The effect of cold storage on the adrenergic mechanisms of intestinal smooth muscle. Br. J. Pharmac. 46 423 - 437.

Hertting G., Potter L.T. & Axelrod J. (1962). Effect of decentralization and ganglion blocking agents on the spontaneous release of H^3 - norepinephrine. J. Pharmac. exp. Ther. 136 289 - 292.

Holman M.E. (1970). Junction potentials in smooth muscle. pp. 244 - 288 In "Smooth muscle" ed. E. Bulbring, A.F. Brading, A.W. Jones & T. Tomita: Edward Arnold; London, 1970.

Holman M.E. & Hughes J.R. (1965). Inhibition of intestinal smooth muscle. Aust. J. exp. Biol. med. Sci. 43 277 - 290.

Holton P. & Perry W.L.M. (1951). On the transmitter responsible for antidromic vasodilatation in the rabbit's ear. J. Physiol. 114 240 - 251.

Holzbauer M. & Sharman D.F. (1972). The distribution of catecholamines in vertebrates. pp. 110 - 185 In "Handbook of experimental pharmacology" Vol. XXXIII "Catecholamines" ed H. Blaschko & E. Muscholl: Springer - Verlag; Berlin, Heidelberg, New York, 1972.

Holzbauer M. & Vogt M. (1956). Depression by reserpine of the noradrenaline concentration in the hypothalamus of the cat. J. Neurochem. 1 8 - 11.

Horton E.W. (1973). Prostaglandins at adrenergic nerve endings. Br. med. Bull. 29 148 - 151.

Howard E.R. & Garrett J.R. (1973). The intrinsic myenteric innervation of the hind - gut and accessory muscles of defaecation in the cat. Z. Zellforsch 136 31 - 44.

Hughes J. & Vane J.R. (1967). An analysis of the responses of the isolated portal vein of the rabbit to electrical stimulation and to drugs. Br. J. Pharmac. 30 46 - 66.

Hukovic S. (1961). Responses of the isolated sympathetic nerve - ductus deferens preparation of the guinea - pig. Br. J. Pharmac. 16 188 - 194.

Hyden H. (1960). The neuron. pp. 215 - 323 In "The cell" IV., ed J. Brachet, A. Mirsky: Academic Press; New York, London, 1960.

Innes I.R. (1962). An action of 5-hydroxytryptamine on adrenaline receptors. Br. J. Pharmac. 19 427 - 441.

Iggo A. & Vogt Marthe (1960). Preganglionic sympathetic activity in normal and in reserpine - treated cats. J. Physiol. 150 114 - 133.

Iversen L.L., Glowinski J. & Axelrod J. (1966). The physiologic disposition and metabolism of norepinephrine in immunosympathectomized animals. J. Pharmac. exp. Ther. 151 273 - 284.

Jacobowitz D. (1965). Histochemical studies of the autonomic innervation of the gut. J. Pharmac. exp. Ther. 149 358 - 364.

Jane F., Planas M.E. & Bonaccorsi A. (1970). Action of desipramine on noradrenaline depletion by reserpine in the rat vas deferens in vivo. J. Pharm. Pharmac. 22 381 - 383.

Jonsson G. (1967). Fluorescence methods for the histochemical demonstration of monoamines (7) Fluorescence studies on biogenic monoamines and related compounds condensed with formaldehyde. Histochemie 8 288 - 296.

Karki N.T., Paasonen M.K. & Vanhakartano P.A. (1959). The influence of pentolinium, isoraunescine and yohimbine on the noradrenaline depleting action of reserpine. Acta pharmac. (Kbh.) 16 13 - 19.

Kopin I.J. & Gordon E.K. (1962). Metabolism of norepinephrine - H^3 released by tyramine and reserpine. J. Pharmac. exp. Ther. 138 351 - 359.

Kopin I.J., Gordon E.K. & Horst W.D. (1965). Studies on uptake of l-norepinephrine-C^{14}. Biochem. Pharmac. 14 753 - 759.

Kosterlitz H.W. & Lees G.M. (1972). Interrelationship between adrenergic and cholinergic mechanisms. Chapter 17 In "Catecholamines" Hnadbook of experimental pharmcology Vol. XXXIII: Springer - Verlag; Berlin, Heidelberg, New York, 1972.

Krebs H.A. und Henseleit K. (1932). Untersechungen uber die Harnstoffbildung im Tierkorper. Hoppe-Seyler's Z. physiol. Chem. 210 33 - 66.

Kukovetz W.R. & Lembech F. (1962). Untersuchungen uber die adrenalinpotenzierende Wirkung von Cocain und Denervierung. N.-S.'s Arch. Exp. Pathol.Pharmakol. 242 467 - 479.

Kuriyama H. (1963). Electrophysiological observations on the motor innervation of the smooth muscle cells in the guinea - pig vas deferens. J. Physiol. 169 213 - 228.

Langley J.N. & Anderson H.K. (1895). The innervation of the pelvic and adjoining viscera. III The external generative organs. J. Physiol. 19 85 - 121.

Langley J.N. (1921). The autonomic nervous system. Cambridge, W. Heffer & Sons, 1921.

Lee F.L. (1967). The relation between norepinephrine content and response to sympathetic nerve stimulation of various organs of cats pretreated with reserpine. J. Pharmac. exp. Ther. 156 137 - 141.

Lewis T. & Marvin H.M. (1927). Observations relating to vasodilatation arising from antidromic impulses, to herpes zoster and trophic effects. Heart 14 27 - 47.

Loewi O. (1918). Uber die Natur der Giftwirkung des Suprarenins. Biochem. Z. 85 295 - 306.

Loffelholz K. & Muscholl E. (1969). A muscarinic inhibition of the noradrenaline release evoked by postganglionic sympathetic nerve stimulation. N.-S.'s Arch. Pharmak. 265 1 - 15.

Lund A. (1949). Fluorometric determination of adrenaline in blood. III. A new sensitive and specific method. Acta pharmac. (Kbh.) 5 231 - 247.

McCrea E.D., McSwiney B.A. & Stopford J.S.G. (1925). The effect on the stomach of stimulation of the peripheral end of the vagus. Q. Jl exp. Physiol. 15 201 - 233.

MacKay D. & McKirdy H.C. (1972). Effect of vasopressin and of adenosine triphosphate on the flat preparation of rabbit rectum. Br. J. Pharmac. 44 366P - 367P.

MacLellan P. (1973). Are there inhibitory nerve fibres in the extrinsic nerves to the rat anococcygeus muscle and if so, what is the effect of drugs on these. B.Sc. Thesis, Dept. of Pharmacology, University of Glasgow.

McSwiney B.A. & Robson J.M. (1929). Reaction of smooth muscle to stimulation of vagus and sympathetic nerves in isolated nerve muscle preparations. Am. J. Physiol. 90 450P.

Malik K.U. & Ling G.M. (1969). Modification by acetylcholine of the response of rat mesenteric arteries to sympathetic stimulation. Circulation Res. 25 1 - 9.

Malmfors T. (1971). The effects of 6OHDA on the adrenergic nerves as revealed by the fluorescence histochemical method. pp. 47 - 58 In "6-hydroxydopamine and catecholamine neurons" ed. T. Malmfors & H. Thoenen: North - Holland; Amsterdam, London, 1971.

Malmfors T. & Sachs Ch. (1968). Degeneration of adrenergic nerves produced by 6-hydroxydopamine. Europ. J. Pharmac. 3 89 - 92.

Malmfors T. & Thoenen H. (1971) (edited by) Discussion p 359 In "6-hydroxydopamine and catecholamine neurons": North - Holland; Amsterdam, London, 1971.

Maxwell R.A. (1965). Concerning the mode of action of guanethidine and some derivatives in augmenting the vasomotor action of adrenergic amines in vascular tissues of the rabbit. J. Pharmac. expl Ther. 148 320 - 328.

Maxwell R.A., Eckhardt S.B. & Wastila W.B. (1968). Concerning the distribution of endogenous norepinephrine in the adventitial and media - intimal layers of the rabbit aorta and the capacity of these layers to bind tritiated norepinephrine. J. Pharmac. exp. Ther. 161 34 - 39.

Maxwell R.A., Wastila W.B. & Eckhardt S.B. (1966). Some factors determining the response of rabbit aortic strips to dl - norepinephrine - 7 - H^3 HCl and the influence of cocaine, guanethidine & methylphenidate on these factors. J. Pharmac. exp. Ther. 151 253 - 261.

Merrilees N.C.R., Burnstock G. & Holman M.E. (1963). Correlation of
fine structure and physiology of the innervation of smooth muscle
in the guinea - pig vas deferens. J. cell. Biol. 19 529 - 550.

Miller M.E., Christensen G.C. & Evans H.E. (1964). Anatomy of the dog.
p.194 Philadelphia & London, W.B. Saunders Co., 1964.

Mirkin B.L. (1961). The effect of synaptic blocking agents on reserpine -
induced alterations in adrenal medullary and urinary catecholamine
levels. J. Pharmac. exp. Ther. 133 34 - 40.

Mitchell G.A.G. (1953). Anatomy of the autonomic nervous system.
Edinburgh, Livingstone, 1953.

Muscholl E. (1970). Cholinomimetic drugs and release of the adrenergic
transmitter pp. 168 - 186 In "New aspects of storage and release
mechanisms of catecholamines" Ed H.J. Schumann & G. Kroneberg:
Springer - Verlag; Berlin, Heidelberg, New York, 1970.

Nachmanson D. (1966). Role of acetylcholine in neuromuscular transmission.
Ann. N.Y. Acad. Sci. 135 136 - 149.

Norberg K.-A. (1964). Adrenergic innervation of the intestinal wall
studies by fluorescence microscopy. Int. J. Neuropharmac. 3
379 - 382.

Norberg K.-A. (1967). Transmitter histochemistry of the sympathetic
nervous system. Brain Res. 5 125 - 170.

O'Donnell S.R. & Hecker S.E. (1967). Contraction of rat vas deferens by
cocaine. J. Pharm. Pharmac. 19 695.

Ohlin P. & Stromblad B.C.R. (1963). Observations on the isolated vas
deferens. Br. J. Pharmac. 20 299 - 306.

Owman Ch. & Sjostrand N.O. (1965). Short adrenergic neurons and
catecholamine - containing cells in vas deferens and accessory
male genital glands of different mammals. Z. Zellforsch. 66 300 -
320.

Paton W.D.M. & Zaimis E.J. (1951). Paralysis of autonomic ganglia by
methonium salts. Br. J. Pharmac. 6 155 - 168.

Pick J. (1970). The autonomic nervous system. pp. 39 - 40.
Philadelphia, Toronto, J.P. Lippincott Co., 1970.

Pluchino S. (1972). Direct and indirect effects of 5HT and tyramine on
cat smooth muscle. N.-M.'s Arch. Pharmak. 272 189 - 224.

Rayner V. (1970). Observations on the functional internal anal sphincter
of the vervet monkey. J. Physiol. 213 27 - 28P.

Read J.B. & Burnstock G. (1969). Adrenergic innervation of the gut
musculature in vertebrates. Histochemie 17 263 - 272.

Richardson K.C. (1962). The fine structure of autonomic nerve endings in
smooth muscle of the rat vas deferens. J. Anat. 96 427 - 442.

Rosenblueth A. & Bard P. (1932). The innervation and functions of the nictitating membrane in the cat. Am. J. Physiol. **100** 537 - 544.

Roth R.H., Stjarne L. & Euler U.S. von (1967). Factors influencing the rate of norepinephrine biosynthesis in nerve tissue. J. Pharmac. exp. Ther. **158** 373 - 377.

Sachs Ch. (1971). Effect of 6-hydroxydopamine *in vitro* on the adrenergic neuron. pp. 59 - 74 In "6-hydroxydopamine and catecholamine neurons" ed. T. Malmfors & H. Thoenen: North-Holland; Amsterdam, London, 1971.

Salzmann R., Pacha W., Taeschler M & Weidmann H. (1968). The effect of ergotamine on humoral and neuronal actions in the nictitating membrane and the spleen of the cat. N.-S.'s Arch. exp. Pathol. Pharmakol. **261** 360 - 378.

Scheving L.E., Harrison W.H. & Pauley J.E. (1968). Daily fluctuation (Circadian) in levels of epinephrine in the rat supra-renal gland. Am. J. Phsyiol. **215** 799 - 802.

Sedvall G. (1964). Short term effects of reserpine on noradrenaline levels in skeletal muscle. Acta phsyiol. scand. **62** 101 - 108.

Sedvall G. & Thorson J. (1965). Adrenergic transmission at vasoconstrictor nerve terminals partially depleted of noradrenaline. Acta physiol. scand. **64** 251 - 258.

Shaw F.H., MacCallum M., Dewhurst D.J. & Mainland J.F. (1951). The possibility of the dual nature of sympathetic ganglion cells III. Aust. J. exp. Biol. med. Sci. **29** 153 - 160.

Sheehan D. & Pick J. (1943). The rami communicantes in the rhesus monkey. J. Anat. **77** 125 - 139.

Shibata S., Hattori K., Sakurai I., Mori J. & Fujiwara M. (1971). Adrenergic innervation and cocaine induced potentiation of adrenergic responses of aortic strips from young and old rabbits. J. Pharmac. exp. Ther. **177** 621 - 632.

Sjostrand N.O. (1962). Inhibition by ganglionic blocking agents of the motor response of the isolated guinea - pig vas deferens to hypogastric nerve stimulation. Acta physiol. scand. **54** 306 - 315.

Sjostrand N.O. (1965). The adrenergic innervation of the vas deferens and the accessory male genital glands. Acta physiol. scand. **65** Suppl. 257.

Sjostrand N.O. & Swedin G. (1967). Effect of chronic hypogastric denervation on the noradrenaline content of the vas deferens and the accessory male reproductive glands of the rat. Experientia (Basel) **23** 817 - 818.

Sjostrand N.O. & Swedin G. (1968). Effect of reserpine on the
noradrenaline content of the vas deferens and the seminal vesicle
compared with the submaxillary gland and the heart of the rat. Acta
physiol. scand. 72 370 - 377.

Steinberg M. & Hilton J.G. (1966). Site of action of atropine in blocking
pressor response to increased intracranial pressure in
chlorisondamine - treated dogs. Tex. Rep. Biol. Med. 24 222 - 227.

Stjarne L. (1973). Prostaglandin - versus α- adrenoreceptor - mediated
control of sympathetic neurotransmitter secretion in guinea - pig
isolated vas deferens. Europ. J. Pharmac. 22 233 - 238.

Stjarne L. & Lishajko F. (1966). Comparison of spontaneous loss of
catecholamines and ATP in vitro from isolated bovine adrenomedullary,
vesicular gland, vas deferens and splenic nerve granules. J.
Neurochem. 13 1213 - 1216.

Straus - Durckheim H. (18845). Anatomie Descriptive et Comparative du
Chat, Type des Mammiferes en Generale et des Carnivores en
Particulier. Vol. II. Paris, 1845.

Swedin G. (1971)a. Studies on neurotransmission mechanisms in the rat and
guinea - pig vas deferens. Acta physiol. scand. Suppl. 369.

Swedin G. (1971)b. Studies on neurotransmission mechanisms in the rat and
guinea - pig vas deferens. Acta physiol. scand. Suppl. 369.

Swedin G. (1971)b. Endogenous inhibition of the mechanical response of the
isolated rat and guinea pig vas deferens to pre and postganglionic
nerve stimulation. Acta physiol. scand. 83 473 - 485.

Thoa N.B., Johnson D.G., Kopin I.J. & Weiner N. (1971). Acceleration of
catecholamine formation in the guinea - pig vas deferens after
hypogastric nerve stimulation: Roles of tyrosine hydroxylase and
new protein synthesis. J. Pharmac. exp. Ther. 178 442 - 449.

Thoenen H., Mueller R. & Axelrod J. (1969). Increased tyrosine hydroxylase
activity after drug induced alteration of sympathetic transmission.
Nature (Lond.) 221 1246.

Thomson J.W. (1958). Studies on the response of the isolated nictitating
membrane of the cat. J. Physiol. 141 46 - 72.

Trendelenburg U. (1972). Classification of sympathomimetic amines.
Chapter 10, pp. 336 - 362 In "Handbook of experimental pharmacology"
Vol. XXXIII "Catecholamines" ed. H. Blaschko & E. Muscholl:
Springer - Verlag; Berlin, Heidelberg, New York, 1972.

Trendelenburg U., Draskoczy P.R. & Pluchino S. (1969). The density of
adrenergic innervation of the cat's nictitating membrane as a factor
influencing the sensitivity of the isolated preparation to 1-
norepinephrine. J. Pharmac. exp. Ther. 166 14 - 25.

Trendelenburg U. & Wagner K. (1971). The effect of 6-hydroxydopamine on the cat's nictitating membrane; degeneration contraction and sensitivity to (-)-noradrenaline. pp. 215 - 224 In "6-hydroxydopamine and catecholamine neurons" ed. T. Malmfors & H. Thoenen : North - Holland; Amsterdam, London, 1971.

Trendelenburg U. & Weiner N. (1962). Sensitivity of the nictitating membrane after various procedures and agents. J. Pharmac. exp. Ther. 136 152 - 161.

Tsai T.H., Denholm S. & McGrath W.H. (1968). Sensitivity of the isolated nictitating membrane of the cat to NA and Ach after various procedures and agents. J. Pharmac. exp. Ther. 164 146 - 157.

Urquilla P.R., Stitzel R.E. & Fleming W.W. (1970). The antagonism of phentolamine against exogenously administered and endogenously released norepinephrine in rabbit aortic strips. J. Pharmac. exp. Ther. 172 310 - 319.

Veach H.O. (1925). Studies on the innervation of smooth muscle. I Vagus effects on the lower end of the oesophagus, cardia and stomach of the cat and the stomach and lungs of the turtle in relation to Wedensky inhibition. Am. J. Physiol. 71 229 - 264.

Volle R.L. (1962). The responses to ganglionic stimulating and blocking drugs of cell groups within a synaptic ganglion. J. Pharmac. exp. Ther. 135 54 - 61.

Volle R.L. & Koelle G.B. (1965). Ganglion stimulating and blocking agents. pp. 578 - 595 In "The pharmacological basis of therapeutics" ed L.S. Goodman & A. Gilman: Macmillan; New York, Toronto, London, 3rd edition, 1965.

Wadsworth R.M. (1973). Abolition of neurally evoked motor responses of the vas deferens by 6-hydroxydopamine. Europ. J. Pharmac. 21 383 - 387.

Woo C.-Y. & Somlyo A.P. (1967). Interaction of magnesium with vasopressin in intestinal smooth muscle. J. Pharmac. exp. Ther. 155 357 - 366.

Wurtman R.J. & Axelrod J. (1966). A 24 hr rythymn in the content of norepinephrine in the pineal and salivary glands of the rat. Life Sci. 5 665 - 669.

Wybauw L. (1936). Transmission humorale de la vaso-dilatation provoquee par l'excitation du bout peripherique des racines posterieures lombaires chz le chat. C.R. Soc. Biol. (paris) 123 524 - 528.

Zaimis E., Berk L. & Callingham B.A. (1965). Morphological, biochemical and functional changes in the sympathetic nervous system of rats treated with nerve growth factor - antiserum. Nature (Lond.) 206 1220 - 1222.

Zuckerman S. (1938). Observations on the autonomic nervous system and neural segmentation in monkeys. Trans. Zool. Soc. London, 23 315 - 378.

Publications

Part of the work of this thesis has been submitted for publication

as follows -

J.S. Gillespie & J.C. McGrath.   The origin of the inhibitory nerve
    pathway to the rat anococcygeus muscle.   J. Physiol.   224   44 - 45 p
    1972.

J.S. Gillespie & J.C. McGrath.   The effect of nerve stimulation on the
    depletion of noradrenaline by reserpine in the heart, vas deferens
    and anococcygeus muscle of the rat.   Br. J. Pharmac.   46   525 - 526 p
    1972.

J.S. Gillespie & J.C. McGrath.   The spinal origin of the motor and
    inhibitory innervation of the rat anococcygeus muscles.   J. Physiol.
    230   659 - 672   1973.

J.S. Gillespie & J.C. McGrath.   The response of the cat anococcygeus
    muscle to nerve or drug stimulation and a comparison with
    the rat anococcygeus.   Br. J. Pharmac.   50   In the press   1974

J.S. Gillespie & J.C. McGrath.   The effect of reserpine on the rat
    anococcygeus, vas deferens and heart.   Submitted for publication to
    Br. J. Pharmac.

Reprints where available are included in a folder at the back of this

thesis.

THE SPINAL ORIGIN OF THE MOTOR AND INHIBITORY INNERVATION OF THE RAT ANOCOCCYGEUS MUSCLES

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SUMMARY

1. A preparation is described whereby the responses of the two anococcygeus muscles can be recorded *in vivo* in the pithed rat and the autonomic outflows to the muscle selectively stimulated in the spinal canal.

2. Motor responses are obtained from stimulation at two levels; an upper extending from T 11 to L 3 and a lower from L 6 to S 2. Stimulation between these levels, i.e. between L 3 and L 6, produces no response. The response to stimulation at both upper and lower levels is abolished by phentolamine. The response to stimulation in the upper region is abolished by hexamethonium and is, therefore, presumably preganglionic; the response to stimulation at the lower level is resistant to hexamethonium and presumably post-ganglionic. Stimulation at levels above T 11 causes contraction after a delay, by liberating catecholamines from the adrenal medulla. This effect is blocked by both phentolamine and hexamethonium.

3. If the adrenergic motor nerves are blocked and the muscle tone raised by a combination of guanethidine and tyramine, stimulation between L 5 and S 2 produces inhibition. The inhibitory outflow, therefore, overlaps the motor outflow but extends one segment more rostral (L 5). Stimulation restricted to this L 5 segment even in the presence of a normal unblocked motor innervation causes inhibition. The inhibitory response is blocked by hexamethonium or mecamylamine but desensitization and 'escape' occurs. This desensitization is less than that observed in the vas deferens when its motor nerves are similarly stimulated in the spinal cord.

4. It is concluded that inhibitory fibres to the anococcygeus arise in the spinal cord and are organized in the pattern of the autonomic nervous system with a peripheral synapse. The site of origin of these inhibitory fibres is different from the motor adrenergic fibres to the muscle.

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INTRODUCTION

Stimulation of intramural nerves in the rat anococcygeus muscle produces contraction from excitation of the dense adrenergic innervation. If muscle tone is first raised, stimulation produces inhibition (Gillespie, 1972). The nature of this inhibitory innervation and of the transmitter involved is unknown. The present experiments were intended to answer two questions. First, do the inhibitory nerves arise in the spinal cord and, if so, does their origin correspond with that of the motor adrenergic nerves? Secondly, if a separate inhibitory innervation exists, is it part of the autonomic nervous system with a characteristic two neurone efferent pathway?
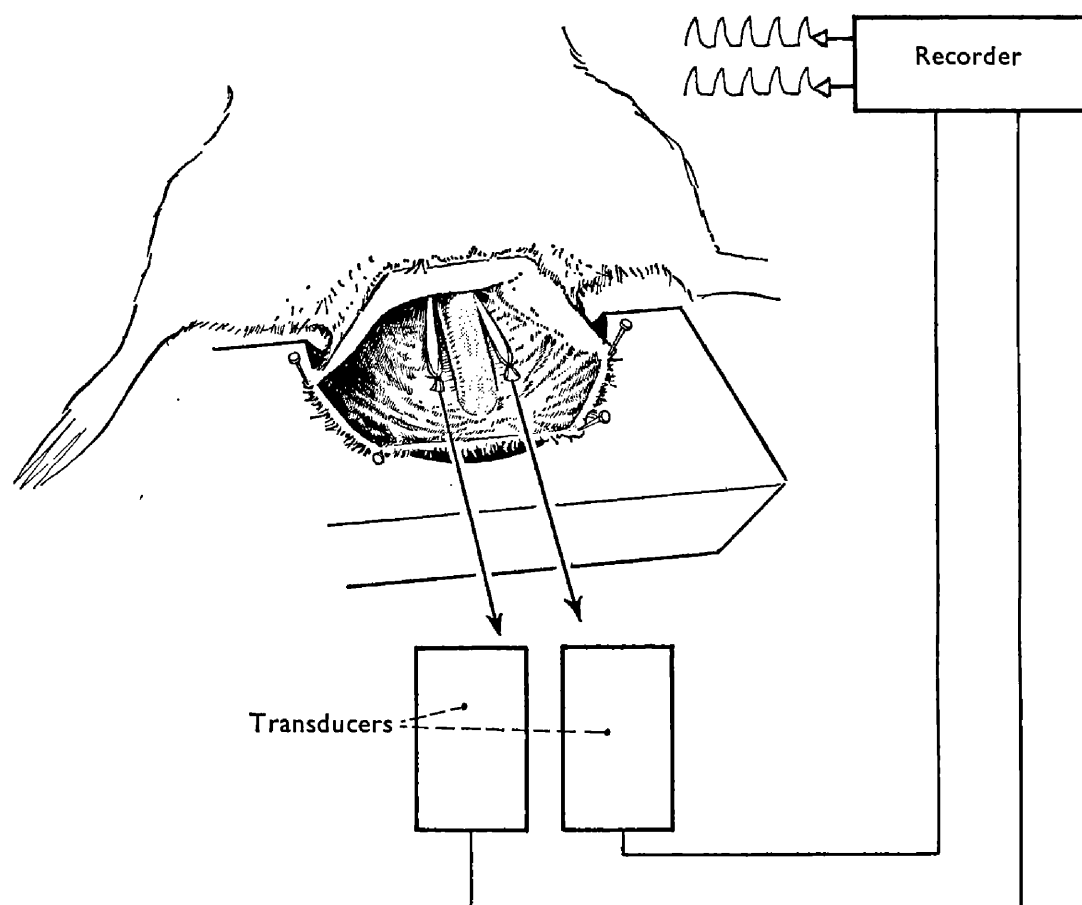

Fig. 1. The arrangement to record *in vivo* the tension response of the anococcygeus muscles in the rat. The scrotum is opened and pinned to a shaped wax block to form a pouch which is filled with liquid paraffin. Each anococcygeus is attached by thread to an isometric transducer.

METHODS

Male Wistar rats (250–300 g) were anaesthetized with a mixture of halothane and nitrous oxide and pithed by the method of Gillespie, MacLaren & Pollock (1970). In this technique the pithing rod is a stainless-steel wire sliding in a fine teflon tube. Both wire and tube can be independently withdrawn to any position in the spinal canal, and the spinal outflow at that region stimulated through the stainless-steel inner wire. The length of wire exposed determines the number of segments stimulated. The position of the steel electrode within the canal was checked by radiography. A silver indifferent electrode was inserted under the skin parallel to the vertebral column. The spinal nerve roots were stimulated electrically by 1 msec pulses of supramaximal voltage at frequencies between 0·1 and 100 Hz, with an electrode length of 5 or 10 mm. Intravenous pancuronium bromide (1 mg/kg) was given to prevent muscle twitching. The two anococcygeus muscles join together on the ventral surface of the colon just short of the anal margin to form a ventral bar. The muscles were exposed by an incision in the scrotum just anterior to the anal margin, and the edges pinned on to a wax block to form a sac which was filled with liquid paraffin. The two muscles were then separated by splitting the ventral bar and each was attached by a thread to a Grass FTO 3 isometric transducer, and a tension of 1 g applied (Fig. 1). In some experiments the muscles were not separated and the tension in the two muscles together was recorded.

The rat's temperature was monitored by a rectal thermometer and maintained at 36° C by a tungsten lamp. Blood pressure was recorded from one carotid artery and one femoral vein was cannulated for drug administration. In some experiments the other femoral vein was cannulated for slow infusion of a tyramine hydrochloride solution (1 mg/ml. solution given at a rate of 10–40 μg/min). Muscle tension, blood pressure and heart rate were displayed on a Grass Polygraph.

The following drugs dissolved in normal saline were used. Doses refer to the salts. Atropine sulphate (B.D.H.); guanethidine sulphate (Ciba); hexamethonium bromide (Koch-Light); D-lysergic acid diethylamide tartrate (Sandoz); mecamylamine hydrochloride (Merck, Sharp and Dohme); (–)-noradrenaline bitartrate (Koch-Light); pancuronium bromide (Organon); phentolamine mesylate (Ciba); tyramine hydrochloride (Sigma).

RESULTS

The motor response

The spinal origin of the motor nerves

Responses to stimulation of successive 5 mm intervals along the spinal canal from S 2 to C 3 are shown in Fig. 2. On withdrawing the stimulating electrode, motor responses were obtained at two positions; first, between S 2 and L 6 and again between L 3 and T 11, with a gap between L 6 and L 3 (Fig. 2a). The maximal response was similar in size in both regions (4·9 g ± 0·2 g per single muscle). Responses were rapid in onset, starting within 1 sec of stimulation, and quick to decline at the end of the stimulation period. Beyond T 11 these rapid responses disappeared to be replaced by delayed responses which began after 10–15 sec, i.e. after end of stimulation. These responses are presumed to be due to

circulating catecholamines released from the adrenal medulla (Fig. 2b). No responses were obtained higher than T 2.

Regions below S 2 could not be separately stimulated since the narrowness of the canal prevented entry of the teflon tube which isolates the stimulating electrode. The precise lower limit of the lower motor outflow could not, therefore, be defined but experiments with finer teflon and the electrode extruded to the end of the vertebral canal suggest that outflows to the anococcygeus do not extend much beyond S 2.
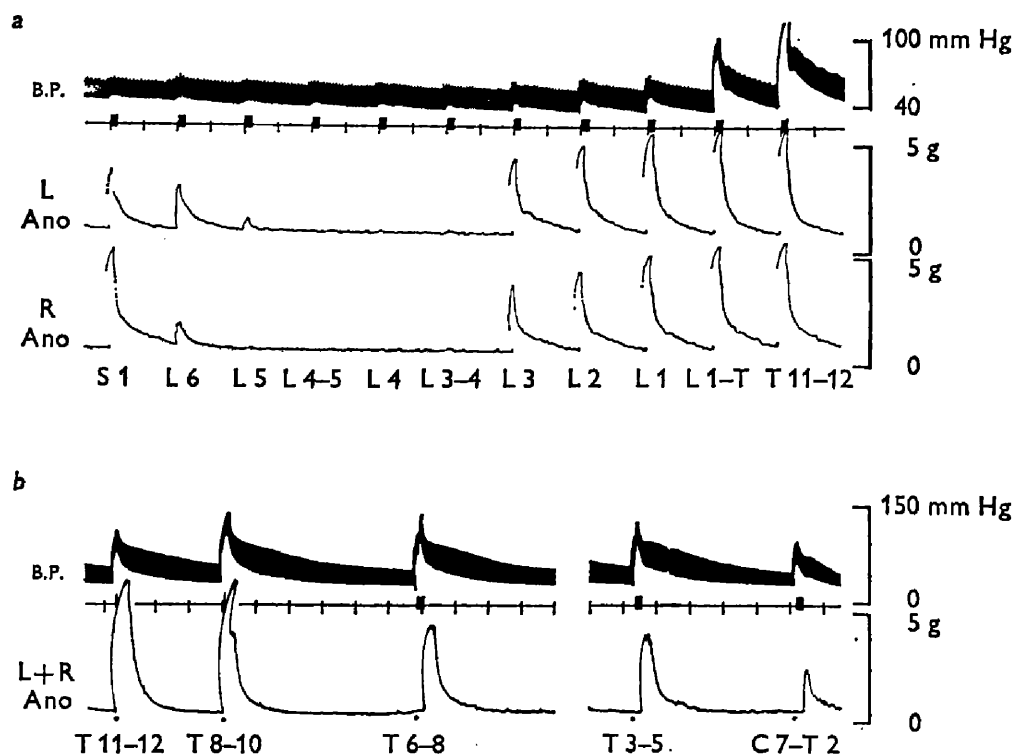
Fig. 2. The effect of stimulating at different levels of the vertebral canal on the response of the anococcygeus muscle and the blood pressure in the pithed rat. Stimulation was at 30 Hz for 10 sec with supramaximal voltage and is indicated by a dot below the trace. *a*, In this rat the tension in the left (L) and right (R) muscles was recorded separately. A 5 min exposure of the stimulating electrode was used, and levels from S 1 to T 11–12 examined. Motor responses were obtained from two regions, at L 6 and S 1 and again at and above L 3. Between L 3 and L 5 no response was obtained from the anococcygeus. *b*, In this rat the combined tension of the two anococcygeus muscles was recorded at higher levels of the vertebral canal explored with a 10 mm electrode exposure. At T 8–10 a second, delayed component appears in the response, and at higher levels this completely replaces the fast response coincident with stimulation seen at lower levels. The delay averages about 15 sec so that the response begins after the end of stimulation and is presumably due to catecholamine release from the adrenals. Time 1 min.

Frequency characteristics

The effect of 10 sec periods of stimulation at frequencies from 0·1 to 100 Hz was examined in both regions giving motor responses. Motor responses were obtained at all frequencies with a maximum at 30 Hz. At frequencies above 10 Hz, the response from the upper and lower
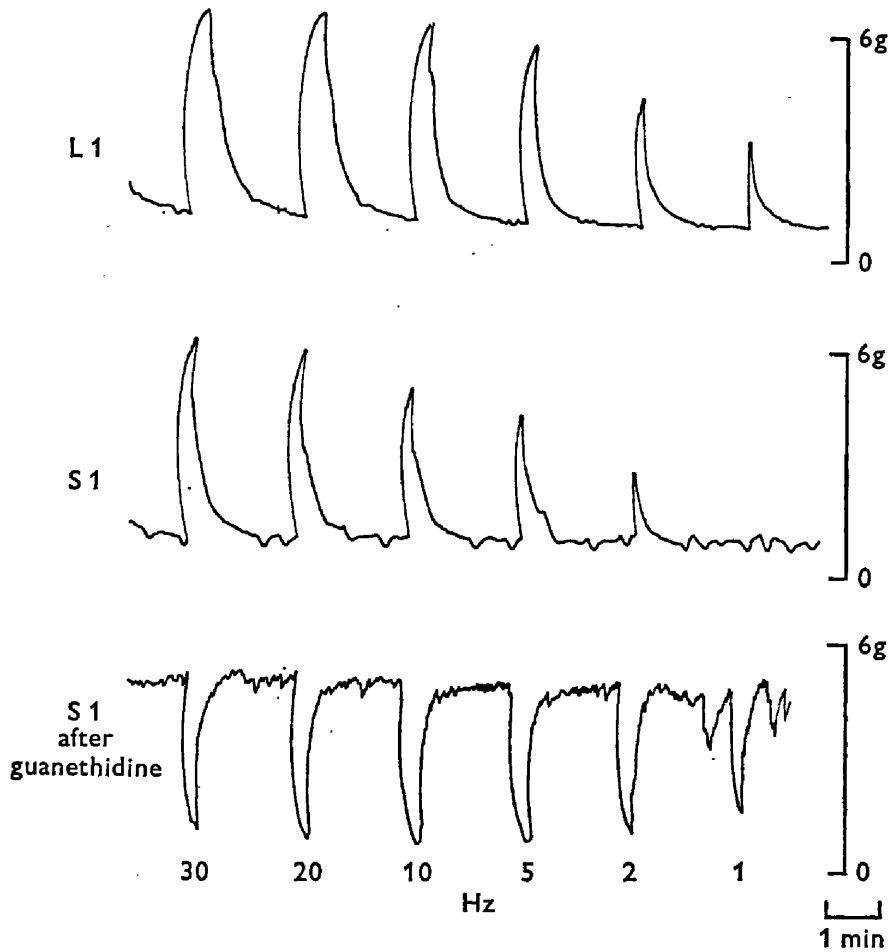

Fig. 3. Frequency characteristics of the motor and inhibitory responses in the anococcygeus muscle of a pithed rat from stimulation in the vertebral canal. The upper record shows the motor response from stimulation at L 1, the middle the motor response from stimulation at S 1, and the lower the inhibitory response from stimulation at S 1 in the presence of guanethidine (10 mg/kg). The frequency of stimulation is shown on the bottom record but applies to all. The duration of stimulation was 10 sec and the electrode exposure 5 mm.

regions were equal and had reached their maximum within the stimulation period of 10 sec. At low frequencies, responses were larger from stimulation in the upper than in the lower region and had not reached their maximum amplitude within the 10 sec of stimulation (Fig. 3).

When longer stimulation periods were used to compensate for this, the most effective frequency remained at 30 Hz, but frequencies as low as 0·5 Hz could eventually produce a plateau tension of some 70 % of the maximum at 30 Hz.

Single pulses produced responses of approximately 10 % of the maximum tension and summation of tension was seen at frequencies at or above 0·1 Hz (Fig. 4). At higher frequencies, up to 50 Hz, repetitive stimulation produced a smooth contraction well maintained for a 3 min period.
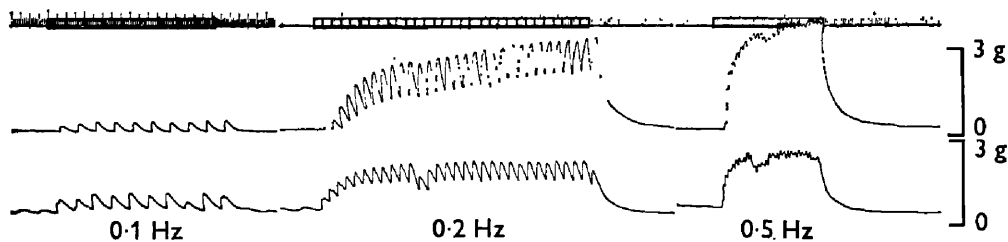

Fig. 4. The separate responses of the left and right anococcygeus muscles in the pithed rat to stimulation in the vertebral canal at L 1–2, at the frequencies shown at the bottom of each panel. At 0·1 Hz the responses to individual stimuli do not summate; at 0·2 Hz and 0·5 Hz summation occurs and if sufficient time is allowed, large tensions are developed. Time marker 1 and 5 sec.

Effect of blocking drugs

Responses from both motor regions were abolished by phentolamine (2 mg/kg) (Fig. 5a).

A short-lived inhibition of the motor response from the upper (L 3–T 11) region was produced by 1 mg/kg of hexamethonium, and the response completely abolished by 5 mg/kg. The motor response from the lower (S 2–L 6) region was unaffected by these doses of hexamethonium (Fig. 5b). Mecamylamine (5 mg/kg) gave similar results to hexamethonium. Atropine (1 mg/kg) and LSD-25 (400 μg/kg) had no effect on the motor responses from either region.

The motor response from stimulation higher in the spinal canal and due to liberation of catecholamines from the adrenals was abolished by phentolamine (2 mg/kg) and reduced by 70 % by hexamethonium (5 mg/kg).

Drugs producing motor responses

In vitro the inhibitory response of the anococcygeus is seen only after raising the tone of the muscle (Gillespie, 1972). The actions of three drugs potentially capable of raising tone were examined *in vivo* to see which

was most able to produce and maintain a steady level of tone on which the inhibitory nerve response could be displayed and studied.

Single injections of noradrenaline (4 μg/kg) and tyramine (500 μg/kg) both produced motor responses which lasted 5–10 min and had no effect on the motor responses to stimulation. A single injection of guanethidine (10 mg/kg) produced a maximal motor response which was well maintained for 25–40 min after which spontaneous activity started and tension
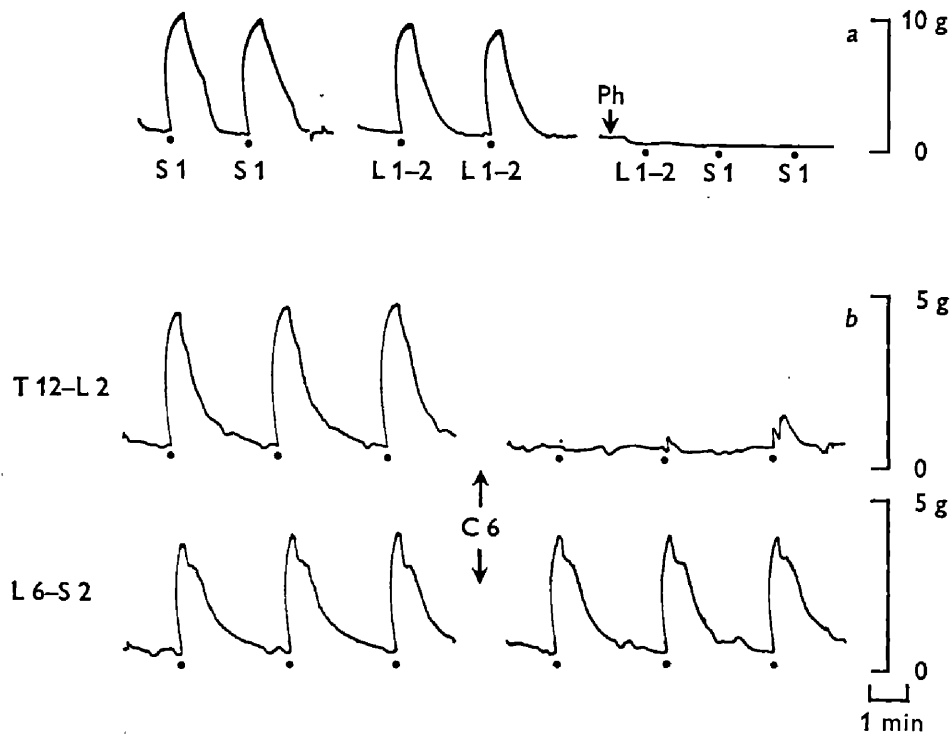

Fig. 5. *a*, This shows the effect of phentolamine (Ph: 2 mg/kg) on the motor response of the rat anococcygeus to stimulation in the vertebral canal at S 1 and at L 1–2. Phentolamine blocks both responses. *b*, This shows the effect of hexamethonium (C 6: 5 mg/kg) on the motor response of the anococcygeus in another rat to stimulation in the vertebral canal in two similar positions, L 6–S 2 and T 12–L 2. Hexamethonium blocks the motor response from the rostral stimulation without affecting that from the caudal. Stimulation was for 10 sec at 30 Hz. Electrode exposure, (*a*) 5 mm, (*b*) 10 mm.

gradually declined. After guanethidine, motor responses to stimulation in both spinal regions were abolished, but adrenal responses were little altered.

Phentolamine (2 mg/kg) abolished motor responses to noradrenaline, tyramine and guanethidine.

A slow infusion of tyramine (1 mg/ml. solution given at a rate of 40 μl./min) produced a maximal motor response well maintained for the

duration of the infusion, even when this was continued up to 2 hr. Slower rates of infusion of tyramine (20 μg/min and 10 μg/min) produced submaximal motor responses which were also well maintained.
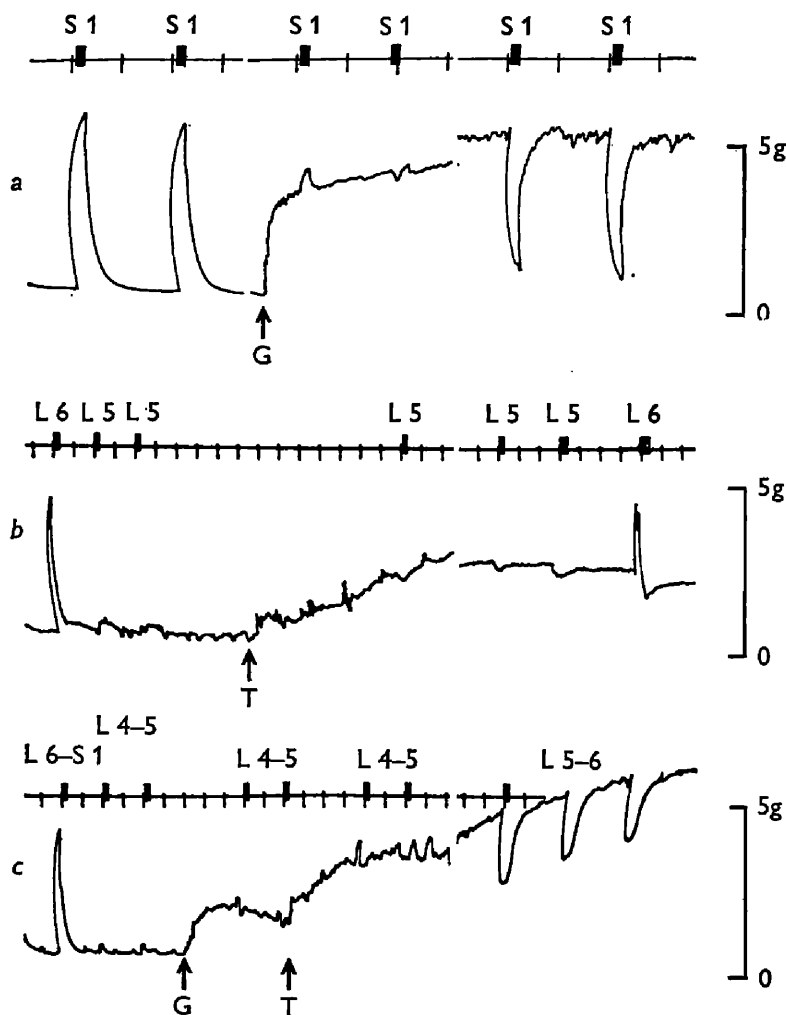

Fig. 6. Effect of guanethidine and tyramine on the anococcygeus muscle of the rat, and its response to nerve stimulation in the vertebral canal at the position shown above the time trace on each record. Time 1 min. *a*, The motor response to nerve stimulation before guanethidine (G: 10 mg/kg) was converted to inhibition in the presence of that drug which itself produced a prolonged rise in muscle tone. Tension was recorded from both muscles. Electrode exposure 5 mm. *b*, In another rat a slow infusion of tyramine (T: 20 μg/min) caused a maintained rise in tone but did not reverse the motor response to stimulation at L 6. Stimulation at L 5, however, which before tyramine was ineffective, caused inhibition in the presence of that drug. Tension was recorded from a single muscle. Electrode exposure 5 mm. *c*, In a third rat guanethidine (G: 5 mg/kg) caused only a small rise in tone; a tyramine infusion (T: 20 μg/min) further increased this. This record also shows the sharp boundary of the inhibitory outflow. Stimulation at L 4–5 produced little inhibition whereas at L 5–6 large inhibitory responses were obtained. Record from a single muscle. Electrode exposure 10 mm.

The inhibitory response

Spinal origin

When the tone of the muscle was raised by guanethidine (10 mg/kg) stimulation in the region L 5–S 2 produced inhibitory responses. In individual experiments, stimulating at 30 Hz for 10 sec, this inhibition
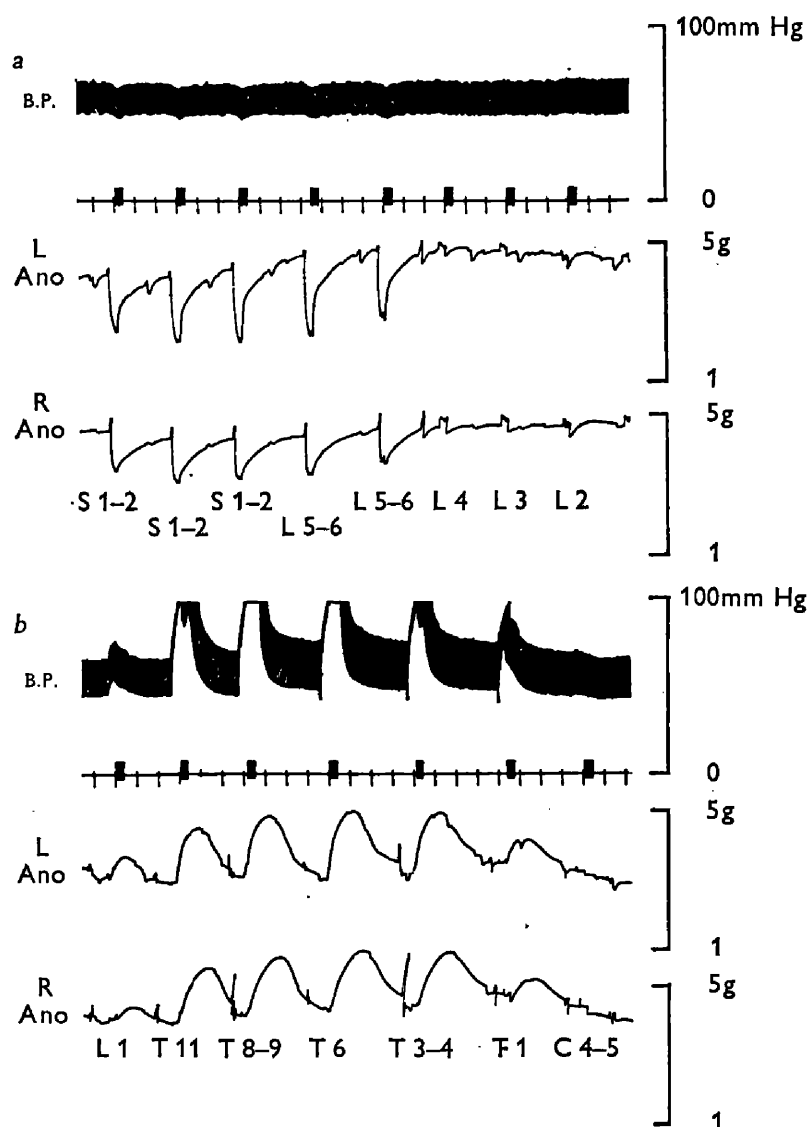

Fig. 7. Responses of the anococcygeus and the blood pressure in the pithed rat to stimulation in the vertebral canal at 30 Hz at the levels shown below each record. An initial injection of guanethidine (G: 5 mg/kg) was given to reverse the response to stimulation to inhibition. The two sets of records are successive and represent exploration of the reponse to stimulation between S 1–2 and C 4–5. *a*, Shows that the inhibitory response is confined to stimulation in the region S 2–L 5. *b*, Shows that more rostral stimulation in the thoracic region can restore the motor response as a consequence of liberating catecholamines from the adrenal gland. At these levels there is also a large vasopressor response. Time 1 min.

could amount to 90 % of the induced tone (Fig. 6a). The average inhibition in all experiments was 60 %.

When the tone was raised with tyramine (20 μg/min), which did not abolish motor responses, stimulation in the upper end of this region (L 5) produced small inhibitory responses, whereas stimulation in the lower part of the same region (L 6–S 2) produced a biphasic response, contraction followed by relaxation (Fig. 6b).

From these results, guanethidine was clearly the better drug on which to study inhibitory responses, presumably because of its neuronal blocking action on the adrenergic motor nerves in addition to its indirect symthomimetic action in raising tone. Guanethidine had one disadvantage; after 25–40 min the tone began to fall and rhythmic activity appeared. In these circumstances it was difficult to demonstrate inhibition. The possibility was, therefore, investigated of reinforcing the motor action of guanethidine with a slow infusion of tyramine, thus combining the neurone blocking action of the former with the steady induced tone of the latter.

Guanethidine (5 mg/kg) was given and a slow infusion of tyramine (20 μg/min) started 5 min later. Tone in the anococcygeus was raised and could be maintained for 2 hr, producing optimum conditions for studying the inhibitory response (Fig. 6c). Under these conditions, the spinal origin of the inhibitory fibres was determined by stimulating at successive 5 mm intervals along the spinal canal as shown in Fig. 7. Inhibitory responses were obtained only between S 2 and L 5. No inhibitory responses were found at the upper region giving motor responses, or at any higher level up to C 4. The region giving inhibitory responses (S 2–L 5) overlapped with the lower region giving motor responses (S 2–L 6) but the former extended one segment more rostral. Stimulation in the region corresponding to the efferent fibres to the adrenals still produced a delayed contraction from catecholamine liberation.

Frequency characteristics

The inhibitory response *in vivo* showed the same high sensitivity to low frequencies of stimulation as was noticed *in vitro* (Gillespie, 1972). Maximum responses were obtained with a 10 sec period of stimulation between 2 and 5 Hz (Fig. 3). Responses were rapid in onset and well maintained over a 20 sec period.

Effect of blocking drugs

Clearly nerve fibres mediating inhibition in the anococcygeus were located in the spinal cord. The question of whether this pathway to the

muscle was interrupted by a ganglion relay was investigated by examining the effects of hexamethonium and mecamylamine.

Hexamethonium or mecamylamine (5 mg/kg) completely abolished the inhibitory response. This is shown for hexamethonium in Fig. 8. When recovery from this inhibition occurred, a second similar dose had little effect in renewing the block. If, initially, a small (1 mg/kg) dose of hexamethonium was given, a transient reduction in the response was observed.
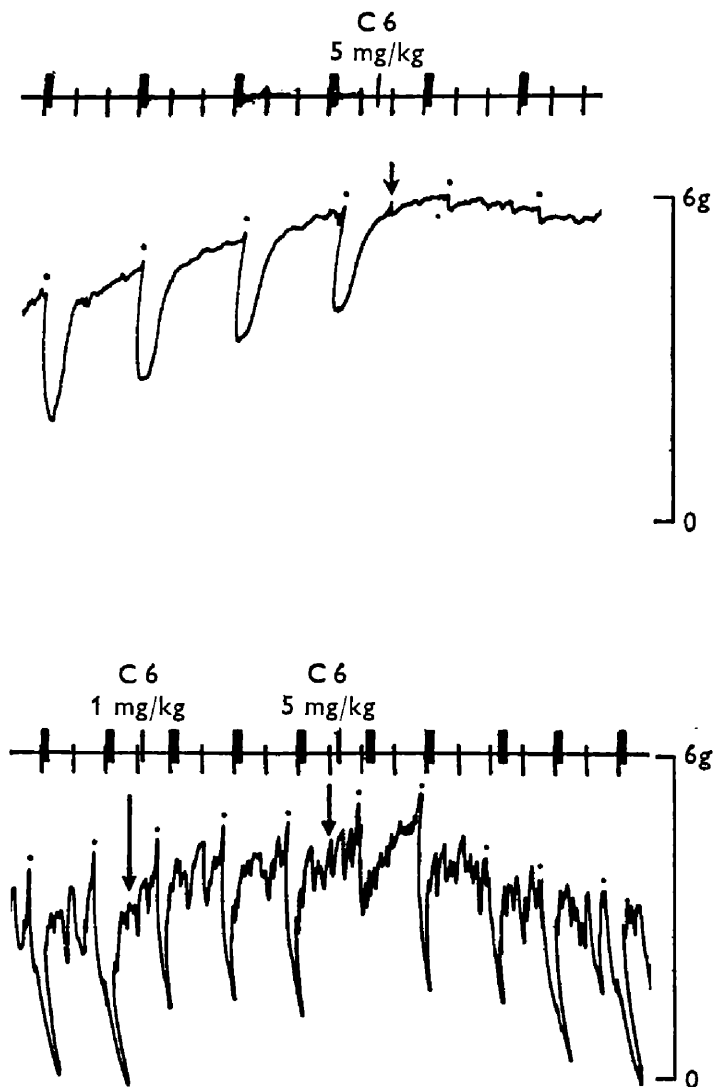

Fig. 8. Effects of hexamethonium (C 6) on the inhibitory responses of the anococcygeus of the pithed rat to stimulation at 30 Hz for 10 sec in the vertebral canal at L 6–S 1. The start of each stimulation period is indicated by a dot and the injection of hexamethonium by an arrow. In the upper record a single dose of hexamethonium (5 mg/kg) almost completely abolishes the inhibitory response. In the lower trace a smaller dose of hexamethonium (1 mg/kg) caused a small reduction in the size of the inhibitory response and a subsequent larger dose (5 mg/kg) produces only a transient block of the response. Time 1 min.

On recovery from this, the tissue was insensitive to subsequent large (5 mg/kg) doses of the drug.

Atropine was also studied since there is the possibility of muscarinic receptors in ganglia (Steinberg & Hilton, 1966). Atropine (1 mg/kg) had no effect on inhibitory responses.

DISCUSSION

Of the two questions posed at the outset of these investigations, the answer to the first is clear. There is an inhibitory nerve outflow from the spinal cord to the anococcygeus muscle whose frequency and drug sensitivity resembles that of the nerves involved in the inhibitory response *in vitro* (Gillespie, 1972). It seems most unlikely that this inhibitory outflow could be the sympathetic adrenergic outflow liberating some additional transmitter whose inhibitory effect is uncovered by blocking the release of noradrenaline by guanethidine, for the following reasons. First, the level of origin of the motor and inhibitory fibres differs. The preganglionic motor fibres arise from L 3–T 11 whereas the inhibitory preganglionic fibres arise from S 2–L 5. Secondly, in the presence of tyramine, when the motor responses to stimulation either of the pre-ganglionic outflow at L 3–T 11 or the post-ganglionic at S 2–L 6 are not blocked, stimulation at L 5 produces a pure inhibitory response, i.e. adrenergic blockade is not an essential requirement before inhibition can be demonstrated. These results are supported by the observation that destruction of the adrenergic innervation by 6-hydroxydopamine abolishes the motor adrenergic response but leaves the inhibitory response unaltered (Gibson & Gillespie, 1973).

The second question, whether the inhibitory outflow is organized in the pattern of the autonomic nervous system, hinges round the action of the ganglion blocking agents hexamethonium and mecamylamine. The effects of these two drugs are similar. They abolish the adrenergic motor response to stimulation in the upper (preganglionic) region. This block is reasonably long lasting and when recovery takes place a second dose of ganglion blocking drug will restore the block. On the inhibitory response the evidence for ganglion blockage was less convincing. If a large dose of drug was given, the inhibitory response was convincingly blocked and remained so for some time. As the response returned, however, a second large dose produced a short-lived inhibition only. If a small initial dose were used, it itself produced only slight transient block but was effective in producing desensitization, so that a second large dose was almost ineffective.

The doubt these results threw on the presence of a ganglion in the

peripheral pathway made us examine the sensitivity of another organ with an adrenergic motor innervation from the lumbar cord – the vas deferens. This was found to be as easily desensitized to the action of hexamethonium as the inhibitory pathway to the anococcygeus muscle. A large dose caused inhibition of the motor response which quickly 'escaped', whereupon a second similar dose had only a slight short-lived action. Since there is certainly a synapse in the motor pathway to the vas deferens (Sjöstrand, 1965) we feel the results consistent with the presence of a synapse in the inhibitory outflow to the anococcygeus muscle.

An observation of some interest was the ability to stimulate the post-ganglionic adrenergic fibres from the lower lumbar–upper sacral region of the spinal canal. In previous investigations of this method of stimulating the spinal autonomic outflows, it had never been possible to stimulate post-ganglionic fibres (Gillespie *et al.* 1970). One explanation for the present exception could have been the position of the anococcygeus muscle directly in front of the vertebral column and having a tendinous origin from the upper coccygeal vertebrae. These conditions might have been particularly favourable for direct stimulation of intramural nerves in a muscle attached to bone overlying the stimulating electrode. This was checked by marking the position of the upper tendinous origin of the anococcygeus muscle with a metal marker and comparing by X-ray the relative positions of this marker and the spinal electrode. There was always a vertical displacement of at least 20 mm so it is unlikely that motor intramural nerves were stimulated within the muscle itself. The ability to stimulate the post-ganglionic motor nerves at least 20 mm from the muscle suggests that in this tissue, unlike the vas deferens, motor innervation is by conventional post-ganglionic 'long' adrenergic neurones.

REFERENCES

GIBSON, A. & GILLESPIE, J. S. (1973). The effect of immunosympathectomy and of 6-hydroxydopamine on the responses of the rat anococcygeus to nerve stimulation and to some drugs. *Br. J. Pharmac.* **47**, 261–267.

GILLESPIE, J. S. (1972). The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br. J. Pharmac.* **45**, 404–416.

GILLESPIE, J. S., MacLAREN, A. & POLLOCK, D. (1970). A method of stimulating different segments of the autonomic outflow from the spinal column to various organs in the pithed cat and rat. *Br. J. Pharmac.* **40**, 257–267.
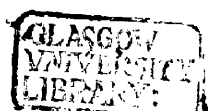
SJÖSTRAND, N. O. (1965). The adrenergic innervation of the vas deferens and the accessory male genital glands. *Acta physiol. scand.* **65**, suppl. 257.

STEINBERG, M. & HILTON, J. G. (1966). Site of action of atropine in blocking pressor response to increased intracranial pressure in chlorisondamine-treated dogs. *Tex. Rep. Biol. Med.* **24**, 222–227.

The effect of nerve stimulation on the depletion of noradrenaline by reserpine in the heart, vas deferens and anococcygeus muscle of the rat

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Most adrenergically innervated peripheral organs are depleted of their noradrenaline (NA) content by reserpine to a roughly similar degree. An exception is the vas deferens which is relatively resistant (Sjöstrand & Swedin, 1968). Two possible explanations are either that the rate of depletion is dependent on the firing frequency in the nerves and this is low in the vas deferens, or that 'short' adrenergic neurones in the vas deferens are less easily depleted than the long adrenergic neurones elsewhere. We have investigated these by studying the effect of artificial stimulation on the rate of depletion of the nerves to the heart, vas deferens and anococcygeus. The latter has a dense adrenergic innervation uniformly distributed throughout the muscle like the vas deferens, but unlike the vas deferens, the neurones are conventional 'long' ones (Gillespie & McGrath, 1972).

Rats were given various doses of reserpine intraperitoneally, the vas deferens and anococcygeus muscles removed 24 h later, and the NA content measured. A dose of 200 μg/kg caused 80–90% depletion of NA in both muscles. The time course of depletion with such a dose was then examined in all three organs. After 6 h the heart was 90% depleted whereas the vas deferens and anococcygeus were only 50% depleted. This dose (200 μg/kg) and time (6 h) were selected to study the effects of nerve stimulation. Six groups of rats were used, control animals; animals pithed but unstimulated; animals pithed and the sympathetic nerves stimulated; animals given reserpine but neither pithed nor stimulated; animals given reserpine, pithed but not stimulated; and animals given reserpine, pithed and stimulated. The appropriate spinal outflows were stimulated by a movable electrode in the spinal canal (Gillespie, MacLaren & Pollock, 1971).

The level of NA at 6 h in animals pithed but unstimulated rose in the anococcygeus muscle in comparison with the controls; stimulation of the nerves at 30 Hz for 10 s periods at 90 s intervals for 2 h lowered NA levels in all organs. Reserpine alone caused a 50% reduction in the vas deferens and anococcygeus and a 90% reduction in the heart.

Pithing the animal after giving reserpine reduced the depletion in the vas deferens and anococcygeus to 36% and 42% respectively and in the heart to 66%. Stimulation of the nerves for 2 h in the reserpinized rat increased the depletion in the vas deferens and anococcygeus to 68% and 82% respectively, but in the heart simply restored the depletion to 90%. The increased depletion with nerve stimulation in the vas deferens and anococcygeus was statistically significant. Since the anococcygeus and vas deferens are equally resistant to reserpine depletion, the length of the adrenergic neurone seems to be unimportant. The reduced depletion, following pithing in all three tissues and the increased depletion by nerve stimulation in the vas deferens and anococcygeus suggest that impulse traffic is an important factor.

REFERENCES

Gillespie, J. S., MacLaren, A. & Pollock, D. (1970). A method of stimulating different segments of the autonomic outflow from the spinal column to various organs in the pithed cat and rat. Br. J. Pharmac., 40, 257–267.

Gillespie, J. S. & McGrath, J. C. (1972). The origin of the inhibitory nerve pathway to the rat anococcygeus muscle. J. Physiol., Proc. Phys. Soc. March.

Sjöstrand, N. O. & Swedin, G., (1968). Effect of reserpine on the noradrenaline content of the vas deferens and the seminal vesicle compared with the submaxillary gland and the heart of the rat. Acta Physiol. Scand., 72, 370–377.

The origin of the inhibitory nerve pathway to the rat anococcygeus muscle

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A new smooth muscle preparation has recently been described (Gillespie, 1971). This preparation has a dense motor adrenergic innervation. When these motor fibres are blocked by guanethidine and the tone of the preparation raised, field stimulation causes a large inhibitory response of rapid onset. This response is abolished by tetrodotoxin and is presumably due to nerve fibres. The transmitter is as yet unknown but is not a catecholamine, acetylcholine or ATP. The present experiments were designed to determine whether these inhibitory fibres had their origin in the spinal cord, whether their position was identical with that of the motor adrenergic fibres and, finally, whether their frequency sensitivity and response to drugs were similar to the adrenergic fibres.

The preparation used was the pithed rat in which the pithing rod is arranged to stimulate selectively different levels of the spinal outflows (Gillespie, MacLaren & Pollock, 1970). The position of the stimulating electrode was identified by X-ray. Isometric tension recordings were made from the anococcygeus muscles which can conveniently, and with minimal operative interference, be approached from below the pelvic arch; heart rate and blood pressure were also recorded.

Stimulation of two regions of the spinal outflow, separated by a definite gap, caused a motor response. The upper corresponded to vertebrae T12-L1 and the lower to L6-S2. The response from stimulation in the upper region was almost completely abolished by hexamethonium 5 mg/kg whereas the lower was little affected; both responses were abolished by phentolamine 2 mg/kg. These results are consistent with an adrenergic sympathetic pathway with a ganglion relay in the sympathetic chain. When this motor response was abolished by guanethidine and the tone of the muscle raised either by giving a large dose of guanethidine or by an infusion of tyramine, stimulation caused inhibition. The position of this inhibitory outflow coincided with the lower motor outflow but extended more rostrally, i.e. to L5. These inhibitory responses were abolished by hexamethonium 5 mg/kg though desensitization to this drug on occasions masked this blocking action. The frequency sensitivity of the two pathways was measured. Optimal motor adrenergic responses were obtained at 30 Hz, optimal inhibitory responses at 5 Hz.

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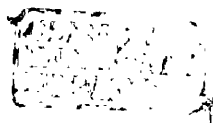
The results show that the inhibitory neural pathway to this muscle does arise from the spinal cord. The difference in localization and frequency sensitivity between the inhibitory and motor outflows suggest these are mediated by different nerves. So far it has not been possible to distinguish a pre- and post-ganglionic component to the inhibitory pathway.

REFERENCES

GILLESPIE, J. S. (1971). *Br. J. Pharmac.* **43**, 430*P*.
GILLESPIE, J. S., MacLAREN, A. & POLLOCK, D. (1970). *Br. J. Pharmac.* **40**, 257–267.

THE RESPONSE OF THE CAT ANOCOCCYGEUS MUSCLE TO NERVE OR DRUG STIMULATION AND A COMPARISON WITH THE RAT ANOCOCCYGEUS

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1   The cat anococcygeus muscle is shown to possess a dual innervation similar to the rat anococcygeus, with a motor adrenergic innervation and an inhibitory innervation whose transmitter is unknown. The pharmacological properties of the cat muscle were investigated and compared with those of the rat muscle.

2   The cat muscle contracts to noradrenaline, 5-hydroxytryptamine, tyramine, amphetamine, guanethidine, cocaine and lysergic acid diethylamide (LSD). The effects of noradrenaline and 5-hydroxytryptamine are blocked by phentolamine and methysergide respectively.

3   The cat anococcygeus is relaxed by acetylcholine, carbachol, isoprenaline, ATP, prostaglandins E_1, E_2 and $F_{2\alpha}$ and vasopressin, all of which contract the rat muscle. The effects of acetylcholine and carbachol are blocked by atropine and those of isoprenaline by propranolol.

4   Field stimulation produces contraction of the cat anococcygeus, which is blocked by phentolamine and guanethidine but unaffected by hexamethonium, atropine or neostigmine.

5   In the presence of guanethidine (10^{-5} M), the tone of the muscle is raised and field stimulation produces relaxation of the muscle. These inhibitory responses are unaffected by phentolamine, hexamethonium, atropine or neostigmine.

6   Neostigmine potentiates the effects of acetylcholine, but not of carbachol in relaxing the cat anococcygeus and in contracting the rat anococcygeus, but has no effect on either motor or inhibitory responses to field stimulation.

7   Cold storage for up to eight days had little effect on either the motor response to noradrenaline or the motor or inhibitory response to field stimulation of the cat anococcygeus. Beyond eight days, the response to field stimulation diminishes more rapidly than the response to noradrenaline.

Introduction

The anatomy and responses to nerve and drug stimulation of the rat anococcygeus have been described (Gillespie & Maxwell, 1971; Gillespie, 1972; Gillespie & McGrath, 1973; Gibson & Gillespie, 1973). The muscle has a dense adrenergic motor innervation, but there is no evidence of cholinergic nerves; it does, however, possess a powerful inhibitory innervation mediated by an unknown transmitter (Gillespie, 1972; Gillespie & McGrath, 1972).

Recently, Garrett, Howard & Lansdale (1972) have examined the similar muscle in the cat and shown that it too has a dense adrenergic innervation but, unlike the rat, it also stains for acetylcholinesterase. It is tempting to interpret this staining as evidence of a cholinergic innervation and to associate the latter with a possible inhibitory mechanism.

The purpose of the present experiments was to determine whether a cholinergic innervation exists in the cat muscle and if so, whether its properties resemble those of the inhibitory innervation in the rat. In the course of the work, certain responses to drugs in the cat muscle were found to differ so radically from those in the rat that a simultaneous comparison was made on muscles from the two species; and these results are described here.

Methods

The arrangement of the cat anococcygeus muscles is similar to that in the rat. The two muscles arise from the upper coccygeal vertebrae close to one another in the mid-line of the pelvic cavity. The muscles run caudally, lying first behind and then to one side of the colon. In the female, the muscles terminate at the sides of the colon. In the

male they continue ventrally to form a bar in front of the colon.

The muscles used in these experiments were from cats primarily intended for experiments with the perfused spleen. For this latter purpose they were anaesthetized with a mixture of nitrous oxide and halothane and given (intravenously) 70 μg of prostaglandin E_1 and 20,000 i.u. of heparin, before removal of the spleen (Blakeley, Brown, Dearnaley & Woods, 1969). The anococcygeus muscles were then immediately dissected out. The animal was first killed by cardiac incision, the abdomen opened in the mid-line as far as the anal margin, the pelvis split and the bladder, urethra and vasa deferentia or uteri removed. The colon was then cut through at the pelvic brim, the pelvic portion pulled forward and the connective tissue behind carefully cleared until the anococcygeus muscles came into view. The muscles were isolated and in females the portion running from the vertebrae to the colon, or in males the entire length to the ventral bar, was removed to a 10 ml bath containing Krebs saline at 36°C and gassed with a mixture of 95% O_2 and 5% CO_2. Rat anococcygeus muscles were removed as described by Gillespie (1972). Tension was measured with Grass FT03 isometric transducers and displayed on a Grass Polygraph. Field stimulation of the intramural nerve fibres was applied after drawing the muscles through a pair of electrodes similar to those described by Burn & Rand (1960). Stimulation of intramural nerves was with 1 ms pulses and supramaximal voltage, at the frequencies indicated in the text.

Most muscles were set up as described above within minutes of removal. On other occasions, this was inconvenient and the muscles were stored in Krebs solution at 4°C for some time before use. For this reason, the effect of cold storage on the responses to field stimulation and to drugs was examined. Muscles were quickly removed from the animal to 100 ml of oxygenated Krebs in a sealed vessel and kept at 4°C for 1-18 days.

The distribution of adrenergic nerves within the muscle was examined histochemically on freeze dried tissue with a slightly modified version of the technique of Hillarp & Falck (Gillespie & Kirpekar, 1966).

Noradrenaline assays were carried out by the trihydroxyindole method of Euler & Lishajko (1961) after absorption on alumina.

Drugs used were: acetylcholine chloride (Koch-Light); (−)-adrenaline bitartrate (B.D.H.); dexamphetamine sulphate; adenosine 5′-triphosphate disodium (Sigma); atropine sulphate; carbachol (Burroughs Wellcome); cocaine hydrochloride; guanethidine sulphate (CIBA); hexamethonium bromide (Koch-Light); histamine acid phosphate

(B.D.H.); 5-hydroxytryptamine creatinine sulphate (Koch-Light); (±)-isoprenaline hydrochloride (Sigma); (+)-lysergic acid diethylamide tartrate (Sandoz); mepyramine maleate (May & Baker); methysergide bimaleate (Sandoz); neostigmine methylsulphate (Roche); (−)-noradrenaline bitartrate (Koch-Light); pancuronium bromide (Organon); phentolamine mesylate (CIBA); propranolol sulphate (Sigma); prostaglandins E_1, E_2, $F_{2\alpha}$; tyramine hydrochloride (Sigma); vasopressin (Parke-Davis). Doses refer to the base, with the exception of prostaglandins and vasopressin.

Results

Anatomically, the rat and cat muscles are similar. Both are about 3 cm in length; in both, the female muscle is shorter than the male because of the absence of the ventral extension. The one macroscopic difference is that the cat muscle is considerably thicker and consequently heavier. Representative weight ranges for a pair of muscles are: cat, male 100-150 mg; cat, female, 50-100 mg; rat, male 25-50 mg. The Falck technique shows that the cat, like the rat, has a terminal plexus of brightly fluorescent fibres scattered uniformly throughout the muscle. Assay of the noradrenaline content gave a value of 2.1 μg/g for the cat compared with 2.56 ± 0.48 μg/g for the rat. Suspended in Krebs solution at 36°C, the cat anococcygeus differs from the rat in developing both spontaneous tone and rhythmic acitivity, greatest immediately after setting up the preparation but usually present to some extent throughout the experiment (e.g. Figures 5 and 8). Papaverine (10^{-5} M) abolished both tone and rhythmic activity.

Effects of drugs

Drugs causing contraction Noradrenaline, adrenaline and 5-hydroxytryptamine all caused a dose-related contraction of the cat anococcygeus. Noradrenaline and adrenaline were approximately equipotent, producing contractions which were antagonized by phentolamine (10^{-6} M). Responses to adrenaline and to a lesser extent noradrenaline, were potentiated by propranolol (10^{-6} M). 5-hydroxytryptamine produced contractions which were antagonized by methysergide (2×10^{-7} M). Dose-response curves for noradrenaline and 5-hydroxytryptamine and for the effects of phentolamine and methysergide, are shown in Figure 1. Muscles from male cats produced larger responses to noradrenaline and 5-hydroxytryptamine than those from females, but the dose-response curves (drawn as a percentage of the
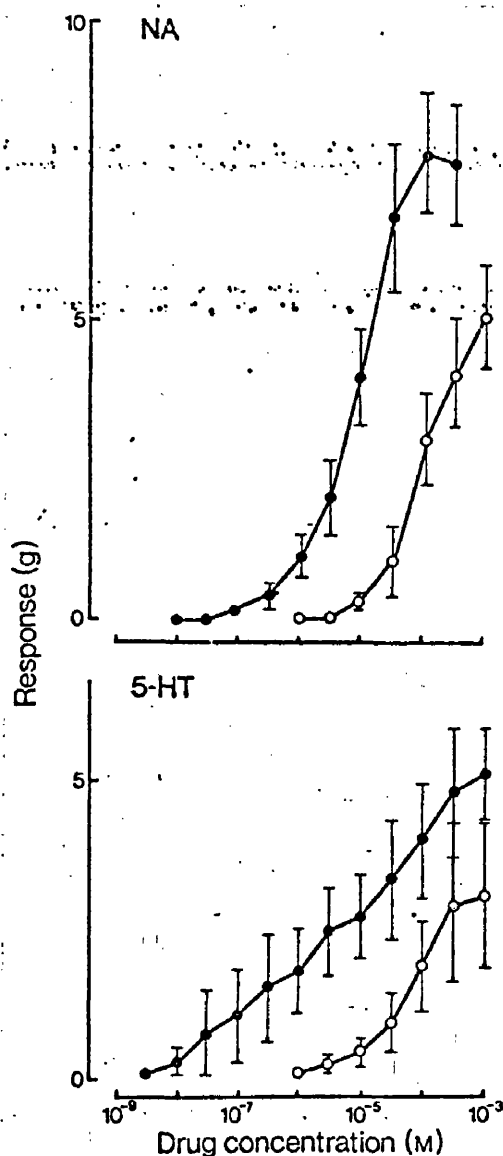
Fig. 1 Dose-response curves of the cat anococcygeus to noradrenaline (NA) and 5-hydroxytryptamine (5-HT) and the effect (•) of phentolamine (10^{-6} M) and methysergide (2×10^{-7} M). Control (o) phentolamine or methysergide. Bars represent $\pm$s.e. mean. Muscles from male and female cats.

high concentrations (10^{-4} M) of the drug, exhibited marked tachyphylaxis and was completely abolished by mepyramine (10^{-6} M).

Four drugs known to act as indirect sympathomimetics · on · other tissues were examined: tyramine, amphetamine, cocaine and guanethidine. All produced contraction, tyramine and amphetamine at or above 3×10^{-7} M, cocaine at or above 10^{-5} M (Fig. 2) and guanethidine at or above 10^{-5} M (Figures 2 and 4). All four drugs produced contractions slower in onset than those to noradrenaline (Fig. 2) and all were reduced or abolished by phentolamine (3×10^{-7} M). One distinguishing feature was that the contraction by tyramine, amphetamine and cocaine was complete within 5 min, whereas guanethidine required up to 30 min to develop its full motor effect even though its blocking effect on the adrenergic motor response developed rapidly and the final tension achieved was equal to that of the other drugs (Figures 2 and 4). Cocaine (3×10^{-5} M) also potentiated and prolonged the motor response to noradrenaline and to field stimulation of the adrenergic nerves.

One unexpected finding was the ability of lysergic acid diethylamide (LSD) to cause contraction. Concentrations as low as 10^{-8} M caused contractions which characteristically were prolonged and persistent even after repeated washing (Figure 2).

In the rat anococcygeus, all five drugs caused contraction and the only minor difference was that the rise in tone induced by guanethidine developed more rapidly than in the cat. LSD was equally effective in the rat and the contraction equally resistant to washing. In both species, some degree of tachyphylaxis was observed with all five drugs.

Drugs causing inhibition A major qualitative difference in the responsiveness to drugs of the cat and rat anococcygeus was the number causing inhibition in the cat. In the rat, no drug capable of causing inhibition was found. In the cat, acetylcholine, carbachol, isoprenaline, ATP, prostaglandins E_1, E_2 and $F_{2\alpha}$ and vasopressin, all of which were motor in the rat, produced relaxation. Because of the presence of spontaneous tone, this inhibitory effect could be demonstrated directly in the cat muscle, but the response was more dramatic if tone was first raised by the addition of 5-hydroxytryptamine, tyramine or guanethidine. Figure 3 contrasts this inhibitory effect on the cat anococcygeus with a simultaneous motor action on the rat for carbachol, isoprenaline, ATP and prostaglandin E_1. Acetylcholine had a similar action. This inhibitory effect on the cat of acetylcholine and carbachol was abolished by

maximum) were similar. Noradrenaline produced a larger maximum response than 5-hydroxytryptamine which had still not reached its maximum at a concentration of 10^{-3} M. This difference was observed in each individual experiment.

The sensitivity of the cat anococcygeus to noradrenaline is similar in the rat, but that to 5-hydroxytryptamine is approx. 100 times greater, as indicated by the contraction produced by it at the start of each panel in Figure 3. The response to histamine as in the rat, was small, was seen only at
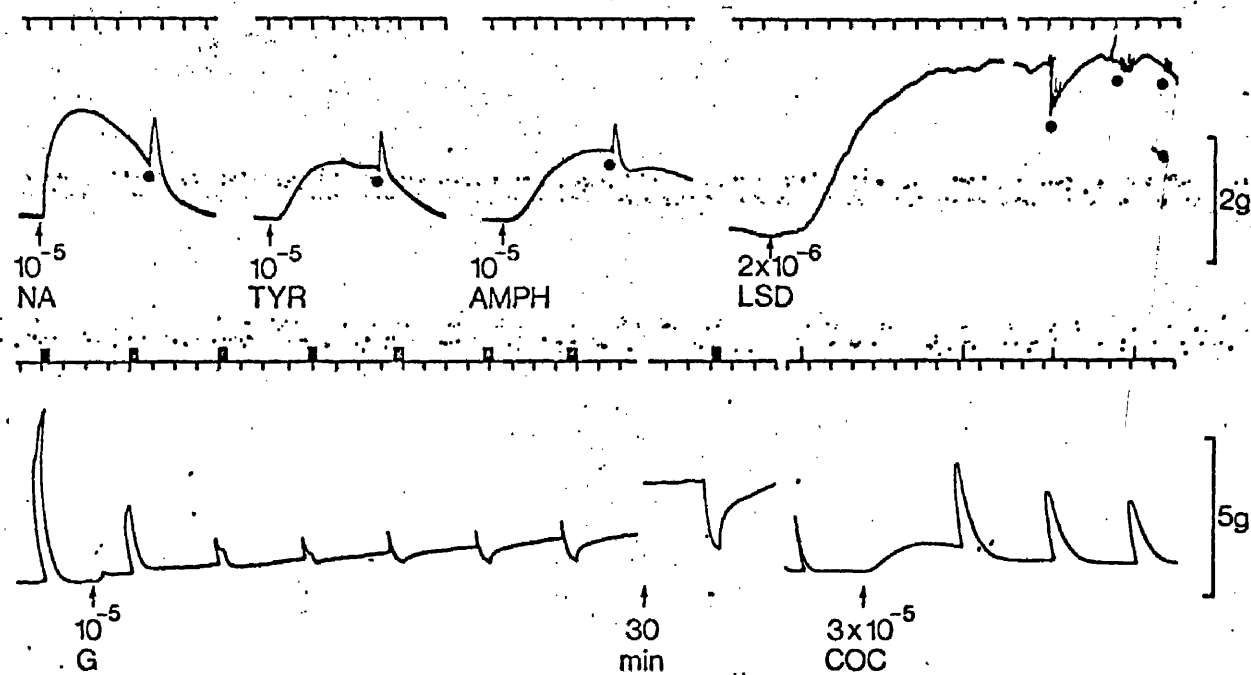
Fig. 2 The effect on the cat anococcygeus of a series of indirect sympathomimetics compared with the action of noradrenaline (NA). Noradrenaline (NA) (10^{-5} M) produced a rapid motor response. Tyramine (TYR) (10^{-5} M), dexamphetamine (AMPH) (10^{-5} M) and lysergic acid diethylamide (LSD) (2×10^{-6} M) produced responses slower in onset. Responses to LSD were persistent even after several washes. Wash at dots. Guanethidine (G) (10^{-5} M) raised the tone slowly, reaching its maximum after 40 min and reversed the response to field stimulation (10 Hz, 20 s) from motor to inhibitory. Cocaine (COC) (3×10^{-5} M) produced a motor response and prolonged and potentiated the motor response to field stimulation (10 Hz, 1 s). Time: 1 minute.
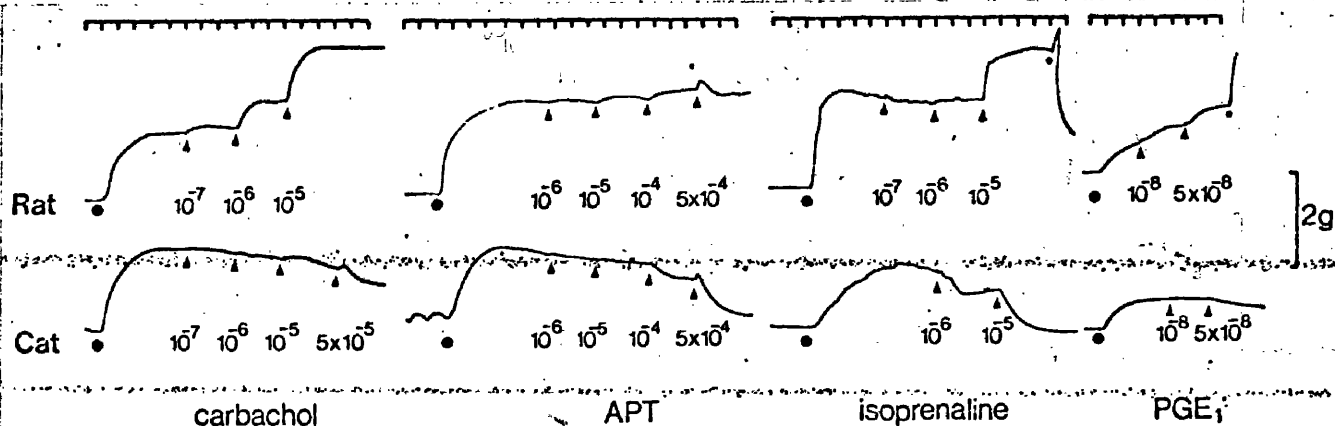

Fig. 3 Comparison of the effects of carbachol, ATP, isoprenaline and prostaglandin E_1 (PGE$_1$) on the rat and cat anococcygeus. In order to detect inhibitory responses, the tone of the muscles was first raised with 5-hydroxytryptamine (5-HT), ●. Because the cat is more sensitive to 5-HT, the dose of 5-HT given was 10^{-7} M compared with 10^{-5} M for rats. In the final rat trace, carbachol (10^{-6} M) replaced 5-HT for this purpose. All four substances contracted the rat muscle but relaxed the cat muscle. Time: 1 minute. Concentrations shown are molar for carbachol, ATP and isoprenaline but g/ml for prostaglandin E_1.
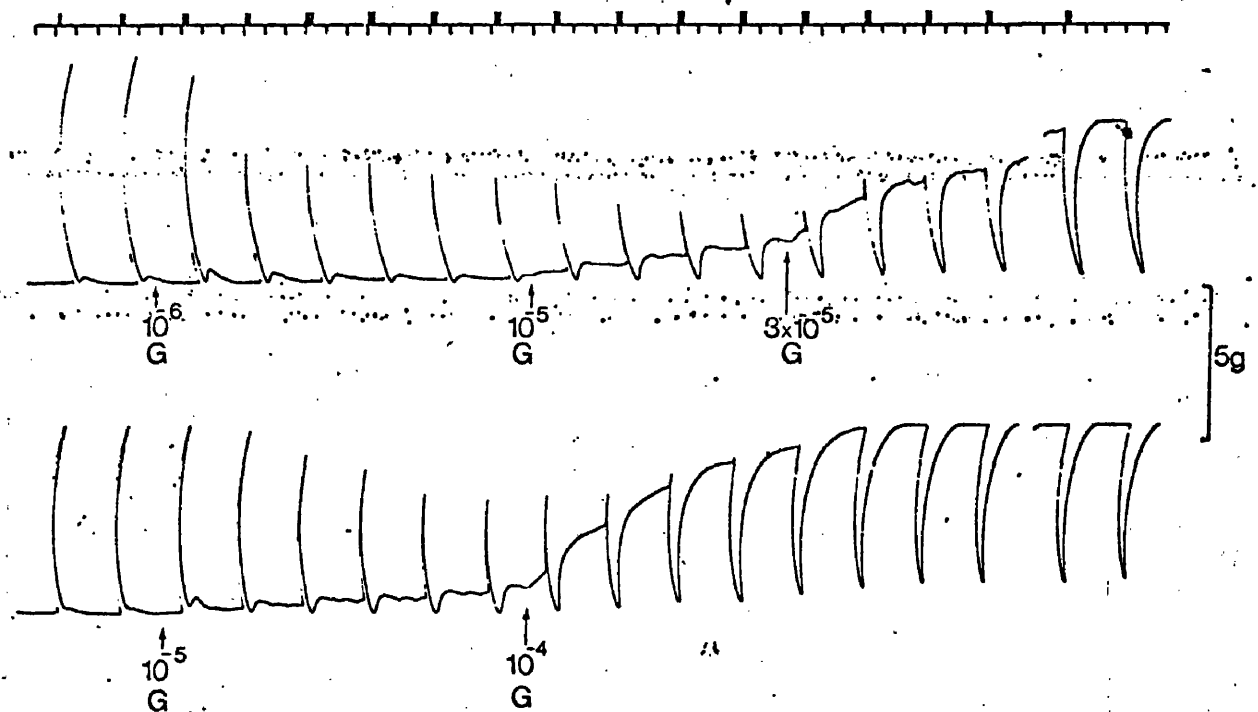
Fig. 4 The effect of guanethidine on the response to field stimulation in the cat anococcygeus muscle. In the upper trace, guanethidine (G) (10^{-6} M) rapidly produced a 50% reduction in the motor response to field stimulation but caused only a slight increase in muscle tone. Guanethidine (10^{-5} M and 3×10^{-5} M) further reduced the motor response and increased the tone more rapidly thus uncovering a large inhibitory response. The lower trace shows that a still larger dose of guanethidine (10^{-4} M) produced a faster increase in tone and reversal of the response from motor to inhibitory. The contractions on the lower trace were truncated by the limits of the recorder, the first two motor responses on the left were 2 g larger than shown and at the right, the maximum tone produced by guanethidine (10^{-4} M) was 1.5 g larger than shown. Stimulation at bars on time trace, 10 s train at 10 Hz. Time: 1 minute.

atropine (10^{-6} M) and of isoprenaline by propranolol (10^{-6} M. Vasopressin (20-200 milliunits/ml) was also examined and found to produce a relaxation in the cat and contraction in the rat muscle.

Response to field stimulation

Field stimulation of the rat anococcygeus produces motor responses due to its adrenergic innervation. When these motor nerves are blocked with guanethidine and the tone of the muscle raised, field stimulation then produces a powerful inhibitory response. Similar motor and inhibitory responses were found in the cat anococcygeus (Figure 4).

Increasing doses of guanethidine gradually depressed the motor response to field stimulation. At 10^{-5} M and above, guanethidine also raised the tone of the muscle and at the same time uncovered an inhibitory response. At 3×10^{-5} M and above, guanethidine completely abolished the motor response leaving a pure inhibitory response which

was capable of abolishing over 80% of the guanethidine-induced tone.

Effects of blocking drugs The effects of phentolamine, atropine, hexamethonium, pancuronium, mepyramine and propranolol were tested on both motor and inhibitory responses in the cat anococcygeus. None of these drugs altered the inhibitory response and only phentolamine blocked the motor response (Figure 5).

In order to examine the effect of phentolamine on the inhibitory response, the tone was raised with a large dose of LSD. This situation allowed inhibitory response to be seen while the tone of the muscle itself was unaffected by the blocking agent phentolamine.

Effects of neostigmine

The report by Garrett, et al. (1972) of a dense cholinesterase staining, together with the present observation that acetylcholine is inhibitory in the cat anococcygeus, suggested that the inhibitory
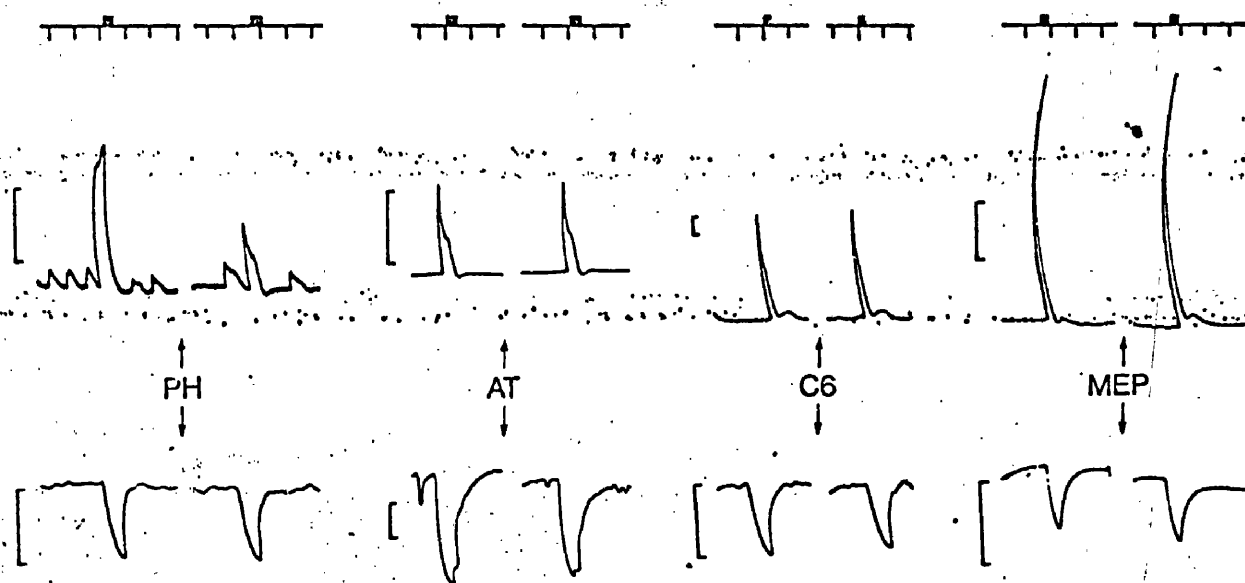
Fig. 5   The effect of phentolamine (PH) (10^{-6} M), atropine (AT) (10^{-6} M), hexamethonium (C6) (10^{-5} M) and mepyramine (MEP) (10^{-5} M) on the motor and inhibitory responses to field stimulation of the cat anococcygeus. Each pair of responses (i.e. before and after drug) was obtained on a different muscle. Motor responses were obtained from stimulation at 10 Hz with the exception of the first pair (30 Hz). Inhibitory responses were produced in the presence of guanethidine (3 x 10^{-5} M) with the exception of the first pair of responses where the tone was raised by LSD (10^{-6} M). Frequency of stimulation for inhibitory responses was 10 Hz with the exception of the first pair (5 Hz). Phentolamine decreased the size of the motor response but did not affect the inhibitory response. Atropine, hexamethonium and mepyramine had no effect on responses whether motor or inhibitory. Time: 1 minute. Vertical calibrations: 1 g.

response might be due to a cholinergic pathway. We therefore examined the effect of an anticholinesterase, neostigmine, to see if this would potentiate either motor or inhibitory responses to field stimulation at frequencies of stimulation ranging from 1-20 Hz. Figure 6 shows the result for the inhibitory response. Neostigmine (10^{-7}M) had no effect on either motor or inhibitory responses to field stimulation at any frequency.

The same dose of neostigmine did, however, potentiate the motor responses to acetylcholine in the rat and the inhibitory responses to the same in the cat (Figure 7). The corresponding responses to carbachol in both species were unaffected by neostigmine.

Effects of cold storage

Since the anococcygeus muscles used were obtained from cats used in other experiments, some were stored in Krebs solution at 4°C for a variable time before their responses were measured. Observations were therefore made on the effect of cold storage on the responses to field stimulation and to agonist drugs. The action of direct and indirect sympthomimetics and the responses to field stimulation proved remarkably

resistant to cold storage. Up to five days, there was little diminution of the motor response to either drug or nerve stimulation.

In another experiment, the effect of noradrenaline and field stimulation was examined in the
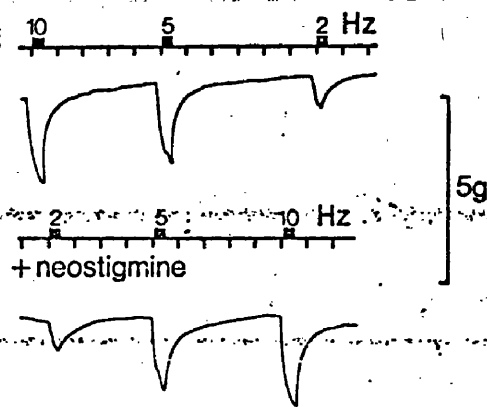

Fig. 6   Effect of neostigmine (10^{-7} M) on the inhibitory response to field stimulation in the cat anococcygeus. In the presence of guanethidine (3 x 10^{-5} M), stimulation at the frequencies indicated above the time trace produced inhibitory responses. Neostigmine (lower trace) had no effect on the size or duration of responses at 2, 5, or 10 Hz. Time: 1 minute.
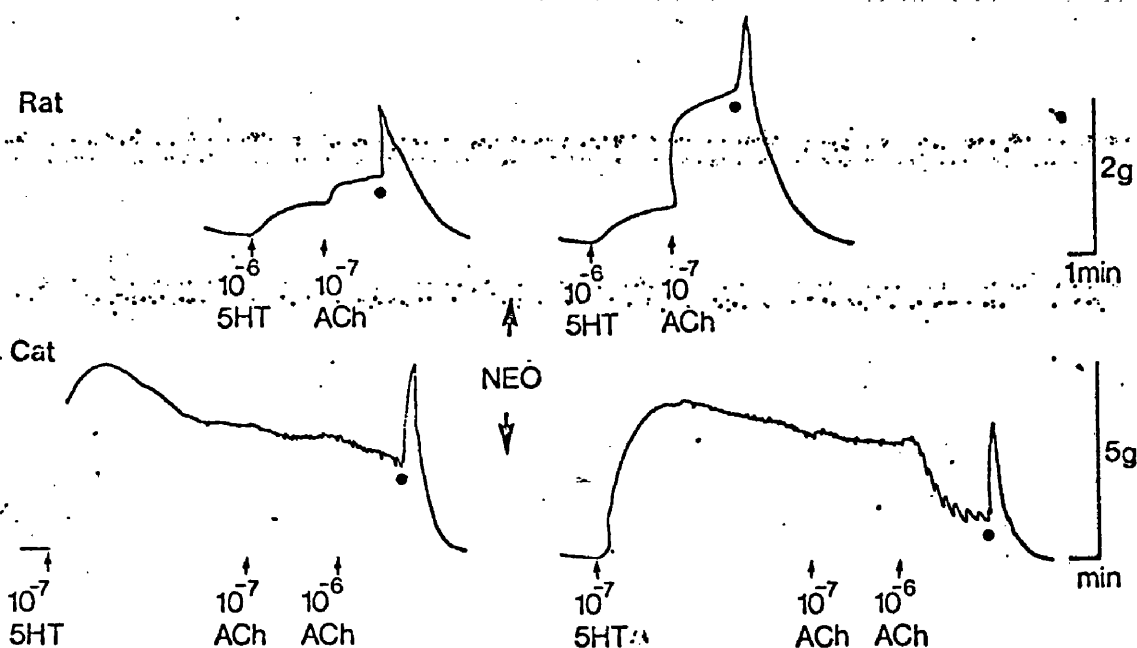
Rat

2g

1min

10^{-6}
5HT

10^{-7}
ACh

10^{-6}
5HT

10^{-7}
ACh

Cat

NEO

5g

min

10^{-7}
5HT

10^{-7}
ACh

10^{-6}
ACh

10^{-7}
5HT

10^{-7}
ACh

10^{-6}
ACh

Fig. 7   Comparison of the effects of neostigmine (NEO) (10^{-7} M) on the response of the rat and cat anococcygeus to acetylcholine (ACh). The tone of the muscles was first raised with 5-hydroxytryptamine, (10^{-6} M) for rat, (10^{-7} M) for cat. Neostigmine enhanced the motor response to acetylcholine (10^{-7} M) in the rat and the inhibitory response to acetylcholine (10^{-6} M) in the cat but did not affect the response to 5-hydroxytryptamine in either species. Wash at dots.

same muscle after 8, 11, 14 and 18 days cold storage (Figure 8). At eight days, the responses to both noradrenaline and field stimulation were similar to those obtained with fresh tissues. After eight days, the responses to noradrenaline declined gradually but were still present at 18 days, the dose-response curve drawn as a percentage of the maximum remaining unchanged. The motor response to field stimulation, on the other hand, declined more rapidly, the greatest change occurring between 8 and 11 days. Motor responses to field stimulation were just detectable at 10 and 30 Hz at 18 days. The effect of cold storage on the inhibitory response was more difficult to examine since guanethidine was necessary to display inhibition and, once given, further examination of the effect of cold storage on the motor response in that muscle was impossible. For this reason, the effect of cold storage on the inhibitory response was examined in only two cats. In one cat, the response was measured in one muscle immediately after removal and in the contralateral muscle from the same animal after five days cold storage. The muscles from the second cat were stored for 11 and 18 days respectively. The percentage inhibition of induced tone in the fresh muscle was 68%, comparable to the average for all cats; at five days, in the same cat, the inhibition was 60%. In the other animal at 11 days, the maximum inhibition

was 27% and at 18 days no response was obtained. The ability of guanethidine (3×10^{-5} M) to raise the tone of the muscle was undiminished at five days, was reduced approximately 50% at 11 days and at 18 days only a small transient response was found.

Histochemical examination of a cat anococcygeus muscle after five days cold storage showed many fluorescent nerve terminals still visible. After 11 days, the terminals were less bright but still present, and at 18 days they were just detectable.

Discussion

The cat anococcygeus muscle shows both similarities and differences with the rat muscle. Both possess a motor adrenergic innervation and an inhibitory innervation whose transmitter is unknown. In both species, the motor responses are blocked by either α-adrenoceptor or neurone blocking agents but, so far, we have found no blocking agent for the inhibitory response. The adrenergic motor nerves seem particularly sensitive to drug-induced release of their transmitter so that not only the classical indirect sypthomimetics, such as tyramine and amphetamine, are effective but also less well known releasing agents, such as guanethidine, cocaine and LSD. The last two are
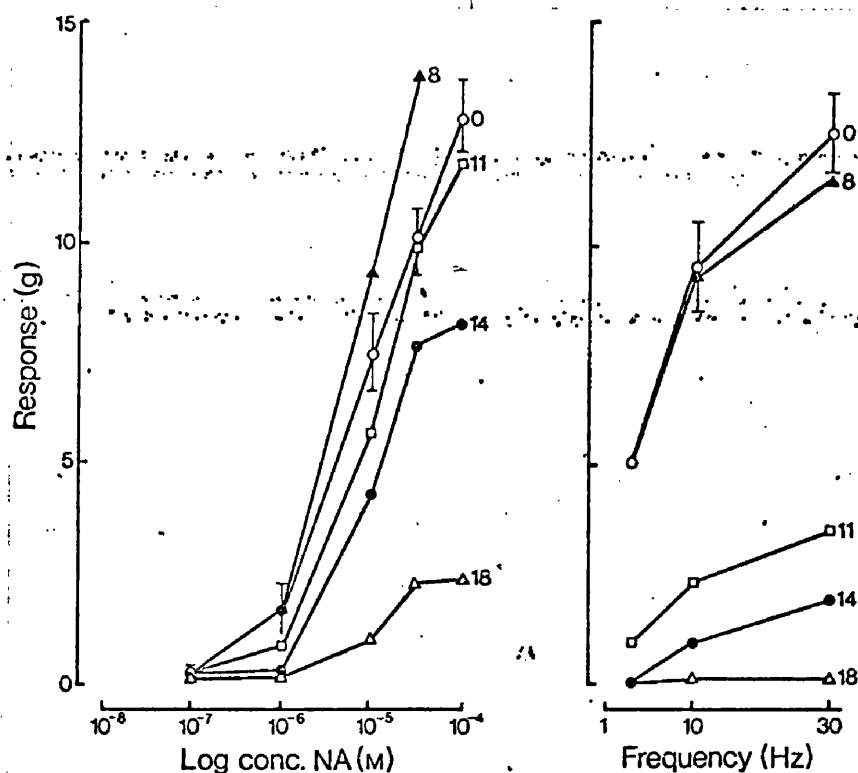
. Fig. 8   Effect of cold storage on the response of a cat anococcygeus to noradrenaline (NA) and to field
stimulation. The mean of the responses in two fresh muscles (o) is compared with responses of a muscle which
was cold stored and intermittently tested between 8-18 days (▲, 8; □, 11; ●, 14; △, 18 days). The responses to
noradrenaline declined steadily after the eighth day but survived longer than those to field stimulation. The
responses to field stimulation showed a sudden decrease between 8 and 11 days which may correspond to nerve
degeneration.

seldom regarded in this light but cocaine has been
shown to be an indirect agonist in several tissues,
including cat nictitating membrane (Kukovetz &
Lembeck, 1962), rat vas deferens (O'Donnell &
Hecker, 1967), rabbit aortic strip (Maengwyn-
Davies & Koppanyi, 1966) and guinea-pig atria
(Trendelenburg, 1968); and LSD contracts the
isolated cat nictitating membrane (Thompson,
1958) in a manner similar to that in our findings
with the rat and cat anococcygeus. In addition, in
rat and cat anococcygeus muscles, after chemical
sympathectomy with 6-hydroxydopamine, the
contractor response from cocaine or LSD is
reduced, suggesting that both drugs act by
releasing noradrenaline from nerves within the
tissue (Gillespie & McGrath, unpublished observa-
tions).

Nevertheless, in terms of receptors, there are
clearly both quantitative and qualitative differ-
ences. The cat apparently has more types of
receptors and several of these induce inhibitory
responses. In the rat, there is good evidence for
only three receptors (α-adrenoceptors, muscarinic
and 5-hydroxytryptamine receptors) and all of
these produce motor responses. Other drugs,

including histamine, ATP, prostaglandins and
isoprenaline, always produce motor effects but are
effective only in high doses. With isoprenaline in
particular, the evidence in the rat is that this is not
an effect on β-adrenoceptors since it is uninflu-
enced by propranolol. In the cat, however,
isoprenaline produces inhibition and the effect is
abolished by propranolol, indicating the presence
of β-adrenoceptors. Even the response to 5-
hydroxytryptamine shows a greater sensitivity by
about two orders of magnitude in the cat over the
rat, though in this instance the response is motor
in both species.

The ability of several potential transmitters to
produce inhibition in the cat muscle raises the
possibility that they are involved in the inhibitory
response to field stimulation. The most likely are
acetylcholine and ATP. Since the inhibitory
response to field stimulation is quite unaffected by
either atropine or neostigmine, in concentrations
which respectively abolish or potentiate the
response to acetylcholine, it does not appear
possible that acetylcholine is the transmitter.
Similar evidence to exclude ATP is lacking but in
view of the high concentrations needed to produce

a small mechanical inhibition in contrast to the intense inhibition produced by field stimulation, it does not seem a likely candidate. Furthermore, it appears more probable that the inhibitory transmitter will be the same in both species and in the rat only motor responses can be obtained with ATP.

Garrett et al. (1972) reported acetylcholinesterase in the cat anococcygeus. No similar staining was observed in the rat muscle, yet neostigmine caused a similar degree of potentiation of the effects of acetylcholine in both. We have no explanation of this discrepancy nor can we suggest a function for the enzyme in this muscle.

These experiments on nerve or drug responses of the cat muscle were carried out on tissues which might be cold stored up to a maximum of two days. The necessary control studies on the effect of cold storage showed that little change in nerve or drug responses occurred over this short period. Less expected was the ability of these nerves, cut off from their ganglion cell body, to be still present and capable of functioning after 11 days, as shown by the histochemical demonstration of adrenergic nerve terminals together with the responses to field stimulation and to the indirect sympathomimetic action of guanethidine. Stimulation of post-ganglionic adrenergic nerves fails to produce responses after two days in mouse, rat and rabbit ileum, three days in guinea-pig colon (Holman & Hughes, 1965) and seven days in guinea-pig taenia caecum (Hattori, Kurahashi, Mori & Shibata, 1972). Adrenergic nerves were not demonstrable histochemically after five days cold storage in guinea-pig caecum (Hattori et al., 1972) or rabbit aortic strips (Shibata, Hattori, Sakurai, Mori & Fujiwara, 1971). The capacity of the anococcygeus muscle to respond to noradrenaline after the nerves had ceased to function is in

agreement with the usual finding that in cold stored tissues the smooth muscle cells remain viable longer than the nerves. Thus intestinal smooth muscle from various species remains responsive to acetylcholine for several days after the cholinergic nerves have ceased to function (Ambache, 1946) and in guinea-pig taenia caecum phenylephrine still relaxes the muscle after 18 days cold storage (Fukuda & Shibata, 1972) despite the complete absence of nerve responses after 5 days (Hattori et al., 1972).

It was not possible, however, to differentiate between motor and inhibitory nerves in the cat anococcygeus by their ability to survive cold storage. In previous studies, where cold storage of a tissue has been employed successfully to differentiate between the components of a multiple innervation, the different anatomical arrangement of the nerves has been a major factor contributing to the preferential survival of one type (Holman & Hughes, 1965; Hattori et al., 1972). However, in the isolated cat anococcygeus muscle as in the rat, (Gillespie, 1972), both motor and inhibitory nerves consist purely of postganglionic fibres since ganglion blockers have no effect on responses to field stimulation. There is therefore no reason to expect differential survival. Despite the long survival after cold storage of the nerve and muscle elements of the cat anococcygeus, we feel that species variation may be at least as important as the properties of the particular tissue used, since the rat anococcygeus muscle does not respond to either field stimulation or noradrenaline after only five or six days (D. Templeton, personal communication).

References

AMBACHE, N. (1946). Interaction of drugs and the effect of cooling on the isolated mammalian tissue. J. Physiol. (Lond.), 104, 266-287.

BLAKELEY, A.G.H., BROWN, SIR L., DEARNALEY, D.P. & WOODS, R.I. (1969). Perfusion of the spleen with blood containing prostaglandin E₁: transmitter liberation and uptake. Proc. Roy. Soc. Lond. B., 174, 281-292.

BURN, J.H. & RAND, M.J. (1960). The relation of circulating noradrenaline to the effect of sympathetic stimulation. J. Physiol. (Lond.), 150, 293-305.

EULER, U.S. VON, & LISHAJKO, F. (1961). Improved techniques for the fluorimetric estimation of catecholamine. Acta. Physiol. Scand., 51, 348-356.

FUKUDA, H. & SHIBATA, S. (1972). The effect of cold storage on the inhibitory action of isoprenaline, phenylephrine and nicotine on the mechanical and membranal activities of guinea-pig taenia caecum. Br. J. Pharmac., 46, 438-448.

GARRETT, J.R., HOWARD, E.R. & LANSDALE, J.M. (1972). Myenteric nerves in the hind gut of the cat. J. Physiol. (Lond.), 226, 103-104.

GIBSON, A. & GILLESPIE, J.S. (1973). The effect of immunosympathectomy and of 6-hydroxydopamine on the response of the rat anococcygeus to nerve stimulation and to some drugs. Br. J. Pharmac., 47, 261-267.

GILLESPIE, J.S. (1972). The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. Br. J. Pharmac., 45, 404-416.

GILLESPIE, J.S. & KIRPEKAR, S.M. (1966). The histological localization of noradrenaline in the cat spleen. *J. Physiol. (Lond.),* 187, 69-79.

GILLESPIE. J.S. & McGRATH, J.C. (1972). The effect of nerve stimulation on the depletion of noradrenaline by reserpine in the heart, vas deferens and anococcygeus muscle of the rat. *Br. J. Pharmac.,* 46, 525-526.

GILLESPIE, J.S. & McGRATH, J.C. (1973). The spinal origin of the motor and inhibitory innervation of the rat anococcygeus muscles. *J. Physiol. (Lond.),* (in the press).

GILLESPIE, J.S. & MAXWELL, J.D. (1971). Adrenergic innervation of sphincteric and non-sphincteric smooth muscle in the rat intestine. *J. Histochem. Cytochem.,* 19, 676-681.

HATTORI, K., KURAHASHI, K., MORI, J. & SHIBATA, S. (1972). The effect of cold storage on the adrenergic mechanisms of intestinal smooth muscle. *Br. J. Pharmac.,* 46, 423-437.

HOLMAN, E. & HUGHES, J.R. (1965). Inhibition of intestinal smooth muscle. *Aust. J. exp. Biol. med. Sci.,* 43, 277-290.

KUKOVETZ, W.R. & LEMBECK, F. (1962). Untersuchungen uber die adrenalinpotenzierende Wirkung von Cocain und Denervierung. *Naunyn-Schmiedeberg's Arch. exp. Path. u Pharmak.,* 242, 467-479.

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

O'DONNELL, S.R. & HECKER, S.E. (1967). Contraction of rat vas deferens by cocaine. *J. Pharm. Pharmac.,* 19, 695.

SHIBATA, S., HATTORI, K., SAKURAI, I., MORI, J. & FUJIWARA, M. (1971). Adrenergic innervation and cocaine-induced potentiation of adrenergic responses of aortic strips from young and old rabbits. *J. Pharmac. exp. Ther.,* 177, 621-632.

THOMSON, J.W. (1958). Studies on the response of the isolated nictitating membrane of the cat. *J. Physiol. (Lond.),* 141, 46-72.

TRENDELENBURG, U. (1968). The effect of cocaine on the pacemaker of isolated guinea-pig atria. *J. Pharmac. exp. Ther.,* 161, 222-231.

SUMMARY

THE INHIBITORY AND MOTOR INNERVATION OF THE

ANOCOCCYGEUS MUSCLE

A thesis presented for the degree of

Doctor of Philosophy

by

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December 1973

(1)   The subject of this thesis was the investigation of the dual

innervation of the anococcygeus muscle.   The rat anococcygeus had

previously been shown _in vitro_ to have a motor adrenergic innervation and

also an inhibitory nerve response whose transmitter was unknown.

(2)   The object of the present study was threefold -

(a)   To determine whether this inhibitory response was due to a nerve

pathway distinct from the motor innervation and with a separate spinal

origin and if so whether this pathway had a ganglion synapse and so could

be considered as part of the autonomic nervous system.

(b)   To compare the pharmacological properties of the anococcygeus

muscle and vas deferens and to determine whether the rate of depletion of

noradrenaline by reserpine in these two tissues was affected by nerve

stimulation.

(c)   To compare the properties of the anococcygeus muscles from the

cat and the rat in order to find whether the dual innervation found in the

rat was represented in this further species and if so whether this

comparison would throw any more light on the nature of the inhibitory

response.

(3)   Using a pithed rat preparation permitting selective stimulation of

the autonomic spinal outflows, it was shown that the inhibitory pathway to

the anococcygeus muscles arose from the spinal canal, that it was

interrupted by a ganglion synapse and that the spinal origin of its pre-

ganglionic nerves was L 5 - S 2 as opposed to T 10 - L 3 for the

preganglionic nerves in the motor pathway.

(4)   Using this same preparation, the pharmacological properties of the

motor nerves to the anococcygeus were examined _in situ_ and compared with

those of the vas deferens.   This comparison demonstrated that the

pharmacological properties of the anococcygeus motor innervation were those

of a classical adrenergic innervation whereas the vas deferens showed responses which were in themselves complex and showed unconventional responses to drugs. A hypothesis is suggested to explain this unconventional nature of the vas deferens response.

(5)   An analysis of the dose dependence and time course of the depletion of tissue noradrenaline by reserpine showed that the rat anococcygeus and vas deferens were depleted to a similar extent and at a similar rate and that this was slower than that found in the heart. Increase in sympathetic nerve activity by spinal stimulation in pithed rats significantly increased the noradrenaline depletion in both anococcygeus and vas deferens. From this it is suggested that nerve impulse traffic may be an important factor in determining the rate of depletion of noradrenaline by reserpine and in the vas deferens may explain the apparent resistance to depletion.

(6)   The cat anococcygeus muscle was investigated in vitro and shown to possess a dual innervation similar to that in the rat. Due to the presence of intrinsic tone, both motor and inhibitory nerve responses could be demonstrated in the absence of blocking drugs and their interaction studied. The pharmacological properties of the cat anococcygeus were similar to those of the rat except that several substances relaxed the cat muscle which contracted the rat including acetylcholine, isoprenaline, prostaglandins and ATP. These substances were therefore assessed as possible inhibitory transmitters but further analysis with blocking drugs suggested that the relaxations produced by these drugs were different from that produced by the inhibitory nerves. The inhibitory effect of acetylcholine on the cat muscle was particularly interesting since it inhibited motor nerve responses as well as relaxing the muscle. Several substances not normally associated with release of noradrenaline from nerves, including guanethidine, cocaine

LSD and 5HT produced indirect sympathomimetic effects in both species.

(7)   It is concluded that the anococcygeus muscle receives a dual innervation consisting of a motor adrenergic pathway originating from the lower thoracic and upper lumbar cord and a separate inhibitory pathway with its preganglionic fibres originating from the lower lumbar and upper sacral region of the vertebral column.   This dual innervation is found in both the rat and cat anococcygeus but in neither species does the inhibitory pathway appear to be adrenergic, cholinergic or purinergic and the transmitter remains unknown.