# THE NATURE OF NON-ADRENERGIC INHIBITION

# IN THE MAMMALIAN INTESTINE

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## SUMMARY

Responses to non-adrenergic inhibitory nerves have been obtained and pharmacologically analysed in isolated intestinal preparations from rabbits, guinea pigs and cats. The nerves were found to differ from adrenergic sympathetic nerves in a number of ways. Their threshold and optimal frequency for electrical stimulation was lower than that of sympathetic nerves and in contrast to sympathetic nerves the peripheral ganglia were situated in or close to the tissue innervated. Responses to non-sympathetic nerves persisted after abolition of sympathetic responses by either adrenergic neurone blocking drugs or by mixtures of α and β adrenoceptor blocking drugs. Non-adrenergic inhibitory responses could be imitated by both nicotine and 5-hydroxytryptamine. Like sympathetic responses the non-adrenergic nerve responses were impaired by reserpine.

In the guinea pig isolated colon preparation, it was demonstrated that non-adrenergic nerves have extrinsic connections via the pelvic nerves. A mixture of pempidine and bufotenine abolished responses to pelvic nerve stimulation, suggesting the involvement of both 5-hydroxytryptamine and acetylcholine as ganglionic transmitters.

Unsuccessful attempts have been made to isolate and identify the postganglionic transmitter substance from non-adrenergic sympathetic nerves. In addition, a variety of biogenic substances have been examined as potential transmitter substances. Particular attention was paid to the adenine nucleotides in view of recent reports suggesting these as likely transmitter substances in non-adrenergic

inhibitory nerves. Little evidence was found to support this concept and it is suggested that the term "purinergic nerves" to describe these nerves is premature.

The phenomenon of "rebound contraction" following transmural stimulation of isolated intestinal preparations has been studied. It is suggested that the major part of the response is due to stimulation of cholinergic nerves within the intestinal wall.

A short examination has been made of the sympathetic nerve blocking action of β-adrenoceptor blocking substances. The evidence suggests that this action is pre-synaptic but differs from that of guanethidine.

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CONTENTS

# PART I

## HISTORICAL INTRODUCTION

## Early investigations concerning the structure and function of autonomic nerves

Up to the end of the eighteenth century no clear distinction had been made between striated and smooth muscles or between somatic and autonomic nerves. The function of nerves innervating the heart and viscera was believed to be to enable them to contract; theories on how they achieved this were imaginative. The widely held belief in the first half of the eighteenth century was that the brain manufactured or received from the blood a fluid known as animal spirits which was forced down small tubes in the nerves to the muscles causing them to contract. Theories on the mechanism by which "animal spirits" caused the muscle to contract included a suggestion that they flowed into the muscle and distended it. Another theory was that the nerves were tense cords and that nerve impulses were vibrations set up in them.

## Classification of the autonomic nervous system

Modern concepts of the structure and function of the autonomic nervous system are based on the work of two nineteenth century scientists, the anatomist W.H. Gaskell and the physiologist J.N. Langley. Both worked towards the end of the century.

Gaskell (1886) classified the efferent nervous system of the body and showed for the first time that the nerves issuing from the central nervous system to supply the plain muscle tissues from the cranial, lumbar-thoracic and sacral regions of the spinal cord had common characteristics which enabled them to be grouped into a common visceral system.

The name "autonomic nervous system" for the "common visceral system" was introduced by Langley (1898) who defined it as "the sympathetic system and the allied nervous system of the gut." He wrote, "the word (i.e. autonomic) implies a certain degree of independent action but exercised under the control of a higher power".

Langley (1905) introduced the term "parasympathetic nerves" for the cranial and sacral autonomic outflow. He introduced the term because "where a tissue is innervated by both sympathetic and parasympathetic nerves they have different effects either in kind or degree". The term "sympathetic nerves" had been introduced much earlier by the Danish anatomist J.B. Winslow in his text book "Exposition Anatomique de la Structure du Corps Humain", published in 1732. Sympathetic nerves are autonomic nerves issuing from the lumbar and thoracic regions of the spinal cord.

Transmission at autonomic nerve endings

By 1900 the concept of the autonomic nervous system as a group of nerves concerned with the regulation of the internal environment of the body was well established and the interest of physiologists became focused on processes at the nerve-muscle junction.

Workers such as Oliver & Schafer (1896), Lewandowsky (1900) and Langley (1901) noted that the action of extracts of the adrenal glands upon plain muscles was similar to that of sympathetic stimulation. In 1901 Takamine announced the isolation of adrenaline from the adrenal glands and identified it as the active principle. Elliot (1905) compared the action of adrenaline with the effect of stimulation of

sympathetic nerves and showed they were similar for a large range of mammalian organs. Thus from 1900 onwards a great deal of interest was shown in the relationship between adrenaline (and later noradrenaline) and the processes of transmission at sympathetic nerve endings.

Parallel discoveries were being made concerning the function of the parasympathetic nerves. The similarity between the action of muscarine and the effects of stimulating parasympathetic nerves on a large number of mammalian plain muscle tissues caused Dixon (1907) to suggest that it was possible that parasympathetic nerves might release a muscarine-like chemical at the nerve endings. This suggestion, together with that of Elliot (1904) that sympathetic nerves might release adrenaline, were very poorly received by physiologists at the time. However, by 1914 the climate of opinion was changing and a distinguished physiologist, Dale, who had originally been critical of Elliot's suggestion, produced a paper in which he made a study of choline derivatives and remarked that the similarity of the action of acetylcholine and the effect of stimulating parasympathetic nerves was of great interest but "there was insufficient evidence to warrant discussion". (Dale, 1914).

Demonstration of neurohumoral transmission

By 1900 physiologists were well aware of the ability of certain naturally occurring substances such as adrenaline and muscarine to mimic the effects of stimulating various autonomic nerves. However, although it was speculated that the effects of the nerves might be due to the release of

these or similar substances at the nerve ending there were no techniques available to investigate events at nerve endings and it was not known whether nerve impulses were carried across the nerve-muscle junction by chemical, electrical or by some other means. The classic paper of Loewi radically changed the position.

In 1921 Loewi published a paper which demonstrated for the first time that transmission of nerve impulses between the nerve endings and the effector organ was (in at least one case, i.e. frog heart) caused by the release of chemical substances. In his first experiment he used the hearts from two frogs; one was perfused with Ringer's solution and the perfused liquid allowed to come into contact with the second heart. When the vagus nerve to the donor frog heart was electrically stimulated the heart rate was slowed and a substance was released which caused the recipient frog heart also to be slowed. This substance Loewi called "Vagusstoff".

The influence of Loewi's work was profound. His experiments determined the direction of research into the autonomic nervous system for decades. Physiologists who had suspected that autonomic nerves might release muscarinic or adrenaline-like substances had had their ideas confirmed, and, moreover, a method for further investigation was introduced to them. The result was that research into processes at autonomic nerve endings became the dominant field in autonomic research. It remains so fifty years later. Evidence was slowly accumulated which at first tentatively and then overwhelmingly indicated that acetylcholine was the

parasympathetic post-ganglionic transmitter and that noradrenaline was the sympathetic post-ganglionic transmitter.

The concentration of interest of research into the autonomic system on the physiology of transmission has been very fruitful and resulted in the introduction into medicine of such drugs as pempidine, α-methyldopa and guanethidine, but one result of this focus of interest is that other areas of autonomic physiology have been ignored. One of these areas - the phenomenon of non-sympathetic inhibition - is the subject of this thesis.

Early reports of non-sympathetic inhibition

The early physiologists working around the turn of the nineteenth century reported many instances where vagal stimulation caused inhibition of gut motility. Langley (1898), in his paper on the inhibitory fibres in the vagus to the end of the oesophagus and the stomach", reported that "the vagus causes not infrequently relaxation of the whole fundus end of the stomach and occasionally relaxation of the whole stomach". He was working with cats and rabbits.

Bayliss & Starling (1899, 1901) stimulated the vagus to the ileum and duodenum of dogs. They reported that the initial effect of stimulation was inhibition but this was followed by excitation. They found that this action of the vagus was not abolished by atropine but was abolished by nicotine. In their summary they stated "The action of the vagus on the intestines is therefore twofold - an initial inhibition followed by augmentation which outlasts the excitation of the nerve".

In 1922 Langley produced a paper in which was included a diagram (Fig. 1) in which he showed the possible arrangement of postganglionic vagal fibres. Inhibitory fibres are included in a 1:1 ratio with motor fibres. Thus it was an accepted fact at this time, that vagal stimulation could cause both excitation and inhibition of intestinal motility.

Veach (1925) concluded that stimulation of the peripheral end of the vagus nerve with currents of relatively low frequencies or intensities had a motor effect on cat stomach while stimulation with considerably higher frequencies or intensities had an inhibitory effect. He suggested that the mechanism by which the vagus could both inhibit and excite the stomach was due to the "Wedensky effect".

The Wedensky effect was a theory of nervously mediated inhibition which was prominent about this time (i.e. 1925). It sought to explain the nervous inhibition by postulating that while with low frequencies of stimulation a nerve would excite a tissue, the effect of increasing the frequency was to make each nerve impulse travel in the relative refractory period of the last, and be therefore reduced to a sub-threshold magnitude. Thus at high frequencies the nerve was blocked and the tissue relaxed.

This theory was disproved in the case of vagal inhibition of the stomach by McSwiney & Wadge (1928). Using cats, they recorded the effects of stimulating the vagus to the stomach. They found that vagal stimulation caused inhibition if the tone was high and contraction if it was low. They also found that it was possible to obtain

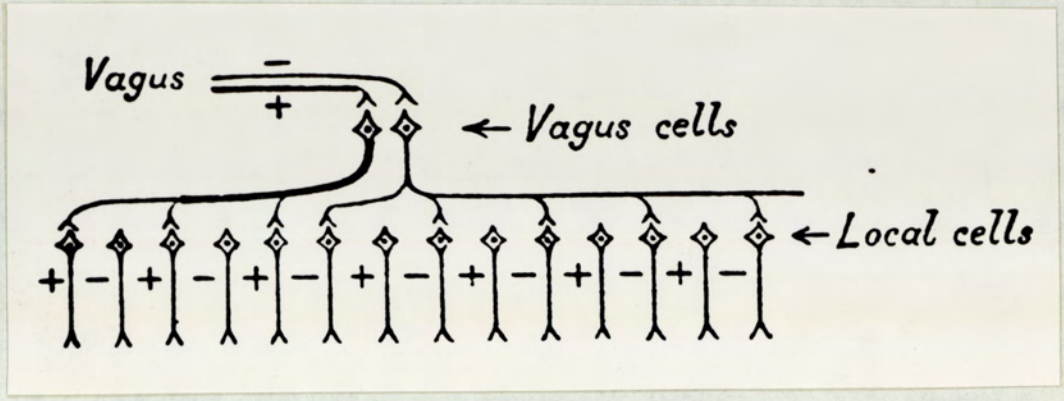


Fig. 1. Possible arrangement of motor and inhibitor vagal fibres to the mammalian intestine (from Langley, J.N. J. Physiol. (Lond.), 56, 39P, 1922).

inhibition at lower frequencies than those used to cause excitation. Thus the "Wedensky inhibition" theory became untenable.

Harrison & McSwiney (1936) also found that stimulation of the vagus could produce either contraction or relaxation. They suggested that the inhibition was caused by adrenergic fibres in the vagus. This theory was tentatively supported by Greeff, Kasperatt & Osswald (1962), and also Paton & Vane (1963). In their original paper, Harrison & McSwiney (1936) wrote, "We are still in doubt as to whether the relaxation of the stomach obtained on stimulation of the vagus nerve is partly or entirely due to the presence of adrenergic fibres... an examination of the records shows that the latent period and character of the responses are unlike the relaxation which may follow sympathetic stimulation".

In 1951 two papers were published (Ambache, 1951; Ambache & Edwards, 1951) in which the effects of botulinum toxin and atropine on the response of isolated small intestine from mice, rabbits and kittens to nicotine were studied. They showed that botulinum toxin abolished the motor action of nicotine without affecting the response to acetylcholine. In the presence of botulinum toxin nicotine caused inhibition of the intestine. Similar results were obtained by using atropine instead of botulinum toxin. In mouse and kitten ileum, atropine reversed the motor effect of nicotine to inhibition. They failed to obtain reversal of nicotine with atropine on rabbit ileum because the motor action was atropine resistant. These workers also showed that the inhibitory action of nicotine in the presence of atropine

or botulinum toxin was blocked by hexamethonium or by large doses of nicotine indicating that nicotine was causing inhibition by acting on inhibitory nerves with synapses locally in the intestinal wall. They found that the inhibitory effect of nicotine could be abolished by high doses of ephedrine. Since ephedrine had been shown to abolish the actions of adrenaline they concluded that the inhibitory nerves were adrenergic. They concluded that there were two kinds of functionally distinct ganglion cells in the myenteric plexus. Stimulation of the one, giving rise to cholinergic fibres, caused contraction; stimulation of the other, giving rise to adrenergic fibres, caused inhibition of the intestine.

Modern work on non-sympathetic inhibitory nerves

The presence of non-sympathetic inhibitory nerves in the vagus was first demonstrated by Martinson and his co-workers in the early sixties (Martinson & Muren, 1960, 1963; Martinson, 1964, 1965a,b; Jansson & Martinson, 1965).

Martinson working with cats recorded the responses of the stomach by inserting a balloon into the stomach and attaching the balloon to a water manometer. He found that vagal stimulation caused the stomach to contract but as the voltage or pulse width was gradually increased the contraction increased to a maximum and then decreased. This suggested that inhibitory fibres were present and that they had a higher stimulation threshold than the excitatory fibres. On giving the animals atropine, he found that the excitatory responses to vagal stimulation were replaced by inhibitory responses, thus clearly demonstrating the presence of

inhibitory fibres in the vagus. Investigations of the inhibitory response showed that vagal inhibitory fibres could cause maximal relaxation of the stomach in the corpus and fundus areas. The prolonged inhibitory responses Martinson obtained caused him to suggest that the vagal fibres either initiate a reverberating activity in local neurones or released a very stable smooth muscle relaxing substance that is not easily removed by the blood stream.

Martinson (1965a b) compared the response to vagal inhibition with responses to infused catecholamines and to sympathetic nerve stimulation and found the response to vagal inhibitory fibres different to the response to the other two stimuli. Thus, the vagal inhibitory responses had a shorter latency period (2-5 seconds) than the responses to sympathetic stimulation (10-30 seconds). The responses to sympathetic stimulation were smaller than those to vagal stimulation, while the response to infused catecholamines were smaller still. He showed that the frequency response curves for vagal and sympathetic stimulation were different; the response to sympathetic stimulation at 16 and 4 Herz gave equivalent responses to vagal stimulation of 4 and 1 Herz respectively. The stomach recovered much more rapidly after being relaxed by sympathetic stimulation than after vagal stimulation. Martinson also showed that when the relaxation of the stomach was seemingly maximal after vagal stimulation, the extent of relaxation could be increased by sympathetic stimulation or by infusing catecholamines. Conversely, when sympathetic inhibition or catecholamine induced inhibition was maximal, a further relaxation could

be obtained by vagal stimulation. Martinson presented evidence that suggested that sympathetic relaxation acted mainly on the pyloric antrum, while vagal inhibition affected only the corpus and fundus.

Martinson also showed that vagal inhibition of the cat stomach was only slightly reduced in the presence of sufficient guanethidine to block or severely reduce sympathetic inhibition. He also showed that vagal inhibition was much less sensitive to pronethalol than was the inhibition caused by infused adrenaline. Martinson also showed that vagal inhibition was blocked by hexamethonium, while sympathetic inhibition was not.

Martinson's experiments strongly suggest that vagal inhibition of the cat stomach is not mediated by adrenergic nerves.

Several workers have reported finding non-sympathetic inhibitory nerves innervating the guinea pig taenia-coli. (The taenia-coli of the guinea pig is a narrow strip of smooth muscle running along the outside of the caecum.) Burnstock, Campbell, Bennett & Holman (1963); Burnstock, Campbell & Rand (1966) and Campbell (1966a,b) reported that when isolated strips of taenia-coli were transmurally stimulated, they responded with either relaxation or contraction or a mixed response with both elements present. The addition of atropine increased the degree of relaxation caused by a given frequency, or revealed an inhibitory response if the original effect had been pure contraction. Neostigmine increased the contractions caused by transmural stimulation and abolished the inhibition.

These workers showed that the taenia-coli was relaxed when a flap of caecal wall connected to the muscle was stimulated and that relaxation could be blocked by ganglion blocking drugs such as pentolinium. Ganglion stimulating drugs caused the taenia-coli to relax in the presence of atropine. The relaxations of the taenia-coli caused by transmural stimulation, by stimulation of a flap of the caecal wall, or by ganglion stimulating drugs, were all unaffected by adrenergic neurone blocking drugs. This contrasted with relaxations obtained by stimulating perivascular nerves which were blocked by adrenergic neurone blocking drugs. The authors concluded that apart from a sympathetic innervation, the taenia-coli was also innervated by intramural inhibitory nerves with their cell bodies in Auerbach's plexus. This conclusion was supported by Bulbring & Tomito (1966) who examined the inhibitory responses after stimulation of strips of taenia-coli and obtained results consistent with stimulation of post-ganglionic nerves only a few millemetres in length, which were distributed longitudinally in the tissue.

A number of workers have reported in recent years (from 1963 onwards), the presence of inhibitory neurones innervating intestinal smooth muscle which are resistant to procedures abolishing sympathetic responses. Thus, Holman & Hughes (1965) obtained inhibition to transmural stimulation and to the addition of the ganglion stimulant dimethylphenylpiperazinium (DMPP) in small and large isolated intestine from the rat, mouse, rabbit and guinea pig. The inhibition was not blocked by guanethidine but was blocked by the β adrenergic receptor blocking agent pronethalol at the high

concentration of 50 µg/ml. Bucknell (1965) showed that transmural stimulation of human isolated colon at frequencies below 8 Herz caused inhibition which was resistant to the blocking action of bretylium and guanethidine and also to a mixture of an α and β blocking agents (dibenamine and pronethalol). Relaxation obtained at frequencies above 8 Herz was sensitive to these blocking drugs and was presumably mediated via sympathetic nerves. The low frequency relaxation was unlikely to be mediated by sympathetic nerves and the author suggested that stimulation of chromaffin cells might be involved. Campbell (1966a,b) supported the earlier work of Martinson and his co-workers (Martinson & Muren, 1960, 1963; Martinson, 1964, 1965a,b,c; Jansson & Martinson, 1965). Campbell, using the guinea pig isolated stomach preparation, compared the responses obtained by stimulating vagal inhibitory nerves with those obtained by stimulating sympathetic nerves. He showed that the vagal inhibitory nerves were more effective at producing inhibitory responses at low rates of stimulation. The responses were of more rapid onset and were unaffected by concentrations of bretylium which abolished sympathetic responses. Campbell (1966a,b) pointed out that although his work provided strong evidence that the vagal inhibitory fibres were non-sympathetic, this did not necessarily mean that they were non-adrenergic.

Crema, Del Tacca, Frigo & Lecchini (1968) investigated the response of human isolated colon to transmural stimulation. The original response was either contraction or inhibition, but after atropine (0.1 µg/ml) only inhibition was seen. The inhibition was not blocked by a mixture of dibenamine and propranolol thus suggesting that non-adrenergic nerves

are responsible. The authors investigated the effects of 5-hydroxytryptamine on the tissue and found that it produced variable responses either contraction, inhibition or a mixture of the two. These workers found that any excitation was blocked by hyoscine, while inhibition persisted and was not affected by the mixture of anti-adrenaline drugs. Tetrodotoxin was found to abolish the response to transmural stimulation but not the inhibition caused by the addition of 5-hydroxytryptamine. Tetrodotoxin is known to prevent conduction in nerve cells and apparently 5-hydroxytryptamine has a direct inhibitory effect on the smooth muscle of the human colon.

Bianchi, Beani, Frigo & Crema (1968) investigated the responses to transmural, parasympathetic and sympathetic stimulation in the guinea pig isolated terminal colon. Transmural stimulation after atropine produced inhibitory responses which were particularly well marked when low frequencies (1-2 Herz) were used. Inhibitions to transmural stimulation were not blocked by a mixture of $\alpha$ and $\beta$ adrenoceptor blocking agents, or by bretylium, whereas sympathetic nerve inhibition was blocked by both. The response to transmural stimulation was abolished by tetrodotoxin thus confirming that it was nervously mediated. These workers also examined the responses to pelvic (parasympathetic) nerve stimulation. Pelvic stimulation produced a rapid contraction (maximal at 10 to 20 Herz) which was blocked by atropine. After atropine pelvic nerve stimulation did not reveal any trace of an inhibitory component. The authors suspected that the inhibition due to transmural stimulation was due to

the release of a non-adrenergic transmitter and attempted to mimic it with a number of known naturally occurring compounds. They tested histamine, bradykinin, glycine, γ-amino butyric acid, ATP, AMP, dopamine, acetylcholine and 5-hydroxytryptamine. The only substances they found to inhibit the gut were ATP, AMP, dopamine and 5-hydroxytryptamine. 5-Hydroxytryptamine, ATP, AMP and dopamine were only mildly relaxant in high concentrations (500 μg/ml) and as the authors remarked it seems unlikely that intramurally stimulated nerve fibres could release such amounts. The response to 5-hydroxytryptamine was more interesting - concentrations of 1 μg/ml contracted the tissue. This contraction was not affected by methysergide or hexamethonium but was abolished by 0.1 μg/ml atropine. Higher doses of 5-hydroxytryptamine produced a mixed response with both motor and inhibitory components. Both of these components persisted in the presence of atropine, methysergide and hexamethonium. However a mixture of dibenamine with propranolol converted the mixed response to pure inhibition by abolishing the atropine insensitive contraction. The authors suggested that not only can 5-hydroxytryptamine excite non-sympathetic inhibitory nerves and cholinergic excitatory nerves, but it can also stimulate non-cholinergic excitatory mechanisms. The authors stated that tetrodotoxin did not abolish the mixed response obtained with high doses of 5-hydroxytryptamine in atropinised preparations but it did abolish the atropine sensitive contraction seen with low doses. They suggest that this contraction is possibly due to 5-hydroxytryptamine releasing acetylcholine from storage sites at the nerve endings, an

effect analogous to that of tyramine in sympathetic nerve endings (Burn & Rand, 1959).

Furness (1969a,b) also investigated the innervation of the guinea pig isolated colon and his results generally support previous findings. He showed that transmural stimulation produced inhibitory junction potentials that persisted in the presence of guanethidine but were blocked by procaine. Sympathetic denervation did not affect the inhibitory response to transmural stimulation. Pelvic nerve stimulation produced excitatory junction potentials. He suggested that the non-adrenergic nerves have no efferent extrinsic connections and are involved in completely intrinsic control of colonic motility.

Bulbring & Gershon (1966, 1967) studied the role of 5-hydroxytryptamine in vagal relaxation of the stomach (using guinea pig and mouse isolated stomachs and recording intra-luminal pressure). They found that the response to 5-hydroxytryptamine was similar to the response to vagal stimulation - both having excitatory and inhibitory components. In both cases hyoscine converted the response to vagal stimulation to pure relaxation. The authors demonstrated that non-depolarising ganglion blocking drugs such as pento-linium, which did not affect responses to 5-hydroxytryptamine, reduced vagal relaxation but did not completely block it. Specific desensitisation of the receptors to 5-hydroxytryptamine caused the inhibitory response to 5-hydroxytryptamine to be abolished, and caused a reduction in vagal inhibition without a complete block being obtained. It was only when - as in the early phase of the blocking action of nicotine - ganglionic

receptors to both acetylcholine and 5-hydroxytryptamine were blocked, that a complete block of vagal inhibition was seen. These results caused the authors to suggest that the vagal inhibitory ganglia receive both cholinergic and 5-hydroxy-tryptaminergic preganglionic fibres. In support of this hypothesis the authors showed that depletion of 5-hydroxy-tryptamine stores by reserpine resulted in a reduction of the vagal inhibitory response and that stimulation of the mouse stomach (after asphyxiation of the mucosa and extrusion of the luminal content) caused the release of 5-hydroxy-tryptamine. The neural origin of the 5-hydroxytryptamine was indicated by the fact that tetrodotoxin blocked the release.

So far all the reports of non-adrenergic inhibition have been concerned with experiments on intestinal smooth muscle. However, Hughes & Vane (1967) reported that trans-mural stimulation of the rabbit isolated portal vein produced inhibitions which persisted in the presence of $\alpha$ and $\beta$ adrenergic blocking drugs. It was not affected by specific antagonists to acetylcholine, 5-hydroxytryptamine and hista-mine. This report indicates that the distribution of non-sympathetic inhibitory nerves may not be confined to intestinal smooth muscle. Everett (1968) showed that non-sympathetic inhibitory nerves occurred in avian as well as mammalian tissues when she demonstrated their presence in chick isolated intestine.

Rebound contraction

An interesting observation that several workers have made is the "after" contraction which is often seen to

immediately follow non-adrenergic inhibition induced by transmural stimulation. Campbell (1966a) and Burnstock, Campbell & Rand (1966) described how in atropinised preparations of guinea pig taenia-coli, transmural stimulation could cause the tissue to relax whilst at the end of the period of stimulation the tissue contracted. Their work was substantiated by Bennett (1966) who, using electrophysiological techniques, showed that stimulation of the intramural inhibitory nerves of the guinea pig taenia-coli gave an inhibitory junction potential which was followed by an increase in the rate of firing of action potentials. He also showed that the rate of firing of action potentials after an inhibitory junction potential and the duration of this enhanced rate, increased with an increase in the mean amplitude of the hyperpolarisation during an inhibitory junction potential. In simple terms, the bigger the inhibition, the bigger the rebound excitation.

Bianchi, Beani, Frigo & Crema (1968) and Del Tacca, Lecchini, Frigo, Crema & Benzi (1968), working with guinea pig colon, also reported the phenomena of "after" contraction. These authors found that in the presence of atropine, transmural stimulation caused inhibition during the period of stimulation which was replaced by contraction immediately after the stimulation ceased.

The fact that all the above authors obtained an "after" contraction to transmural stimulation in the presence of atropine has led them to question whether the contraction could be due to activation of cholinergic nerves. They have suggested that after contraction is a "rebound" contraction

of myogenic origin that is a result of the preceding inhibition. As Bennett suggested:-

"The most likely explanation of rebound excitation is that as a result of hyperpolarisation of the cell membrane, there follows a period in which the membrane tends to become depolarised beyond its normal value and this is responsible for an increase in the rate of action potential firing."

## Recent investigations into the nature of the non-adrenergic inhibitory transmitter substance

Some workers have recently suggested that the non-adrenergic inhibitory transmitter substance might be an adenosine nucleotide such as adenosine triphosphate (ATP) or adenosine diphosphate (ADP). Thus, Satchell, Burnstock & Campbell (1969) detected adenosine after the electrical stimulation of the inhibitory nerves to the stomach of the toad Bufo marinus. Similarly, after stimulation of Auerbach's plexus from turkey gizzard considerable amounts of adenosine monophosphate (AMP) were released. They wrote "It is likely that adenosine in the stomach perfusate and AMP from nerve have been metabolized from ATP, and ATP or an analogue is the inhibitory transmitter substance." These findings were later extended by Burnstock, Campbell, Satchell & Smythe (1970) who collected ATP, ADP and AMP after electrical stimulation of the isolated Auerbach's plexus from turkey gizzard. They also perfused isolated stomachs from guinea pigs and toads and found that stimulation of the vagal non-adrenergic inhibitory innervation caused the release of adenosine and inosine. They showed that when the stomachs were perfused with ATP it was broken down into adenosine and inosine.

These workers concurred with the hypothesis that ATP or a related nucleotide was the transmitter substance released from non-sympathetic inhibitory nerves.

Satchell & Burnstock (1971) claimed that ATP or a related analogue broadly satisfied the criteria suggested by Eccles (1964) for substances to be considered neurotransmitters. Eccles (1964) argued that any successful candidate for the role of neurotransmitter must be able to satisfy a number of conditions namely:-

(a) The transmitter and enzymes capable of its synthesis must be present in the nerve.

(b) The transmitter must be released when the nerves are stimulated.

(c) The transmitter given extrinsically must mimic the effect of nerve stimulation.

(d) An enzyme or enzyme system capable of inactivating the transmitter must be present in the tissue.

(e) Drugs which alter the response to nerve stimulation should alter the response to the transmitter substance in the same way.

Using tritium-labelled nucleotides, Su, Bevan & Burnstock (1971) showed that guinea pig isolated taenia-coli would take up adenosine and convert it to ATP. They found that ATP was released when non-adrenergic inhibitory nerves were electrically stimulated. Unfortunately their hypothesis was very seriously weakened by the finding that ATP was also released on electrical stimulation of sympathetic adrenergic nerves.

Owing to the ubiquitous distribution of ATP in the body the finding that it satisfies the criteria (a), (b) and (d)

suggested by Eccles (1964) does not necessarily imply that it is a neurotransmitter substance. The evidence presented in favour of the hypothesis by Burnstock and his co-workers, although suggestive that adenosine nucleotides are in some way involved in autonomic nerve transmission, is not yet sufficiently strong to establish their hypothesis. Similarly evidence presented in this thesis argues against the hypothesis.

The work of the various authors reviewed in this survey shows that non-adrenergic inhibitory nerves are distributed to a number of tissues. Apart from Hughes & Vane (1967), all the workers have found these nerves in tissues taken from the stomach, ileum, and colon of a number of different species; namely the cat, rabbit, guinea pig, dog and human. Apart from the existence of these nerves, very little is known about them. The nature of the post-ganglionic transmitter (or transmitters) has not been established with certainty, neither has the function of the nerves.

Recently, the whole subject of non-adrenergic inhibition of smooth muscle has been reviewed by Burnstock (1972).

# PART 2

## EXPERIMENTAL METHODS

Transmural stimulation of rabbit isolated ileum

Rabbits weighing 1 - 3 Kg were killed by a blow on the back of the neck and bled out. Sections of ileum about 3 cm long were removed and carefully cleared of any contents from the lumen. Sections chosen were free of lymph nodes and were attached to a mesenteric artery. The tissues were usually set up in aerated Tyrode's solution and the longitudinal contractions recorded with isotonic writing levers on a smoked drum. The bath temperature varied in different experiments between 28 and 37°C and details are given in the Experimental Results section. In some experiments Kreb's bicarbonate or McEwen's (1956) solution was used gassed with 5% carbon dioxide in oxygen; responses were no different to those obtained using aerated Tyrode's solution.

The periarterial (sympathetic) nerves were stimulated with an electronic stimulator delivering rectangular pulses through bipolar platinum electrodes of the type described by Burn & Rand (1960). Transmural stimulation was effected with bipolar intraluminal electrodes of the type described by Paton (1955). The arrangement of electrodes used to stimulate nerve innervating the tissue is illustrated in Fig.2.

Stimulation parameters varied according to the aims of the particular experiment and details are given in the Experimental Results section. Sympathetic or transmural stimulation was usually applied for 20 sec periods at 3 minute intervals with pulses of supramaximal strength (usually 20V) and duration (usually 2 m.sec.)

Reserpine pretreatment

Rabbits were pretreated by injecting reserpine intravenously either dissolved in 50% aqueous ascorbic acid or in

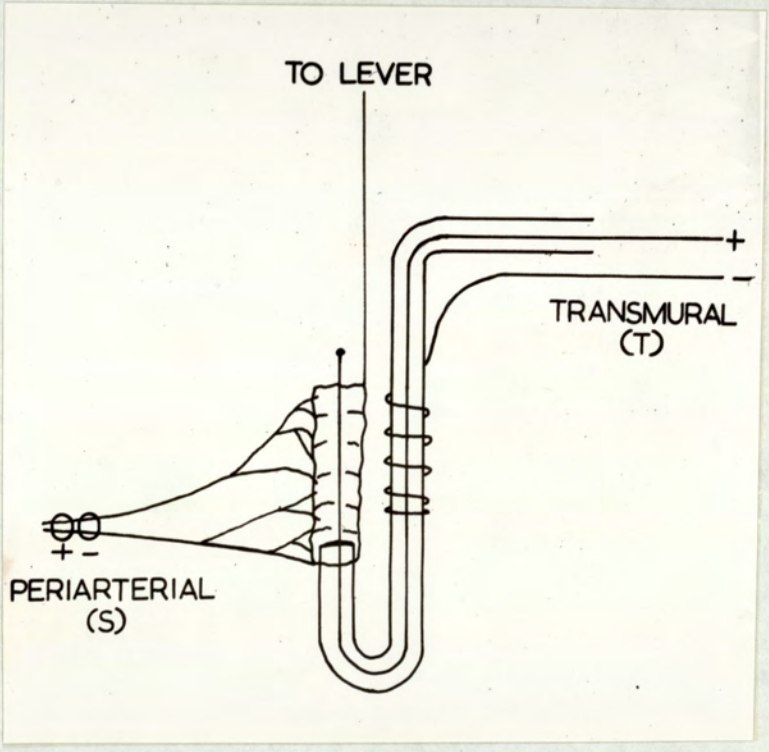


Fig. 2. Electrode for transmural stimulation of isolated intestinal preparations. Electrode is constructed of glass, poles of electrode are made from platinum wire. Intraluminal pole is sealed into glass with epoxy resin.

the form of the commercially available injection (Halewood Chemical Company) into the marginal ear vein. The following dosage schedule was employed, 0.25 mg/Kg for 4 days, 1 mg/Kg for one day, miss a day, then 1 mg/Kg for one day with the animal killed and used on the following day.

Chronic sympathetic denervation of segments of rabbit ileum

Rabbits were anaesthetized with pentobarbitone (35 mg/Kg) injected into a marginal ear vein. A mid-line abdominal incision was made and the periarterial nerves and blood vessels serving a suitable segment of ileum were sectioned between ties. 3 inch lengths of thread were left attached to the sectioned nerves to serve as a marker. The incision was closed with cotton sutures and the animal left for 14 days. The animals were killed on day 15 and segments of ileum identified by the marker threads were removed and set up in the usual way.

Rabbit isolated colon

The preparation with both sympathetic and parasympathetic nerves attached was used as first described by Garry & Gillespie (1954). The rabbit was killed, bled and the abdomen opened in the mid-line. The section of colon supplied by the inferior mesenteric artery was identified as shown in Fig. 3. The sympathetic nerve runs with the inferior mesenteric artery whilst the parasympathetic nerves run in the mesentery just below the inferior mesenteric artery. Both sets of nerves were identified and ligatured. The surrounding mesentery was gently removed and about 4-5 cm of colon together with the two sets of nerves were removed. The tissue was attached to a transmural electrode and set up

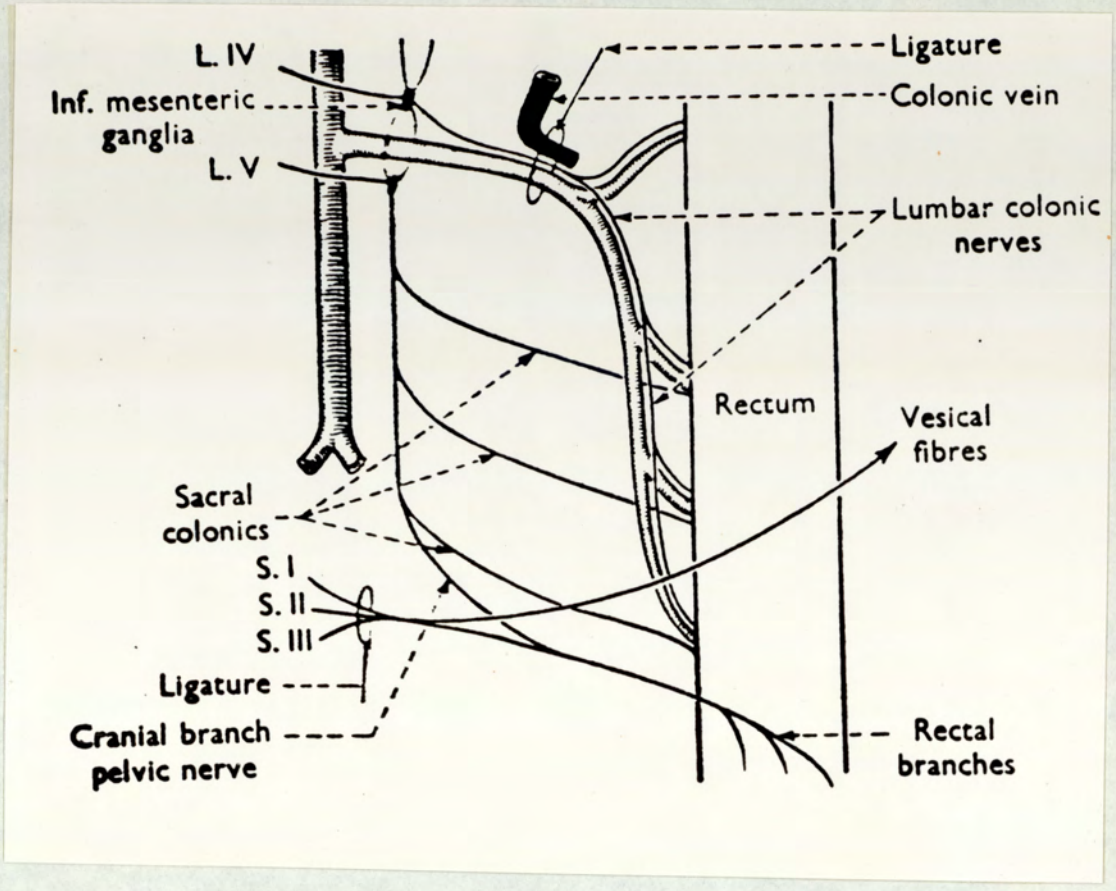


Fig. 3. Arrangement of sympathetic and parasympathetic extrinsic nerves to the colon of the rabbit (from Garry, R. & Gillespie, J.S. J.Physiol.(Lond.), 123, 60-61P, 1954).

in an organ bath containing McEwen's (1956) solution gassed with 5% carbon dioxide in oxygen. Longitudinal contractions were recorded with an isotonic lever writing on a smoked drum. The bath temperature was usually 37°C unless otherwise stated in the Experimental Results section. Sympathetic and parasympathetic nerves were separately threaded through bipolar platinum electrodes of the type described by Burn & Rand (1960). Stimulation of nerves was by rectangular pulses of supramaximal strength (usually 10 to 20 V) and pulse duration (1 to 2 msec). Stimulation was applied for 20 second periods at intervals of 3 minutes.

Cat and kitten isolated ileum

The same technique used for working with isolated rabbit ileum was used with these tissues. Cats or kittens were killed by a blow on the back of the neck, bled and 3-5 cm sections of ileum were removed. The sections were mounted on transmural electrodes, placed in an organ bath, and the periarterial nerves threaded through bipolar platinum electrodes. Isotonic frontal writing levers recorded the longitudinal contractions of the tissue on a smoked drum.

Cat ileum is composed of much thicker muscular layers than is rabbit ileum and it was found to be much more difficult to keep viable in an organ bath presumably due to difficulty in maintaining a suitable level of oxygen in the tissue. A variety of different bathing media were tried (Tyrode's gassed with air, Kreb's and McEwen's solution each gassed with 5% carbon dioxide in oxygen, and Locke's solution gassed with oxygen). No significant differences in the responses of the tissue to drugs or to the effective "life" of the preparation

were noted between these bathing media.

A number of experiments were performed using isolated intestine taken from 9-week-old kittens in the hope that the muscular wall thickness would be less and therefore viability easier to maintain. Unfortunately, the intestinal preparations were in appearance no different to those taken from adult cats and did not respond differently or for a longer period when set up in isolated organ baths.

Guinea pig isolated colon

This preparation of doubly-innervated guinea pig isolated colon was first used by Hukovic and cited by Rand & Ridehalgh (1965). The preparation is essentially similar to the doubly-innervated rabbit isolated colon preparation described by Garry & Gillespie (1954). Guinea pigs were killed by a blow on the back of the head and bled. An incision was made along the mid-line of the abdomen and the intestines gently retracted to one side to reveal the colon. The sympathetic nerves run with the inferior mesenteric artery and the pelvic (parasympathetic) nerves run just below the artery as shown in Fig. 3. The nerves were ligatured, the surrounding mesentery detached and a 4-5 cm segment of colon removed and mounted on a transmural electrode. Bipolar platinum electrodes were used to stimulate the extrinsic nerves as described for the rabbit colon. The preparation was placed in an organ bath containing McEwen's (1956) solution at 37°C gassed with 5% $CO_2$ in oxygen. An isotonic frontal writing lever recorded the longitudinal contractions on a smoked drum.

Perfusion of segments of ileum in anaesthetized rabbits

Rabbits weighing approximately 2 Kg were anaesthetized by a mixture of 6 ml per Kg 25% aqueous urethane and 30 mg/Kg pentobarbitone injected into a marginal ear vein. The trachea was cannulated and 5,000 units of heparin given intravenously. An artery and vein serving a suitable segment of ileum were cannulated with fine polyethylene tubing. Aerated Tyrode's solution at 37°C was perfused through the artery by means of a constant flow pump at a rate of 2 ml per minute. The Tyrode solution contained 5 µg per litre of neostigmine. Both vagi in the neck were mobilised, tied and cut and the peripheral ends threaded through a bipolar platinum electrode. The perfusate from the mesenteric vein was collected and 0.2 ml samples added to isolated and sensitized guinea pig ileum.

Guinea pig isolated ileum sensitized to acetylcholine

The method first described by Paton (1957) and modified by Paton & Aboo Zar (1968) was used. Isolated segments of guinea pig ileum were set up in an organ bath filled with Tyrode's solution containing morphine (10 mg/litre) and neostigmine (5 µg/litre), gassed with air and maintained at 37°C. After setting up, the tissue was left undisturbed for 2 hours, was then taken down and cleaned and set up again in the organ bath. It was found that if guinea pigs were given 0.5% ascorbic acid in their drinking water for 3 to 10 days prior to the experiment then the sensitivity of the ileum was markedly increased such that responses to as little as 10 pg/ml acetylcholine could be obtained in some experiments.

Rat isolated vas deferens preparation

Rats were killed by a blow on the head and both vas deferens removed and threaded through bipolar platinum electrodes. Preparations were set up in organ baths containing aerated Tyrode's solution at 32°C. Electrical stimulation was by rectangular pulses of supramaximal strength (usually 20V) and pulse width (usually 2 msec). Stimulation was applied for periods of 15 seconds at 5 minute intervals.

Finkleman (1930) preparation

The preparation was set up in the same way as for transmural stimulation of rabbit isolated ileum except that instead of a transmural electrode a simple glass or metal tissue holder was used.

Rabbit isolated ear artery preparation

The preparation was set up and electrically stimulated as described by De la Lande & Rand (1965). Rabbits were killed by a blow on the back of the neck and bled out. An ear was removed and a polyethylene cannula inserted into the central artery as near to the base as possible. The blood remaining within the vasculature of the ear was flushed out using Kreb's solution and the artery was then dissected free of the surrounding tissues and a length of 4-5 cm taken for perfusion. The artery was suspended in air and perfused with Kreb's solution at 37°C gassed with 5% carbon dioxide in oxygen. Perfusion pressures were recorded by means of a rat blood pressure mercury manometer (Palmer & Co.) Drugs were dissolved in the perfusion solution and either injected into the cannula or added to the resevoir of perfusion fluid.

The vascular sympathetic nerves were stimulated by threading the artery through bipolar platinum electrodes and delivering rectangular pulses of supramaximal voltage and pulse width (usually 50-60V and 2 msec). The frequencies of nerve stimulation are given in the Experimental Results section.

## Drugs used

The following drugs were used in this thesis:-

acetylcholine chloride (B.D.H.)

adenosine 5'- monophosphoric acid (AMP - Sigma)

adenosine 5'- diphosphate (ADP - Sigma)

adenosine 5'- triphosphate (ATP - Sigma)

adrenaline acid tartrate (B.D.H.)

L-ascorbic acid (B.D.H.)

γ-amino-n-butyric acid (GABA - Sigma)

atropine sulphate (B.D.H.)

barium chloride (B.D.H.)

bethanidine sulphate (B.W.)

2-bromo-lysergic acid diethylamide (BOL - Sandoz)

bufotenine mono oxalate $H_2O$ (Sigma)

carbachol (B.D.H.)

cinchocaine hydrochloride (Ward Blenkinsop)

cocaine hydrochloride (B.D.H.)

cyproheptadine hydrochloride (Merck)

dexamphetamine sulphate (Ward Blenkinsop)

dimethylphenylpiperazinium chloride (DMPP - B.D.H.)

dopamine hydrochloride (B.D.H.)

ephedrine hydrochloride (B.D.H.)

ergometrine maleate (B.D.H.)

ergotamine tartrate (Sandoz)

glycine (B.D.H.)

guanethidine sulphate (Ciba)

histamine acid phosphate (B.D.H.)

5-hydroxytryptamine creatinine sulphate (Sigma)

5-hydroxytryptophan (Sigma)

hyoscine hydrobromide (B.D.H.)

lignocaine hydrochloride (Therapharm.)

lysergic acid diethylamide (LSD - Sandoz)

mepyramine maleate (May & Baker)

methysergide (Sandoz)

morphine hydrochloride (May & Baker)

neostigmine methyl sulphate (Roche)

nicotine hydrogen (4) - tartrate (B.D.H.)

( -noradrenaline (Sigma)

pempidine tartrate (May & Baker)

pentobarbitone sodium (Macarthy)

pentolinium tartrate (May & Baker)

phentolamine mesylate (Ciba)

physostigmine sulphate (eserine - B.D.H.)

picrotoxin (Sigma)

procaine hydrochloride (B.D.H.)

propranolol (I.C.I.)

quinidine sulphate (Macarthy)

quinine sulphate (B.D.H.)

reserpine (B.D.H.)

reserpine injection (Halewood Chemical Co.)

strychnine sulphate (Sigma)

tetrodotoxin (Sankyo)

tetramethylammonium chloride (TMA - B.D.H.)

triethylcholine (B.D.H.)

urethane (B.D.H.)

xylocholine bromide (May & Baker)

All concentrations and doses given in the text of this thesis refer to the forms and salts listed above.

# PART 3

# EXPERIMENTAL RESULTS

## Chapter 1.

## The response to transmural electrical stimulation of rabbit isolated ileum

Inhibitory responses elicited by electrical stimulation of the vagal efferent nerves to the gastro-intestinal tract were first described towards the end of the nineteenth century by Langley (1898) and by Bayliss & Starling (1899). Similar observations have been reported at regular intervals over the years (see Historical Introduction for literature review). The nature of the inhibitory responses is not clear, but the original suggestion of Harrison & McSwiney (1936) that they are caused by admixture of vagal efferent with sympathetic efferent fibres is not substantiated by recent pharmacological evidence. Various workers (for instance Martinson & Muren, 1963; Burnstock, Campbell & Rand, 1966) have found these inhibitory responses to be unaffected by the specific adrenergic neurone blocking agents bretylium and guanethidine, thus suggesting a non-sympathetic origin.

Holman & Hughes (1965) have obtained biphasic responses consisting of inhibitory and motor components after transmural electrical stimulation of isolated intestinal preparations taken from mice, rats, guinea pigs and rabbits.

The first chapter of this thesis is concerned with characterising the response to transmural electrical stimulation in segments of rabbit isolated ileum. This tissue was chosen since it displays regular myogenic activity and its responses to various drugs and procedures have been well documented. In addition a preliminary comparison of the inhibitions to transmural and sympathetic stimulation has been made.

## RESULTS

### Transmural stimulation of rabbit isolated intestine

The response to transmural stimulation in segments of rabbit isolated ileum set up at 37°C usually consisted of mixed inhibitory and motor components. There was some variation between different preparations, but in general there were four main types of response. These are illustrated in Fig. 4. The most usual was a rapid and complete inhibition of spontaneous activity which changed, during the stimulation period, into a contraction which subsided at the end of the stimulus and was followed by inhibition of variable extent and duration (Fig. 4a). The second type of response commonly seen was identical, except that the secondary inhibition was absent or very slight (Fig. 4b).

The third type, seen in a few preparations, consisted of an immediate contraction followed, after the stimulation, by inhibition (Fig. 4c). The fourth type, seen in only about ten of an initial group of more than two hundred preparations, consisted solely of a contraction lasting throughout the stimulation period, followed by the return of normal spontaneous activity (Fig. 4d).

### Effect of local anaesthetics

A comparison was made between the action of local anaesthetic agents on the response to transmural and to sympathetic (periarterial) stimulation to determine whether the response to transmural stimulation was likely to be nervously mediated. The local anaesthetics used were lignocaine, procaine, cinchocaine and cocaine. These, with the occasional exception of cocaine, depressed the spontaneous

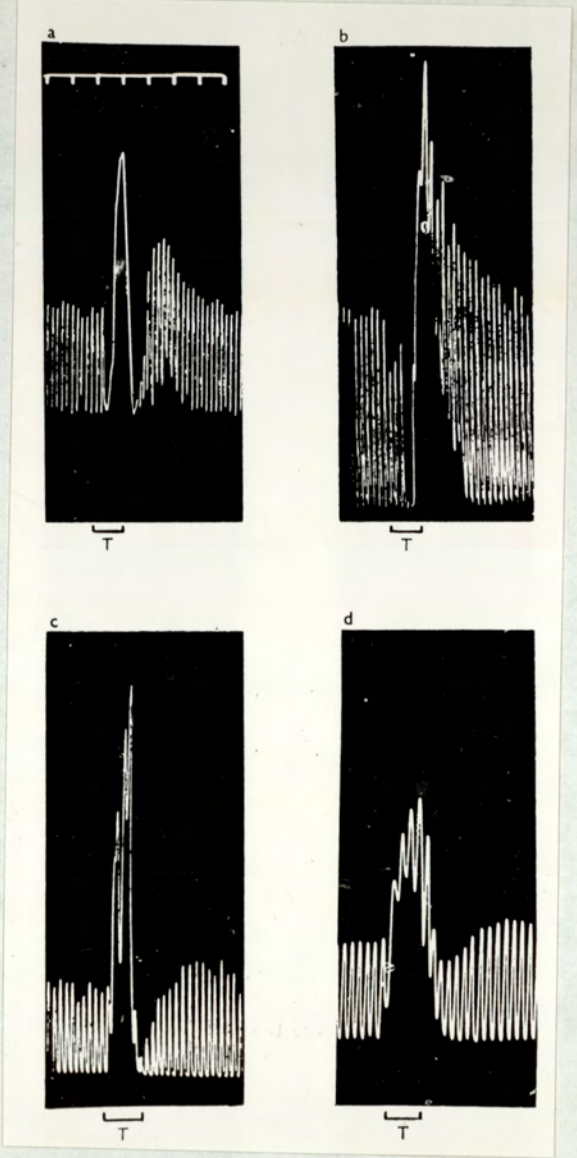


Fig. 4. Four main types of response to transmural stimulation (T) in different segments of rabbit isolated ileum in aerated Tyrode solution at 37°C. Stimulation applied for 20 sec with 1 msec 20V pulses at 20 Herz. Time marker in 30 sec intervals.

activity of the ileum in relatively low concentrations (4 to 50 µg/ml), thus making it impossible to establish with certainty whether the responses were impaired. In six experiments out of twelve in which cocaine (20-50 µg/ml) was used there was little impairment of the pendular movements, but the inhibitory and motor components of the transmural response were abolished as was the inhibition to sympathetic stimulation. The impairment of the responses caused by cocaine was partially reversed by washing. Fig. 5 illustrates one of the experiments in which cocaine (20 µg/ml) abolished the responses to periarterial sympathetic stimulation and to transmural stimulation without causing any impairment of the myogenic contractions of the preparation.

Effect of tetrodotoxin

Tetrodotoxin is known to cause a powerful yet reversible impairment of nervous conduction whilst producing little effect on smooth or skeletal muscle contractility. Tetrodotoxin (0.1 to 1 µg/ml) was tested on the responses to sympathetic and transmural stimulation in five experiments. In each experiment it abolished both responses but also reduced the height of the spontaneous contractions. This is illustrated in Fig. 6 where in the presence of tetrodotoxin (1 µg/ml) the responses to both sympathetic and transmural stimulation were abolished, but the pendular movements of the preparation were considerably reduced. The effects of tetrodotoxin on both nervous and muscular activity were only partially reversed by repeated washing of the preparation over a period of 45-60 minutes.

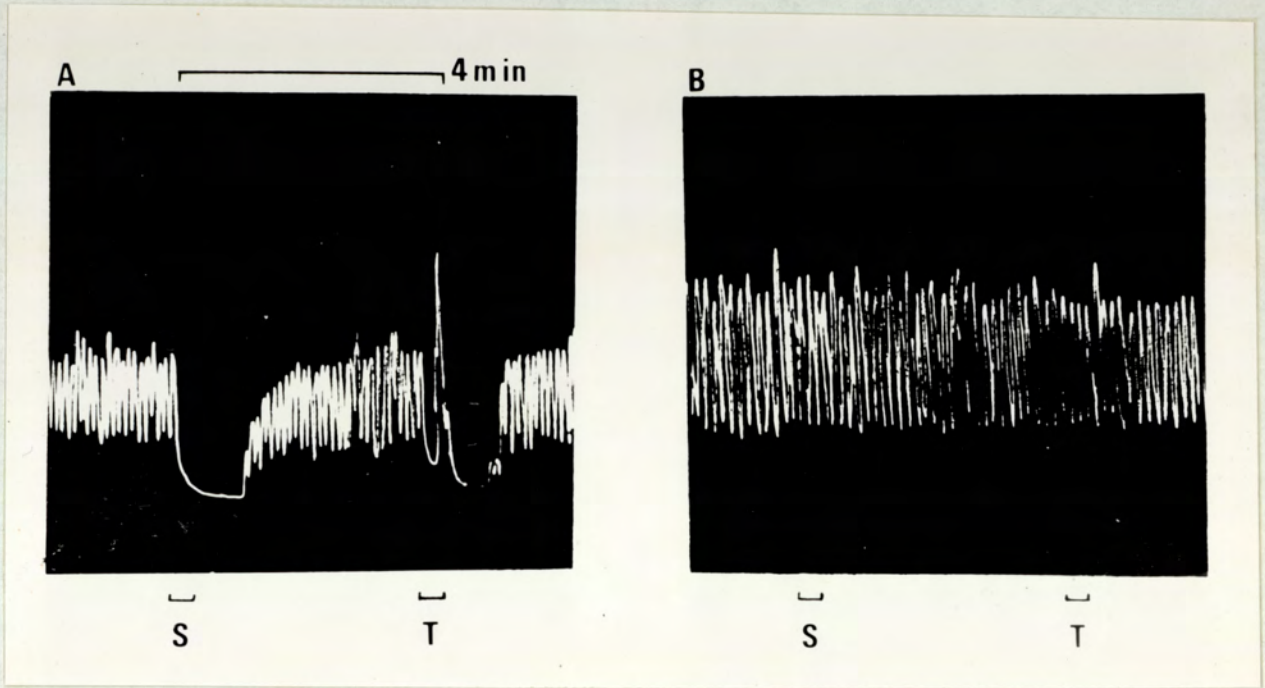


Fig. 5. Rabbit isolated ileum suspended in aerated Tyrode solution at 37°C. In A, responses to sympathetic (S) and transmural (T) stimulation applied for 20 sec in each case with 2 msec 20V pulses at 20 Herz. In B, responses repeated 25 min after the addition of cocaine (20 µg/ml) to the bathing solution.

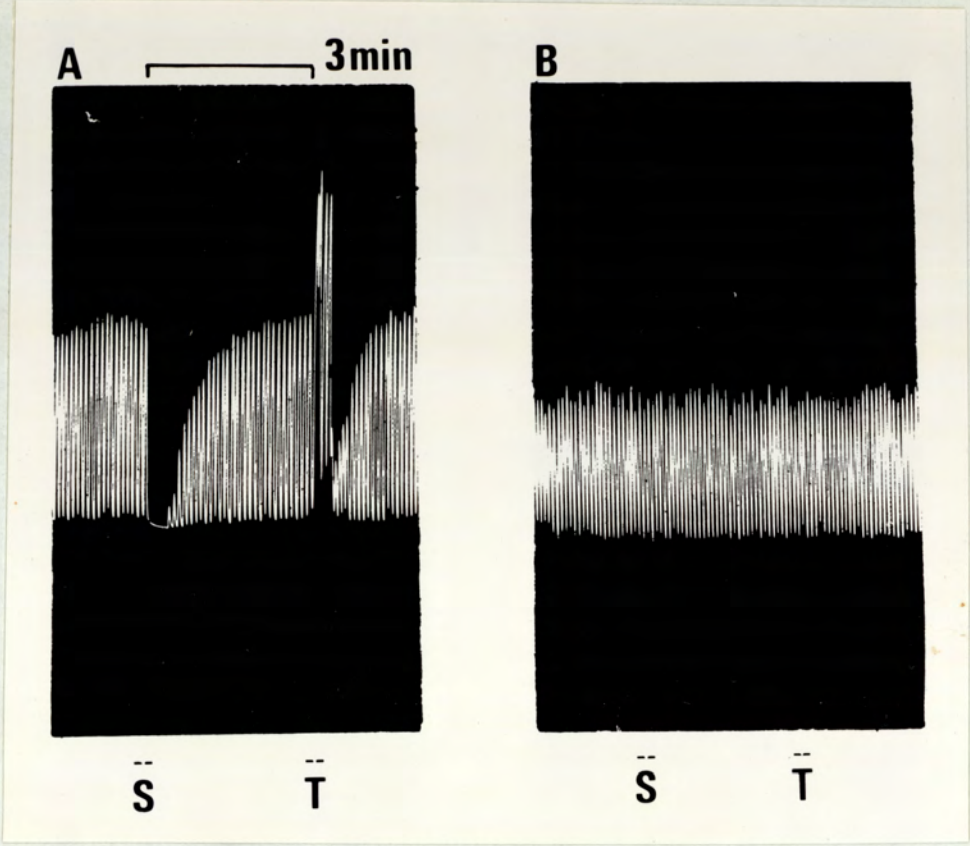


Fig. 6. Rabbit isolated ileum suspended in aerated Tyrode solution at 37°C. In A, responses to sympathetic (S) and transmural (T) stimulation applied for 20 sec with 2 msec 20V pulses at 20 Herz. In B, responses repeated 20 min after the addition of tetrodotoxin (1 µg/ml) to the bath.

Effect of altering the bath temperature

When the bath temperature was progressively lowered from 37°C to 28°C the response to transmural stimulation changed; the inhibitory phase became more prolonged while the motor phase was greatly reduced. In many experiments at 28°C the response to transmural stimulation was pure inhibition whereas at 37°C the motor component was more marked. This is illustrated in Fig. 7. At 28°C transmural stimulation produced an inhibitory response which outlasted the stimulation period. At 33°C a small motor component appeared in the response and the inhibitory phase was less prolonged, while at 37°C the main response during stimulation was contraction followed by inhibition. The motor responses to acetylcholine (0.01 to 0.04 µg/ml) were altered in the same way by lowering the bath temperature as was the motor component of the transmural response. Thus the responses were reduced by lowering the temperature from 37°C and were sometimes almost abolished at 28°C. This depression was completely reversed by returning the bath temperature to 37°C.

It was found that a more complete inhibition of the motor component to transmural stimulation was obtained by starting an experiment at 28°C rather than by reducing the temperature from 37°C or 33°C in preparations in which motor responses were already well developed. Thus, in those experiments in which the temperature was raised from 28°C to 37°C and in which a strong motor component developed, the reduction of the motor component on returning the bath temperature to 28°C was usually not as complete as that

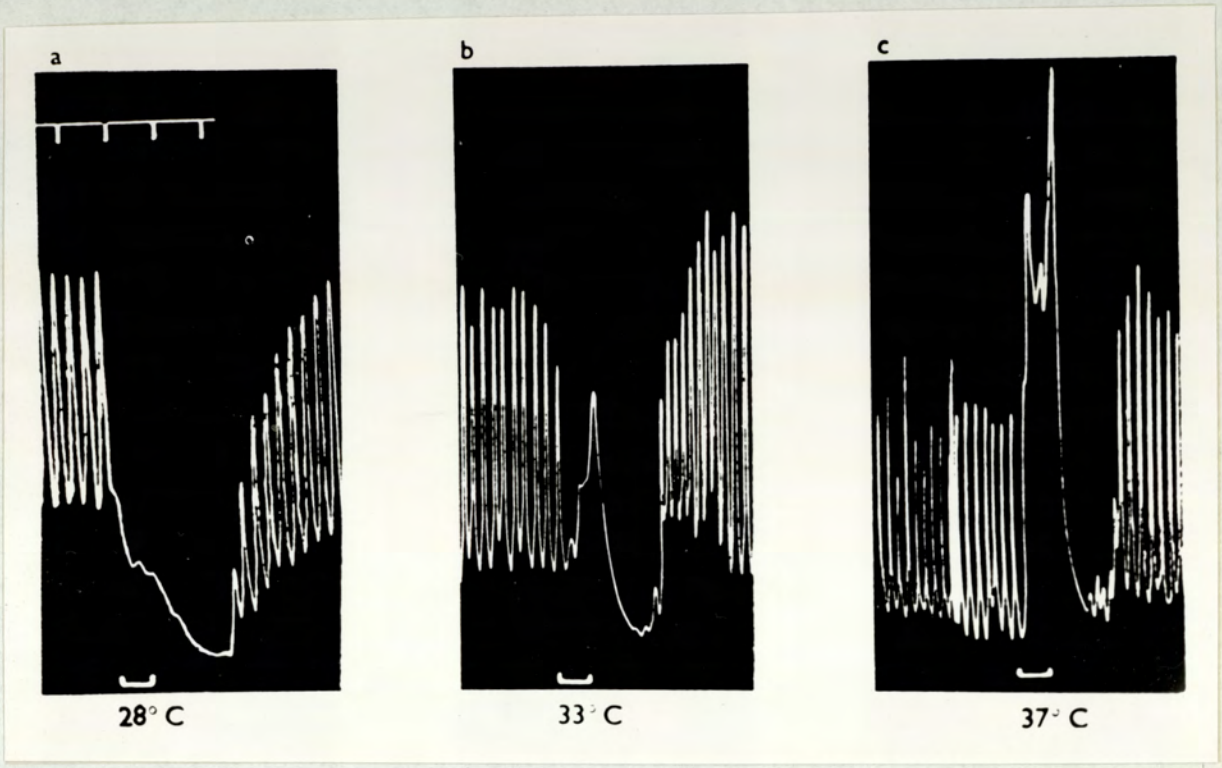


Fig. 7. Effect of raising the bath temperature on the response to transmural stimulation in a segment of rabbit isolated ileum suspended in aerated Tyrode solution. Transmural stimulation (at ⎵) applied for 20 sec with 2 msec pulses of supramaximal strength at a frequency of 20 Herz. Time marker in 30 sec intervals.

observed at this temperature at the beginning of the experiment.

## Comparison between the stimulus parameters for transmural and sympathetic inhibition

In some preparations the relaxations obtained by stimulating the sympathetic nerves were compared with those obtained by transmural stimulation in order to investigate the possibility of a common origin for these responses. It was found that the optimal frequency of stimulation for transmural inhibition was lower (10 to 20 Herz) than for sympathetic inhibition (50 Herz). It was, however, difficult to obtain a valid comparison between the two responses because the motor component of the transmural response varied in different preparations and at different frequencies and may therefore have influenced the inhibitory response in a variable manner.

In several preparations the relaxation to transmural stimulation was larger at a stimulus frequency of 50 Herz than it was at 20 Herz. In these experiments the addition of guanethidine (1-10 µg/ml) to the bath abolished the effects of sympathetic stimulation and reduced the optimal frequency for transmural inhibition to 20 Herz. Thus, in some experiments at least, there may have been a sympathetic component to the transmural inhibition. Submaximal inhibitions to transmural stimulation could be elicited by frequencies of stimulation (1 to 5 Herz) which in most experiments were too low to cause inhibition to sympathetic stimulation.

The motor component of the response to transmural

stimulation was fully developed usually at a frequency of 20 Herz. In most experiments increasing the frequency to 50 Herz did not increase this part of the response.

The threshold pulse-width for both sympathetic and transmural inhibition was of the order of 0.1 msec. Pulse-widths of 0.5 msec - 0.1 msec tended to increase the inhibitory component and decrease the motor component of the transmural response. In most preparations a pulse-width of 1 msec was used for transmural stimulation because this was supramaximal for both inhibitory and motor components of the response.

To obtain maximal responses at any given pulse-width and frequency for both sympathetic and transmural stimulation the necessary voltage was usually between 5 and 10. A supramaximal voltage (20 V) was used in all experiments for both sympathetic and transmural stimulation.

## DISCUSSION

The results presented in this chapter confirm the observations of Holman & Hughes (1965) that transmural electrical stimulation elicits a complex response consisting usually of both motor and inhibitory components. The fact that inhibitions of myogenic activity elicited by either periarterial sympathetic nerve stimulation or by transmural stimulation were abolished by similar concentrations of local anaesthetics and tetrodotoxin is convincing evidence that both responses are neurogenically mediated.

The observation that lowering the bath temperature significantly depressed the motor component of the transmural response provides a useful mechanism whereby the transmural

inhibition can be revealed more clearly. Moreover, the parallel depression of the contractile action of acetylcholine and the transmural motor response suggests that the motor effect may be mediated, partly at least, by cholinergic neurones within the muscle wall.

Some evidence has been presented which suggests that the inhibitions caused by sympathetic and transmural stimulation are mediated via different sets of neurones. Thus, the optimal frequency of stimulation for transmural inhibition was usually lower than that for sympathetic inhibition. These experiments were complicated firstly, by the motor component of the transmural response which was of variable size and duration, and secondly, by what appeared to be a sympathetic, i.e. partly guanethidine-sensitive, component of the transmural response. However, in a few experiments inhibitions to transmural stimulation were elicited by very low frequency stimulation (1 or 2 Herz) which was always ineffective in causing sympathetic inhibition.

In summary, transmural electrical stimulation of rabbit isolated ileum appears to activate at least three nervous pathways, i.e. motor neurones causing intestinal contraction, sympathetic and non-sympathetic neurones causing inhibition of gastro-intestinal activity.

## Chapter 2

## Pharmacological analysis of the responses to transmural stimulation of rabbit isolated ileum: (a) Comparison of inhibitory responses to transmural and sympathetic stimulation

In the previous chapter the complex response to transmural stimulation in rabbit isolated ileum was described and evidence presented which strongly suggested that the response was nervously mediated. The present chapter is devoted to experiments designed specifically to determine the contribution of sympathetic activation to the transmural inhibitory responses and to further characterise the nature of the apparently non-adrenergic inhibitory responses in this preparation.

### RESULTS

#### Effect of chronic sympathectomy

Preparations were taken from rabbits in which the blood vessels and nerves to segments of ileum were sectioned at operation 12-14 days previously. It was observed at operation that these segments of ileum maintained an adequate blood perfusion from small vessels branching within the muscle wall from mesenteric vessels perfusing adjacent segments of ileum. The "denervated" segment was identified by leaving threads attached to the sectioned mesenteric vessels. Six preparations set up in this way from three operated rabbits showed normal myogenic activity but in each preparation stimulation of the sectioned periarterial bundle produced no inhibition of the myogenic activity. Fig. 8 illustrates one such experiment. Transmural stimulation in all these preparations produced the usual mixed response consisting of inhibitory and motor components.

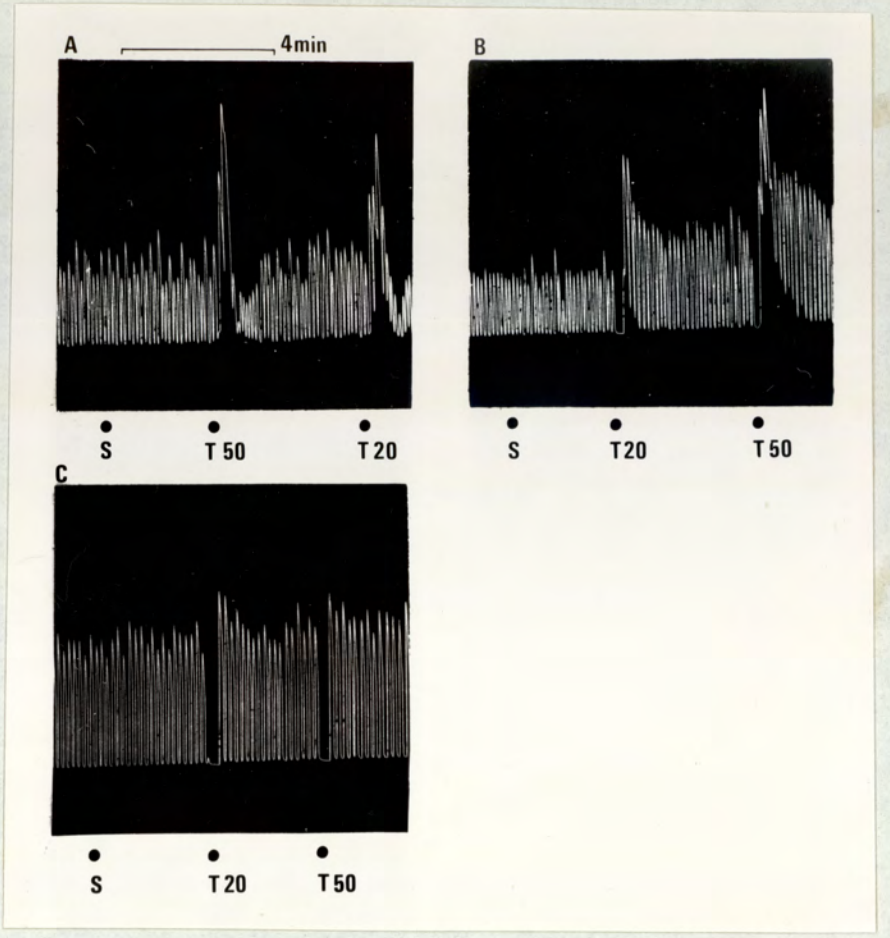


Fig. 8. Isolated ileum taken from rabbit in which the periarterial (sympathetic) nerves to the test segment were sectioned under anaesthesia 14 days previously. Preparation set up in aerated Tyrode solution at 28°C. In A, responses to stimulation of the sectioned periarterial bundle (S) with 2 msec 20V pulses at 50 Herz and transmural stimulation (T) with the same parameters at 50 and 20 Herz. The responses were repeated 20 min after adding a mixture of propranolol and phentolamine (2 µg/ml of each) to the bath (panel B) and again 20 min after the further addition of hyoscine (2 µg/ml) (panel C).

## Experiment to test for "overlap" of sympathetic innervation from one segment of ileum to another

One objection to the chronic denervation type experiment described above is that post ganglionic sympathetic neurones could follow the same course as the blood vessels which perfuse the test segment of ileum even after all the visible mesenteric vessels have been sectioned. If this were so then it would still be possible for transmural stimulation to release noradrenaline from these neurones even after chronic section of all the extrinsic neuronal connections.

The simple experiment illustrated in Fig. 9 was designed to test this hypothesis. It was found in three separate experiments that when the periarterial nerves to each part of a double normal length segment of ileum were stimulated in turn at a frequency (50 Herz) normally causing complete inhibition of myogenic activity, there was a similar but incomplete inhibition in each case. One periarterial nerve bundle was then stimulated at maximal frequency until the inhibition produced completely subsided, presumably due to exhaustion of transmitter output. At this time stimulation was re-applied through the other electrode and the inhibitory response produced was identical to that elicited initially with this electrode. This experiment would seen to indicate that each periarterial nerve bundle was innervating the segment of ileum to which it was attached and that no significant "overlap" occurred.

### Depletion of sympathetic transmitter

Two methods of abolishing sympathetic effects by removal of the sympathetic transmitter were attempted. The

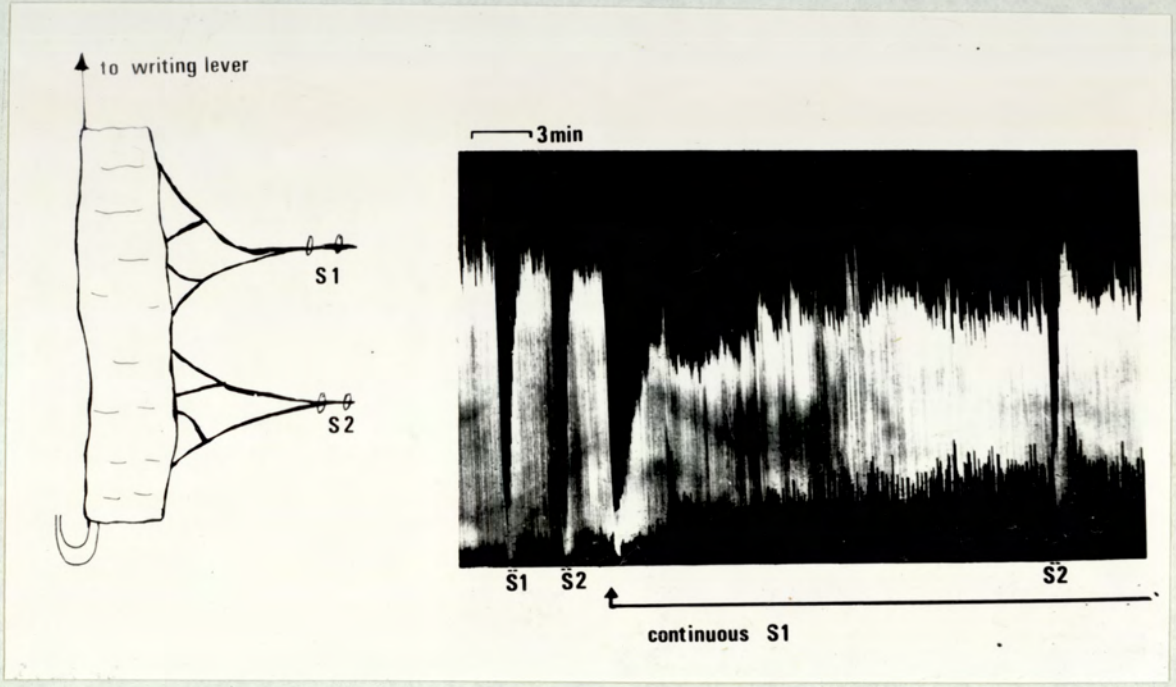


Fig. 9. Rabbit isolated ileum "double" segment set up as per diagram on left in aerated Tyrode solution at 37°C. Stimulation with 2 msec 20V pulses at 50 Herz through either electrode S1 or S2 produced partial inhibition of pendular movements. Continuous stimulation through electrode S1 produced failure of inhibitory response but did not affect the response to stimulation through S2 applied simultaneously.

first was by prolonged stimulation of the sympathetic (periarterial) nerves until the inhibitory effects on the spontaneous activity subsided; the second was by using the noradrenaline-depleting agent reserpine.

(a) Prolonged sympathetic stimulation

In these experiments failure of sympathetic inhibition was produced by stimulating the periarterial sympathetic nerves at the optimal frequency for inhibition of myogenic activity (50 Herz) until the pendular movements returned despite the continued stimulation. The failure of sympathetic responses produced in this way is unlikely to be due to nervous damage since undiminished inhibitory responses to sympathetic stimulation could be obtained again after resting the preparation. Similarly, the sympathetic failure was not due to desensitization of the tissue to transmitter substance since noradrenaline produced an undiminished inhibitory effect on the tissue at a time when sympathetic stimulation was ineffective. One such experiment is illustrated in Fig. 10 which is the record of a preparation maintained at 28°C. Control inhibitions were obtained to added noradrenaline and to sympathetic and transmural stimulation. There was an initial motor component to the transmural response in this preparation which was sometimes seen at this bath temperature. However, this motor component was of much briefer duration than the usual response at 37°C. When continuous sympathetic stimulation was applied there was virtually complete inhibition of the pendular movements for about 40 minutes and then they slowly recovered towards normal. At 65 minutes the response to transmural stimulation

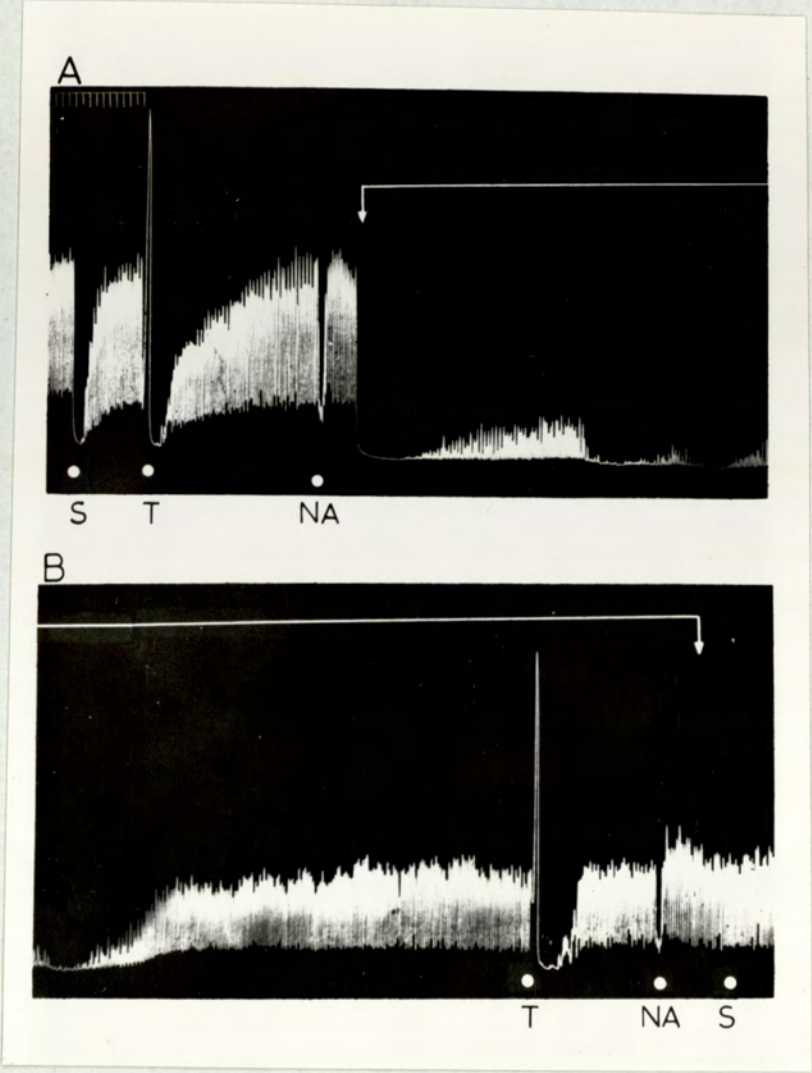


Fig. 10. Rabbit isolated ileum in aerated Tyrode solution at 28°C. In A control responses to sympathetic stimulation (at S) applied for 20 sec with 2 msec 20 V pulses at frequency of 50 Herz, transmural stimulation (at T) with 2 msec 20V pulses at 20 Herz, and added noradrenaline (NA) in concentration of 0.01 µg/ml with a 30 sec contact time. The sympathetic nerves were stimulated with the same parameters as at S throughout the period enclosed by the white bar. In B the responses to transmural stimulation and to noradrenaline were repeated during continuous stimulation of the sympathetic nerves. Another 20 sec period of sympathetic stimulation (at S) was applied a few minutes after the end of the prolonged stimulation in B. Time marker in one minute intervals.

was retested; the motor component was unaffected and the inhibitory component was slightly prolonged in duration. In three other experiments a similar result was obtained and in each case a small but definite increase in the duration of the transmural inhibition occurred after failure of the sympathetic responses.

A criticism of the above type of experiment (W.A. Bain, personal communication 1967) is that the return of the spontaneous contractions during the prolonged period of sympathetic stimulation could have been caused by local nerve damage at the point of stimulation rather than by the depletion of transmitter from sympathetic nerve terminals. This possibility was investigated in a further series of 12 experiments in which either the electrode was moved nearer the muscle after nerve failure or two electrodes were placed on the same periarterial bundle and stimulation applied through the distal electrode until the myogenic contractions returned and then the effects of stimulating through the proximal electrode were tested. In 4 experiments from this series of 12 some evidence of nerve "damage" was produced since moving the electrodes closer to the muscle or stimulating through the proximal electrodes increased the responses. However, there were two main differences between the experiments in which nervous damage occurred and the other experiments such as that illustrated in Fig. 10. Firstly, when nervous damage occurred it did so after only 5-10 min. of stimulation whereas in the experiment illustrated in Fig. 10 the response declined slowly over an hour or so. Secondly, the local nerve effects were irreversible whereas the failure produced in the other experiments was reversible after resting the preparation.

(b) Reserpine treatment

Reserpine was either administered intravenously to rabbits for several days before the experiment (for details see Experimental Methods), or was added to the bath containing the tissues.

In 32 preparations of ileum set up at 32°C or 37°C and taken from 11 rabbits treated with reserpine, the inhibitory responses to sympathetic stimulation were impaired but not abolished. The inhibitory component of the transmural response also seemed to be impaired, but this could have been caused by an apparent enhancement of the motor component noticed in these preparations which might tend to mask the inhibitory component of the response.

Clearer results were obtained in 16 other preparations taken from untreated rabbits and set up at 32°C in order to reduce the motor phase of the response. In these experiments reserpine (0.5 -1.0 µg/ml) was added to the bath and caused a slowly developing impairment of the inhibitory responses to both sympathetic and transmural stimulation which were both, in most experiments, completely abolished after contact with the drug for 3-4 hours. In these experiments dopamine (50 µg/ml) partially reversed the sympathetic nerve block but produced little or no enhancement of the inhibitory component of the transmural response.

This observation is illustrated in Fig. 11. In this experiment the inhibitory component of the transmural response and the sympathetic inhibition were abolished after contact for 225 min. with reserpine (0.5 µg/ml). Dopamine (50 µg/ml) was added to the bath and left for 45 min. after which time the sympathetic but not the transmural inhibition was largely restored.

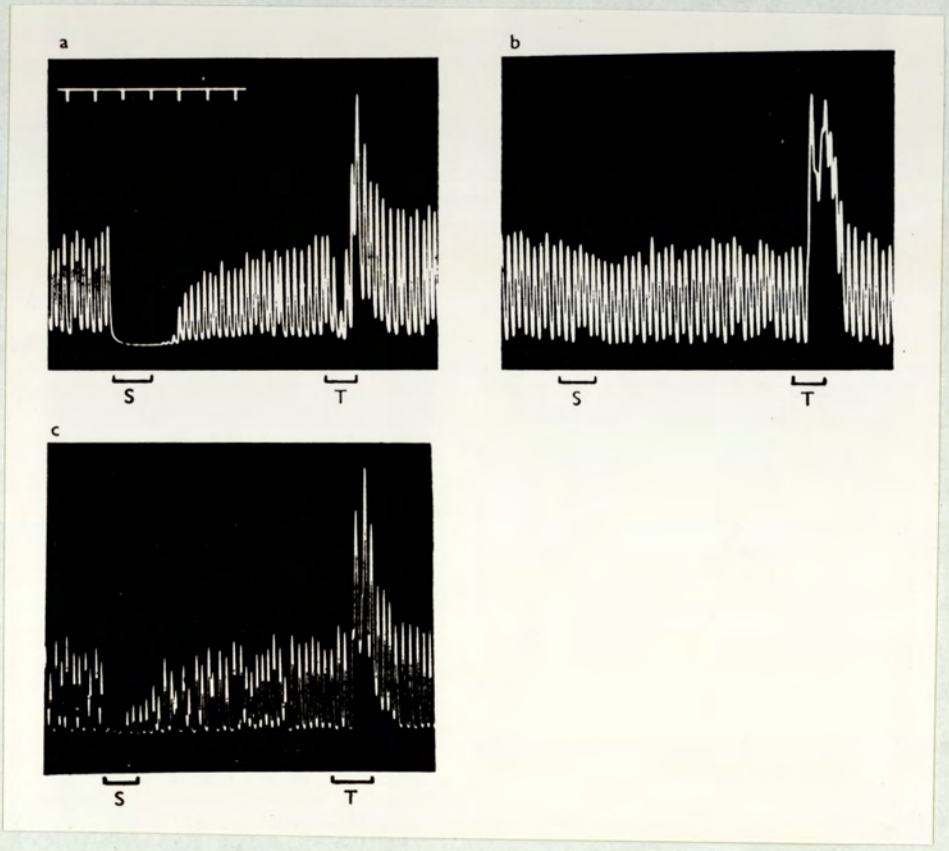


Fig. 11. Rabbit ileum at 32°C. A: control responses to sympathetic stimulation (S) with 1 msec 20V pulses at a frequency of 50 Herz and transmural stimulation (T) with 1 msec 20V pulses at 20 Herz each applied for 20 sec periods. B: the same responses repeated 225 min after the addition of reserpine (0.5 µg/ml) to the bath. Between B and C dopamine (50 µg/ml) was added to the bath and the responses were repeated 45 min later in C. Hyoscine (1 µg/ml) was present in the bath throughout the experiment. Time marker in 30 sec intervals.

Another difference between the inhibitions to sympathetic and transmural stimulation noticed in these experiments lay in the difference in onset of the blocking action of reserpine. Although as previously mentioned the time for complete abolition of both responses was similar (3-4 hr.) the onset of the impairment of the transmural inhibition was usually considerably more rapid. This is illustrated in Fig. 12 which is a preparation set up at 32°C and additionally treated with hyoscine (1 µg/ml) to further reduce the motor component of the transmural response. The addition of reserpine (1 µg/ml) to the bath caused a significantly more rapid impairment of the inhibitory responses to transmural stimulation.

Adrenergic neurone blocking agents

Guanethidine (1-10 µg/ml) or xylocholine (3-20 µg/ml) when added to the bath abolished the inhibitory responses to sympathetic stimulation while in most preparations producing little or no impairment of the inhibitory component of the transmural response. This is shown in the experiment illustrated in Fig. 13 which is of a preparation maintained at 32°C in order to enhance the inhibitory component and depress the motor component of the transmural response.

Between Fig. 13a and 13b the preparation was left in contact with a high concentration (10 µg/ml) of guanethidine for 45 min. In Fig. 13b the sympathetic response was abolished while the inhibition to transmural stimulation was only slightly reduced. In preparations maintained at 37°C the motor response to transmural response was more prominent than at lower temperatures and it was not significantly altered by even high concentrations of guanethidine or xylocholine.

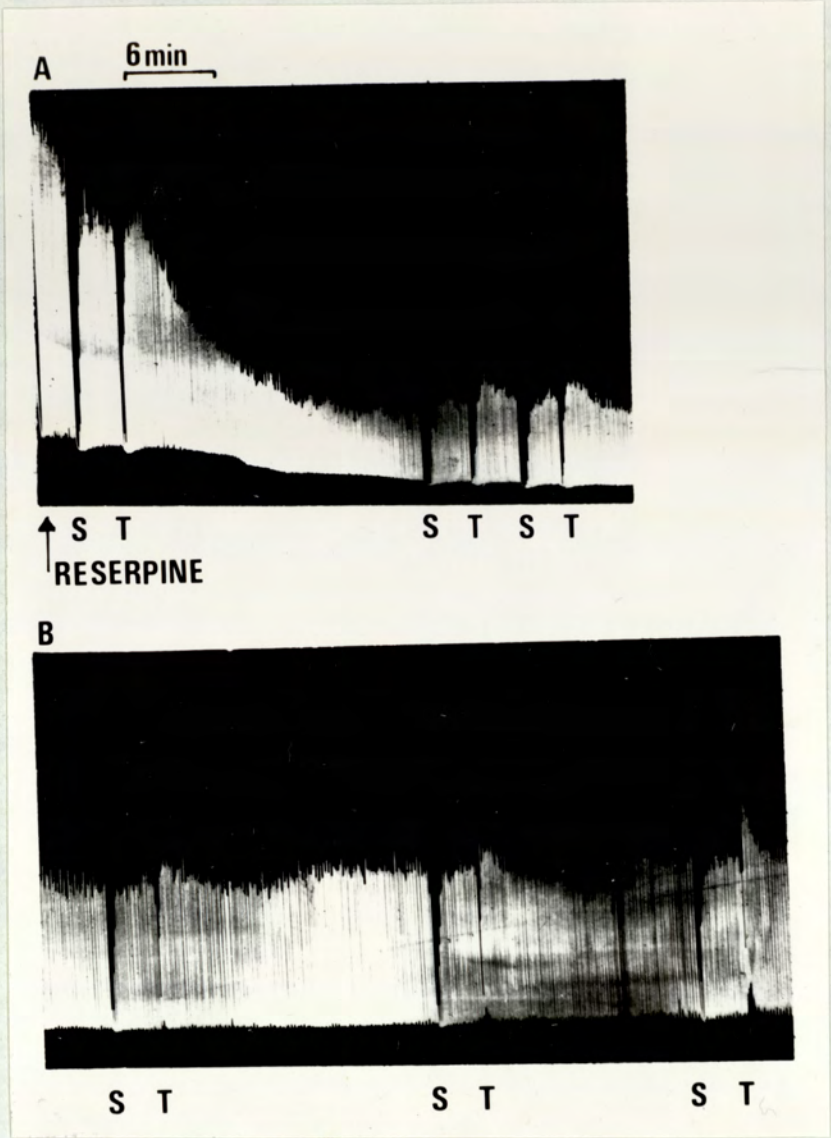


Fig. 12. Rabbit isolated ileum suspended at 33°C in McEwen's solution gassed with 5% $CO_2$ in $O_2$.
Sympathetic (S) and transmural (T) stimulation applied for 20 sec with 2 msec 20V pulses at 20 Herz. Reserpine (1 µg/ml) added in A and the record in B was commenced 40 min from the end of A. Hyoscine (1 µg/ml) was present throughout the experiment.

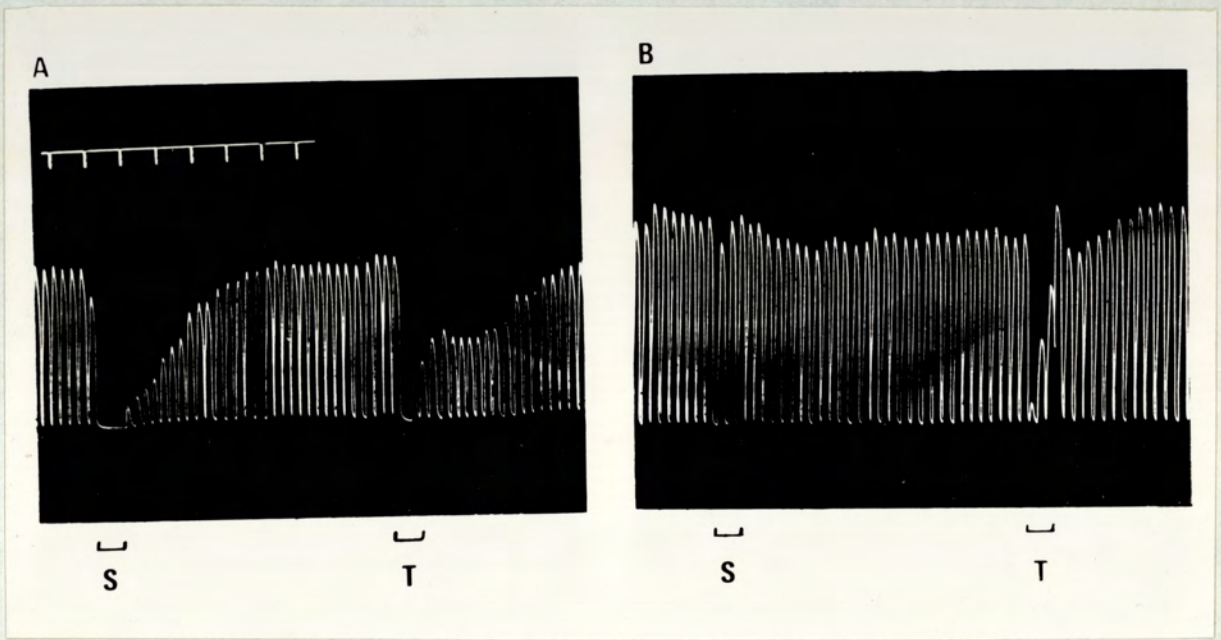


Fig. 13. Rabbit isolated ileum at 32°C. In A, sympathetic stimulation (S) with 2 msec 20V pulses at a frequency of 50 Herz applied for 20 sec, and transmural stimulation (T) for 20 sec with 0.5 msec 20V pulses at a frequency of 20 Herz. In B, same responses 45 min after adding guanethidine (10 µg/ml) to the bath. Time marker in 30 sec intervals.

In a few preparations at 37°C, guanethidine caused a marked impairment of the inhibitions to transmural stimulation. The block could, however, be distinguished from the sympathetic nerve blockade by the fact that it could be reversed by lowering the bath temperature by 4° - 7°C, whereas this did not reverse the sympathetic blockade.

Adrenoceptor blocking substances

Inhibition of the isolated intestine by sympathetic stimulation and by added noradrenaline is caused by the activation of both α and β receptors. Thus, a mixture of both α and β receptor blocking substances is necessary to abolish these effects (Furchgott, 1960). An experiment in which a mixture of adrenoceptor blocking substances was tested on the inhibitory responses to transmural and sympathetic stimulation and to added noradrenaline is shown in Fig. 14. In this experiment the bath temperature was maintained at 32°C and the response to transmural stimulation was predominantly inhibitory. The addition to the bath of a mixture of phentolamine (1 μg/ml) and propranolol (2 μg/ml) abolished the inhibitory responses to sympathetic stimulation and to added noradrenaline, but the transmural inhibition was unaffected in size and slightly more prolonged in duration than before the blocking drugs.

In some experiments propranolol (1-3 μg/ml) was added to the bath alone. It was found that this resulted in a slowly developing impairment of the responses to sympathetic stimulation which closely resembled the effects of the adrenergic neurone blocking agents. In these experiments the inhibitory responses to added noradrenaline were not reduced at a time when the sympathetic inhibitions were

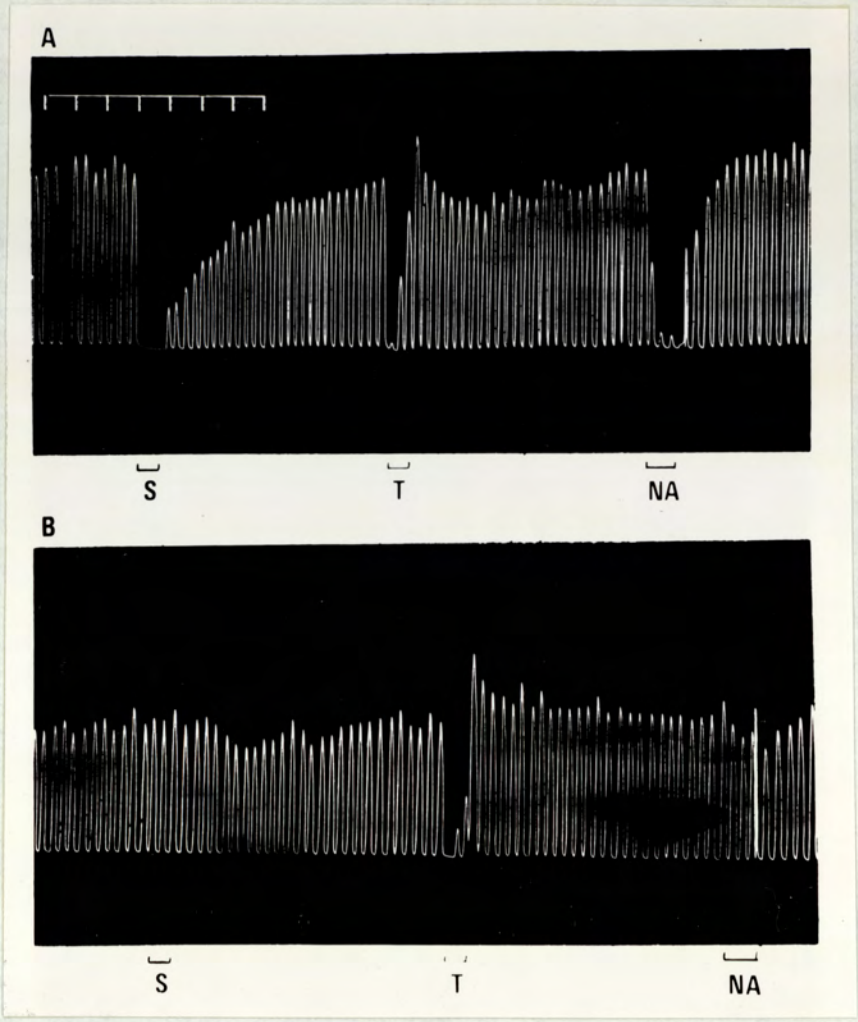


Fig. 14. Rabbit isolated ileum in aerated Tyrode solution at 32°C. A: control responses to sympathetic stimulation (S) applied for 20 sec with 2 msec 20V pulses at 50 Herz, transmural stimulation (T) with 0.5 msec 20V pulses at 20 Herz and added noradrenaline (NA) in a concentration of 0.02 µg/ml left in contact for 30 sec. B: same responses repeated 30 min after adding a mixture of propranolol (2 µg/ml) and phentolamine (1 µg/ml) to the bath. Time marker in 30 sec intervals.

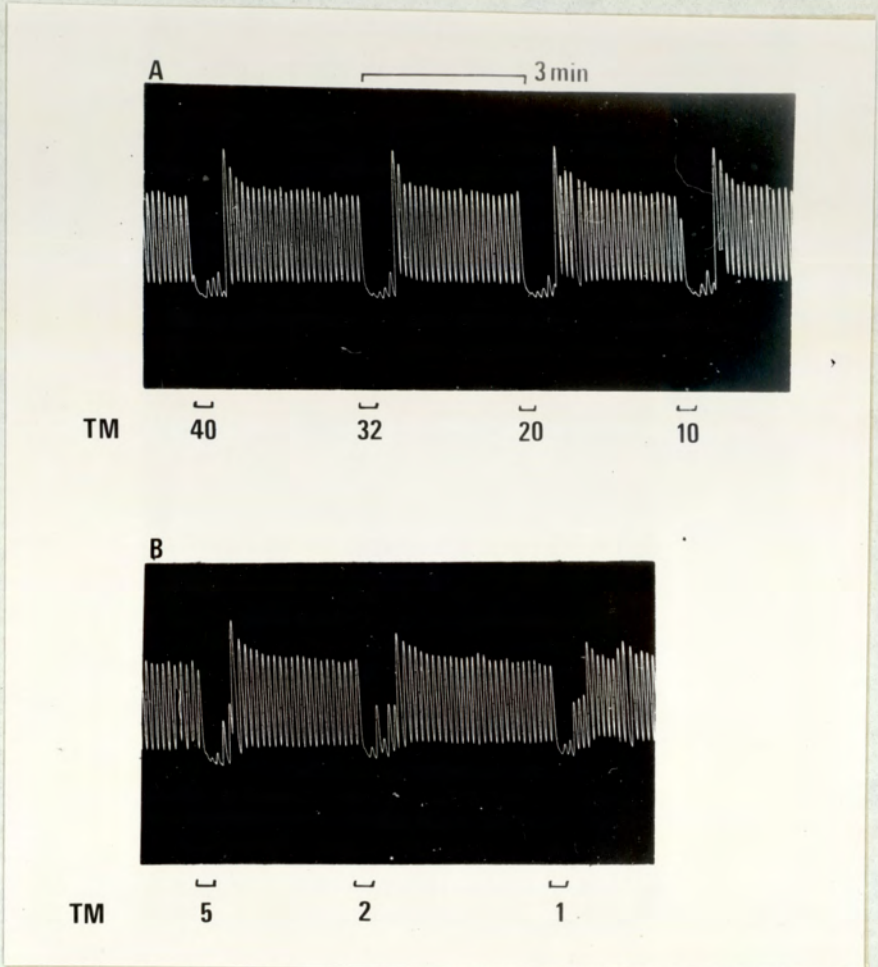


Fig. 15. Rabbit isolated ileum suspended in aerated Tyrode solution at 32°C. Hyoscine (1 µg/ml), propranolol (1 µg/ml) and phentolamine (1 µg/ml) were present throughout the experiment. Transmural stimulation was applied at the frequency indicated for 20 sec periods with 2 msec 20V pulses.

abolished. Thus, propranolol exerts a pre-synaptic as well as post-synaptic effect in this preparation. Since this action of propranolol may be of importance in both its experimental and clinical use a further analysis of it has been made (Chapter 10).

Following treatment with a mixture of α and β adrenoceptor blocking substances a re-examination of the variation of the transmural inhibitions at different frequencies was undertaken. In general, the results reported in Chapter 1 were confirmed, i.e. the threshold for transmural inhibition was much lower than that for sympathetic inhibition in control preparations.

This is illustrated in Fig. 15 which is the record from a preparation set up at 32°C and treated throughout with hyoscine to reduce the motor component and a mixture of α and β adrenoceptor substances to remove sympathetic influence. Maximal inhibitions of the myogenic activity occurred with transmural stimulus frequencies of 5 Herz but the response was very well developed at only 1 Herz.

## DISCUSSION

The results presented in this chapter strongly suggest a different origin for the inhibitory responses to transmural and sympathetic (periarterial) nerve stimulation. The denervation experiments are open to the usual criticism of this type of experiment, namely, that denervation may not have been complete for a variety of reasons. No attempt has been made to examine the "denervated" tissues histochemically for the absence of sympathetic innervation. However, sympathetic stimulation following chronic section

of the periarterial bundle was totally without effect on the pendular movements whilst transmural stimulation caused an inhibitory response very similar to those recorded in control preparations. Similarly, the experiments in which prolonged sympathetic stimulation was used to exhaust transmitter supplies are also open to criticism. The most important (Bain, 1967 personal communication) being the possibility of local nerve damage caused by the very prolonged period of stimulation. In fact some evidence of a local effect on sympathetic nerve transmission was obtained in a minority of experiments and therefore the possibility exists that nerve damage could have been a contributing factor to the sympathetic failure in all these experiments. Nevertheless, the evidence from this group of experiments is strongly suggestive of a different origin for the sympathetic and transmural inhibitions. The results obtained using drugs having a known effect on sympathetic neuro-effector transmitter mechanisms were even more strongly in support of this hypothesis.

Preparations taken from reserpine-treated animals showed markedly reduced inhibitory responses to sympathetic stimulation and also to transmural stimulation. However, the motor component of the transmural response appeared to be enhanced and this could have lead to an obscuring of the transmural inhibitory response rather than to a true blockade. Somewhat clearer results were obtained in the experiments in which reserpine was added to the bath containing the tissue. In these preparations the motor component was reduced by maintaining the bath temperature at 32°C and by using hyoscine

in the bathing solution (see Chapter 3 for effects of atropine-like substances on transmural stimulation). Acutely administered reserpine caused a slowly developing block of the inhibitory responses to both sympathetic and transmural stimulation which could however be distinguished in two ways. Firstly, the responses to transmural stimulation were more rapidly impaired by reserpine, and secondly, only sympathetic responses were restored by dopamine. However, the fact that transmural inhibition was reduced by reserpine suggests the possibility that neuro-effector transmission in this system may be mediated via a biogenic substance depleted by reserpine.

The adrenergic neurone blocking substances guanethidine and xylocholine which specifically prevent noradrenaline release from sympathetic nerve terminals were highly effective in preventing sympathetic inhibition whilst having, in most experiments, virtually no effect on transmural inhibition. However, as reported in Chapter 1, guanethidine sometimes reduced the magnitude of the transmural inhibition and reduced the optimal frequency for inhibitory responses. It was found that the most selective removal of sympathetic influence was treatment with a mixture of $\alpha$ and $\beta$ adrenoceptor blocking substances. When this treatment was combined with a bath temperature of 32°C and in the presence of hyoscine, the transmural inhibition was very fully revealed (for instance, Fig. 15). Under these conditions the sensitivity of the transmural inhibition to differing frequencies of stimulation was strikingly revealed. The optimal and threshold stimulation frequencies for transmural inhibition were both clearly much lower than for sympathetic inhibition.

## Chapter 3

### Pharmacological analysis of the responses to transmural stimulation of rabbit ileum: (b) Drugs affecting the motor component

As described in the preceding chapters the inhibitory response to transmural stimulation can be quite well differentiated from that to sympathetic stimulation by the use of drugs and other procedures. However, it is clear that in many of the experiments so far described that the response to transmural stimulation is complex consisting of several components including a major motor effect. Similarly, it is apparent that the inhibitory component of the transmural response may be changed by factors affecting predominantly the motor component. For instance, in preparations taken from reserpinised rabbits the motor component of the transmural response was enhanced and consequently it was difficult to determine whether the inhibitory component was impaired by reserpine or merely obscured by the more prominent motor component.

It has long been known that motor fibres innervating the intestine and having their cell bodies in Auerbach's plexus are predominantly cholinergic. However, the contractile response of the intestine elicited by indirect stimulation of these cell bodies with nicotine shows varying susceptibility to atropine. Thus, Ambache & Edwards (1951) found that the contractile action of nicotine on isolated intestinal preparations from the kitten were abolished by atropine but not similar responses in preparations from the rabbit. However, in both species the motor effect of nicotine was abolished by botulinum toxin suggesting a major cholinergic component (Ambache 1951).

More recently evidence has been published which suggests that atropine-insensitive responses to transmural stimulation of isolated intestinal preparations may be due to release of spasmogens from non-cholinergic neurones in Auerbach's plexus (Ambache & Freeman, 1968). Another hypothesis which has been proposed to explain the atropine-resistant responses to transmural stimulation is that the contractions are a "rebound" phenomenon and are a consequence of the initial inhibitory phase (Campbell, 1966a). This interesting suggestion is examined more closely later in this thesis (Chapter 9).

The work in this chapter was based on the assumption, formed during many early experiments, that the contractile response to transmural stimulation was mediated predominantly via cholinergic neurones. Accordingly, procedures and drugs have been used to reduce the spasmogenic effects of acetylcholine with the object of revealing more clearly the nature of the transmural inhibitory responses.

## RESULTS

### Effect of atropine and hyoscine

The effects of both atropine and hyoscine in concentrations ranging from 0.1 to 100 µg/ml were tested on the responses to transmural stimulation. Atropine in concentrations above 0.1 µg/ml frequently inhibited spontaneous activity of the gut as noticed by Holman & Hughes (1965). Hyoscine rarely caused this effect and was therefore used in most experiments. In preparations maintained at 37°C in which the motor component of the transmural response was well marked, hyoscine potentiated the initial

inhibitory phase of the response but usually had little effect on the motor component. In those preparations in which an initial inhibition was absent (for instance Fig. 4c), or in which the response was entirely motor (for instance Fig. 4d), hyoscine revealed an initial inhibitory component. This observation is illustrated in Fig. 16 where the transmural response was converted by hyoscine (1 µg/ml) from pure motor to initial inhibition followed by a reduced motor effect. In this experiment there was a slight impairment of the response to sympathetic stimulation after hyoscine.

In preparations maintained below 37°C the motor component of the transmural response was usually much less well marked and was relatively more inhibited by hyoscine. The experiment illustrated in Fig. 17 shows the effect of hyoscine on the response to added acetylcholine and to transmural stimulation in a preparation maintained at 32°C. In Fig. 17a the stimuli for the transmural response were altered in order to get graded motor effects. In Fig. 17b, after contact for 15 min. with hyoscine (1 µg/ml), the response to added acetylcholine was abolished and transmural stimulation then produced only inhibition.

Anticholinesterases

The addition of physostigmine (eserine) or neostigmine to the bath in concentrations of 0.05 to 0.1 µg/ml markedly enhanced the motor component of the transmural response with a consequent masking of the inhibitory component. Fig. 18 illustrates an experiment in which the response to transmural stimulation was either purely inhibitory or biphasic according to the intensity of the stimuli used. The addition

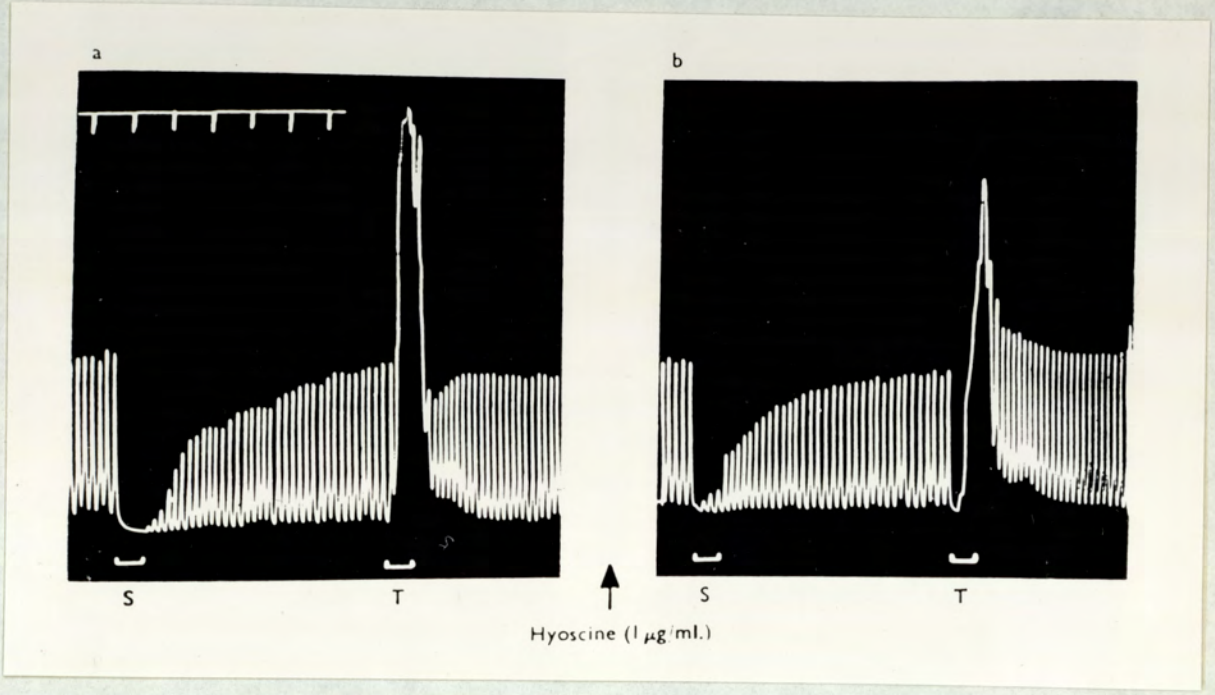


Fig. 16. Rabbit ileum at 37°C. A: control responses to sympathetic stimulation (S) with 2 msec 20V pulses at 50 Herz and transmural stimulation (T) with 2 msec 20V pulses at 20 Herz each applied for 20 sec. B: responses repeated 15 min after adding hyoscine (1 μg/ml) to the bath. Time marker in 30 sec intervals.

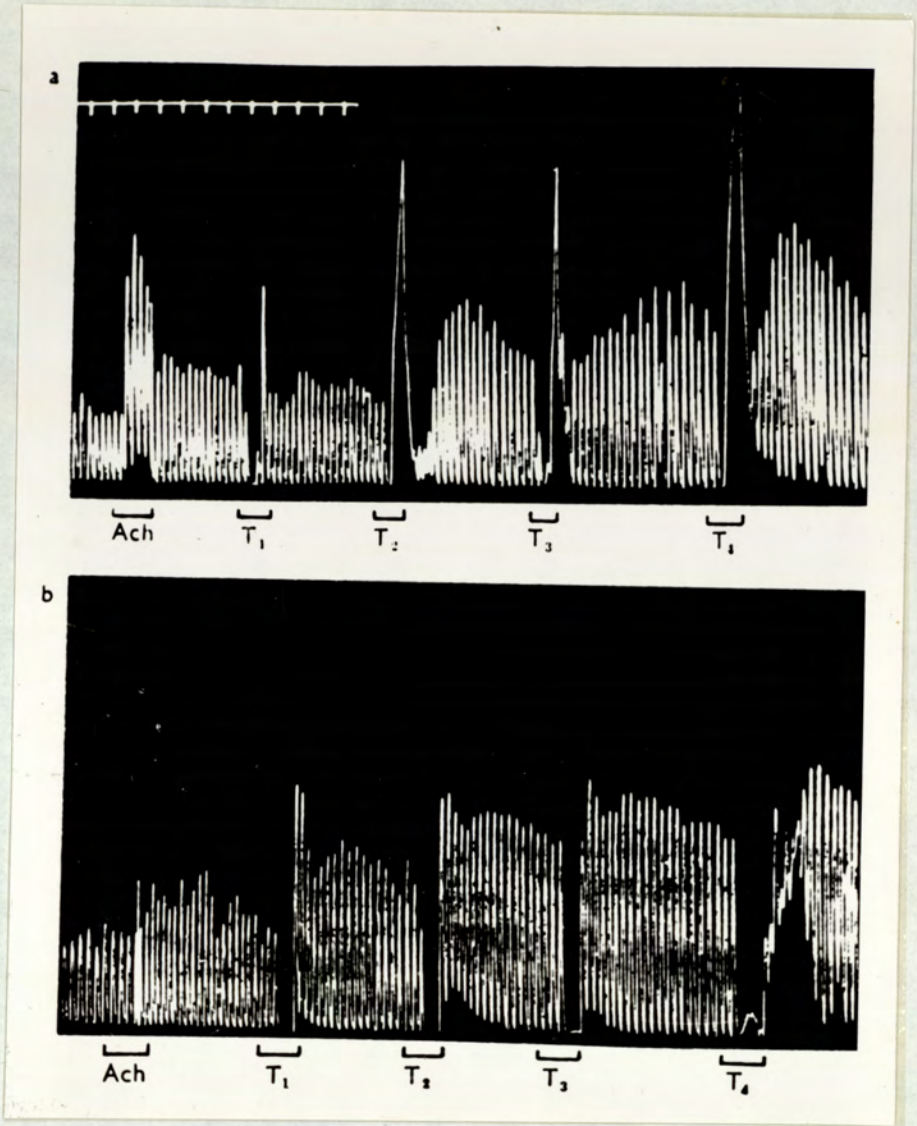


Fig. 17. Rabbit ileum at 32°C. A: control responses to added acetylcholine (0.02 µg/ml) (Ach) and transmural stimulations applied for 20 sec periods with supramaximal strength pulses. $T_1$ pulse width of 0.5 msec and a frequency of 20 Herz. $T_2$ 0.5 msec and 50 Herz. $T_3$ 2 msec and 20 Herz. $T_4$ 2 msec and 50 Herz. B: same responses repeated in the presence of hyoscine (1 µg/ml). Time marker in 30 sec intervals.

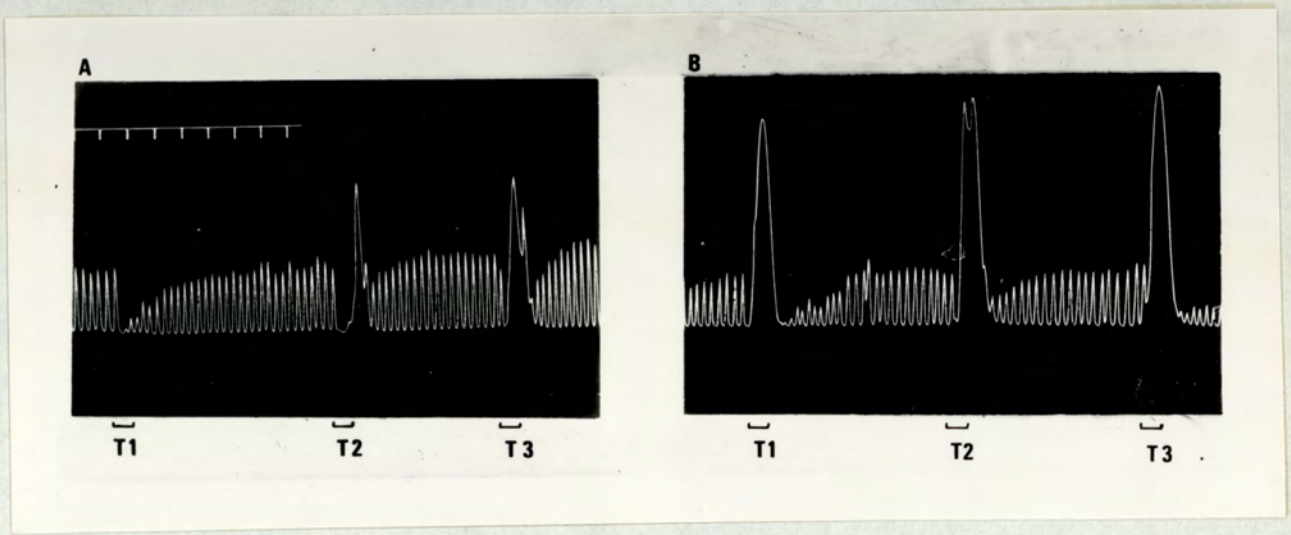


Fig. 18. Rabbit ileum at 32°C. A: control responses to transmural stimulation with varying stimuli. $T_1$ pulse width 0.5 msec, frequency 50 Herz; $T_2$ 2 msec and 20 Herz; $T_3$ 2 msec and 50 Herz. Each pulse of supramaximal strength and with a stimulus period of 20 sec. B: the same responses are repeated 14 min after the addition of physostigmine (0.1 µg/ml) to the bath. Time marker in 30 sec intervals.

of physostigmine (0.1 µg/ml) to the bath revealed a large motor component to the response which had previously been purely inhibitory and the motor components of the other two responses were enhanced and the inhibitory components inhibited.

Triethylcholine

In concentrations of 100 to 400 µg/ml triethylcholine produced variable effects on the motor component of the transmural response. Thus, in 9 out of 16 experiments triethylcholine reduced the motor component of the response whilst increasing the inhibitory response. This is illustrated in Fig. 19 where in a preparation maintained at 37°C triethylcholine (100 µg/ml) caused a marked reduction in the motor component of the response after 2 hr. contact with the preparation and a parallel increase in the inhibitory component (Fig. 19b). The motor component of the response to transmural stimulation recovered after repeated washing of the preparation without stimulation over a 45 min. period (Fig.19c). However, in 7 other experiments it was found that triethylcholine either had no effect on the motor component of the response, or caused a transient increase in the motor response or, in 4 experiments, resulted in a reduction in the motor response not significantly different from the spontaneous variation in the response seen in control tissues set up in parallel experiments but not treated with the drug.

Autonomic ganglion stimulating substances

The ganglion stimulating substances tetramethylammonium bromide (TMA), nicotine and dimethylphenylpiperazinum iodide (DMPP) were all used in concentrations ranging from 1 to 5 µg/ml in preparations of ileum maintained at 37°C. These

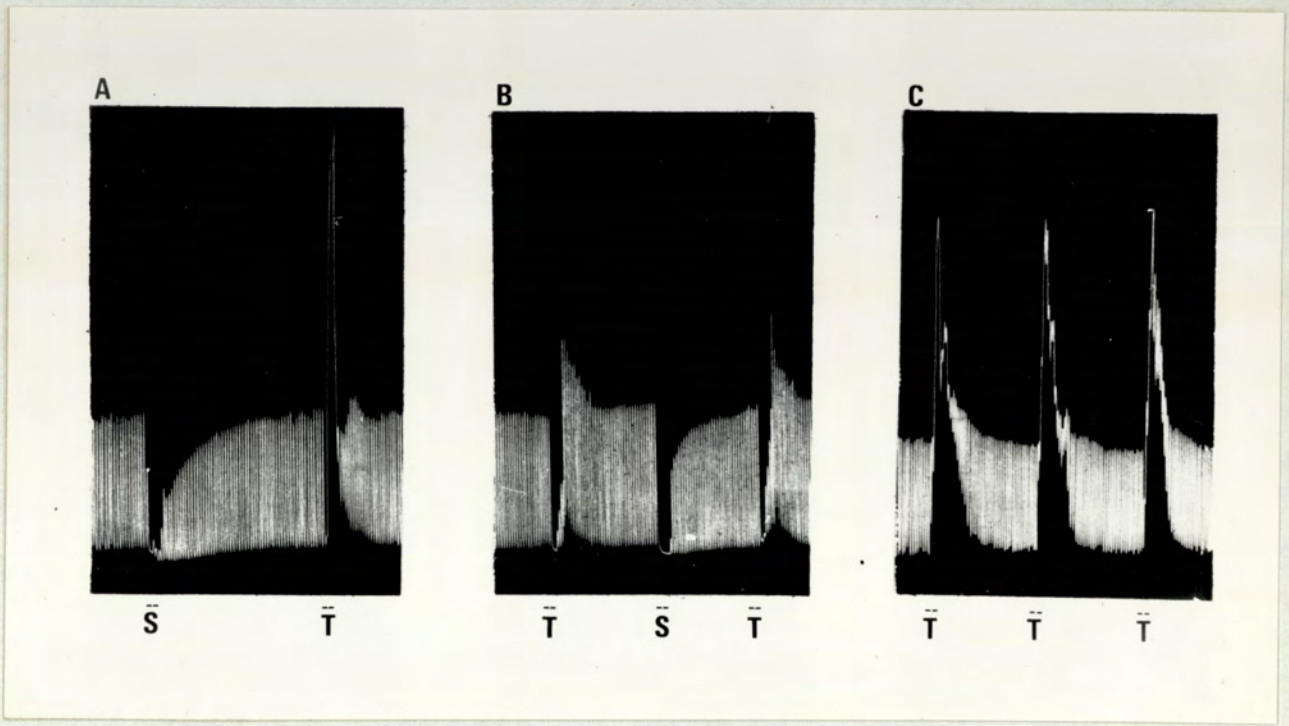


Fig. 19. Rabbit isolated ileum suspended at 37°C in McEwen's solution gassed with 5% $CO_2$ in $O_2$.
Sympathetic (S) and transmural (T) stimulation applied for 20 sec periods with 2 msec 20V pulses at 50 Herz. Between A and B, triethylcholine (100 µg/ml) was added to the bath and remained for the remainder of the experiment. 120 min interval between A and B and 45 min between B and C.

substances produced qualitatively similar responses consisting predominantly of a marked motor response. However, in some experiments there was an initial inhibition such that the complete response closely resembled that to transmural stimulation. The responses of the rabbit isolated ileum to ganglion stimulating substances will be further examined in the next chapter.

Ganglion blocking substances

The ganglion blocking drugs pentolinium and pempidine (each 1 to 100 μg/ml) did not significantly alter the responses to either sympathetic (periarterial) or transmural stimulation but abolished both inhibitory and motor components of the responses to ganglion stimulating substances.

## DISCUSSION

The evidence presented in this chapter strongly suggests that a major part of the motor response to transmural stimulation in rabbit isolated ileum, despite its relative insensitivity to blockade by atropine-like drugs, is mediated via cholinergic nerves. Thus, atropine-like drugs always potentiated the inhibitory component of the transmural response and in most experiments depressed the motor component. The responses to acetylcholine were depressed in the same way as the transmural contraction by lowering the bath temperature. Moreover, in some experiments, at lower bath temperatures atropine and hyoscine completely abolished the residual motor response to transmural stimulation revealing a purely inhibitory response. The results using triethylcholine were equivocal yet pointed in the same direction. In most experiments this substance depressed

the motor component whilst enhancing the inhibitory component of the transmural response. However, in some experiments it produced no greater impairment of the responses to transmural stimulation than occurred in control preparations subjected to the same degree of electrical stimulation. This may be due to the previously reported finding with triethylcholine that it impairs responses to cholinergic nerve stimulation much more effectively when higher frequencies of nerve stimulation were used (Bowman & Rand, 1961). In the present experiments stimulation was at 50 Herz repeated at 3 minute intervals for several hours and undoubtedly this caused nerve fatigue in some, but not all, control preparations.

The evidence with anticholinesterase agents also lends support to the concept that the motor component of the transmural response is mediated largely by acetylcholine. Thus, anticholinesterases potentiated the motor component of the responses and apparently reduced its latency, both factors tending to obscure the inhibitory component. The ganglion stimulating substances produced responses which in some experiments consisted of mixed inhibitory and motor components which strikingly resembled the responses to transmural stimulation. The presence of inhibitory ganglia in the intestinal wall was first suggested by Langley (1922) and has since been confirmed by several workers (Ambache, 1951; Ambache & Edwards, 1951; Gillespie & Mackenna, 1960). Ambache (1951) was unable to detect an inhibitory effect of nicotine on rabbit isolated ileum in his experiments even after atropine. However, Gillespie & Mackenna (1960) revealed inhibitory effects of nicotine on rabbit isolated colon and these were markedly potentiated by atropine.

The next chapter deals with a further examination of autonomic ganglion stimulating substances with a view to determining the ganglion mediator of non-adrenergic inhibition in the rabbit isolated ileum.

## Chapter 4

### Possible mediators of ganglionic transmission in non-adrenergic inhibitory neurones

Nicotine and some other autonomic ganglion stimulants are known to cause mixed inhibitory and motor effects on isolated intestinal preparations (Ambache, 1951; Ambache & Edwards, 1951; Gillespie & Mackenna, 1960; Holman & Hughes, 1965). The evidence produced by these workers, and also that described in this thesis using drugs which modify the action of acetylcholine, suggest that the motor component of the response is predominantly cholinergic. The origin of the inhibitory component is not clear. Ambache & Edwards (1951) found that it was reduced by ephedrine but unaffected by xylocholine, whilst Gillespie & Mackenna (1960) reported that it was reduced by reserpine. If, as seems likely, the inhibitory response to nicotine is caused by activation of ganglion cells in Auerbach's plexus giving rise to non-adrenergic inhibitory post ganglionic fibres, then the lack of effect of xylocholine and the partial block by reserpine would be in close agreement with the results previously described in this thesis for the inhibitory component of the transmural response.

Bulbring & Gershon (1966, 1967) produced evidence suggesting that 5HT may act as a ganglionic transmitter in vagal ganglia from which arise non-adrenergic inhibitory fibres in the guinea pig isolated stomach. These workers suggested that some preganglionic fibres innervating inhibitory ganglia release acetylcholine whilst others release 5HT. This hypothesis might explain the observation of Gillespie & Mackenna (1960) that reserpine treatment which is known to

deplete 5HT as well as catecholamines from the intestine, reduced the inhibitory response to nicotine. In the experiment previously described (this thesis, chapter 2, Day & Warren, 1968) reserpine reduced the inhibitory responses to transmural stimulation and these were not restored by application of dopamine which increased the previously impaired sympathetic responses.

In this chapter a further examination has been made of the action of nicotine, other ganglion stimulants and 5HT on preparations of isolated ileum in an attempt to determine the nature of possible mediators of ganglionic transmission in non-adrenergic inhibitory neurones.

## RESULTS

### Effect of nicotinic drugs on rabbit isolated ileum

As mentioned in the previous chapter the autonomic ganglion stimulating substances nicotine, TMA and DMPP (1-5 µg/ml of each) caused qualitatively similar responses consisting predominantly of a large motor response in preparations of rabbit isolated ileum maintained at 37°C. In a few preparations there was a slight transient inhibition which was increased in the presence of hyoscine (0.1 µg/ml). Hyoscine, in concentrations up to 5 µg/ml, caused a variable effect on the motor response to ganglion stimulants, in some preparations only slightly reducing it, whilst in others the motor component was virtually abolished. A similar variability in susceptibility to atropine and hyoscine was found for the motor component of the transmural response as reported previously.

The response to ganglion stimulants was changed by reducing the bath temperature in the same way as the response to transmural stimulation. This is illustrated by the experiment in Fig. 20. In this experiment sympathetic responses were abolished by guanethidine and the responses to acetylcholine, nicotine and transmural stimulation were then tested at 37, 33 and 28°C. At 37°C nicotine and acetylcholine produced purely motor effects whilst there was an initial inhibition to transmural stimulation followed by a large motor component. At 33°C the motor response to all three procedures was reduced and a small initial inhibition was seen with nicotine. At 28°C the response to transmural stimulation was predominantly inhibitory, acetylcholine was without effect and nicotine caused a small inhibitory effect.

The results using TMA or DMPP were essentially similar. Fig. 21 illustrates an experiment performed at 28°C. Sympathetic and transmural stimulation each caused predominantly inhibitory responses at this temperature as did the addition of TMA at two dose levels. After the addition of a mixture of bethanidine (40 µg/ml) and hyoscine (5 µg/ml) to the bath only sympathetic inhibitions were abolished. The inhibitory responses to TMA were greatly reduced in the presence of pempidine (10 µg/ml) in Fig. 21c.

It was regularly found in these experiments (as also reported in chapter 1) that the inhibitory response to sympathetic stimulation was considerably increased as the bath temperature was lowered. In addition it was found that much higher concentrations of adrenergic neurone blocking drugs were required to block sympathetic inhibition at 28°C as compared with 37°C. Thus, in Fig. 20a at 37°C guanethidine

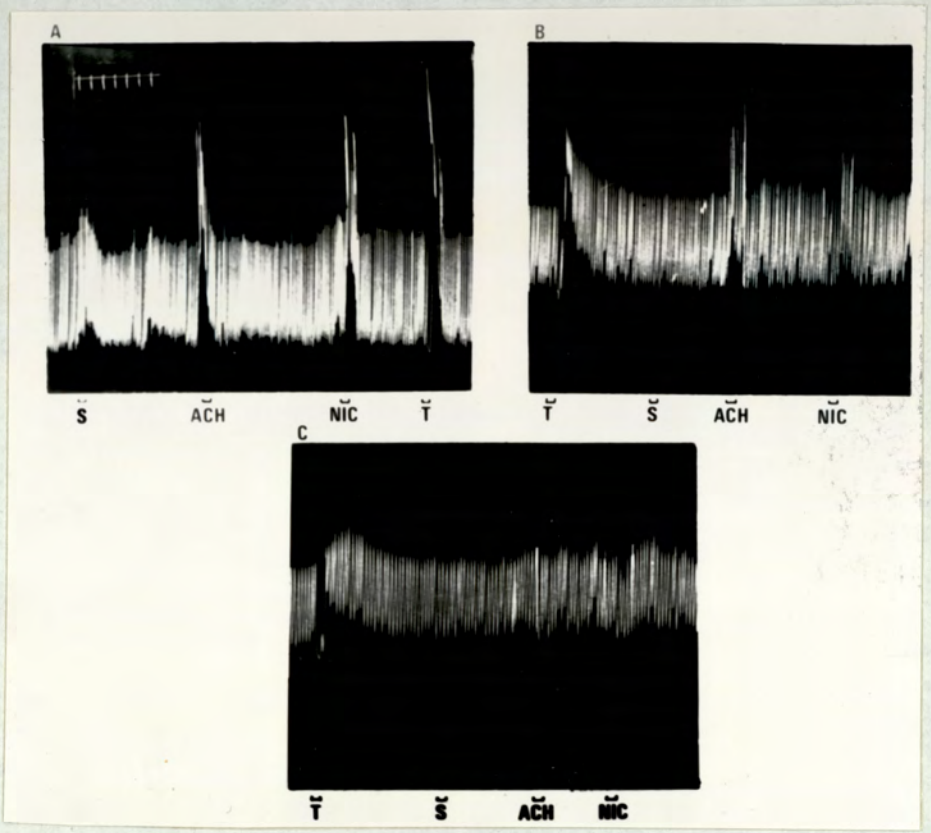


Fig. 20. Rabbit isolated ileum in aerated Tyrode solution. In A at 37°C, responses to sympathetic (S) stimulation for 20 sec period with 2 msec 20V pulses at 50 Herz, 0.5 µg/ml acetylcholine (ACH) and 1 µg/ml nicotine (NIC) each applied for 30 sec and transmural stimulation (T) applied as per sympathetic stimulation. In B, the responses were repeated at 33°C and in C at 28°C. Guanethidine (1 µg/ml) was added to the bath 30 min before A and remained throughout the experiment. Time marker in 30 sec intervals.

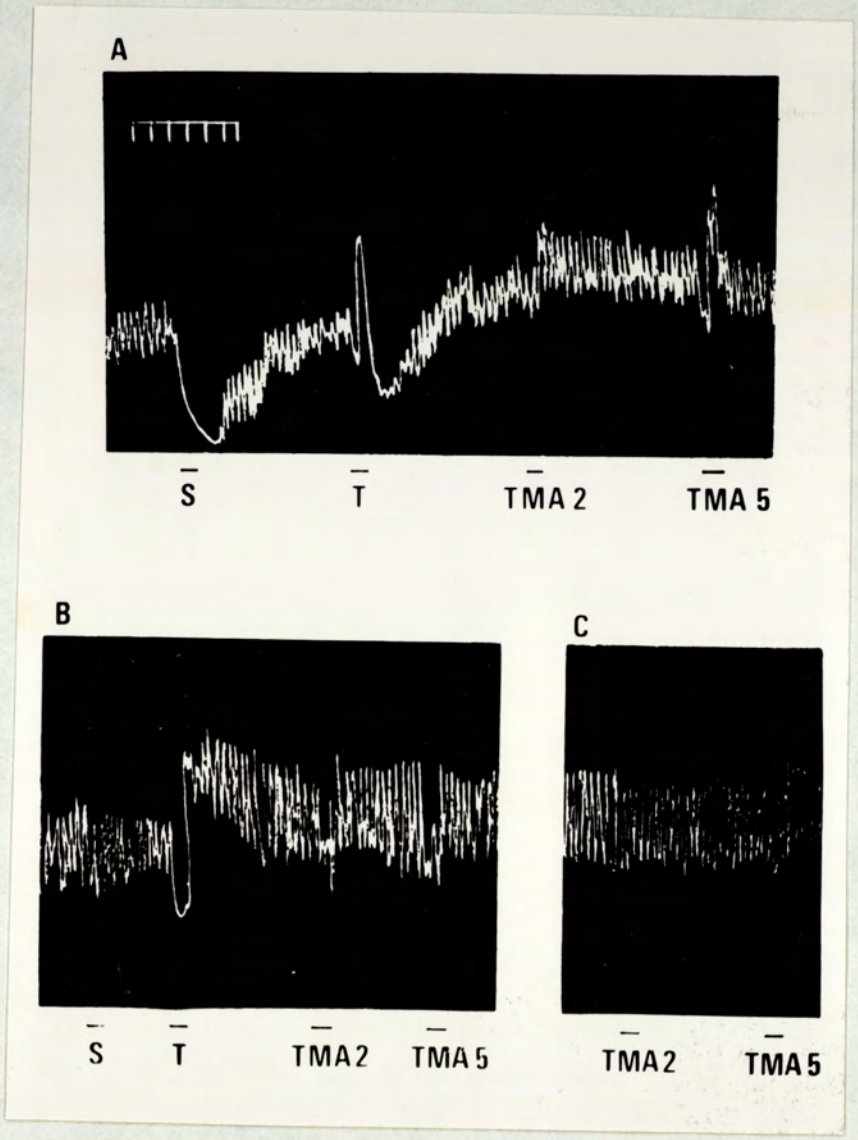


Fig. 21. Rabbit isolated ileum in aerated Tyrode solution at 28°C. In A, sympathetic (S) and transmural (T) stimulation with 2 msec 20V pulses at 20 Herz applied for 20 sec periods, tetramethylammonium bromide (TMA) 2 and 5 µg/ml in contact for 30 sec periods. Between A and B bethanidine (40 µg/ml) and hyoscine (5 µg/ml) were added to the bath and in B the responses were repeated 30 min later. Between B and C pempidine (10 µg/ml) was added and the responses repeated in C 25 min later. Time marker in 30 sec intervals.

(1 µg/ml) abolished sympathetic responses whilst in Fig. 21 in a preparation maintained at 28°C, 40 µg/ml of bethanidine were necessary to abolish sympathetic responses.

These results strongly suggest that nicotinic drugs stimulate inhibitory ganglion cells within the gut and furthermore add weight to the previous suggestion that the motor component of the response to nicotinic drugs like that to transmural stimulation is mediated via cholinergic nerves.

Effect of 5-hydroxytryptamine (5HT)

5HT is known to occur in the intestine and has been shown to possess a variety of pharmacological actions including both excitation and inhibition of intestinal smooth muscle (for review see Kosterlitz & Lees, 1964).

The addition of 5HT (0.1 - 5 µg/ml) to preparations of rabbit isolated ileum maintained at 37°C produced variable results. Thus, in 9 out of 24 experiments it caused a purely motor effect; in 9 other experiments it produced a biphasic response consisting of initial inhibition followed by a motor response; whilst in 6 experiments it produced only inhibition of motility. The inhibitory component of the response was more prominent in experiments in which the bath temperature was lowered to 32 or 28°C. In general, in any particular experiment the response to 5HT closely resembled that to transmural stimulation. This is demonstrated in the experiment illustrated in Fig. 22 which is of a preparation maintained at 33°C. In Fig. 22a two biphasic responses to transmural stimulation at different frequencies of stimulation were obtained and these responses are closely mimicked in Fig. 22b by two different concentrations of 5HT.

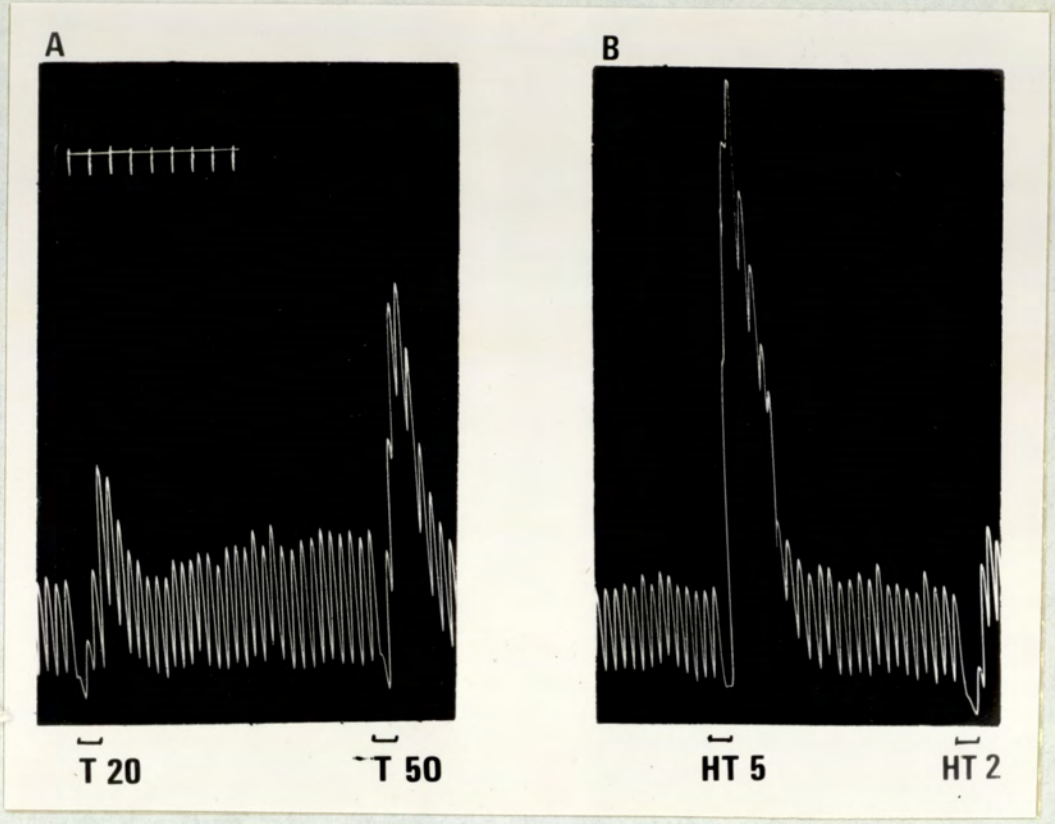


Fig. 22. Rabbit isolated ileum in aerated Tyrode solution at 32°C. In A, transmural stimulation (T) for 20 sec periods with 2 msec 20V pulses at 20 and 50 Herz. In B, responses to added 5-hydroxytryptamine (5 µg/ml) and (2 µg/ml) in contact for 30 sec. Time marker in 30 sec intervals.

The motor component of the response to 5HT was affected in the same variable way as was the motor components of the responses to nicotine and transmural stimulation suggesting a common origin for all these responses. Although hyoscine (0.1 to 5 μg/ml) did not regularly abolish the motor component of the 5HT response, it did regularly reveal initial inhibitory responses in those preparations in which they were initially absent.

The inhibitory component of the 5HT response persisted in the presence of guanethidine or mixtures of α and β adrenoceptor blocking substances in concentrations sufficient to abolish responses to sympathetic stimulation.

In three experiments it was shown that both components of the 5HT response were abolished in the presence of tetrodotoxin (1 μg/ml).

Effect of 5-hydroxytryptamine antagonists

The responses to 5HT, nicotine and transmural stimulation were in many experiments strikingly similar. However, whereas the responses to nicotine and related drugs were selectively abolished by ganglion blocking substances 5HT antagonists did not selectively inhibit either component of the 5HT response. The following 5HT antagonists were used, BOL (0.1 - 2 μg/ml), LSD (2 μg/ml), ergotamine (1-40 μg/ml), ergometrine (2-10 μg/ml), methysergide (1-32 μg/ml) and cyproheptadine (0.1 - 3 μg/ml). Each of these substances failed to produce a selective blockade of either the response to transmural stimulation or to 5HT. At the higher concentrations mentioned above all the 5HT antagonists produced a similar non-specific blocking action on the

responses to both sympathetic and transmural stimulation. This is illustrated in Fig. 23 which shows the non-selective blocking action of BOL (2 µg/ml) on responses to sympathetic and transmural stimulation.

Effect of 5-hydroxytryptophan (5HTP)

In 6 experiments 5HTP (2-20 µg/ml) the amino-acid precursor of 5HT was added to preparations of rabbit isolated ileum maintained at 37°C.

It was found that 5HTP in these concentrations produced no significant effect on either inhibitory or motor components of the transmural response.

Effect of histamine

Histamine (1-5 µg/ml) was found to produce a weak motor response on preparations of isolated ileum at 37°C. In two experiments out of 7 it produced a weak initial inhibition after hyoscine (0.1 µg/ml). The anti-histamine drugs cyproheptadine (0.1 µg/ml) and mepyramine (0.1 µg/ml) each abolished the responses to histamine but did not affect the responses to transmural stimulation.

Dexamphetamine and ephedrine

Dexamphetamine (1-60 µg/ml) and ephedrine (1-50 µg/ml) were used because of the report by Ambache & Edwards (1951) that ephedrine reduced the inhibitory response to nicotine seen in isolated intestinal preparations after atropine. The results obtained with these substances were similar, both caused a similar partial block of the responses to both sympathetic and transmural stimulation usually at a concentration of 10-30 µg/ml. The blocking action on each response was readily reversed by washing the preparation.

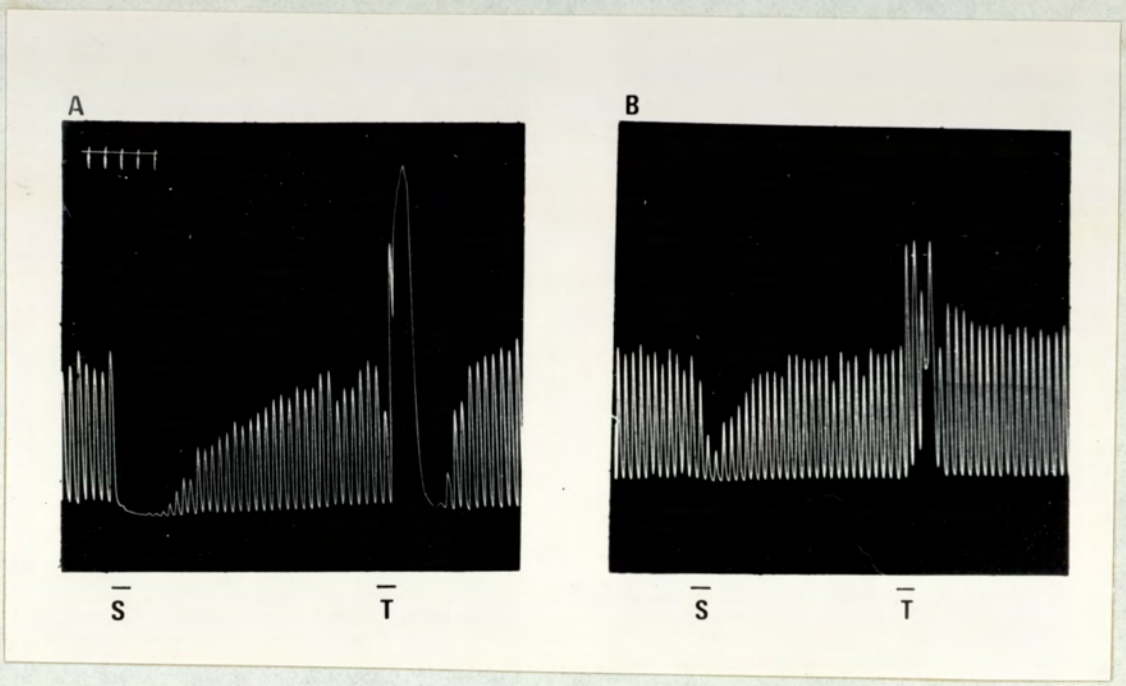


Fig. 23. Rabbit isolated ileum in aerated Tyrode solution at 32°C. In A, sympathetic (S) stimulation with 0.5 msec 20V pulses at 50 Herz and transmural (T) stimulation with 0.5 msec 20V pulses at 20 Herz. In B, the responses are repeated 20 min after the addition of BOL (2 µg/ml) to the bath. Time marker in 30 sec intervals.

## DISCUSSION

The results reported in this chapter strongly suggest the concept that ganglion cells exist in the intestine which give rise to non-adrenergic inhibitory post-ganglionic fibres. Clearly, the ganglionic transmitter for some of these inhibitory neurones is acetylcholine. Thus, nicotinic drugs produce non-adrenergic inhibitory responses which closely mimick the inhibitory component of the response to transmural stimulation. The inhibitory responses to nicotinic drugs and those to transmural stimulation are similarly affected by lowering the bath temperature and by hyoscine. It is also apparent that the motor component of the response to nicotinic drugs like that to transmural stimulation and to acetylcholine is most prominent at 37°C and all these responses are progressively reduced by lowering of the bath temperature. This is further evidence that the motor components are ultimately mediated via a common mechanism, i.e. the action of acetylcholine on muscarinic receptors in the intestine.

The action of nicotine and related substances in causing non-adrenergic inhibition explains the earlier observations of Ambache & his co-workers (Ambache, 1951; Ambache & Edwards, 1951) and of Gillespie & Mackenna (1960) who noticed inhibitory effects of nicotine on intestinal preparations from rabbits and cats. The observation of Gillespie & Mackenna (1960) that the inhibitory action of nicotine on rabbit isolated colon is susceptible to the action of reserpine is in close agreement with the results in this thesis that reserpine impaired the inhibitory component of the transmural response.

5HT has long been thought to be concerned in the control of intestinal motility (for review see Kosterlitz & Lees, 1964).

Bulbring & Gershon (1966, 1967) have produced evidence which strongly suggests that 5HT as well as acetylcholine may be a ganglionic mediator of non-adrenergic inhibition in guinea pig isolated stomach. Their results in which they stimulated the vagus nerves support the original concept of Langley (1922) of preganglionic vagal efferent fibres innervating both excitatory and inhibitory ganglia within the stomach and intestinal wall. The results in this chapter confirm the observations of Bulbring & Gershon (1966, 1967). Thus, 5HT is able to produce non-adrenergic inhibitory responses, which closely mimick the responses to transmural stimulation of rabbit ileum. However, neither inhibitory nor motor components of the 5HT response were abolished by 5HT antagonists. A possible explanation of this observation (Gershon, 1967) is that the 5HT receptors concerned in the inhibitory response are situated on autonomic ganglia and are not blocked by 5HT antagonists which exert their effects on receptors in smooth muscle.

The observation of Ambache & Edwards (1951) that ephedrine abolished the inhibitory effect of nicotine in the isolated ileum from the cat or rabbit was thought to be worthy of further examination since it might provide a selective blocker of non-adrenergic inhibition. However, it was found that neither ephedrine nor dexamphetamine produced a selective blockade of the responses to transmural stimulation in rabbit ileum. It is possible that the blocking action of ephedrine

on nicotine responses reported by Ambache & Edwards (1951) may have been the result of a non-selective, or possibly ganglionic blocking action of high concentrations of ephedrine.

## Chapter 5

## Non-adrenergic inhibitory responses in isolated preparations of rabbit colon and cat ileum and colon

The experiments to be described in this chapter were undertaken for two main reasons. Firstly, to extend the search for non-adrenergic inhibitory neurones to other parts of the intestine and to another species, and secondly, to attempt to find a preparation in which non-adrenergic inhibitory neurones could be stimulated without simultaneous stimulation of motor neurones. The rabbit isolated colon was chosen because it is possible to obtain separate responses to extrinsic nerve stimulation of sympathetic and parasympathetic innervations (Garry & Gillespie, 1954) and because the observations of Gillespie & Mackenna (1960) using nicotine strongly suggest the presence of non-adrenergic inhibitory neurones within the tissue.

The cat ileum and colon were used because of the reports of Ambache (1951) and Ambache & Edwards (1951) that the motor component of the nicotine response in this tissue was readily susceptible to the blocking action of atropine revealing an inhibitory response. If the motor response to transmural stimulation were similarly abolished by atropine then this may provide a more convenient preparation for the pharmacological analysis of non-adrenergic inhibition.

### RESULTS

### Rabbit isolated colon

Preparations were set up as described by Garry & Gillespie (1954) with the addition of a transmural electrode

so that all extrinsic and intrinsic nerves innervating the tissue could be stimulated. The effects of transmural, sympathetic and parasympathetic stimulation are described separately.

(a) Transmural stimulation

The responses to transmural stimulation varied according to the frequency of stimulation. At low stimulus frequencies (0.5 - 5 Herz) the response was predominantly inhibitory usually with a small secondary motor component. As the frequency of stimulation was increased so the motor response increased until at about 20 Herz the response was either purely or predominantly motor.

Attempts were made to stimulate non-adrenergic inhibitory neurones preferentially by altering the stimulus parameters. However, it was found that the threshold for transmural inhibition for pulse-width (0.05 - 0.2 msec) and voltage (2-5 volts) were very similar to those for transmural contraction. The optimal frequency of stimulation for transmural inhibition varied between 1 and 5 Herz and in 14 of 20 experiments was approximately 3 Herz. This is similar to the optimal frequency for transmural inhibition in the ileum after complete abolition of the sympathetic component (this thesis, chapter 2).

Atropine and hyoscine

The motor component of the transmural response was similarly resistant to atropine and hyoscine as was the motor component in the ileum. Atropine and hyoscine (each 0.1 - 100 µg/ml) produced variable degrees of inhibition of the motor component to transmural stimulation partly related

to the stimulus frequency. Thus, at stimulus frequencies up to 10 Herz inhibitory responses predominated and in these experiments atropine or hyoscine abolished any small motor component and enhanced the inhibition. However, at higher stimulus frequencies (10-50 Herz) the motor component predominated and this was only slightly reduced by even high (50-100 μg/ml) concentrations of atropine and hyoscine.

Effect of lowering bath temperature

The myogenic contractions of rabbit isolated colon at 37°C were in general much less regular than those previously seen in ileal preparations. Progressive lowering of the bath temperature down to 28°C made the contractions larger, slower and even more irregular. In 6 experiments started at 37°C in which the responses to transmural stimulation were inhibitory, lowering of the bath temperature to 31°C increased the inhibitions in both extent and duration thus paralleling the results previously described in the ileum.

(b) Sympathetic stimulation

Stimulation of the periarterial nerves at frequencies ranging from 5 to 50 Herz produced purely inhibitory responses as reported by Garry & Gillespie (1954). These responses were clearly sympathetically-mediated since they were abolished by guanethidine (1-10 μg/ml) or by a mixture of propranolol (2 μg/ml) and phentolamine (2 μg/ml) and were unaffected by atropine or hyoscine (each 0.1-100 μg/ml) or by pempidine (1-50 μg/ml).

The transmural inhibitions were virtually unaffected by sympathetic blocking doses of either guanethidine or a mixture of α and β adrenoceptor blocking substances.

Autonomic ganglion stimulants

The ganglion stimulants nicotine and TMA (each in concentrations 0.5 - 5 µg/ml) caused responses on isolated colon which were either purely inhibitory (10 experiments), purely excitatory (3 experiments), or biphasic with initial inhibition followed by motor (7 experiments). The excitatory effect of nicotine, like the motor response to transmural stimulation, was resistant to hyoscine or atropine. However, in those preparations in which nicotine caused a purely excitatory effect an initial inhibitory component was revealed after atropine or hyoscine.

The experiment illustrated in Fig. 24 shows the striking similarity between the responses to transmural stimulation at three stimulus frequencies and to the inhibitory response to TMA. As in the ileum inhibitory responses to nicotine were resistant to sympathetic nerve blocking concentrations of either guanethidine or a mixture of α and β adrenoceptor blockers, but were abolished by pempidine (10 µg/ml).

Acetylcholine and carbachol

Both acetylcholine (0.1 - 1.0 µg/ml) and carbachol (0.4 - 2 µg/ml) caused motor effects on rabbit isolated colon preparations at temperatures between 28°C and 37°C. However, in the presence of hyoscine (0.1 - 5 µg/ml) the responses were converted to inhibitions which were resistant to guanethidine and to mixtures of α and β adrenoceptor blockers in sufficient concentration to abolish responses to sympathetic stimulation.

Fig. 25 illustrates an experiment performed at 28°C in which at the start of the experiment transmural stimulation produced a biphasic response whilst acetylcholine was

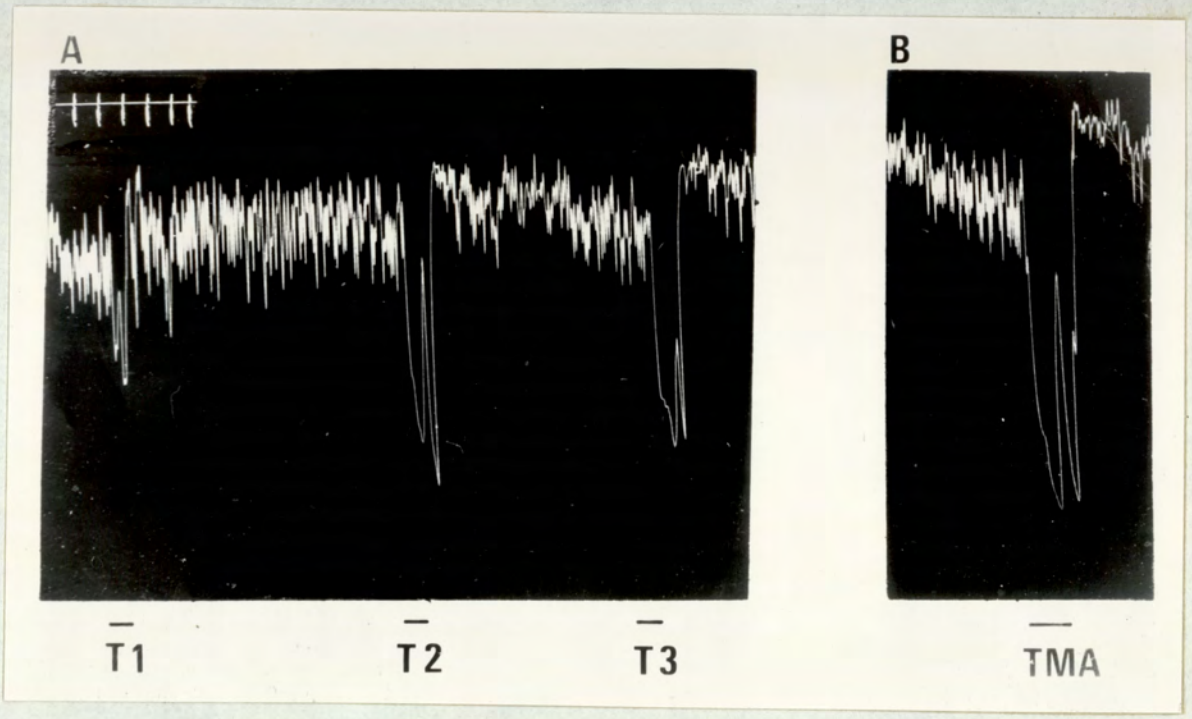


Fig. 24. Rabbit isolated colon suspended in McEwen's solution gassed with 95% $O_2$ and 5% $CO_2$ and maintained at 37°C.
In A, transmural (T) stimulations for 20 sec periods with 2 msec 20V pulses at 1, 2 and 3 Herz. In B, 1 µg/ml tetramethylammonium bromide (TMA) added for 30 sec. Time marker in 30 sec intervals.

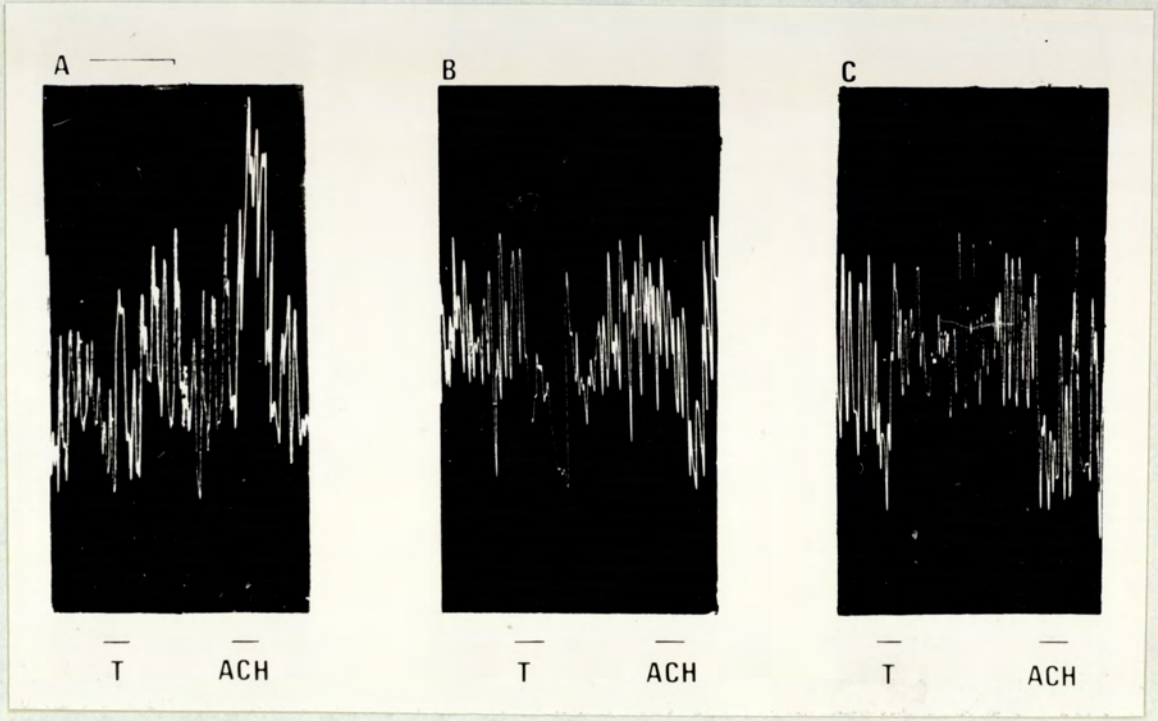


Fig. 25. Rabbit isolated colon maintained at 28°C and suspended in Kreb's solution gassed with 95% $O_2$ and 5% $CO_2$. In A, transmural stimulation (T) with 2 msec 20V pulses at 10 Herz for 20 sec and response to 1 µg/ml acetylcholine (ACH). Between A and B 1 µg/ml hyoscine added to the bath and left for remainder of the experiment and in B responses repeated 20 min later. Between B and C 10 µg/ml guanethidine added to the bath and responses in C are 30 min later.

motor (Fig. 25a). In the presence of hyoscine (Fig. 25b) the inhibition to transmural stimulation was increased and the response to acetylcholine was reversed to inhibition. Neither response was significantly altered in the presence of guanethidine (Fig. 25c).

(c) Parasympathetic nerve stimulation

Stimulation of the pelvic parasympathetic nerves to the rabbit isolated distal colon produced motor effects with an optimal effect reached with frequencies of between 10 and 20 Herz as described previously by Gillespie & Mackenna (1960, 1961). The motor effect to parasympathetic stimulation was only slightly reduced or, in some experiments, unaffected by hyoscine (0.1 - 10 µg/ml), again confirming the observations of Gillespie & Mackenna (1960). However, in about half the experiments hyoscine treatment revealed an initial inhibitory component to the transmural response. This inhibitory response was usually poorly developed, was unaffected by guanethidine (1-2 µg/ml) which abolished sympathetic inhibitions, but was abolished by pempidine (1-8 µg/ml).

Experiments using cat and kitten intestine

Segments of ileum taken from adult cats and set up at 37°C showed regular spontaneous contractions which tended to become smaller after a period of 1 - 2 hours and eventually ceased altogether. Various attempts were made to lengthen the effective life of the preparation without any success. The preparations initially responded well to sympathetic and to transmural stimulation. Transmural stimulation frequently produced very pronounced inhibitory responses which greatly outlasted the stimulation period. This is illustrated in

Fig. 26 which is the record of an ileal preparation taken from an adult cat. Transmural stimulation at 20 Herz produced a very pronounced inhibitory response without any motor component whilst sympathetic stimulation produced a response very similar to those seen in rabbit ileum. In Fig. 26b the responses were repeated after 35 minutes contact with guanethidine (2 µg/ml) which had abolished sympathetic responses. In Fig. 26b the myogenic contractions were reduced in size and the tone of the preparation was lower but transmural stimulation still produced a large inhibitory response.

In other preparations it was found that at frequencies of transmural stimulation below 20 Herz a motor component was present in the response. However, unlike in rabbit preparations, this motor response was completely abolished by low concentrations of hyoscine (0.1 µg/ml). Fig. 27 shows an experiment using ileum from a 9 week old kitten. Sympathetic stimulation produced a typical inhibitory response whilst the response to transmural stimulation was either substantially motor (at 5 Herz) or substantially inhibitory (at 20 Herz). Fig. 27b shows the responses repeated after 30 minutes contact with a mixture of guanethidine (10 µg/ml) and hyoscine (0.1 µg/ml). Sympathetic inhibitions were abolished and the response to transmural stimulation at 5 Herz was now inhibition, whilst transmural stimulation at 20 Herz produced a similar long-lasting inhibition as it did before drug treatment.

A few experiments were attempted using cat isolated colon but this preparation became anoxic and failed even

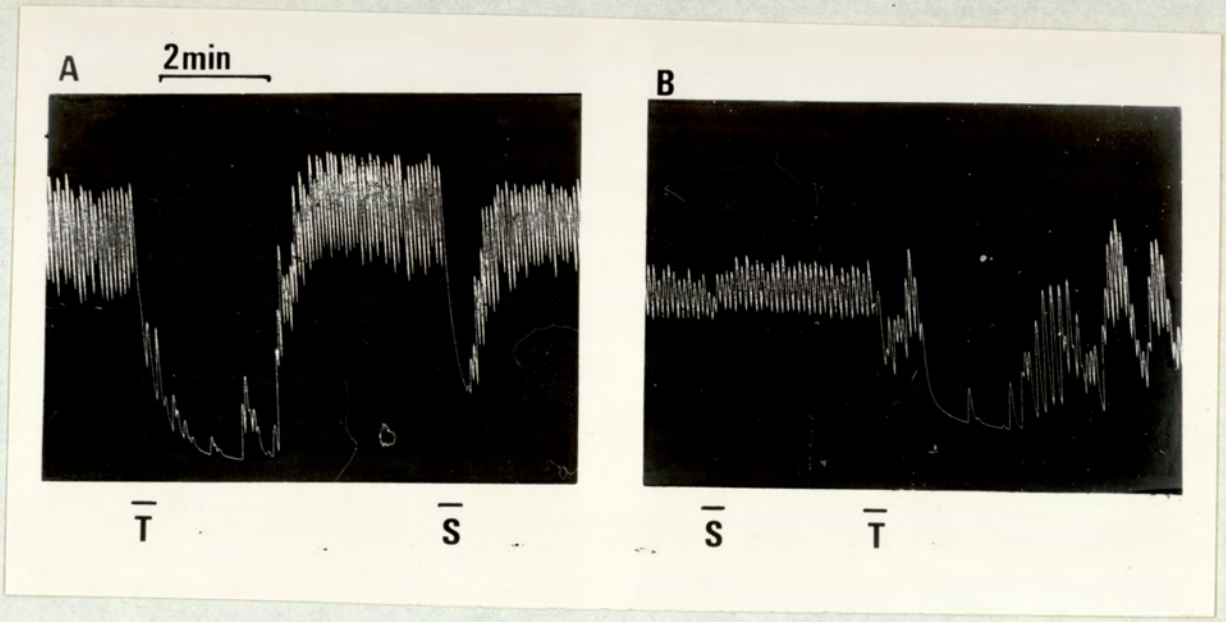


Fig. 26. Isolated ileum preparation taken from adult cat (2.4 Kg) maintained at 37°C in McEwen's solution gassed with 95% $O_2$ and 5% $CO_2$.

In A, transmural stimulation (T) with 2 msec 60V pulses at 50 Herz and to sympathetic stimulation (S) with 2 msec 20V pulses at 50 Herz each applied for 20 sec periods. In B, the responses were repeated 30 min after the addition of guanethidine (2 µg/ml) to the bath.

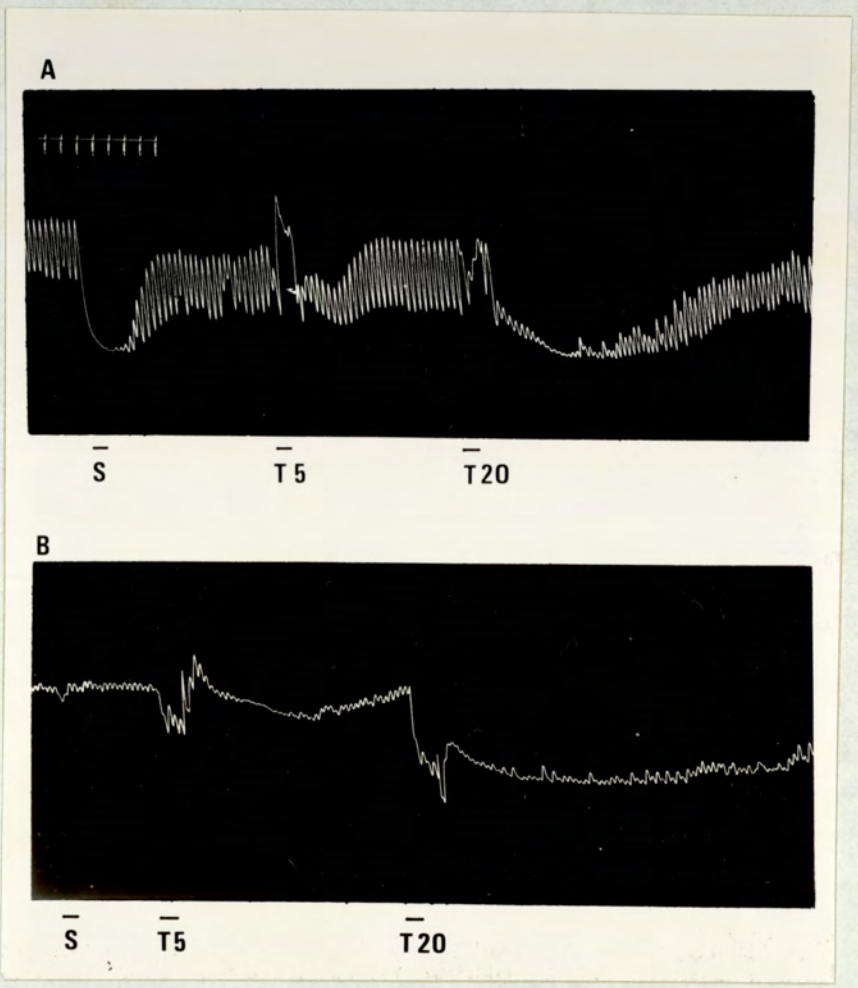


Fig. 27. Isolated ileum preparation taken from a nine-week old kitten. Preparation maintained at 37°C in McEwen's solution gassed with 95% $O_2$ and 5% $CO_2$.
In A, responses to sympathetic stimulation (S) with 20V 2 msec pulses at 20 Herz and transmural stimulation (T) with 50V 2 msec pulses at 5 and 20 Herz; stimulations applied for 20 sec periods. In B, the responses were repeated 35 min after the addition of a mixture of guanethidine (10 µg/ml) and hyoscine (0.1 µg/ml) to the bath. Time marker 30 sec intervals.

more quickly than preparations of ileum. Transmural stimulation produced mixed inhibitory and motor responses. However, the motor component was less sensitive to hyoscine than in the ileum and the records obtained were complicated by large spontaneous changes in the height and frequency of the myogenic contractions.

## DISCUSSION

The results described in this chapter indicate the presence of non-adrenergic inhibitory responses in the isolated colon of the rabbit and in the isolated ileum of the cat. The properties of the non-adrenergic inhibitory responses in the rabbit colon preparations were strikingly similar to those already described in the rabbit isolated ileum. Thus, they were unaffected by adrenergic neurone blocking drugs or by mixtures of $\alpha$ and $\beta$ adrenoceptor blocking substances, were enhanced by a lowered bath temperature, and were mimicked by autonomic ganglion stimulants and by cholinergic substances in the presence of hyoscine. The responses to pelvic nerve stimulation in the rabbit isolated colon were predominantly motor as first described by Garry & Gillespie (1954) and by Gillespie & Mackenna (1960, 1961), and only a small transient inhibitory component was seen in some experiments. This inhibitory response was somewhat increased by hyoscine but the motor component of the response was not significantly reduced by even high concentrations of hyoscine. This observation, together with the irregular nature of the myogenic contractions, suggested that this preparation would not have any significant advantages over the rabbit isolated ileum for the detailed study of non-adrenergic inhibitory responses.

The cat and kitten isolated ileum preparations both suffered from the severe limitation that they could not be kept viable for more than 1 - 2 hours. However, very pronounced and long-lasting inhibitory responses to transmural stimulation were recorded which were easily differentiated from the sympathetic responses by their total insensitivity to guanethidine. In cat isolated ileum preparations a motor component to transmural stimulation was only evident at higher frequencies of stimulation and was readily susceptible to the blocking action of low concentrations of hyoscine. This is consistent with the observation of Ambache & Edwards (1951) that the motor response to nicotine was converted to inhibition in the presence of atropine in cat isolated intestinal preparations.

## Chapter 6

### Experiments using the doubly-innervated isolated colon of the guinea pig

The preparations so far used in this thesis for the study of non-adrenergic inhibitory responses have involved the use of transmural electrical stimulation which activates at least three types of neurones simultaneously. The resulting complex response has to some extent been analysed mainly by the use of blocking substances such as hyoscine and guanethidine. The evidence from the work so far described as well as that from other workers beginning with Langley (1922) suggests that the non-adrenergic inhibitory neurones are part of the parasympathetic branch of the autonomic nervous system with ganglion cells in Auerbach's plexus. An ideal preparation to study these nerves would be one in which the extrinsic parasympathetic nerves could be stimulated and in which any cholinergic response was susceptible to the blocking action of atropine. In the previous chapter the rabbit isolated colon preparation as first described by Garry & Gillespie (1954) was used but suffered from the drawback that the motor response to parasympathetic (pelvic nerve) stimulation was almost totally resistant to the blocking action of hyoscine or atropine.

Paton (1954) showed that transmural stimulation of guinea pig isolated ileum with single supramaximal electrical shocks produced contractile responses which were readily abolished by low concentrations of atropine. In view of this observation it was considered worthwhile to attempt to set up preparations of guinea pig isolated colon with both

sympathetic and parasympathetic nerves attached as described by Garry & Gillespie (1954) for rabbit colon. Since completing the work in this chapter Bianchi and her colleagues (Bianchi, Beani, Frigo & Crema, 1968) have made a similar study using the same preparation. The results of this group differ in several important respects from those described here and will be discussed at the end of the chapter.

## RESULTS

### Transmural stimulation of guinea pig colon

The isolated colon of the guinea pig showed regular myogenic contractions when set up at 37°C. The response to transmural stimulation (0.5 - 50 Herz) was either biphasic consisting of initial inhibition followed by a motor component (12 experiments), or purely inhibitory (4 experiments), or purely motor (3 experiments). The motor component of the response was always greatly reduced and frequently abolished in the presence of low concentrations of hyoscine (0.05 - 1 μg/ml). This is illustrated in Fig. 28 which shows the record obtained in one of the experiments in which the initial response to transmural stimulation was purely motor. In the presence of hyoscine (1 μg/ml) the responses became predominantly inhibitory (Fig. 28b) and these responses were only slightly reduced in the presence of 1 μg/ml guanethidine (Fig. 28c). In other experiments the inhibitory responses to transmural stimulation were found to be unaffected by a mixture of α and β adrenoceptor blocking drugs.

In some preparations attempts were made to separate the motor and inhibitory components of the transmural response

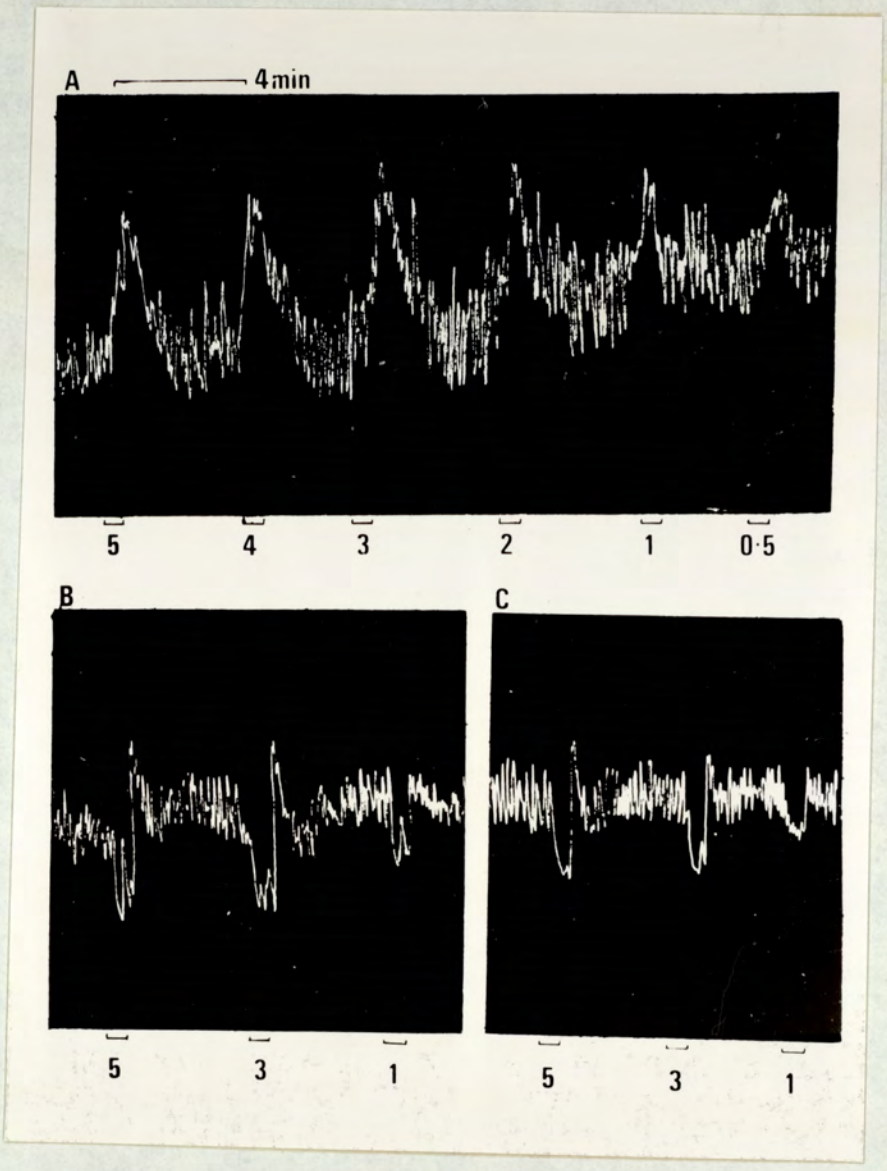


Fig. 28. Guinea pig isolated colon maintained at 37°C in McEwen's solution gassed with 95% $O_2$ and 5% $CO_2$.
All responses are to transmural stimulation for 20 sec periods with 2 msec 20V pulses at the frequency indicated. Between A and B hyoscine (1 µg/ml) was added to the bath and remained for the rest of the experiment. The responses in B were repeated 20 min later and those in C 30 min after the further addition of guanethidine (1 µg/ml) to the bath.

by altering the stimulus parameters. However, it was found that the threshold frequency (0.2 - 0.5 Herz), voltage (1 to 5 volts) and pulse-width (0.05 - 0.2 msec) were indistinguishable for both motor and inhibitory components of the transmural response.

Effect of parasympathetic (pelvic) nerve stimulation

Pelvic nerve stimulation at frequencies ranging from 0.5 to 50 Herz produced responses which were virtually identical to those produced by transmural stimulation. In about 25% of these experiments pelvic nerve stimulation produced purely inhibitory responses as shown in the experiment illustrated in Fig. 29, in which the responses were illicited by frequencies of 5, 10, 20 and 50 Herz. In those preparations in which pelvic nerve stimulation produced either purely motor or mixed effects the responses became inhibitory in the presence of hyoscine and were not reduced by drugs abolishing the effects of sympathetic stimulation. This is illustrated in Fig. 30 showing the responses to pelvic nerve stimulation which were initially motor but became inhibitory in the presence of hyoscine (Fig. 30b) and were not reduced by guanethidine in sufficient concentrations to abolish sympathetic inhibitory responses (Fig. 30c). $\alpha$ and $\beta$ adrenoceptor blocking drug mixtures were similarly ineffective at reducing inhibitory responses to parasympathetic nerve stimulation.

Effect of autonomic ganglion blockade

The inhibitory responses to pelvic nerve stimulation were reduced but not abolished in the presence of autonomic ganglion blocking substance pempidine (1 to 50 µg/ml).

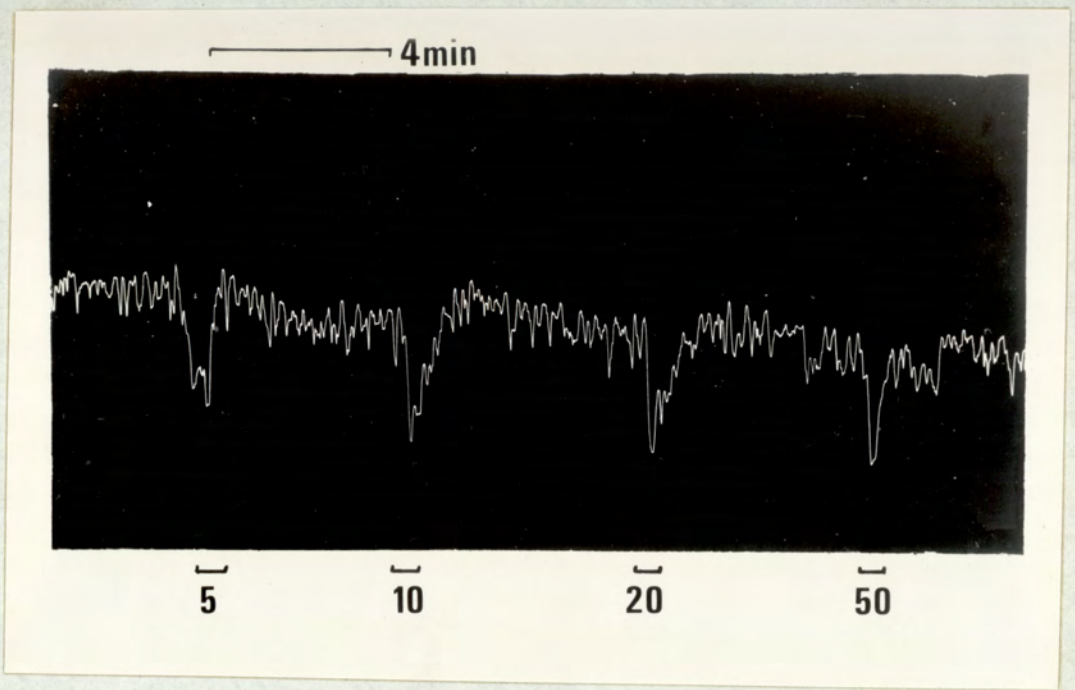


Fig. 29. Guinea pig isolated colon maintained at 37°C in McEwen's solution gassed with 95% $O_2$ and 5% $CO_2$. Pelvic (parasympathetic) stimulation with 2 msec 20V pulses applied for 20 sec periods at the frequency indicated.

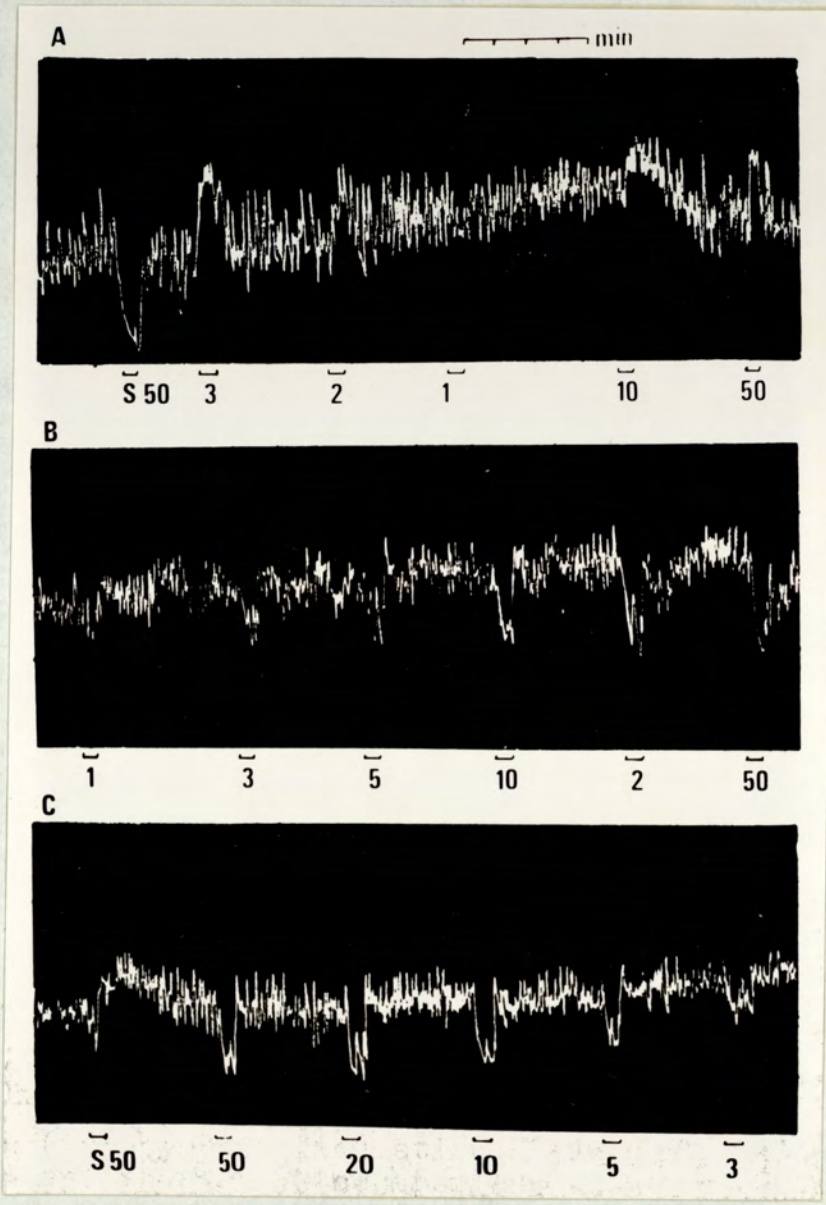


Fig. 30. Guinea pig isolated colon maintained at 37°C in McEwen's solution gassed with 95% $O_2$ and 5% $CO_2$.

In A, the first response is to sympathetic stimulation (S) applied for 20 sec with 2 msec 20V pulses at 50 Herz, remaining responses are to pelvic (parasympathetic) stimulation with 2 msec 20V pulses at the frequency indicated.

In B, parasympathetic responses were repeated 25 min after the addition of hyoscine (0.1 µg/ml) to the bath, and in C, sympathetic and parasympathetic responses were repeated 30 min after the further addition of guanethidine (1 µg/ml) to the bath.

This is shown in the experiment illustrated in Fig. 31 in which pelvic nerve stimulation in the presence of hyoscine produced inhibitory responses which were only partially blocked even in the presence of 50 µg/ml pempidine (Fig. 31b). In general the motor component of the response to pelvic nerve stimulation seemed more susceptible to pempidine than did the inhibitory component. Thus, in the experiment illustrated in Fig. 32, the initial predominantly motor response to parasympathetic stimulation was converted to an inhibitory response in the presence of 10 µg/ml pempidine.

Effect of autonomic ganglion stimulants

Nicotine and TMA (each in concentrations of 0.5 to 5 µg/ml) produced responses which closely resembled those to either transmural or pelvic nerve stimulation in that particular preparation being most often mixed motor and inhibitory but sometimes either purely motor or purely inhibitory. Fig. 33 illustrates an experiment in which TMA produced biphasic responses consisting of initial inhibitory followed by motor components. These responses were converted in the presence of a mixture of hyoscine and guanethidine into purely inhibitory effects (Fig. 33b). The inhibitory responses to nicotine and TMA were abolished by pempidine (1 to 10 µg/ml) but were unaffected by guanethidine or by mixtures of $\alpha$ and $\beta$ adrenoceptor blockers.

Effect of 5HT

5HT (0.5 to 1 µg/ml) caused biphasic responses consisting usually of initial motor followed by inhibitory components. In a few experiments either purely inhibitory or purely motor responses were obtained. The motor component

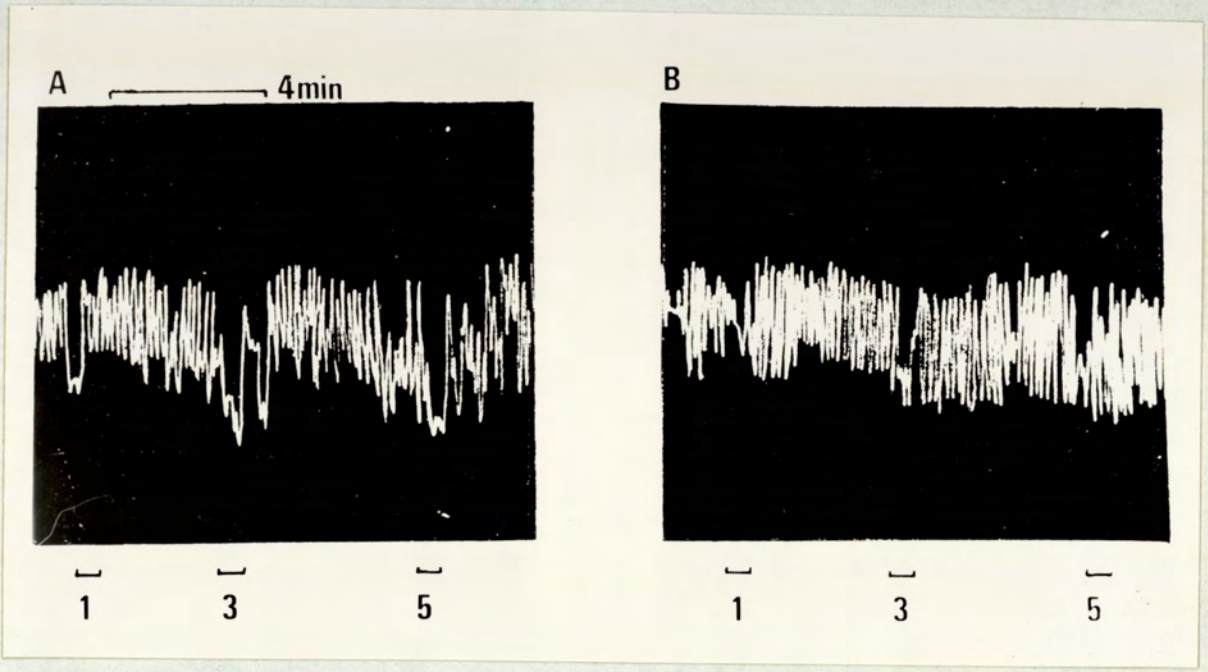


Fig. 31. Guinea pig isolated colon maintained at 37°C in McEwen's solution gassed with 95% $O_2$ and 5% $CO_2$.

In A, the responses are produced by 20 sec periods of pelvic (parasympathetic) nerve stimulation with 2 msec 20V pulses at the frequencies indicated and in the presence of hyoscine (10 µg/ml).

The responses in B were repeated 20 min after the further addition of pempidine (50 µg/ml) to the bath.

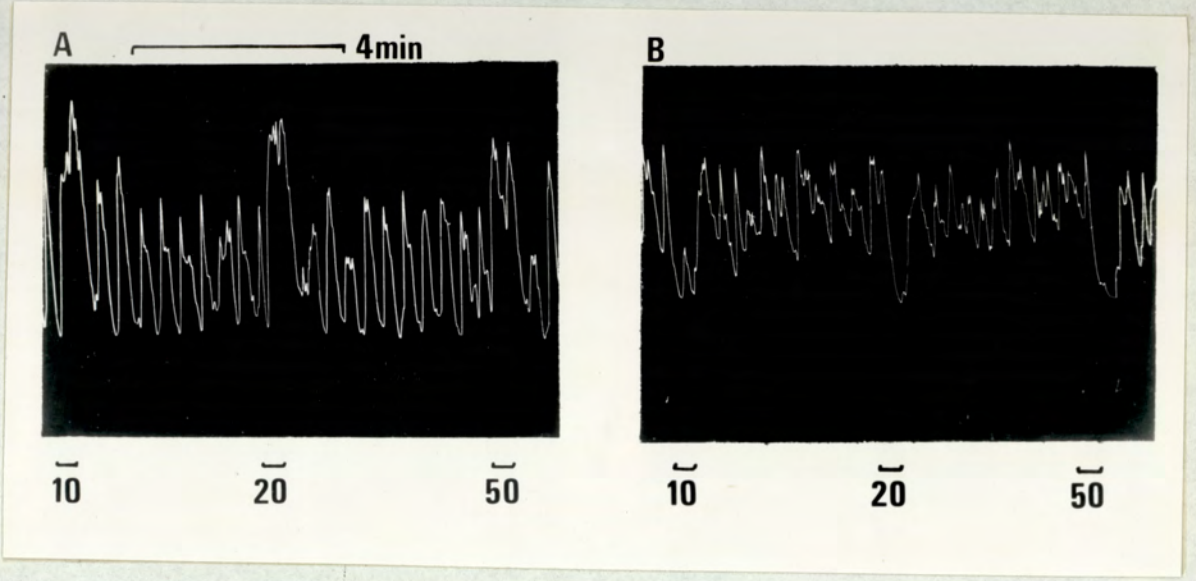


Fig. 32. Guinea pig isolated colon maintained at 37°C in McEwen's solution gassed with 95% $O_2$ and 5% $CO_2$.

In A, motor responses were elicited by 20 sec periods of pelvic (parasympathetic) nerve stimulation with 2 msec 20V pulses at the frequencies indicated.

In B, the responses were mainly inhibitory when repeated 25 min after the addition of pempidine (10 µg/ml) to the bath.

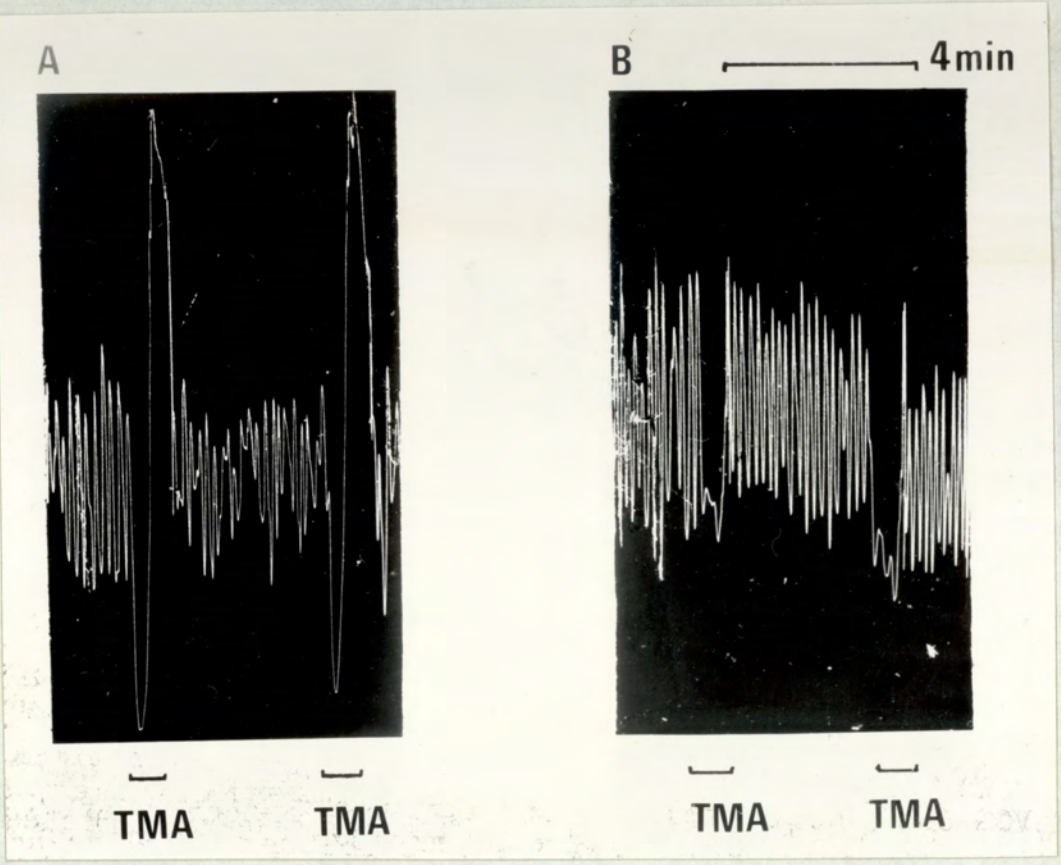


Fig. 33. Guinea pig isolated colon at 37°C in McEwen's solution gassed with 95% $O_2$ and 5% $CO_2$.

In A, the biphasic responses ere produced by the addition of 4 µg/ml tetramethylammonium bromide (TMA) to the bath for 30 sec periods.

In B, the responses became inhibitory when repeated 30 min after the addition of a mixture of hyoscine (0.1 µg/ml) and guanethidine (1 µg/ml).

of the 5HT response was markedly reduced or abolished in the presence of hyoscine (0.1 to 1 μg/ml). The inhibitory component of the 5HT response was unaffected by either hyoscine or by a mixture of α and β adrenoceptor blocking substances which abolished sympathetic inhibitions. Both phases of the 5HT response were abolished in the presence of bufotenine (0.5 to 1 μg/ml) indicating that the actions of 5HT on this preparation are probably neurally mediated (Bulbring & Gershon, 1967).

When a large concentration of 5HT (1 mg/ml) was added to the bath the tissue contracted for about 2 min. after which it relaxed for a further 5 to 10 min. and then resumed normal spontaneous activity. Further amounts of 5HT added at this time produced no effect showing that tachyphylaxis had occurred. In preparations in which tachyphylaxis to 5HT had been induced the inhibitory responses to parasympathetic nerve stimulation were greatly reduced. This is shown in the experiment illustrated in Fig. 34. In this preparation which is hyoscine-treated the responses to parasympathetic stimulation at three frequencies were purely inhibitory (Fig. 34a). However, after tachyphylaxis to 5HT had been induced the inhibitory responses to parasympathetic stimulation were greatly reduced (Fig. 34b). In other experiments it was shown that tachyphylaxis to 5HT did not affect the responses to acetylcholine, noradrenaline or the inhibitory responses to transmural stimulation.

Effect of Bufotenine

As mentioned previously bufotenine (0.5 to 1 μg/ml) abolished all phases of the response to 5HT. At a concen-

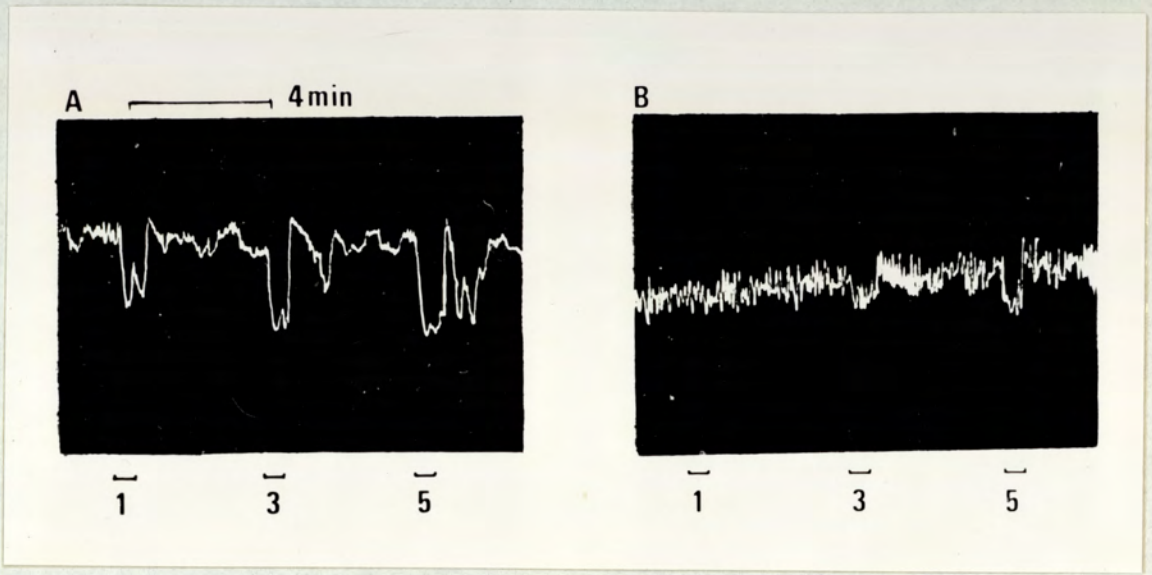


Fig. 34. Guinea pig isolated colon at 37°C in McEwen's solution gassed with 95% $O_2$ and 5% $CO_2$.

In A, responses are to 20 sec periods of pelvic (parasympathetic) stimulation with 2 msec 20V pulses at the frequencies indicated. Between A and B, 5-hydroxytryptamine (5HT) (1 mg/ml) was added to the bath and left in contact with the preparation until the end of the experiment.

In B, the parasympathetic stimulations were repeated after tachyphylaxis to 5HT. Hyoscine (0.1 µg/ml) was present throughout.

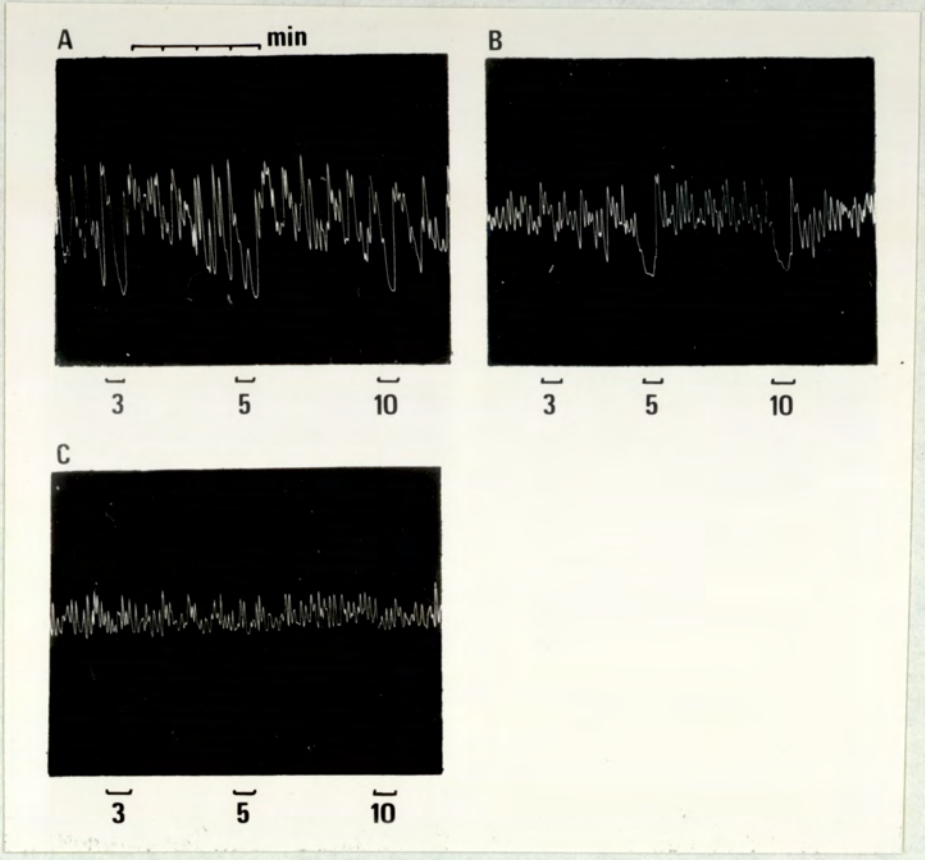


Fig. 35. Guinea pig isolated colon at 37°C in McEwen's solution gassed with 5% $CO_2$ in $O_2$.

In A, control responses to parasympathetic (pelvic) nerve stimulation for 20 sec periods with 2 msec 20V pulses at the frequencies indicated. The responses were repeated 20 min after the addition of pempidine (10 µg/ml) to the bath (in B) and again 20 min after the further addition of 1 µg/ml bufotenine to the bath (panel C).

tration of 1 µg/ml it did not affect responses to acetylcholine, noradrenaline, transmural electrical stimulation or to sympathetic stimulation. However, this concentration invariably reduced inhibitory responses to parasympathetic stimulation and in two experiments of 12 completely abolished it. In each of the experiments in this series motor effects of parasympathetic nerve stimulation were abolished by pempidine (10 µg/ml) revealing inhibitory responses which were usually abolished in the presence of bufotenine (1 µg/ml). In the experiment illustrated in Fig. 35 parasympathetic stimulation produced inhibition even in the absence of hyoscine. After pempidine (10 µg/ml) the inhibitory responses were slightly reduced (Fig. 35b) but were completely abolished after the addition of 1 µg/ml bufotenine (Fig. 35c).

## DISCUSSION

The results presented in this chapter clearly indicate the presence of non-adrenergic inhibitory neurones in the guinea pig colon. Moreover, in this preparation non-adrenergic inhibitory responses were obtained by stimulation of the pelvic parasympathetic nerves. The motor component of the parasympathetic responses when present, and the motor component of the transmural response, were both readily susceptible to the blocking action of hyoscine indicating their probable cholinergic nature. Bianchi, Beani, Frigo & Crema (1968) in their investigations using the same preparation were unable to obtain inhibitory responses to parasympathetic stimulation either before or after atropine. They stated that "the pelvic nerves behave almost like a pure parasympathetic pathway". This fundamental difference

between the results described here and those of Bianchi et al (1968) is very difficult to explain. The characteristics of pelvic inhibition described here were strikingly similar to transmural inhibition in terms of sensitivity to electrical stimulation and to blocking drugs and were easily distinguished from sympathetic inhibition.

The involvement of acetylcholine as a ganglionic mediator of non-adrenergic inhibition is suggested by the action of nicotine and TMA which both before and after hyoscine produced responses clearly mimicking those produced by either parasympathetic or transmural stimulation. Bianchi et al (1968) were unable to produce a nicotinic relaxation in this preparation using acetylcholine after atropine. The results using pempidine suggested that more than one transmitter substance may be involved in ganglionic transmission of the pelvic nerve responses. Thus, the motor component of the pelvic response was more readily blocked by pempidine than was the inhibitory component. However, the inhibitory response was partially susceptible to the blocking action of bufotenine which is known to block neurally-mediated 5HT responses (Bulbring & Gershon, 1967). Moreover a combination of ganglion blocker and bufotenine completely abolished parasympathetic inhibition. Further evidence that 5HT is involved in ganglionic transmission of pelvic responses was gained from the observation that after desensitization of the preparation to 5HT by producing 5HT tachyphylaxis then inhibitory parasympathetic responses were greatly reduced, but not sympathetic inhibitions. These results with 5HT in the isolated colon are in very close agreement with the results of Bulbring & Gershon (1967) who

used the guinea pig isolated stomach and obtained inhibitory responses by vagal (parasympathetic) stimulation.

The motor effect of 5HT was found in the present experiments to be hyoscine-sensitive which is in agreement with Bianchi et al (1968). However, these workers found that after atropine 5HT produced a further complex response consisting of rapid inhibition followed by a more sustained contraction and finally a long-lasting inhibition. They suggested that 5HT may activate neuronal pathways in the colon causing cholinergic and non-cholinergic excitation as well as non-adrenergic inhibition.

## Chapter 7

### Attempts to isolate and identify the non-adrenergic inhibitory transmitter substance

The evidence presented in this thesis has confirmed the presence of non-adrenergic neurones having an inhibitory function in all parts of the gastro-intestinal tract from several species. These neurones appear to be part of the parasympathetic system and have ganglia in Auerbach's plexus. There is a considerable body of evidence implicating both acetylcholine and 5HT in ganglionic transmission of non-adrenergic neurones. Little is known of the nature of the final transmitter substance of these neurones and this chapter is devoted to a review and discussion of possible transmitter substances. In addition a brief account is given of some unsuccessful attempts to isolate the transmitter substance from rabbit ileum.

## RESULTS AND DISCUSSION

### Possible candidates for the role of non-adrenergic transmitter substance

5HT. This substance is present in all parts of the mammalian intestine and the results described in this thesis and by Bulbring & Gershon (1967) strongly suggest that it may play a role in ganglionic transmission. Most of the effects of 5HT on the gastro-intestinal tract appear to be mediated via effects on other neurones and are abolished by bufotenine but not by methysergide. Reserpine was shown (this thesis, chapter 2) to reduce non-adrenergic as well as sympathetic inhibitory responses in rabbit isolated ileum. However,

neither 5HT (1 to 50 µg/ml) nor 5HTP (1 to 100 µg/ml) increased the inhibitory responses to transmural stimulation after reserpine.

Histamine. This substance is also present in the mammalian gut and is depleted by reserpine. However, in rabbit isolated ileum it produced weak motor effects which were abolished by mepyramine. Bianchi et al (1968) found histamine to be weakly motor in guinea pig isolated colon and therefore discounted it as a possible mediator of non-adrenergic inhibition.

Glycine. This amino-acid in concentrations of 0.1 to 100 µg/ml produced no effect on spontaneous contractions or tone of rabbit isolated ileum confirming the negative result of Bianchi et al (1968) in the guinea pig colon preparation.

Y-aminobutyric acid (GABA). This substance is thought to be an inhibitory transmitter in the mammalian brain and has been reported to be specifically antagonised by picrotoxin and by strychnine (Van der Kloot, Robbins & Cooke, 1958).
In preparations of guinea pig isolated colon GABA (0.5 - 100 µg/ml) produced inhibitory responses. The maximal inhibition which was obtained with a concentration of 40 µg/ml was smaller than the maximal inhibition obtained by either transmural or extrinsic parasympathetic stimulation.
Picrotoxin (50 µg/ml) abolished the inhibitory responses to GABA but not those to transmural stimulation or to noradrenaline. In 10 of 16 experiments the inhibitory responses to transmural stimulation were increased in the presence of picrotoxin (50 µg/ml) which had abolished GABA inhibitions.

Thus it would appear that GABA is unlikely to be the mediator of transmural inhibition.

Strychnine (20 to 40 µg/ml) also abolished the inhibitory effects of GABA but its action was apparently less specific than picrotoxin since the effects of sympathetic and transmural stimulation (both motor and inhibitory) were also partly blocked.

Adenine nucleotides. ATP, ADP and AMP (all in concentrations of 1 to 100 µg/ml) were found to produce inhibitory responses on rabbit isolated ileum. These substances are known to be depleted by reserpine treatment. However, they did not increase the inhibitory responses to transmural stimulation after these had been blocked by reserpine treatment. In general, the adenine nucleotides appeared to be too weak as inhibitory substances to be serious candidates for the role of non-adrenergic inhibitory substance. Bianchi et al (1968) also tested ATP and AMP as potential inhibitory transmitters in guinea pig isolated colon but found that they produced significant inhibitory responses only at a concentration of 500 µg/ml.

However, since the work described above was completed Burnstock and his co-workers have published a number of papers in which they suggest that an adenine nucleotide is the non-adrenergic transmitter substance (for review see Burnstock, 1972). For this reason these substances have been re-examined in the next chapter.

Attempts to isolate the non-adrenergic inhibitory transmitter

(a) "In vivo" experiments. In these experiments segments of ileum of anaesthetised rabbits were perfused with aerated

and eserinised Tyrode solution at 37°C through the mesenteric arteries. The perfusate was collected from the veins draining the tissue before, during and after electrical stimulation of both vagi in the neck. The perfusate was assayed on isolated guinea pig ileum sensitized to acetylcholine by the method of Paton (1957). Although contractile responses of the tissue were initially obtained acetylcholine could not be consistently detected in the perfusate and the preparations rapidly deteriorated with the animals going into a shocked condition. In view of the sensitivity of anaesthetized rabbits to even slight handling of the intestines and the irregular recoveries of acetylcholine from the perfusate attempts to develop the technique further with a view to isolating the non-adrenergic transmitter substance were abandoned.

(b) "In vitro" experiments. In these experiments samples of Tyrode solution were removed from the lumen of a piece of rabbit isolated ileum before or after transmural electrical stimulation of the preparation and were assayed on a segment of guinea pig ileum. Transmural stimulation caused the appearance of a considerable amount of material having a stimulant effect on guinea pig ileum. It was found that hyoscine (0.001 µg/ml) abolished control responses to acetylcholine in the recipient preparation but only reduced the contractile effect of the perfusate. The hyoscine-resistant contraction was abolished by mepyramine (0.01 µg/ml) indicating the probable presence of histamine as well as acetylcholine in the perfusate.

In a further series of preparations the tone of the

recipient guinea pig ileum was raised with 5HT (1 µg/ml) and barium chloride (50 µg/ml), hyoscine (0.001 µg/ml) and mepyramine (0.01 µg/ml) added to abolish any contractile effects. Using this preparation no inhibitory responses were elicited after the addition of perfusate from rabbit ileum subjected to transmural stimulation.

## Chapter 8

## Adenine nucleotides as potential mediators of non-adrenergic inhibition

The results described in the previous chapter appeared to have discounted the adenine nucleotides as well as several other naturally-occurring substances as likely mediators of post-ganglionic non-adrenergic nerve transmission. Bianchi et al (1968) reached a similar conclusion after finding that concentrations of ATP or AMP of 500 μg/ml were required to produce even mild relaxation of guinea pig isolated terminal colon in their experiments.

However, Burnstock and his co-workers have recently produced a considerable amount of evidence which suggests that the post-ganglionic transmitter in non-adrenergic inhibitory nerves is ATP or a related compound. For instance, Satchell, Burnstock & Campbell (1969) suggested that ATP or a closely related substance is the inhibitory transmitter of vagal inhibitory nerves to the stomach of the toad, Bufo Marinus. These workers perfused toad stomach with nutrient media and characterized substances released into the perfusate after stimulation of the vagus nerves. They found that adenosine and inosine, presumably from the breakdown of ATP, were the principal substances released into the perfusate. Burnstock, Campbell, Satchell & Smythe (1970) confirmed these results and in addition showed that stimulation of Auerbach's plexus in the turkey isolated gizzard caused the release of ATP, ADP and AMP. They showed that quinidine abolished the inhibitory effects of both ATP and non-adrenergic inhibitory stimulation and further that after tachyphylaxis to ATP

responses to stimulation of non-adrenergic nerves were depressed. Su, Bevan & Burnstock (1971), using tritium labelled adenine nucleotides, showed that guinea pig isolated taenia coli concentrated AMP, ADP, ATP and adenosine in preference to inosine and adenine. Tritium was released from taenia coli previously exposed to tritium-labelled adenosine after stimulation of the non-adrenergic inhibitory nerves. Satchell, Lynch, Bourke & Burnstock (1972) showed that dipyridamole and hexobendine potentiated responses to both ATP and to non-adrenergic inhibitory nerve stimulation. In his review of non-adrenergic inhibitory nerves, Burnstock (1972) suggested that ATP or a related nucleotide "broadly satisfies" the criteria proposed by Eccles (1964) for a neurotransmitter substance.

In view of the above data it was considered necessary to re-examine adenine nucleotides as potential mediators of non-adrenergic inhibitory nerves to the intestine. The results previously presented in this thesis suggested that the guinea pig isolated terminal colon would be the most suitable tissue for this study.

## RESULTS

### Action of ATP and related purine derivatives on the tone and motility of guinea pig isolated terminal colon

The actions of ATP, ADP and AMP were investigated on isolated segments of guinea pig colon. AMP was weakly inhibitory, the threshold concentration being 0.4 to 1 μg/ml. It was found that higher concentrations of AMP (up to 20 μg/ml) did not produce significantly greater inhibitory responses than the threshold concentrations and no concentration used

was capable of producing a full relaxation of the tissue as was readily obtained by either sympathetic or non-adrenergic nerve stimulation. ADP had a similar threshold concentration to produce a response as did AMP but in contrast to this substance the response obtained was biphasic consisting of inhibition followed by contraction. The inhibitory phase was usually poorly developed and the motor component was resistant to hyoscine (10 μg/ml). Since, in many preparations it has been found possible to elicit purely inhibitory responses to extrinsic parasympathetic nerve stimulation, then it would appear unlikely that ADP is the non-adrenergic inhibitory transmitter.

ATP produced purely inhibitory responses when added to preparations of guinea pig isolated colon. In most preparations a concentration of 1-2 μg/ml were necessary to imitate a response although in some experiments the threshold concentration was as low as 0.1 μg/ml. The nature of the inhibition to added ATP was similar to that observed with AMP in that the maximal inhibitory response which could be achieved was not as complete as could be readily obtained with either transmural or extrinsic parasympathetic stimulation. In addition, the inhibitory responses to transmural or parasympathetic stimulation were of more rapid onset and recovery after stimulation was quicker than when ATP was added to the bath. This is illustrated in Fig. 36. In this experiment the response to a maximally-effective concentration of ATP (1 μg/ml in this preparation) is compared to that produced by submaximal transmural stimulation at 5 Herz. The response to ATP was slower in onset and recovery slower than that to transmural stimulation and in

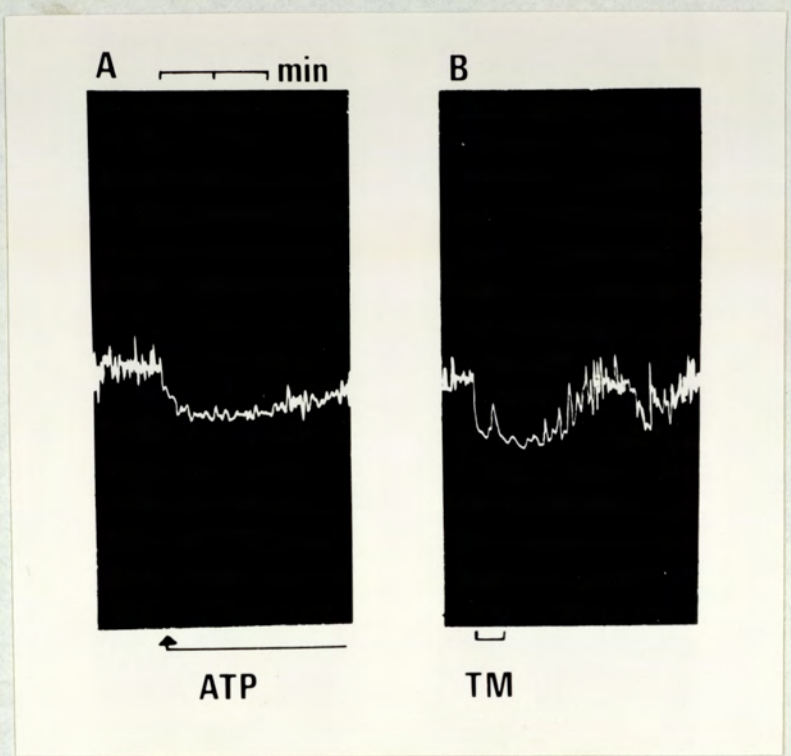


Fig. 36. Guinea pig isolated colon in McEwen's solution gassed with 5% $CO_2$ in oxygen and maintained at 37°C.

In A, the response to a maximally effective concentration of ATP (1 µg/ml) is compared (in B) to the submaximal response obtained by transmural stimulation (TM) for 20 sec with 2 msec 20V pulses at 5 Herz.

addition, despite the greater degree of inhibition, recover from transmural stimulation was more rapid.

Effect of quinidine

The action of quinidine in blocking the responses to both ATP and to non-sympathetic inhibitory nerves has been cited as evidence in support of the hypothesis that the nerves release ATP as the inhibitory transmitter substance (Burnstock, Campbell, Satchell & Smythe, 1970; Burnstock, Dumsday & Smythe, 1972; Snedden, Smythe & Burnstock, 1972; Burnstock, 1972).

In the majority of the present experiments quinidine (0.1 to 20 μg/ml) was found to have a reversible but non-selective blocking action on the responses to added ATP as well as to sympathetic, parasympathetic and transmural stimulation. However, in a few experiments the responses to ATP were impaired whilst those to non-adrenergic inhibition were unaffected. This was the case in the experiment illustrated in Fig. 37. In this experiment, in the presence of quinidine (1 μg/ml) the inhibitory responses to sympathetic and transmural stimulation were increased, the response to extrinsic parasympathetic stimulation was reversed from motor to inhibition, whilst the response to ATP was slightly reduced. In the presence of a higher concentration of quinidine (20 μg/ml) the responses to parasympathetic and sympathetic stimulation and that to ATP were virtually abolished, whilst the inhibition to transmural stimulation is no different to the control response (Fig. 37b).

The results with quinidine lend no support to the concept that ATP is the mediator of transmural and/or parasympathetic inhibition in this preparation.

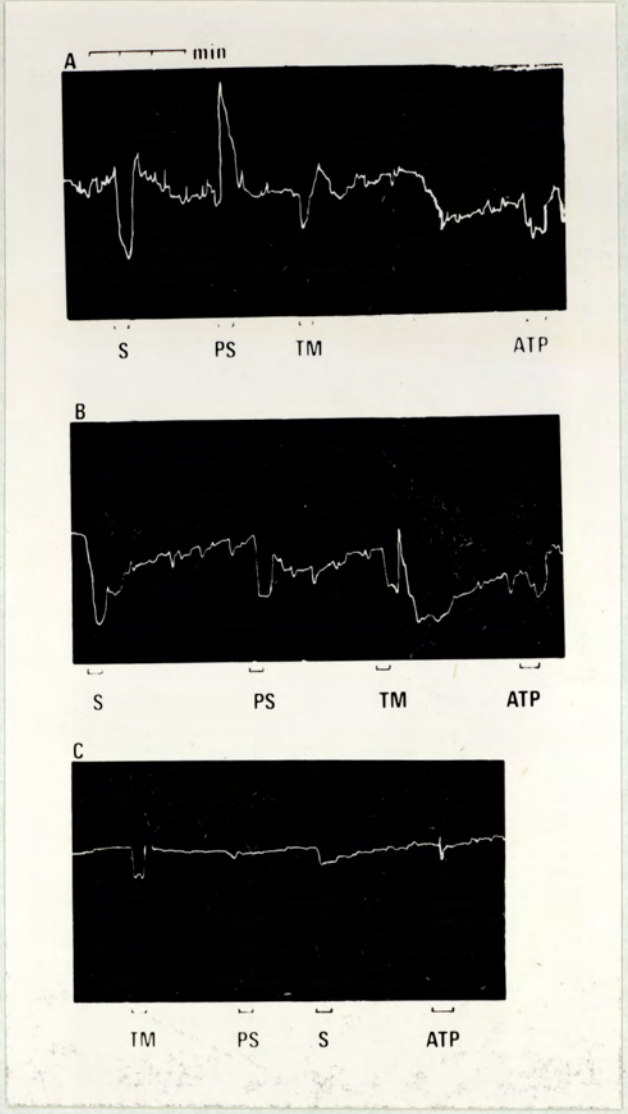


Fig. 37. Guinea pig isolated colon in McEwen's solution at 37°C and gassed with 95% $O_2$ and 5% $CO_2$.

In A, responses to sympathetic (S), parasympathetic (PS) and transmural stimulation (TM) each for 20 sec periods with 2 msec 20V pulses at 5 Herz, and to 30 sec contact period with ATP (1 µg/ml). In B, the responses were repeated 25 min after the addition of quinidine (1 µg/ml) to the bath and in C, 15 min after increasing the concentration of quinidine to 20 µg/ml.

Quinine

Quinine has been shown to antagonize the action of ATP (Wayne, Goodwin & Stoner, 1949) and was therefore used in the present series of experiments. It was found to be non-specific in its action. Thus, the lowest concentration of quinine which abolished the inhibitory action of ATP was found to be 100 µg/ml and this concentration also abolished responses to both sympathetic and parasympathetic stimulation. This is illustrated in Fig. 38.

Digoxin

Digoxin was reported by Rand, Stafford & Thorp (1958) to antagonize the action of ATP. In the present experiments it was found that digoxin (0.1 µg/ml) sometimes slightly impaired responses to added ATP but when this occurred it was usually accompanied by an increase in the inhibitory response to parasympathetic and/or transmural stimulation. One such experiment is illustrated in Fig. 39 in which digoxin (0.1 µg/ml) increased the inhibitory responses to low frequency parasympathetic stimulation whilst partially inhibiting the responses to added ATP. Higher concentrations of digoxin (1 to 10 µg/ml) produced a non-specific impairment of responses to sympathetic, parasympathetic and transmural stimulation and to added ATP.

Dipyridamole

Burnstock (1972) has pointed out that many workers have shown that responses to ATP are potentiated by dipyridamole. Recently, Satchell, Lynch, Bourke & Burnstock (1972) showed that inhibitions of guinea pig isolated taenia coli caused by either ATP or transmural electrical stimulation

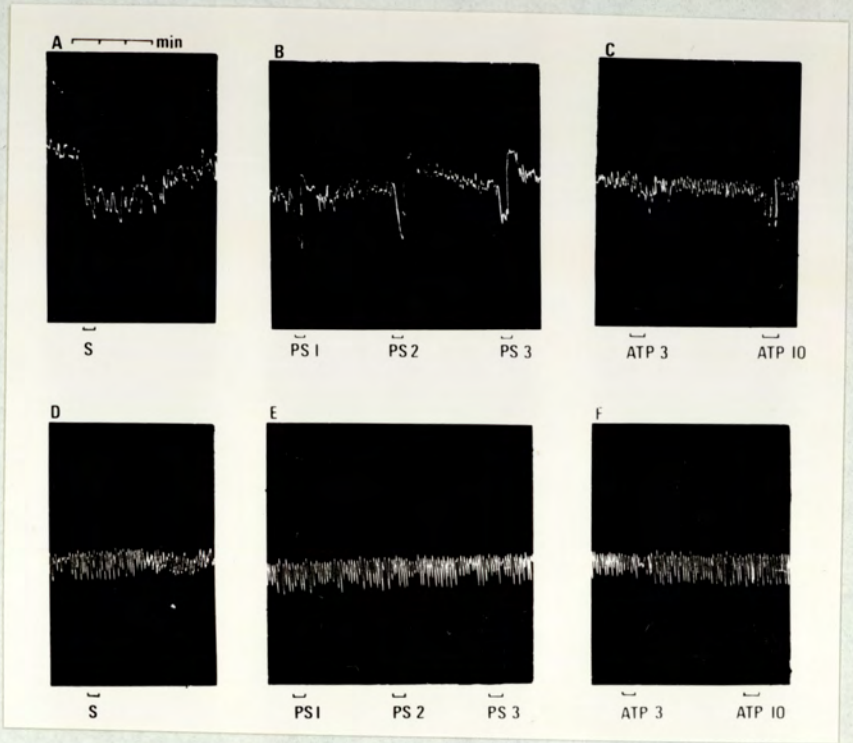


Fig. 38. Guinea pig isolated colon in McEwen's solution gassed with 95% $O_2$ and 5% $CO_2$ and maintained at 37°C.

In A, response to sympathetic stimulation (S) for 20 sec period with 2 msec 20V pulses at 50 Herz. In B, responses to parasympathetic stimulation (PS) with similar strength parameters at frequencies indicated and in C, responses to 30 sec contact periods with ATP (3 and 10 µg/ml).

In panels D, E and F the above responses were repeated 20 min after adding quinine (100 µg/ml) to the bath.

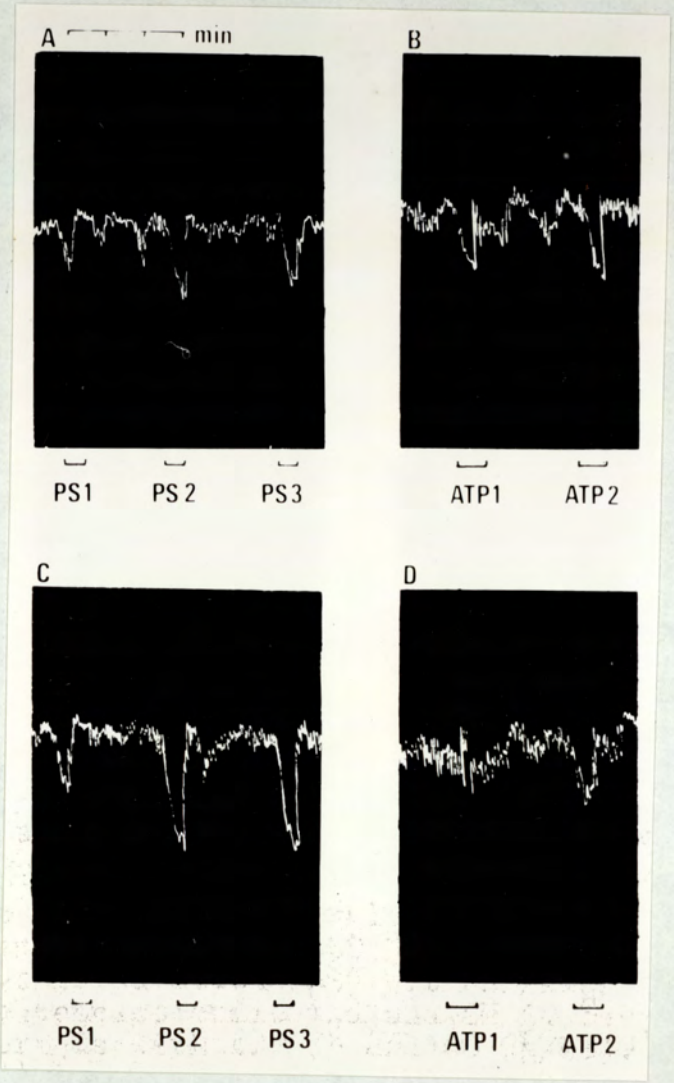


Fig. 39. Guinea pig isolated colon in McEwen's solution gassed with 95% $O_2$ and 5% $CO_2$ at 37°C.

In A, responses to pelvic (parasympathetic) stimulation for 20 sec periods with 2 msec 20V pulses at the frequencies indicated.

In B, responses to 30 sec contact period with ATP (1 and 2 µg/ml).

In C and D the responses were repeated 20 min after the addition of digoxin (0.1 µg/ml).

were increased in the presence of dipyridamole, thus suggesting that the transmural inhibition may be due to ATP release. These workers have pointed out that this action of dipyridamole is probably independent of its known action as an inhibitor of 3'5'AMP phosphodiesterase since other inhibitors of this enzyme (e.g. aminophylline) do not potentiate ATP responses.

In the present experiments it was found that dipyridamole produced very variable effects on responses to sympathetic, parasympathetic and transmural stimulation and to added ATP. Thus, in the experiment illustrated in Fig. 40 dipyridamole (1 μg/ml) reduced the inhibitory responses to transmural, sympathetic and parasympathetic stimulation whilst slightly increasing the response to added ATP. Dipyridamole regularly reduced the motor component of the transmural response as shown in Fig. 40B. In other experiments dipyridamole increased the inhibitory responses to both parasympathetic and transmural stimulation. However, in these experiments dipyridamole also increased the inhibitory responses to sympathetic stimulation. This is shown in Fig. 41 where in the presence of dipyridamole (1 μg/ml) the inhibitory response to parasympathetic stimulation was markedly enhanced, that to transmural stimulation marginally increased, whilst the response to sympathetic stimulation was increased most of all.

Reserpine

When preparations of rabbit isolated ileum were set up at 37°C and treated with reserpine (1 - 5 μg/ml) the inhibitory responses to both sympathetic and transmural stimulation were

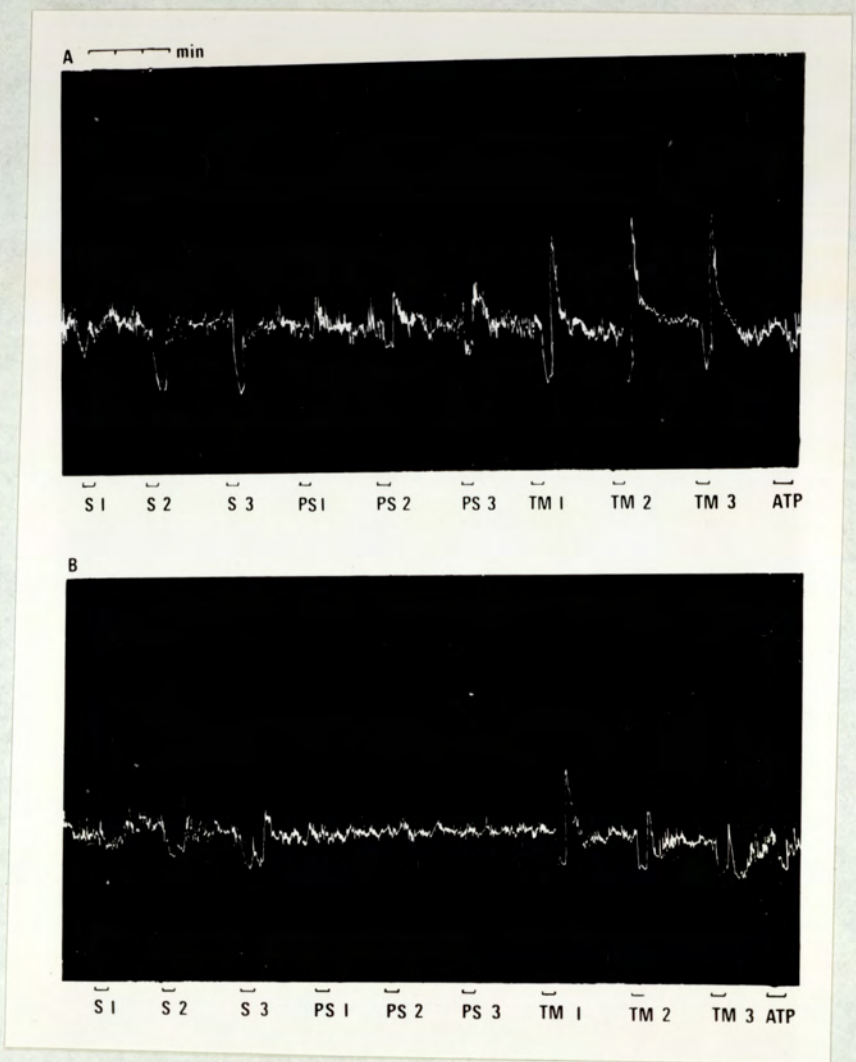


Fig. 40. Guinea pig isolated colon in McEwen's solution gassed with 5% $CO_2$ in $O_2$ and maintained at 37°C.

In A, control responses to sympathetic (S), parasympathetic (PS) and transmural (TM) stimulation each for 20 sec periods with 2 msec 20V pulses at the frequencies indicated and to ATP (1 µg/ml) left in contact with the tissue for 30 sec.

In B, the responses were repeated 30 min after the addition of dipyridamole (1 µg/ml) to the bath.

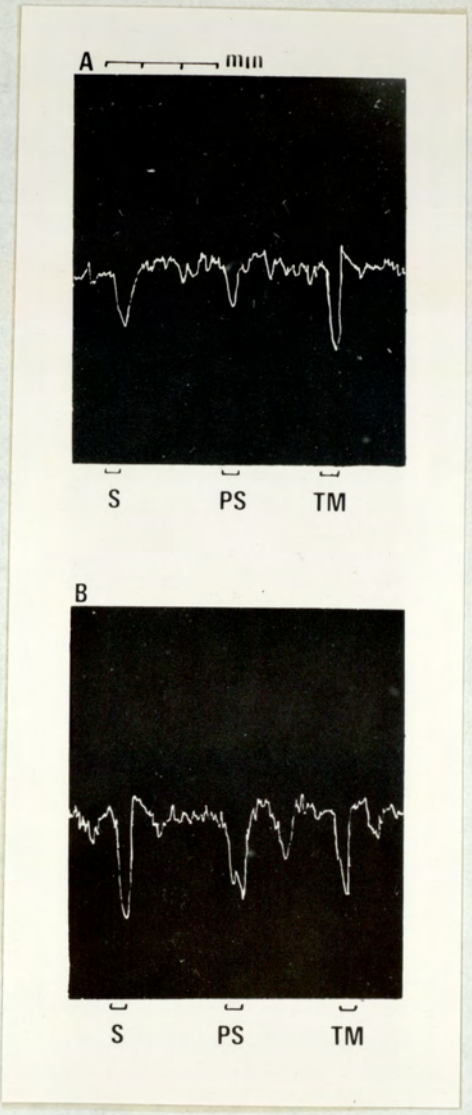


Fig. 41. Guinea pig isolated colon in McEwen's solution at 37°C, gassed with 5% $CO_2$ in oxygen. In A, responses to sympathetic (S), parasympathetic (PS) and transmural (TM) stimulation all for 20 sec periods with 2 msec 20V pulses at 1 Herz. In B, responses repeated 30 min after the addition of dipyridamole (1 µg/ml) to the bath.

slowly abolished (see Chapter 2). Incubation at 37°C with ATP (50 µg/ml) for 20 minutes did not restore the inhibitory responses to transmural stimulation whilst similar treatment with dopamine (50 µg/ml) markedly increased the responses to sympathetic stimulation. In other preparations taken from rabbits pretreated with reserpine incubation with a mixture of ATP (50 µg/ml) and dopamine (50 µg/ml) for 20 minutes at 37°C partly restored sympathetic inhibitory responses but not those to transmural stimulation. As in the previous experiments incubation of tissues from these animals with ATP (50 µg/ml) alone was ineffective at increasing either sympathetic or transmural responses.

The failure of ATP to reverse the reserpine block of transmural inhibition cannot be taken as positive evidence that ATP is not the post-ganglionic transmitter in non-adrenergic inhibitory nerves since the mechanism of reserpine block of non-adrenergic nerves is not established and it is possible therefore that incubation with transmitter would not restore the response. However it is in general agreement with the other data reported thus far in this chapter that no clear correlation exists between the inhibitory responses produced by transmural and parasympathetic stimulation and those produced by added ATP.

Tachyphylaxis to ATP

Burnstock, Campbell, Satchell & Smythe (1970) reported that when segments of rabbit isolated ileum were desensitized to ATP by adding it in concentrations of 10 to 100 µg/ml to the bath, then the inhibitory component of the transmural

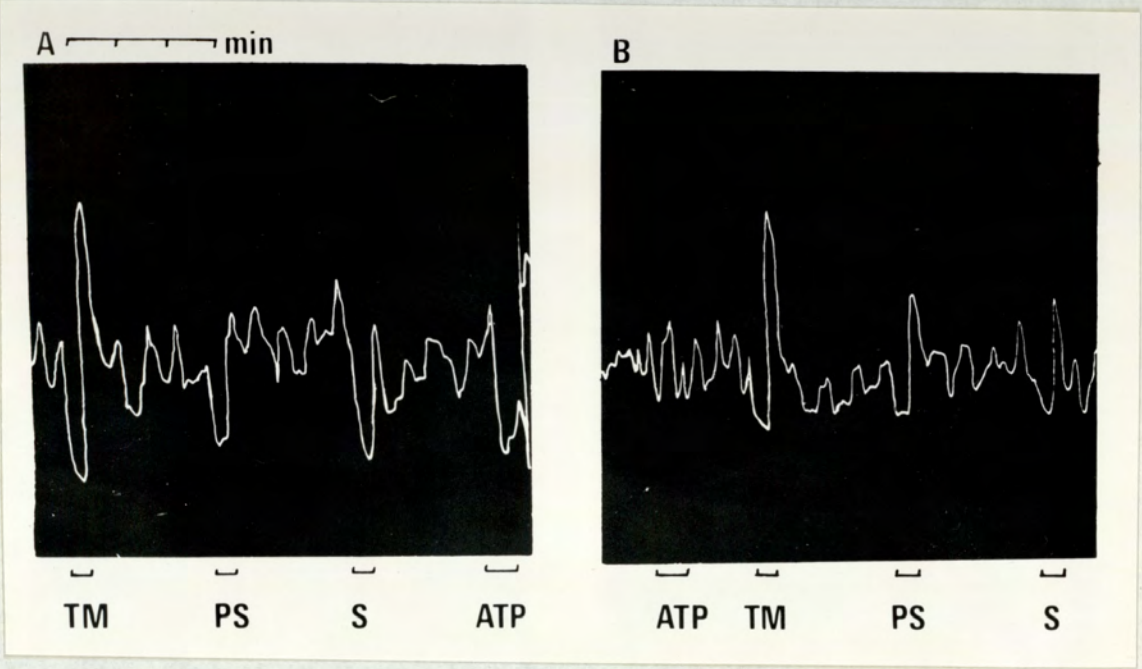


Fig. 42. Rabbit isolated colon in McEwen's solution gassed with 5% $CO_2$ and 95% $O_2$ and maintained at 37°C.

In A, responses to transmural (TM), parasympathetic (PS) and sympathetic (S) stimulation each for 20 sec periods with 2 msec 20V pulses at 3 Herz, and to ATP (1 µg/ml) left in the bath for 30 sec. Between A and B ATP (10 µg/ml) was added to the bath and left for the remainder of the experiment. In B, the responses were repeated as in A in the tissue which was desensitized to ATP.

response was reduced or abolished. Attempts were made to repeat this observation in segments of guinea pig isolated colon. It was found in this preparation that tachyphylaxis to ATP was readily produced by a single dose of ATP (10 µg/ml) allowed to remain in the bath until the tone and motility of the preparation returned. After such treatment previously inhibitory concentrations of ATP (usually 1 µg/ml) were no longer effective. In 4 preparations out of 6 treated in this way, tachyphylaxis to ATP did not reduce the inhibitory responses to either sympathetic, parasympathetic or transmural stimulation. In the other two preparations in this series the response to all three nervously-mediated inhibitory responses were similarly reduced (Fig. 42) after ATP tachyphylaxis. Thus again no specific effects linking transmural inhibitory responses with the effects of added ATP could be found.

## DISCUSSION

Burnstock, Campbell, Satchell & Smythe (1970) and again Burnstock (1972) have suggested that "in broad outline" ATP satisfies the criteria suggested by Eccles (1964) for a substance to be established as a neurotransmitter. These criteria are:-

(1) The substance together with the enzymes necessary in its formation must be present in the nerves.

(2) The substance must be released when the nerves are stimulated.

(3) The substance must mimic the effects of nervous stimulation when applied exogenously.

(4) A mechanism for the inactivation of the potential neurotransmitter must be present.

(5) Drugs which reduce or potentiate the effects of nervous stimulation should similarly affect the exogenously applied substance.

Burnstock (1972) in his review entitled "Purinergic Nerves" has described a number of sophisticated experiments which have been designed to demonstrate that ATP meets the above criteria as the neurotransmitter from non-adrenergic inhibitory nerves. For instance, Su, Bevan & Burnstock (1971) demonstrated that guinea pig isolated taenia coli concentrate tritiated ATP and release it when submitted to electrical stimulation. However, their observation that under similar experimental conditions sympathetic nerves also release labelled ATP casts serious doubt upon ATP as a potential non-adrenergic inhibitory transmitter substance. Moreover this evidence taken with the ubiquitous occurrence of ATP in mammalian tissues does not lend support to the claim of Burnstock and his co-workers that non-adrenergic nerves synthesise, store and release ATP, which is thus a likely candidate for the role of non-adrenergic neurotransmitter. Given the widespread distribution of ATP it is hardly surprising that (1) and (4) of Eccles (1964) criteria are satisfied. Similarly, the fact that sympathetic nerves also release ATP means that compliance with criteria (2) is also not conclusive evidence.

The experiments which have been described in this chapter concentrate mainly on (3) and (5) of Eccles (1964) criteria. That is, on investigating whether ATP mimics the response to non-adrenergic nerve stimulation and on the effect of drugs on responses to ATP and to stimulation of

non-adrenergic nerves. The results in general do not support the hypothesis. ATP (also AMP and ADP) does not closely mimic the response to non-adrenergic nerve stimulation. The time of onset and recovery from the effects of added ATP were both slower than for non-adrenergic nervous stimulation. Furthermore, ATP was incapable of producing as complete a relaxation in any concentration as was non-adrenergic inhibitory stimulation.

The experiments using the blocking drugs quinidine, quinine and digoxin indicated that these drugs were, in the main, unspecific in their effects. Despite this in a number of experiments with quinidine and digoxin the responses to ATP and non-adrenergic stimulation were affected in different ways arguing strongly against both responses being mediated by a common substance. Results using dipyridamole which is known to potentiate ATP responses in a number of tissues also questioned the hypothesis of purinergic nerves. Dipyridamole in concentrations necessary to potentiate ATP also potentiated sympathetic inhibitory responses in some experiments, whilst in others the ATP inhibitions were increased, whilst inhibitions to non-adrenergic stimulation were decreased. Finally, complete desensitization of the tissue to ATP did not regularly depress responsiveness to non-adrenergic nervous stimulation.

Thus the results in this chapter indicate that ATP poorly imitates the effects of non-adrenergic nervous stimulation and moreover drugs which increase or decrease the effects of ATP do not regularly produce parallel effects on non-adrenergic inhibitory responses.

To summarise the evidence which conflicts with the hypothesis of Burnstock (1972) that ATP is the non-adrenergic neurotransmitter:-

(a) Evidence that the nerves take up ATP or precursors and synthesize and store ATP is unconvincing due to the ubiquitous nature of ATP.

(b) Evidence that the nerves release ATP is unconvincing as sympathetic nerves do the same.

(c) Evidence from pharmacological analysis as described in this chapter suggests a lack of parallelism between the effect of drugs on ATP responses and on the responses to non-adrenergic stimulation.

Thus, the question of the nature of the non-adrenergic neurotransmitter is still open and the term "purinergic" (Burnstock, 1972) to describe the effects of stimulation of these nerves is probably premature.

## Chapter 9

### The motor component of the response to transmural stimulation of isolated intestine - neurally mediated or "rebound" contraction?

The term "rebound" contraction was first used by Burnstock, Campbell, Bennett & Holman (1963) to describe the contractile response following the inhibition of guinea pig isolated taenia coli caused by electrical stimulation of the intramural nerve plexus. This contraction has since been reported by most other workers who have used transmural stimulation of isolated intestinal preparations. The origin of the contraction, which is more or less resistant to atropine, has been the subject of much speculation and some experiment, but in general has tended to be overshadowed by the non-sympathetic inhibition which usually precedes it.

Campbell (1966a) and Bennett (1966a) both investigated the nature of the "rebound" contraction and both concluded that it resulted from, and was caused by, the preceding inhibition. They considered the excitation to be of myogenic origin and due to the fact that after hyperpolarization of the muscle cells there follows a period in which the muscle membrane becomes depolarized beyond its normal value and thus initiates the contraction.

The view proposed in this thesis (Chapter 3) and by Day & Warren (1968) that the contraction could be due to atropine resistant cholinergic fibres has received little support from other workers. Bianchi, Beani, Frigo & Crema (1968), Del Tacca, Lecchini, Frigo, Crema & Benzi (1968), and Furness (1969a,b, 1970) all used guinea pig isolated

terminal colon and all supported the "rebound" explanation.

Ambache & Freeman (1968) working with strips of longitudinal muscle from guinea pig ileum obtained contractions to transmural stimulation which persisted in the presence of atropine but were abolished by tetrodotoxin and strychnine, thus suggesting a neurogenic origin for the contractions. Beani, Bianchi & Crema (1971) using guinea pig isolated stomach, also produced evidence that the contractile component which followed the inhibition produced by vagal stimulation was nervously mediated. They found that they could record excitatory junction potentials following vagal stimulation in cells which had shown no inhibitory junction potentials during the stimulation period. Similarly, no excitatory junction potentials were recorded in some cells which had shown inhibitory junction potentials during vagal stimulation.

Thus, although transmural stimulation of isolated intestinal preparations has been used mainly in the last few years as a means of studying non-adrenergic inhibitory effects the nature of the motor phase that is usually seen is the subject of speculation and varying explanations. The results presented here were obtained from several different isolated preparations and suggest an explanation for this interesting phenomenon.

## RESULTS

### Rabbit isolated ileum

#### Different types of response to transmural stimulation at 37°C.

The effects of transmural stimulation of rabbit ileum can be broadly classified into four types as shown in Fig. 43 Whilst (a) and (b) type responses which consist essentially

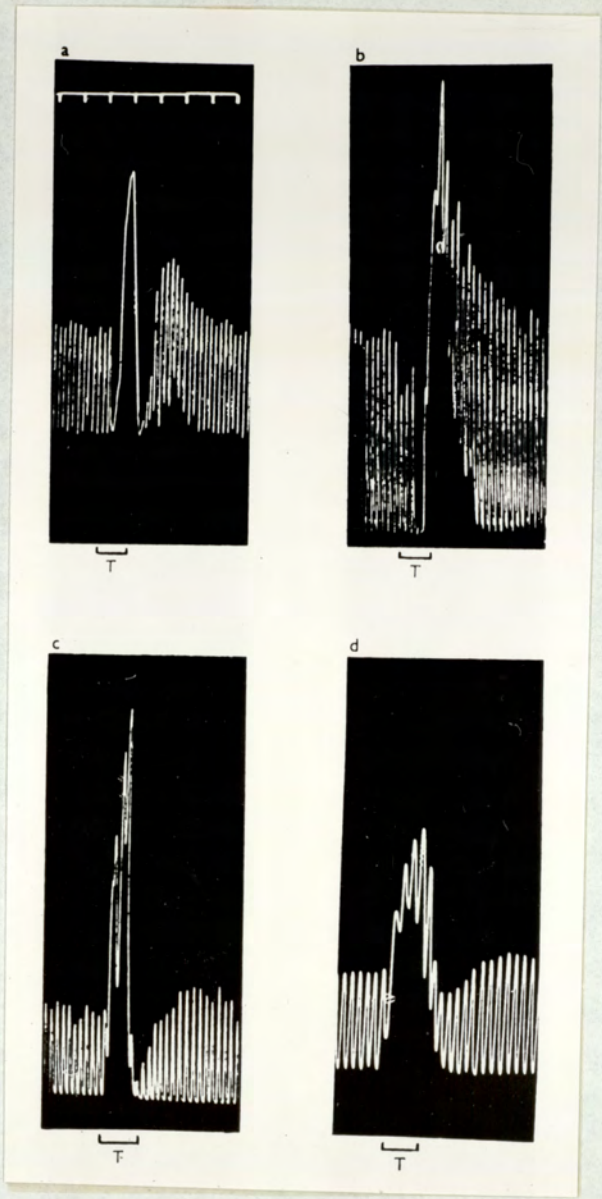


Fig. 43. (also Fig. 4, Chapter 1). Four main types of response to transmural stimulation (T) in different segments of rabbit isolated ileum in aerated Tyrode solution at 37°C. Stimulation applied for 20 sec with 1 msec 20V pulses at 20 Herz. Time marker in 30 sec intervals.

of inhibition followed by contraction are by far the most common types, types (c) and (d) are sometimes seen. In these the motor phase precedes the inhibitory phase as in (c) or occurs in the absence of any inhibition as in (d). Both these latter responses are difficult to explain if the contraction is in fact "rebound" and therefore dependent upon the presence of an initial inhibitory phase.

Effect of altering bath temperature. When the bath temperature is progressively lowered from 37°C to 28°C the response to transmural stimulation changes as in Fig. 44 At lower temperatures the inhibitory part of the response was much increased and was accompanied by a decrease in the motor phase. At 28°C a purely inhibitory response was sometimes seen. The contractile response to acetylcholine (0.01 to 0.04 µg/ml) were affected in the same way as the motor component of the transmural response being depressed at lower bath temperatures and recovering at 37°C. Fig. 45 illustrates this point. It shows how lowering the bath temperature produced a similar depressant effect on the contractile responses to nicotine, acetylcholine and transmural stimulation.

Effect of atropine and hyoscine. At 37°C both atropine and hyoscine potentiated the inhibitory phase of the transmural response without significantly altering the motor phase. It is this atropine-resistant nature of the motor component which has led to various workers doubting its cholinergic nature and explaining it instead as a "rebound" contraction. However, if the rebound explanation were correct it would be expected that hyoscine treatment would tend to increase the

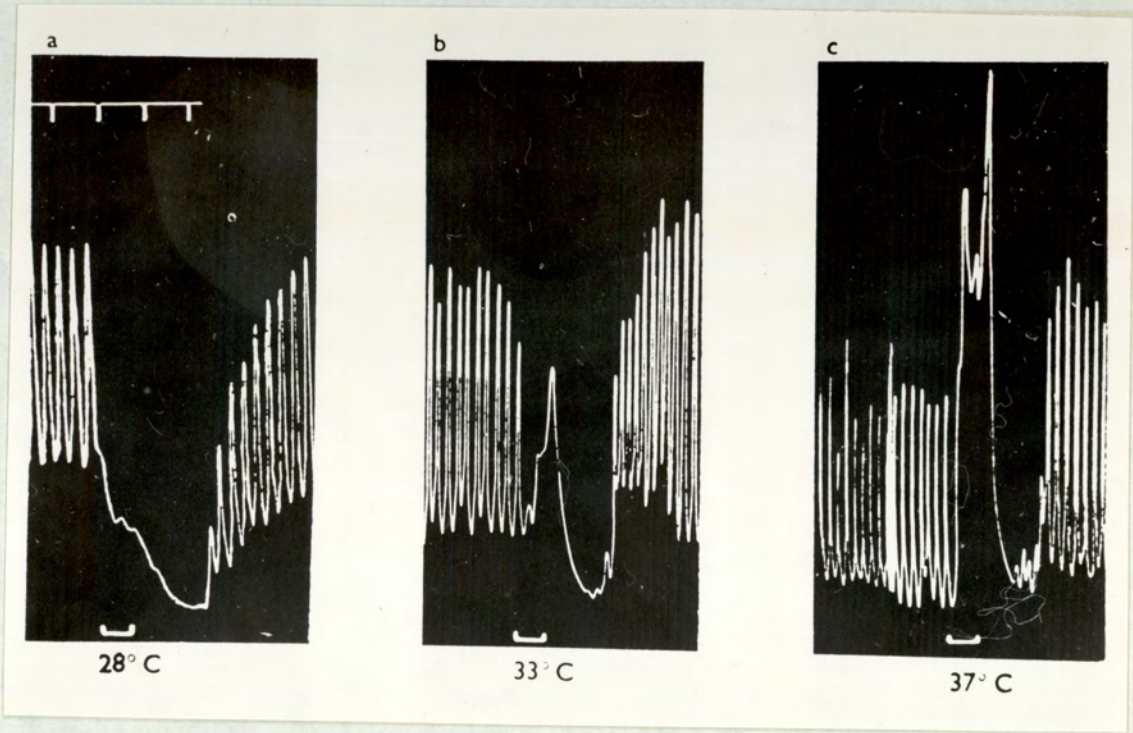


Fig. 44. (also Fig. 7, Chapter 1). Effect of raising the bath temperature on the response to transmural stimulation in a segment of rabbit isolated ileum suspended in aerated Tyrode solution. Transmural stimulation (at ⎵ ) applied for 20 sec with 2 msec pulses of supramaximal strength at a frequency of 20 Herz. Time marker in 30 sec intervals.

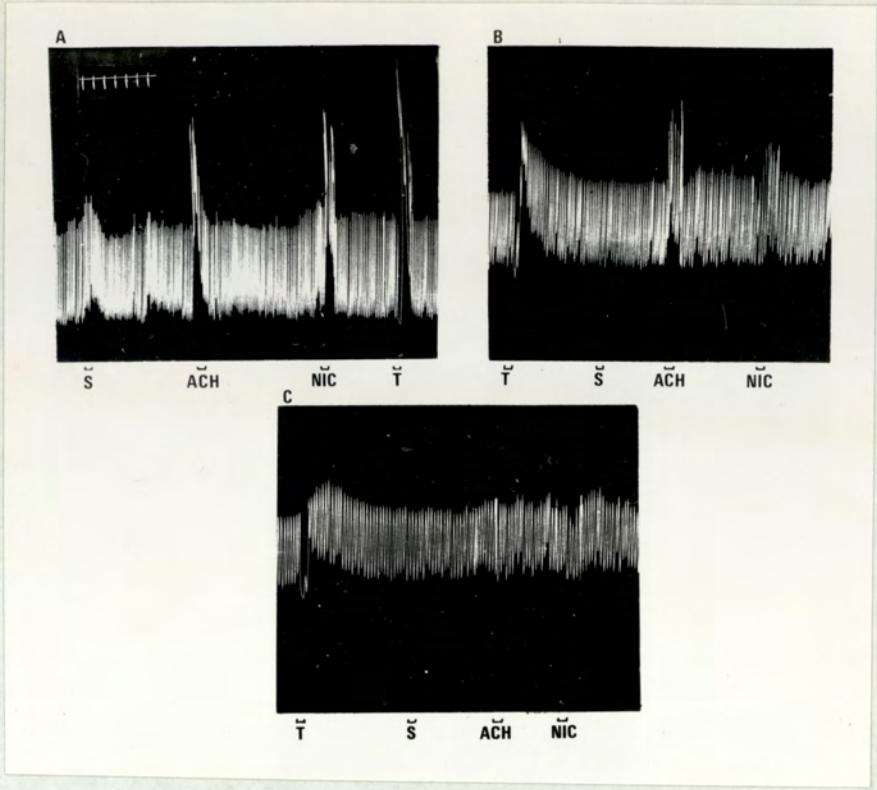


Fig. 45. (also Fig. 20, Chapter 4). Rabbit isolated ileum in aerated Tyrode solution. In A at 37°C, responses to sympathetic (S) stimulation for 20 sec period with 2 msec 20V pulses at 50 Herz, 0.5 µg/ml acetylcholine (ACH) and 1 µg/ml nicotine (NIC) each applied for 30 sec and transmural stimulation (T) applied as per sympathetic stimulation.
In B, the responses were repeated at 33°C and in C at 28°C. Guanethidine (1 µg/ml) was added to the bath 30 min before A and remained throughout the experiment. Time marker in 30 sec intervals.

motor phase since the initial inhibition is potentiated as in Fig. 46.

In preparations maintained below 37°C the motor component of the transmural response was usually less well developed and relatively more susceptible to the blocking action of hyoscine. This is illustrated in Fig. 47 which shows a preparation maintained at 32°C in which motor responses to both acetylcholine and transmural stimulation were abolished by hyoscine.

Effect of anticholinesterases. The effect of anticholinesterases on the response to transmural stimulation is shown in the experiment illustrated in Fig. 48. The responses to transmural stimulation were markedly altered in the presence of eserine (0.1 µg/ml). A motor component appeared in the response which was initially inhibitory and the motor components of the other responses were potentiated whilst the inhibitory components were reduced.

Effect of reserpine. In experiments in which reserpine (0.1 to 1 µg/ml) was added to the bath the inhibitory components of the responses to transmural stimulation were reduced whilst the motor responses were either unaffected, or more often, increased. In the experiment illustrated in Fig. 49 the responses to transmural stimulation were initially inhibitory but became entirely motor in the presence of reserpine (1 µg/ml).

The above results do not support the concept that the motor phase of the transmural response is a "rebound" contraction dependent on an initial inhibitory response. The main evidence against the "rebound" contraction is as follows:-

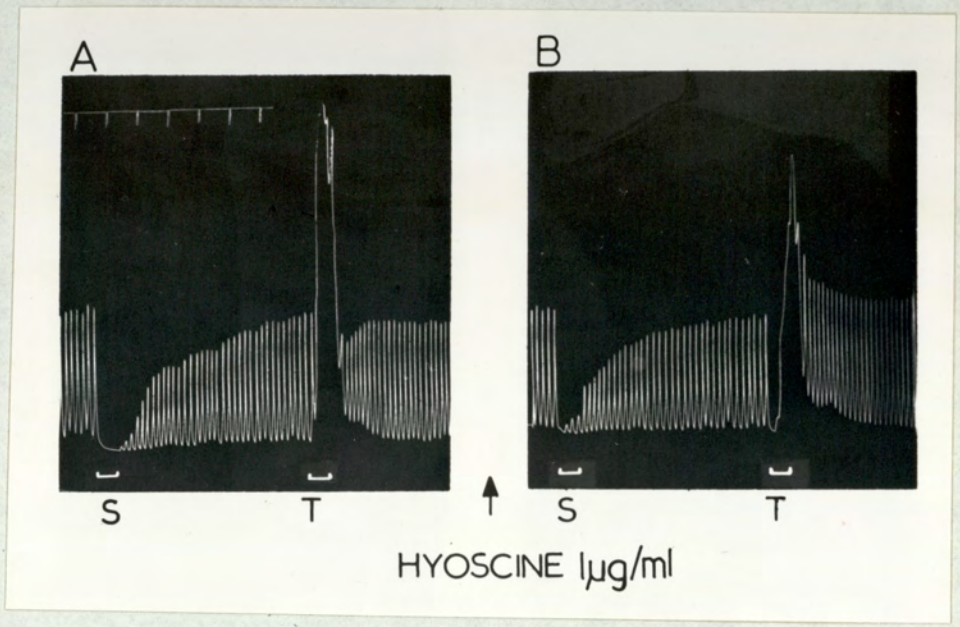


Fig. 46. (also Fig. 16, Chapter 3). Rabbit ileum at 37°C. A: Control responses to sympathetic stimulation (S) with 2 msec 20V pulses at 50 Herz and transmural stimulation (T) with 2 msec 20V pulses at 20 Herz each applied for 20 sec. B: Responses repeated 15 min after adding hyoscine (1 µg/ml) to the bath. Time marker in 30 sec intervals.

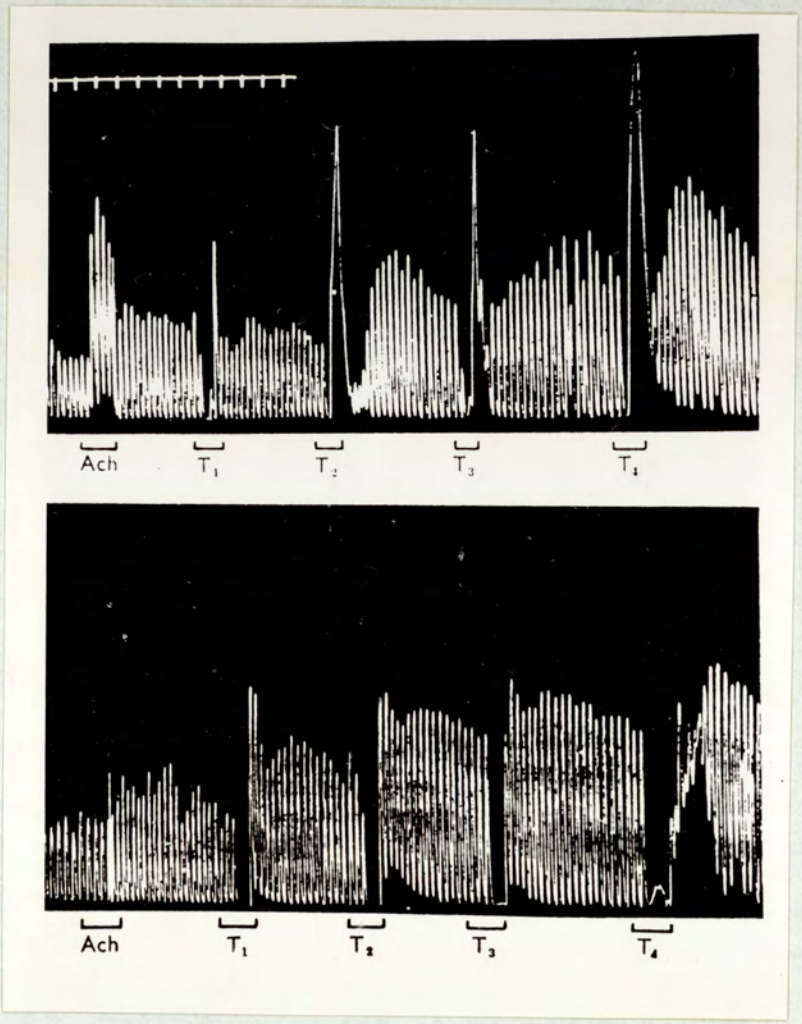


Fig. 47. (also Fig. 17, Chapter 3). Rabbit ileum at 32°C.
A: Control responses to added acetylcholine (0.02 µg/ml) (Ach) and transmural stimulations applied for 20 sec periods with supramaximal strength pulses: $T_1$, pulse width of 0.5 msec at 20 Herz; $T_2$, 0.5 msec at 50 Herz; $T_3$, 2 msec and 20 Herz; $T_4$, 2 msec and 50 Herz.
B: Same responses repeated in the presence of hyoscine (1 µg/ml). Time marker in 30 sec intervals.

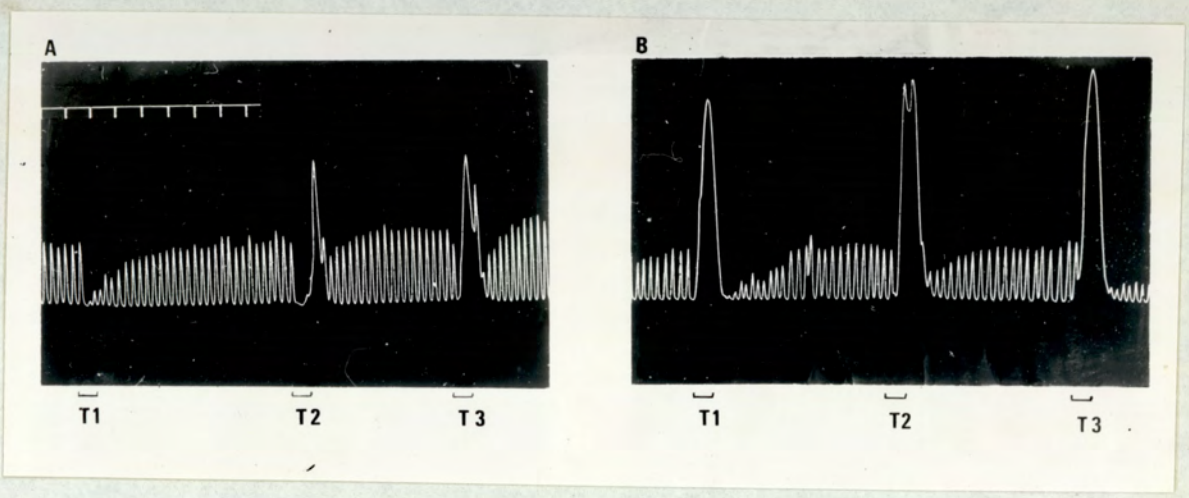


Fig. 48. (also Fig. 18, Chapter 3). Rabbit ileum at 32°C.
A: Control responses to transmural stimulation with varying stimuli. $T_1$, pulse width 0.5 msec, frequency 50 Herz; $T_2$, 2 msec and 20 Herz; $T_3$, 2 msec and 50 Herz. Each pulse of supramaximal strength and with a stimulus period of 20 sec.
B: The same responses are repeated 14 min after the addition of physostigmine (0.1 µg/ml) to the bath.
Time marker in 30 sec intervals.

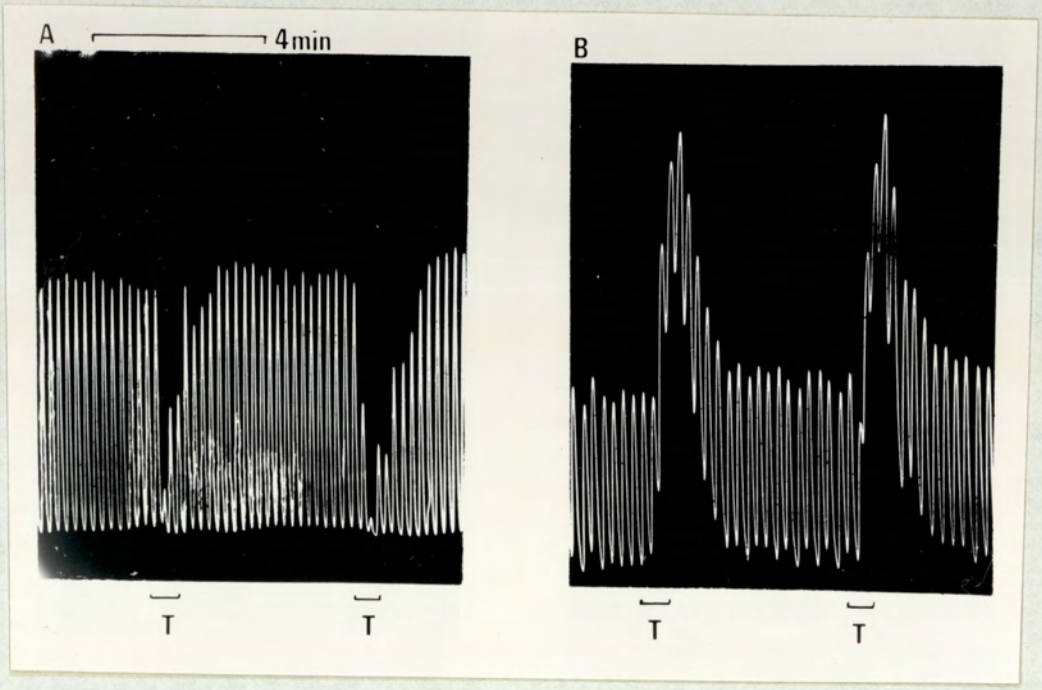


Fig. 49. Rabbit isolated ileum maintained at 32°C in aerated Tyrode's solution. In A, the responses to transmural stimulation (T) for 20 sec periods with 2 msec 20V pulses at 20 Herz. Between A and B reserpine (1 µg/ml) was added to the bath and the transmural responses were repeated 180 min later in B.

(a) The motor component of the transmural response like the response to added acetylcholine was depressed by lowering the bath temperature whilst the inhibitory component was enhanced.

(b) In some experiments the motor component preceded the inhibition or occurred in its absence.

(c) The initial inhibitory phase of the transmural response but not the motor component was impaired by reserpine.

(d) The motor component of the response was often partly blocked by hyoscine particularly at bath temperatures below 37°C whilst the inhibitory response was enhanced.

(e) The motor component of the transmural response was potentiated by anticholinesterases.

These observations taken together suggest that the inhibitory and motor phases of the response to transmural stimulation are separate phenomena mediated through different nervous pathways.

Although the motor phase of the transmural response in rabbit isolated ileum at 37°C is relatively insensitive to hyoscine it may still be mediated via cholinergic nerves. Thus, Ambache & Edwards (1951) showed that the motor effect of nicotine in rabbit ileum persisted in the presence of high concentrations of atropine but was reversed to inhibition by botulinum toxin which blocks cholinergic nerves. Ambache & Edwards (1951) showed that the motor response to nicotine in kitten isolated ileum was atropine sensitive and it was this observation that prompted the following experiments.

Cat and kitten isolated ileum

As reported previously (this thesis, chapter 5) these

experiments were curtailed by the relatively short period that these tissues remained viable. However, in the experiment illustrated in Fig. 50 it can be seen that this tissue did not respond in the same way to transmural stimulation as did preparations of rabbit intestine. In this experiment transmural stimulation produced strongly inhibitory responses and the weak motor response when present was readily abolished by hyoscine.

Guinea pig isolated colon

In this tissue non-adrenergic nerves could be stimulated in two ways, (a) by pelvic nerve stimulation, and (b) by transmural stimulation.

Pelvic nerve stimulation. As described in chapter 6 the responses to pelvic nerve stimulation were either purely motor, purely inhibitory or mixed motor and inhibitory. However, at low frequencies of stimulation (1 to 5 Herz) inhibitory responses predominated whilst at high frequencies (20 to 50 Herz) motor responses were more common.

The action of pempidine. In those preparations in which pelvic nerve stimulation produced predominantly motor responses such as that illustrated in Fig. 51 pempidine abolished the motor component leaving a purely inhibitory response.

Effect of transmural stimulation. This closely resembled the effect of pelvic nerve stimulation; stimulation at low frequencies favouring inhibitory responses, any motor component appearing being abolished with hyoscine, whilst stimulation at higher frequencies produced predominantly motor responses.

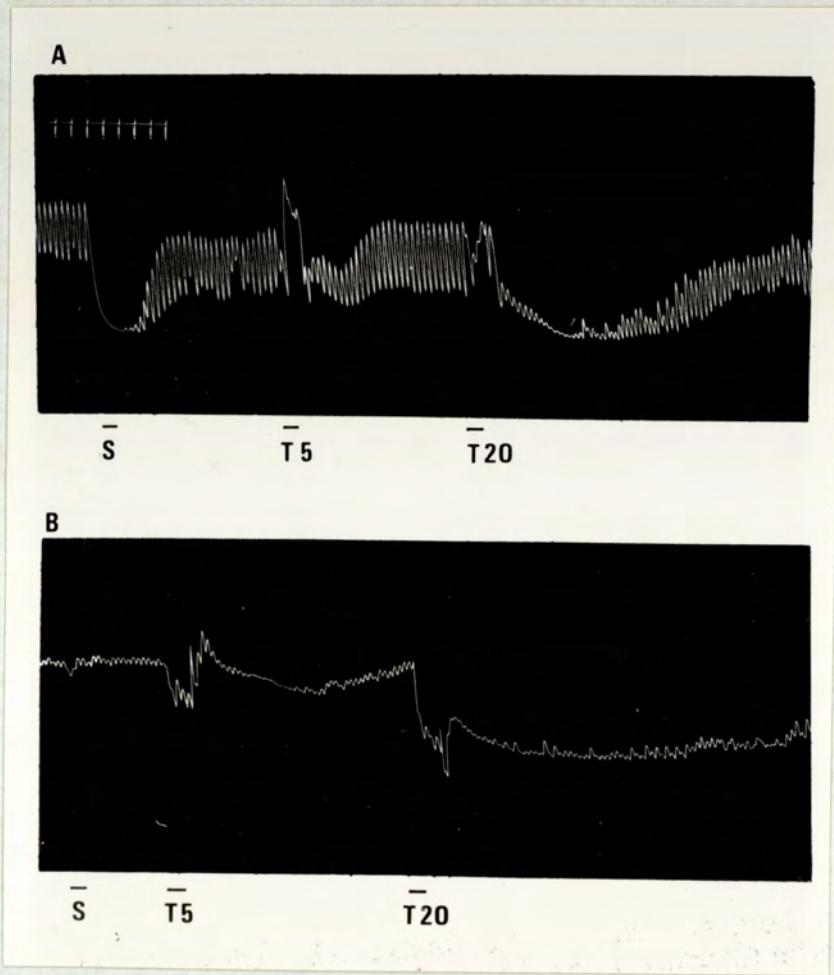


Fig. 50. (also Fig. 27, Chapter 5). Isolated ileum preparation taken from a nine-week old kitten. Preparation maintained at 37°C in McEwen's solution gassed with 95% $O_2$ and 5% $CO_2$.

In A, responses to sympathetic stimulation (S) with 20V 2 msec pulses at 20 Herz and transmural stimulation (T) with 50V 2 msec pulses at 5 and 20 Herz; stimulations applied for 20 sec periods.

In B, the responses were repeated 35 min after the addition of a mixture of guanethidine (10 µg/ml) and hyoscine (0.1 µg/ml) to the bath. Time marker 30 sec intervals.

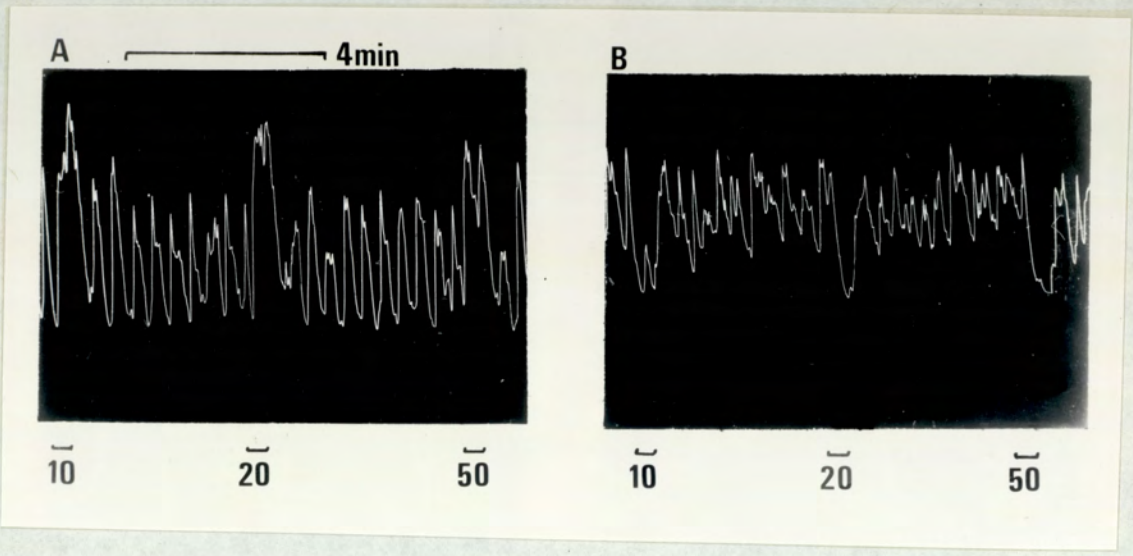


Fig. 51. (also Fig. 32, Chapter 6). Guinea pig isolated colon maintained at 37°C in McEwen's solution gassed with 95% $O_2$ and 5% $CO_2$.

In A, motor responses were produced by 20 sec periods of pelvic (parasympathetic) nerve stimulation with 2 msec 20V pulses at the frequencies indicated.

In B, the responses were mainly inhibitory when repeated 25 min after the addition of pempidine (10 µg/ml) to the bath.

## DISCUSSION

The evidence concerning the nature of the "rebound" contraction is conflicting. For instance, Campbell (1966a) reported that transmural stimulation of the guinea pig isolated taenia coli in the presence of atropine caused inhibition invariably followed by an "after" contraction which was assumed to be "rebound" in nature and a direct consequence of the initial inhibition. However, Satchell, Lynch, Bourke & Burnstock (1972) working with the same tissue, obtained purely inhibitory responses in the presence of hyoscine. Furness (1970,b) also produced conflicting evidence from his experiments using guinea pig isolated terminal colon in which he recorded intracellular muscle membrane potentials. He reported that secondary ("rebound") excitation of muscle cells was never observed without initial hyperpolarization. This contrasts with the findings of Beani, Bianchi & Crema (1971) who studied rebound contraction in guinea pig isolated stomach. These workers detected "rebound" excitation in cells which showed no change in membrane potential during the stimulation period.

The results in this chapter which have been collected together from various parts of this thesis suggest that the so called "rebound" contraction described by many other workers is due to stimulation of motor nerves which are probably cholinergic in nature.

It was the hyoscine-resistant nature of the after contraction of rabbit isolated ileum to transmural stimulation which first suggested that it could be a myogenic "rebound" response. However, subsequent experiments do not substantiate

this view. The contraction is not completely hysocine-resistant especially at bath temperatures below 37°C. Moreover the contraction on occasion can precede the inhibition or even occur in the absence of prior inhibition. Reserpine added to the bath abolished the inhibitory phase without increasing the motor phase. Finally, anticholinesterases increased the contraction and reduced or abolished the inhibitory phase.

The above observations seem to be more compatible with a scheme involving two sets of nerves, one inhibitory and one excitatory. The responses to transmural stimulation would then constitute a summation of their effects and would explain the variability of the responses obtained under different conditions and by the use of drugs. It would also explain why the motor phase appears to increase at the expense of the inhibitory phase and vice versa. The explanation that the after contraction is "rebound" in nature as postulated by other workers requires the motor phase to increase when the inhibitory phase is diminished; the results presented in this thesis in fact show the reverse to be generally the case.

The results using tissues other than rabbit ileum also support the concept that the contraction is mediated via separate neurones to the inhibition. Thus, in preparations of cat and kitten ileum the response to transmural stimulation was often purely inhibitory and motor components when present were atropine sensitive. Similarly, in the guinea pig isolated colon preparation the response to transmural stimulation varied according to the frequency of stimulation,

lower frequencies tending to cause inhibitory responses and higher frequencies motor effects. This observation is very difficult to reconcile with the concept that the contraction is a direct result of the initial inhibition. Also, in this preparation it was found that pelvic nerve stimulation gave responses qualitatively similar to those obtained with transmural stimulation thus strongly suggesting the existence of separate motor and inhibitory fibres within the pelvic nerves. The ability of pempidine to abolish the motor effect of pelvic nerve stimulation is evidence that the motor nerves are parasympathetic and cholinergic in origin. This supports the observations of Nakazato, Sato & Oliga (1970) who used the vagally innervated chicken isolated proventriculus preparation. They obtained identical responses to vagal and transmural inhibition consisting of initial inhibition followed by an after contraction which was atropine-resistant. However, the after contraction was abolished by hexamethonium suggesting that the contractions to both vagal and transmural stimulation were due to vagal cholinergic postganglionic fibres.

Thus the results presented in this chapter are consistent with the motor phase being cholinergic in nature but somewhat resistant to the blocking action of hyoscine. Ambache & Freeman (1968) suggested that the motor phase which they noticed after transmural stimulation in their experiments might be mediated via non-cholinergic motor nerves since it was resistant to blockade by hyoscine. However, the results described here as well as those of Ambache & Edwards (1951) and Nakazato et al (1970) suggest that hyoscine-resistance may be insufficient evidence on which to classify a nerve as non-cholinergic.

## Chapter 10

## A pre-synaptic adrenergic neurone blocking action of β-adrenoceptor antagonists in isolated tissues

Propranolol is a potent and specific β-adrenoceptor blocking agent with little intrinsic sympathomimetic activity (Black, Crowther & others, 1964). Propranolol also has potent local anaesthetic activity (Morales-Aguilera & Vaughan-Williams, 1965) and clinically has been shown to exhibit antifibrillatory (Rowlands, Howitt & Markman, 1965), anti-anginal (Gillam & Prichard, 1965) and antihypertensive properties (Prichard & Gillam, 1964).

It has been suggested that propranolol lowers arterial blood pressure by impairing cardiac sympathetic tone and thus reducing cardiac output (Prichard, 1968). An anti-hypertensive agent with this mode of action is of particular interest since it might be free from many side-effects caused by non-selective sympathetic blockade such as occurs with the adrenergic neurone-blocking drugs (Green, 1962). The adrenergic neurone-blocking drugs xylocholine, bretylium and guanethidine have antihypertensive properties in common with propranolol and are potent local anaesthetics (Green, 1962).

In Chapter 2 of this thesis it was noticed that propranolol impaired the responses to sympathetic nerve stimulation in the rabbit isolated ileum without apparently reducing the responses to extrinsic noradrenaline. In view of the widespread experimental and clinical use of the β-adrenoceptor blocking agents it was decided to examine this action further.

## RESULTS

Rat isolated vas deferens.

In this preparation propranolol (1 to 5 μg/ml) caused a progressive impairment of the responses to sympathetic nerve stimulation whilst the responses to added noradrenaline were either unaffected, or more usually, increased. The result of an experiment in which the sympathetic nerve blocking action of propranolol was compared with that of guanethidine is shown in Fig. 52. In this experiment propranolol (3 μg/ml) caused a similar degree of impairment of the responses to sympathetic stimulation as did guanethidine (1 μg/ml). In each experiment the response to added noradrenaline (2 μg/ml) was slightly increased after establishment of the block. Whereas the adrenergic neurone blocking action of guanethidine was reversed 1 hour after adding dexamphetamine (0.05 μg/ml) to the bath (Fig. 52B), this treatment did not restore the responses to sympathetic stimulation after propranolol (Fig. 52D). The adrenergic neurone blocking action of propranolol was persistent and was only very slowly reversed by repeated washing of the preparation over several hours.

In other experiments, attempts were made to reverse the blocking action of propranolol with either noradrenaline (1 to 2 μg/ml) or desipramine (0.1 to 0.5 μg/ml). These concentrations of noradrenaline initially contracted the tissue but caused no increase in the sympathetic responses after propranolol left in contact for up to 45 minutes. Desipramine caused a large increase in the sensitivity to added noradrenaline but had no effect on the response to

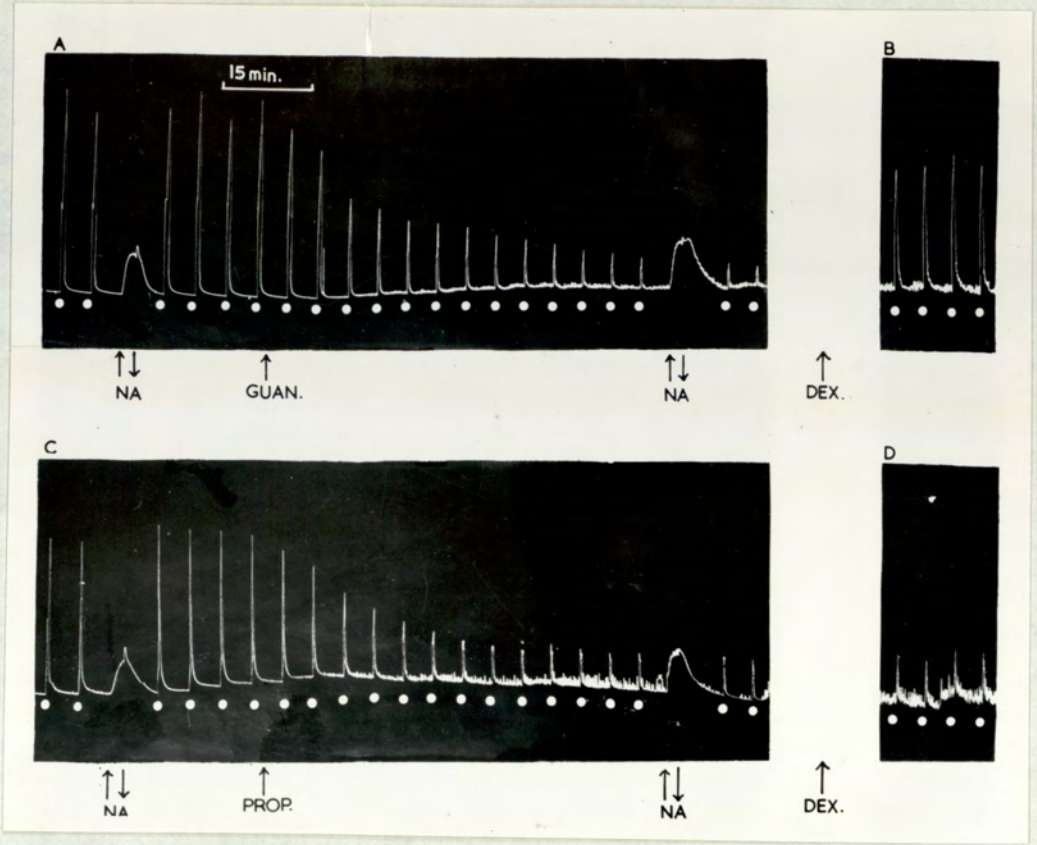


Fig. 52. Rat vas deferens preparations. At white dots stimulation of intramural sympathetic nerves with 2 msec 20V pulses at 10 Herz. 2 µg/ml noradrenaline (NA) added to the bath at arrows and left in contact with the preparations for 2 min.

Upper record: 1 µg/ml guanethidine caused sympathetic block which was partly reversed in B 60 min after adding dexamphetamine (DEX) (0.05 µg/ml) to the bath.

Lower record: contralateral preparation from the same rat; sympathetic blockade produced by 3 µg/ml propranolol was not reversed (in D) 60 min after adding dexamphetamine (DEX) (0.05 µg/ml) to the bath.

sympathetic stimulation when added before or after the establishment of a propranolol block.

In a few preparations pronethalol was used instead of propranolol and was found to have a similar action in blocking nervously-mediated responses without reducing the responses to added noradrenaline. Pronethalol was approximately half as potent as propranolol in producing nerve block and was more readily reversed by washing.

Finkleman preparation

This preparation was chosen to test the effects of propranolol on inhibitory sympathetic responses because the responses are mediated by an action of neuronal noradrenaline on both α- and β-adrenergic receptors (Furchgott, 1960). The results using this preparation were essentially the same as those obtained using the isolated vas deferens preparation. Thus, propranolol (3 μg/ml) produced a similar impairment of the responses to sympathetic nerve stimulation as did guanethidine (1 μg/ml). Fig. 53 illustrates an experiment in which propranolol (3 μg/ml) produced a rapidly developing impairment of the responses to sympathetic stimulation although the inhibitory responses to added noradrenaline were virtually unaffected. As in the vas deferens preparation, the blocking action of propranolol was not reversed by dexamphetamine (0.1 to 0.5 μg/ml) and was only slowly reversed by repeated washing of the preparation. The blocking action of guanethidine was even more persistent after washing the preparation but was readily reversed by dexamphetamine. Pronethalol had a similar effect in this preparation to

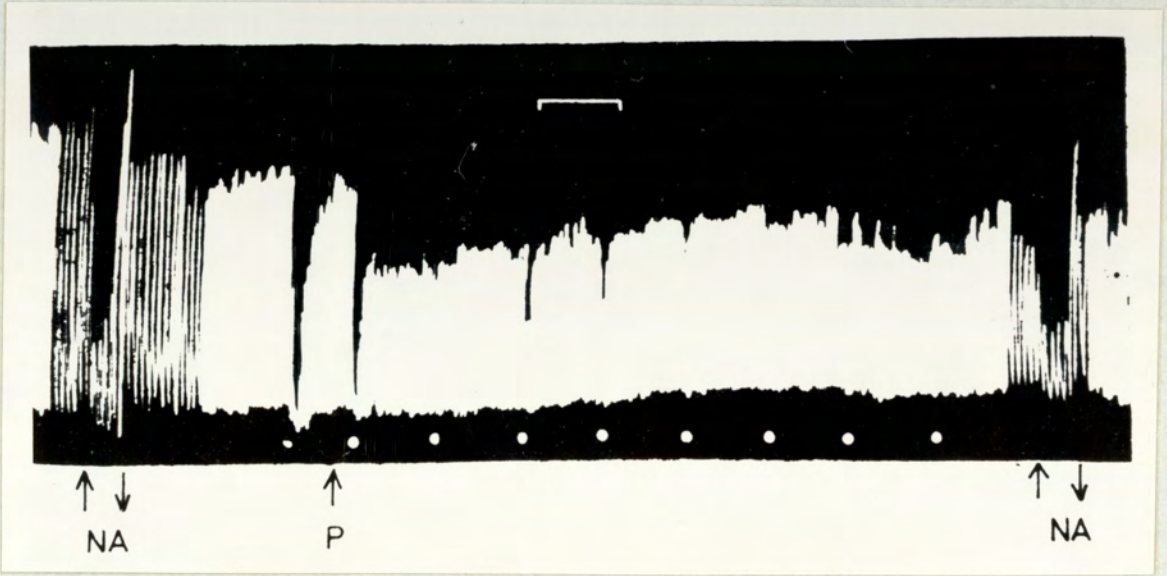


Fig. 53. Finkleman preparation of rabbit ileum in aerated Tyrode's solution at 37°C. At white dots periarterial sympathetic nerves stimulated for 20 sec with 2 msec 10V pulses at 50 Herz. Noradrenaline (0.05 µg/ml) added to bath (at NA) and left in contact with preparation 30 sec. Propranolol (3 µg/ml) (at P) added to bath. Recording speed increased during noradrenaline responses. Horizontal white bar represents time between stimulation periods (4 min).

propranolol but again was less potent, was more easily reversed, and itself inhibited the spontaneous activity of the preparation.

Rabbit isolated ear artery preparation

This preparation was chosen to determine whether propranolol had a similar adrenergic neurone blocking action on sympathetically innervated vascular smooth muscle as it did in other smooth muscle preparations tested, since this may have some bearing on its use as an antihypertensive agent. It was found that propranolol (0.25 to 1 µg/ml) produced a slowly-developing but persistent impairment of the constrictor responses to sympathetic stimulation whereas the responses to injected noradrenaline were enhanced. In this preparation, unlike the other preparations tested, propranolol was at least as potent as guanethidine in producing adrenergic neurone blockade.

Comparison of the nerve blocking actions of propranolol and lignocaine

Propranolol has similar local anaesthetic potency to lignocaine (Morales-Aguilera & Vaughan-Williams, 1965) and it was thought possible that this action could explain its effects on adrenergic neurones. For this reason the blocking action of propranolol was compared with that of lignocaine in the Finkleman preparation of rabbit ileum and in the rat isolated vas deferens. In the rabbit ileum preparation lignocaine usually caused impairment of the pendular movements of the preparation in concentrations (10 to 30 µg/ml) which did not significantly affect the responses to sympathetic stimulation. Propranolol on the other hand

caused a complete abolition of the nervously mediated responses at a concentration of 1 to 3 μg/ml which did not affect the spontaneous activity of the preparation.

In the isolated vas deferens preparation lignocaine did not affect the responses to sympathetic stimulation at a concentration (30 μg/ml) ten times higher than that of propranolol needed to cause an almost complete block of the responses. At a concentration of 50 to 100 μg/ml, lignocaine caused a partial nerve blockade which unlike the propranolol block was readily reversed by washing.

## DISCUSSION

The results described indicate that propranolol has a potent blocking action on adrenergic sympathetic neurones in isolated smooth muscle preparations. The adrenergic neurone blocking action of propranolol appears to be pre-synaptic and independent of its post-synaptic effect on β-adrenergic receptors. Thus, at a time when the block was at a maximum the responses to exogenous noradrenaline were either unaffected or increased; in addition the block occurred in tissues such as the rat vas deferens and rabbit ear artery in which only α-adrenoceptors are involved.

The potency of propranolol in blocking adrenergic neurones was only slightly less than that of guanethidine to which it has a similar time of onset and was almost equally persistent in its blocking action after changing the bath fluid. However, the blocking action of propranolol could be distinguished from that of guanethidine by the fact that only that of guanethidine was reversed by dexamphetamine. Antagonism occurs with dexamphetamine and other adrenergic

neurone blocking agents and is probably competitive in nature (Day, 1962; Day & Rand, 1963). Similarly it is unlikely that the blocking action of propranolol is caused by depletion of noradrenaline from the sympathetic nerves, as occurs with reserpine, since the block was not reversed by noradrenaline. Desipramine was tested as a potential propranolol antagonist because of the recent report that it partially antagonized the action of propranolol in preventing the increase in rate of beating of isolated atria in response to sympathetic stimulation (Shimamoto & Toda, 1968). No such antagonism was found in the rat vas deferens preparation despite a large increase in sensitivity of the preparation to added noradrenaline caused by desipramine.

Thus the most likely explanation of the blocking action of propranolol is to be found in its potent local anaesthetic property. However, in a direct comparison with lignocaine, with which it has been reported to be approximately equipotent as a local anaesthetic (Morales-Aguilera & Vaughan-Williams, 1965), propranolol was found to be much more potent and persistent in its blocking action on adrenergic neurones. The possibility that the sympathetic blocking action of propranolol is a consequence of its local anaesthetic activity cannot be precluded since it may be that it exerts this action on sympathetic nerve endings more effectively than lignocaine possibly as a result of more complete penetration into the tissue.

The antihypertensive effect of propranolol in man is of slow onset (Prichard & Gillam, 1964) and this is consistent with the hypothesis that the drug is slowly accumulated in

peripheral adrenergic neurones thus causing a reduction in sympathetic vasomotor tone which would tend to reinforce its better known β-blocking action on cardiac receptors in lowering arterial blood pressure.

# PART 4

# GENERAL DISCUSSION

The results section of this thesis record an investigation into the nature of non-sympathetic inhibitory nerves. The aim of the investigation was to examine their nature and if possible identify the post-ganglionic transmitter. A number of isolated intestinal tissues taken from several species were used of which the most useful preparation was the dually innervated guinea pig terminal colon. Non-sympathetic nerves were stimulated in one of three ways; by stimulation of parasympathetic nerves with which they are anatomically associated, by transmural stimulation, and by ganglion stimulating drugs.

Although widespread use was made of transmural stimulation in this work and by other workers investigating the same subject, it should be pointed out that results obtained by analysis of responses obtained to transmural stimulation should always be treated with reserve. Paton (1955) showed that a single transmural pulse of 50 msec duration activated parasympathetic nerves in guinea pig ileum. Thus, a disadvantage of transmural stimulation is that it is liable to activate several nervous networks and cause the release of several transmitters. Furthermore, transmural stimulation could well release physiologically active substances which are not transmitters. Buchnell (1965) has suggested that inhibitory responses to transmural stimulation in human isolated colon could be due to release of catecholamines from chromaffin tissues.

The essentially non-physiological nature of transmural stimulation has been pointed out by Weisenthal, Hug, Weisbrodt & Bass (1971), working with isolated guinea pig taenia coli

they suggested that in the intact animal perivascular nerves might exert their influence on muscle tone via the inhibitory cholinergic ganglia while in isolated preparations it is possible that transmural stimulation results in the release of enough transmitter from perivascular nerves to overflow the ganglionic sites and act directly on muscle receptors. Thus the fact that transmural stimulation is essentially non-physiological method of activating nerves must always be kept in mind when examining results obtained using this method.

The initial work of this investigation was carried out on rabbit isolated ileum. Transmural stimulation produced a response which had both inhibitory and motor components. By use of adrenergic neurone blocking drugs, adrenoceptor blocking drugs and noradrenaline desensitization, it was shown that the response to transmural stimulation had an inhibitory component which was probably due to stimulation of non-adrenergic nerves. Due to the large motor response which was almost inseparable from the inhibitory component it was difficult to investigate the nature of these nerves. However, results were obtained which indicated that the non-adrenergic nerves had a lower threshold frequency and optimal frequency than sympathetic nerves and that their responses could be impaired with reserpine. Inhibitory responses could be obtained to ganglion stimulating drugs such as TMA or 5HT which persisted in the presence of adrenoceptor blocking drugs. This suggested that the non-adrenergic inhibitory nerves synapse locally and is consistent with the work of Bulbring & Gershon (1966, 1967). These workers studied vagal inhibitory nerves innervating the stomach and

suggested that both 5HT and ACH are released by the pre-ganglionic fibre and act as ganglionic transmitters. This view has been contradicted by the work of Beani, Bianchi & Crema (1971) who showed that while hexamethonium reduced (but did not block) vagal relaxation the residual inhibitions persisted during 5HT tachyphylaxis.

Rabbit isolated ileum is clearly of limited use in the study of non-adrenergic inhibition and attempts were made to find a more suitable tissue. Rabbit colon and cat and kitten ileum were tried and although it was possible to show that all three tissues contained non-adrenergic inhibitory nerves they were not ideal tissues for studying the neurones. The feline tissues proved impossible to keep viable for more than about an hour, while the rabbit isolated colon was prone to such wildly irregular pendular movements that it was difficult to interpret responses to nervous stimulation.

The dually innervated guinea pig isolated colon proved to be a satisfactory tissue for studying the nerves and experiments with this tissue yielded a number of interesting results. For instance, it was found that non-adrenergic nerves run with the pelvic nerves to the tissue and have an optimal frequency of about 3 Herz. This result is of great interest as previous workers (Furness, 1969; Bianchi, Beani, Frigo & Crema, 1968) had found no extrinsic connections to the non-adrenergic inhibitory nerves to the guinea pig colon. Bianchi et al (1968) had observed an inhibitory component to follow the contraction caused by stimulation of the pelvic nerves and had suggested that it might be a

"myogenic rebound." Furness (1969) had suggested that non-adrenergic inhibitory nerves play a purely intrinsic role in regulating the colon's activity. However, the evidence in Chapter 6 of this thesis shows that non-adrenergic inhibitory nerves to the guinea pig isolated colon do have extrinsic connections via the pelvic nerve, so presumably the non-adrenergic inhibitory nerves are to some extent subject to control from the central nervous system.

An interesting point about inhibitions obtained by stimulation of the pelvic nerve is that although either pempidine or bufotenine alone produced a partial block of the response, both drugs had to be present to completely block the inhibitions. This strongly suggests firstly that the post-ganglionic nerves are short and synapse locally, and secondly, that both ACH and 5HT act as ganglionic transmitters. This supports the idea first suggested by Bulbring & Gershon (1966, 1967) who studied vagal inhibition of the mouse and guinea pig stomach, and suggested that both ACH and 5HT were released by the pre-ganglionic fibres as ganglionic transmitters.

As pointed out previously, Weisenthal, Hug, Weisbrodt & Bass (1971) have questioned the presence of non-adrenergic neurones in guinea pig taenia coli on the grounds that evidence for their existence was obtained by analysing responses to transmural stimulation. They suggest that the difference between inhibitory responses to sympathetic nerves and to transmural stimulation is due to the artificial nature of transmural stimulation rather than to the existence of

non-adrenergic nerves. While accepting their criticism of transmural stimulation, and acknowledging that much of the work on non-adrenergic nerves has used this technique, inhibitory responses obtained to the extrinsic stimulation of the pelvic nerve as obtained in Chapter 6 of this thesis are not subject to this criticism. Similarly, non-adrenergic inhibitory responses of the stomach mediated via vagal stimulation cannot be criticised on the grounds of using transmural stimulation.

Thus, if the work presented in the preceding chapters is considered with that summarized in the historical introduction it can be seen that it is difficult to resist the conclusion that in addition to the sympathetic and parasympathetic nerves there is another division of autonomic nerves - non-adrenergic inhibitory nerves. These nerves appear to innervate a large number of smooth muscle tissues, and have been shown to run with the parasympathetic nerves in two cases, namely, with the vagus nerve to the stomach and the pelvic nerve to the guinea pig colon. The nerves are similar to parasympathetic nerves in that the post-ganglionic nerve is short; the ganglionic transmitters may well be both ACH and 5HT.

Inevitably evidence that a large number of tissues are innervated with non-adrenergic nerves leads to speculation as to the nature of the transmitter. At first sight there is apparently no shortage of candidates as there are a number of physiologically active substances such as 5HT and histamine which are known to occur in the gut and whose exact physiological role in this tissue is uncertain. Unfortunately,

it is only too easy to demonstrate, with one exception, that the various candidates for the role of transmitter are unacceptable. Chapter 7 of this thesis investigates the possibility that 5HT, histamine, glycine or GABA is the post-ganglionic transmitter and (regretfully) concludes that none could play the role. Similarly, Bianchi, Beani, Frigo & Crema (1968) examined non-adrenergic inhibitory responses in guinea pig isolated colon; they investigated the possibility of histamine, bradykinin, glycine, GABA, ATP, AMP, dopamine, ACH or 5HT could be the final transmitter and concluded none was. The only naturally occurring substance which can seriously be considered on the evidence now available (i.e. March 1973) as a candidate for the role of post-ganglionic transmitter of non-adrenergic inhibitory nerves is ATP. Initial work as reported in Chapter 7 of this thesis, and by Bianchi et al (1968), seem to rule out ATP as a possible transmitter. However, recent work by Burnstock and his co-workers (see Chapter 8 for references) has produced a mass of evidence that ATP is the final transmitter of these nerves. Burnstock (1972) has reviewed the evidence in detail and it is discussed in Chapter 8 of this thesis.

Briefly, Burnstock and his co-workers, using several different tissues, have shown that non-adrenergic neurones take up adenosine and ATP, are capable of synthesising ATP, store ATP and release it when stimulated. They have shown that dipyridamole potentiates responses to both ATP and nervous stimulation and have blocked responses to nervous stimulation and ATP with quinidine. They suggest that their evidence broadly satisfies the five criteria suggested

by Eccles (1964) for examination of potential neuro-transmitters and have named non-adrenergic nerves "purinergic nerves."

The evidence that non-adrenergic inhibitory nerves release ATP has been investigated in Chapter 8 of this thesis. In this chapter, in which the guinea pig isolated colon preparation was used, it was found impossible to relax the tissue with ATP, however high the concentration, as fully as it is by stimulation of extrinsic non-adrenergic inhibitory nerves or by sympathetic stimulation. Also the rate of relaxation following nervous stimulation is quicker than following the addition of ATP while the recovery of normal tone following nervous stimulation occurs sooner than after the addition of ATP even though the extrinsic ATP induced a smaller response. Discrepancies between the response to nervous stimulation and the response to extrinsically added neuro-transmitter have been observed before (for instance atropine-resistant cholinergic nerves - see Ambache & Edwards, 1951) and are usually explained by assuming that the nerve endings which release the transmitter are in close proximity to receptor sites or to receptors that are unavailable to extrinsically added drugs. However, the differences between extrinsically added ATP and stimulation of non-adrenergic nerves seem to be too great to be explained in this manner. The likelihood of ATP being the transmitter recedes when the action of quinine, quinidine and digoxin is considered. These drugs have been shown to block ATP but, as shown in Chapter 8 of this thesis, their action is quite unspecific and evidence obtained with them designed to

indicate that ATP is the transmitter is unacceptable. This is especially so in the case of quinidine which in some experiments enhanced responses to non-adrenergic inhibitory nerves while reducing responses to extrinsic ATP. The action of dipyridamole has been cited by Burnstock and his colleagues as evidence that non-adrenergic inhibitory nerves release ATP. However, the potentiation of ATP by dipyridamole is not shared only by non-adrenergic nerves, as the above authors have suggested, but also by sympathetic nerves. Also as explained in Chapter 8, in some experiments it was found possible to enhance the ATP response while reducing inhibitory responses to pelvic inhibitory nerve stimulation. When it is considered that in addition to the results which have just been discussed, ATP will not reverse the reserpine block of non-adrenergic nerves and responses to non-adrenergic nerves persist in the presence of ATP tachyphylaxis, it is fair to state that it seems unlikely that the post-ganglionic transmitter of non-adrenergic nerves is ATP. The carefully performed experiments of Burnstock and his co-workers which show that non-adrenergic nerves can synthesise and store ATP and release it when stimulated, arouse admiration for the sophisticated techniques used, but control experiments by the same workers showing that sympathetic nerves also synthesise and store ATP and release it when stimulated, cast serious doubt on the concept that the functional transmitter released from non-adrenergic nerves is an adenine nucleotide. Thus it is suggested that the claim by Burnstock and his co-workers that ATP "broadly satisfies the criteria of Eccles" is not acceptable and that

the term "purinergic nerves" is premature and probably incorrect.

A phenomenon that has been closely associated with non-adrenergic inhibition and in fact overshadowed by it is that of rebound contraction. Workers studying non-adrenergic inhibition have found that an atropine resistant contraction usually follows the inhibitory response. Day & Warren (1968) suggested that the contraction was due to excitation of cholinergic fibres which were resistant to atropine but this explanation has not been favoured by most subsequent workers who have preferred the explanation that the contraction is a rebound phenomenon as first suggested by Burnstock, Campbell, Bennett & Holman (1963). As elaborated by Bennett (1966a, 1966b) and Campbell (1966a) the concept of rebound excitation is that after hyper-polarization of the muscle cells the muscle membrane becomes depolarized beyond its normal value. Thus recovery from the inhibition initiates a contraction. Proponents of this view have thus far omitted to explain why extrinsically added inhibitory agents, e.g. noradrenaline, do not initiate similar rebound contractions.

In Chapter 9 of this thesis the phenomenon of rebound contraction was examined in preparations of rabbit isolated ileum. It was shown that the excitatory component of the transmural response sometimes preceded the inhibition or even on occasion occurred in its absence. It was also found that when the bath temperature was lowered the inhibitory phase of the transmural response was increased whilst the contractile phase was decreased. Similarly, in some

experiments the inhibitory phase was abolished leaving the contraction unimpaired. In some experiments the motor component was susceptible to the blocking action of hyoscine or atropine and could be potentiated by anticholinesterases. In preparations of isolated ileum taken from the cat the contractile component of the transmural response was sensitive to hyoscine. When guinea pig isolated colon was used and responses consisting of inhibition followed by contraction obtained, the contraction was abolished by pempidine leaving purely inhibitory responses. The above results considered together with the evidence of Nakazato, Sato & Oliga (1970) render the hypothesis of rebound excitation untenable. Nakazato and his co-workers obtained responses of the chicken isolated proventriculus muscle consisting of inhibition followed by "rebound" contraction to both vagal and transmural stimulation. Hexamethonium abolished the contractile phase of the vagal response leaving a purely inhibitory response. Thus it would appear that the contraction that usually follows non-adrenergic inhibition is not a rebound phenomenon but is due to stimulation of a separate set of nerves than those responsible for the inhibitory response. The evidence suggests that these nerves are cholinergic but partly resistent to hyoscine in some tissues.

At the start of this discussion the non-physiological nature of transmural stimulation was commented upon and the difficulty this led to when interpreting the result of experiments when using this technique. The evidence for rebound contraction was obtained by transmural stimulation and in view of the ease with which the hypothesis could be

disproved once suitable innervated preparations had been found is an example of the disadvantages of the technique.

To summarize then, it would appear that another division of the autonomic nervous system exists which on anatomical grounds, i.e. position of peripheral efferent ganglia, may well be classified as a sub-division of the parasympathetic system. In the opinion of the author of this thesis the identity of the post-ganglionic neuro-transmitter substance has not yet been discovered. The physiological significance of this system awaits identity of the transmitter substance and the production of specific antagonists to it.

# PART 5

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# An adrenergic neuron blocking action of propranolol in isolated tissues

M. D. DAY, D. A. A. OWEN AND P. R. WARREN

Propranolol was tested for adrenergic neuron blocking activity in three isolated sympathetically-innervated smooth muscle preparations; the rat vas deferens, rabbit ileum and rabbit ear artery. In each preparation propranolol impaired the responses to sympathetic stimulation without reducing the responses to added noradrenaline. This blocking action of propranolol resembled that of guanethidine in time of onset and persistence of blocking activity but, unlike blocking by guanethidine, was not reversed by (+)-amphetamine. Desipramine and noradrenaline also failed to reverse the blocking action of propranolol. In the rat vas deferens preparation lignocaine had a weaker and more transient sympathetic blocking action than propranolol. It is suggested that the sympathetic blocking action of propranolol may contribute to its antihypertensive effect in man.

PROPRANOLOL is a potent and specific $\beta$-adrenergic receptor blocking agent with little intrinsic sympathomimetic activity (Black, Crowther & others, 1964). Propranolol also has potent local anaesthetic activity (Morales-Aguilerá & Vaughan-Williams, 1965) and clinically has been shown to exhibit antifibrillatory (Rowlands, Howitt & Markman, 1965), anti-anginal (Gillam & Prichard, 1965) and antihypertensive properties (Prichard & Gillam, 1964).

It has been suggested that propranolol lowers arterial blood pressure by impairing cardiac sympathetic tone and thus reducing cardiac output (Prichard, 1968). An antihypertensive agent with this mode of action is of particular interest since it might be free from many side-effects caused by non-selective sympathetic blockade such as occurs with the adrenergic neuron-blocking drugs (Green, 1962). The adrenergic neuron-blocking drugs xylocholine, bretylium and guanethidine have antihypertensive properties in common with propranolol and are potent local anaesthetics (Green, 1962). Propranolol was therefore tested for a possible pre-synaptic blocking action on peripheral adrenergic neurons.

## Experimental

*Rat isolated vas deferens.* Both vasa deferentia removed from recently killed rats were threaded through bipolar platinum electrodes and were set up in organ baths containing aerated Tyrode solution at 32° in separate but simultaneous experiments. Electrical stimulation of the intramural sympathetic nerve endings was with pulses of supramaximal strength (usually 20 V) of 2 msec duration and at a frequency of 5 to 20 pulses/sec delivered from a constant voltage electronic stimulator for periods of 15 sec repeated every 5 min.

*Finkleman preparation of rabbit ileum.* Preparations were set up and electrically stimulated as described by Day & Rand (1961) except that the Ringer solution was replaced by aerated Tyrode at 37°.

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*Rabbit isolated ear artery preparation.* This preparation was set up and electrically stimulated as described by De la Lande & Rand (1965).

## Results

*Rat isolated vas deferens.* In this preparation propranolol (1 to 5 $\mu$g/ml) caused a progressive impairment of the responses to sympathetic nerve stimulation whilst the responses to added noradrenaline were either unaffected, or more usually, increased. The result of an experiment in which the sympathetic nerve blocking action of propranolol was compared with that of guanethidine is shown in Fig. 1. In this experiment propranolol (3 $\mu$g/ml) caused a similar degree of impairment of the responses to sympathetic stimulation as did guanethidine (1 $\mu$g/ml). In each experiment the response to added noradrenaline (2 $\mu$g/ml) was slightly increased after establishment of the block. Whereas the adrenergic neuron blocking action of guanethidine was reversed 1 hr after adding (+)-amphetamine (0·05 $\mu$g/ml) to the bath (Fig. 1B), this treatment did not restore the responses to sympathetic stimulation after propranolol

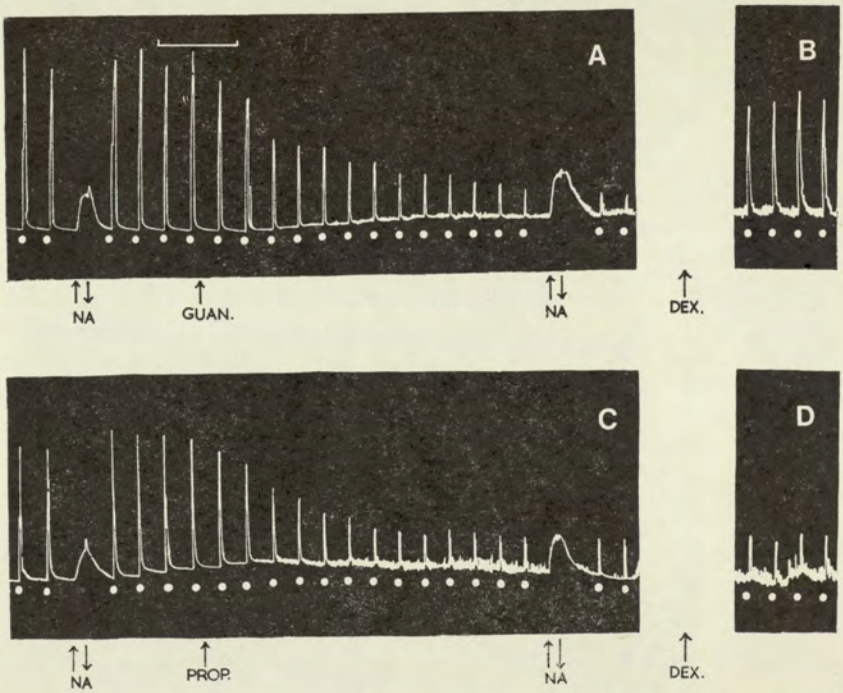


FIG. 1. Rat vas deferens preparations. At white dots stimulation of intramural sympathetic nerves with 2 msec 20 V pulses at frequency of 10 pulses/sec. 2 $\mu$g/ml noradrenaline (NA) added to bath at arrows and left in contact with the preparations for 2 min. Upper record: 1 $\mu$g/ml guanethidine caused sympathetic block which was partly reversed in B 60 min after adding (+)-amphetamine (DEX) (0·05 $\mu$g/ml) to the bath. Lower record: contralateral preparation from same rat sympathetic blockade produced by 3 $\mu$g/ml propranolol was not reversed (in D) 60 min after adding (+)-amphetamine to the bath.

(Fig. 1D). The adrenergic neuron blocking action of propranolol was persistent and was only very slowly reversed by repeated washing of the preparation over several hours.

In other experiments, attempts were made to reverse the blocking action of propranolol with either noradrenaline (1 to 2 $\mu$g/ml) or desipramine (0·1 to 0·5 $\mu$g/ml). These concentrations of noradrenaline initially contracted the tissue but caused no increase in the sympathetic responses after propranolol left in contact for up to 45 min. Desipramine caused a large increase in the sensitivity to added noradrenaline but had no effect on the response to sympathetic stimulation when added before or after the establishment of a propranolol block.

In a few preparations pronethalol was used instead of propranolol and was found to have a similar action in blocking nervously-mediated responses without reducing the responses to added noradrenaline. Pronethalol was approximately half as potent as propranolol in producing nerve block and was more readily reversed by washing.

*Finkleman preparation.* This preparation was chosen to test the effects of propranolol on inhibitory sympathetic responses because the responses are mediated by an action of neuronal noradrenaline on both $\alpha$- and $\beta$-adrenergic receptors (Furchgott, 1960). The results using this preparation were essentially the same as those obtained using the isolated vas deferens preparation. Thus, propranolol (3 $\mu$g/ml) produced a similar impairment of the responses to sympathetic nerve stimulation as did guanethidine (1 $\mu$g/ml). Fig. 2 illustrates an experiment in which propranolol (3 $\mu$g/ml) produced a rapidly developing impairment of the responses to sympathetic stimulation although the inhibitory responses to added noradrenaline were virtually unaffected. As in the vas deferens preparation, the blocking action of propranolol was not reversed by (+)-amphetamine (0·1 to 0·5 $\mu$g/ml) and was only slowly reversed by repeated washing of the preparation. The blocking action of guanethidine was even more persistent after washing the preparation but was readily

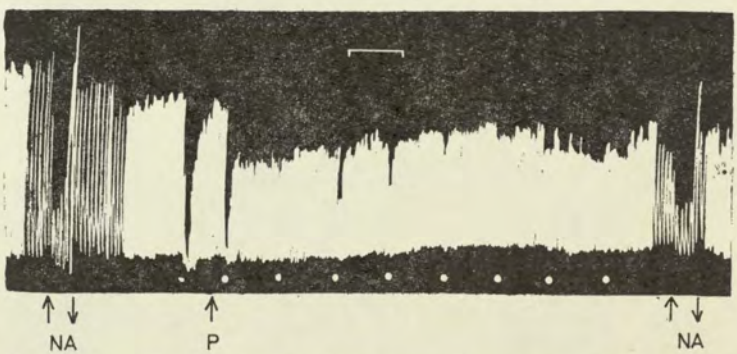


FIG. 2. Finkleman preparation of rabbit ileum. At white dots periarterial sympathetic nerves stimulated with 2 msec 10 V pulses at frequency of 50 pulses/sec. Noradrenaline 0·05 $\mu$g/ml added to bath (at NA) and left in contact with preparation 30 sec. Propranolol 3 $\mu$g/ml (at P) added to bath. Drum speed increased during noradrenaline responses.

reversed by (+)-amphetamine. Pronethalol had a similar effect in this preparation to propranolol but again was less potent, was more easily reversed, and itself inhibited the spontaneous activity of the preparation.

*Rabbit isolated ear artery preparation.* This preparation was chosen to determine whether propranolol had a similar adrenergic neuron blocking action on sympathetically innervated vascular smooth muscle as it did in other smooth muscle preparations tested, since this may have some bearing on its use as an antihypertensive agent. It was found that propranolol (0·25 to 1 $\mu$g/ml) produced a slowly-developing but persistent impairment of the constrictor responses to sympathetic stimulation whereas the responses to injected noradrenaline were enhanced. In this preparation, unlike the other preparations tested propranolol was at least as potent as guanethidine in producing adrenergic neuron blockade.

*Comparison of the nerve blocking actions of propranolol and lignocaine.* Propranolol has similar local anaesthetic potency to lignocaine (Morales-Aguilerá & Vaughan-Williams, 1965) and it was thought possible that this action could explain its effects on adrenergic neurons. For this reason the blocking action of propranolol was compared with that of lignocaine in the Finkleman preparation of rabbit ileum and in the rat isolated vas deferens. In the rabbit ileum preparation lignocaine usually caused impairment of the pendular movements of the preparation in concentrations (10 to 30 $\mu$g/ml) which did not significantly affect the responses to sympathetic stimulation. Propranolol on the other hand caused a complete abolition of the nervously mediated responses at a concentration of 1 to 3 $\mu$g/ml which did not affect the spontaneous activity of the preparation.

In the isolated vas deferens preparation lignocaine did not affect the responses to sympathetic stimulation at a concentration (30 $\mu$g/ml) ten times higher than that of propranolol needed to cause an almost complete block of the responses. At a concentration of 50 to 100 $\mu$g/ml, lignocaine caused a partial nerve blockade which unlike the propranolol block was readily reversed by washing.

## Discussion

The results described indicate that propranolol has a potent blocking action on adrenergic sympathetic neurons in isolated smooth muscle preparations. The adrenergic neuron blocking action of propranolol appears to be pre-synaptic and independent of its post-synaptic effect on $\beta$-adrenergic receptors. Thus, at a time when the block was at a maximum the responses to exogenous noradrenaline were either unaffected or increased; in addition the block occurred in tissues such as the rat vas deferens and rabbit ear artery in which only $\alpha$-adrenergic receptors are involved.

The potency of propranolol in blocking adrenergic neurons was only slightly less than that of guanethidine to which it has a similar time of onset and was almost equally persistent in its blocking action after changing the bath fluid. However, the blocking action of propranolol could be distinguished from that of guanethidine by the fact that only that of

guanethidine was reversed by (+)-amphetamine. Antagonism occurs with (+)-amphetamine and other adrenergic neuron blocking agents and is probably competitive in nature (Day, 1962; Day & Rand, 1963). Similarly it is unlikely that the blocking action of propranolol is caused by depletion of noradrenaline from the sympathetic nerves, as occurs with reserpine, since the block was not reversed by noradrenaline. Desipramine was tested as a potential propranolol antagonist because of the recent report that it partially antagonized the action of propranolol in preventing the increase in rate of beating of isolated atria in response to sympathetic stimulation (Shimamoto & Toda, 1968). No such antagonism was found in the rat vas deferens preparation despite a large increase in sensitivity of the preparation to added noradrenaline caused by desipramine.

Thus the most likely explanation of the blocking action of propranolol is to be found in its potent local anaesthetic property. However, in a direct comparison with lignocaine, with which it has been reported to be approximately equipotent as a local anaesthetic (Morales-Aguilerá & Vaughan-Williams, 1965), propranolol was found to be much more potent and persistent in its blocking action on adrenergic neurons. We cannot preclude the possibility that the sympathetic blocking action of propranolol is a consequence of its local anaesthetic activity since it may be that it exerts this action on sympathetic nerve endings more effectively than lignocaine possibly as a result of more complete penetration into the tissue.

The antihypertensive effect of propranolol in man is of slow onset (Prichard & Gillam, 1964) and this is consistent with the hypothesis that the drug is slowly accumulated in peripheral adrenergic neurons thus causing a reduction in sympathetic vasomotor tone which would tend to reinforce its better known $\beta$-blocking action on cardiac receptors in lowering arterial blood pressure.

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## Inhibitory responses to transmural stimulation in isolated intestinal preparations

SIR,—Transmural electrical stimulation of guinea-pig isolated ileum elicits contractile responses due to activation of parasympathetic nerve elements within the muscle wall (Paton, 1955, 1957). During the course of experiments to determine the nature of the cholinergic fibres associated with the periarterial nerves in the rabbit intestine (Gillespie & Mackenna, 1961; Day & Rand, 1961; Bentley, 1962) we used transmural stimulation in segments of rabbit isolated intestine. We were surprised to note that in most of the preparations transmural stimulation caused a complex response consisting of immediate inhibition of spontaneous activity followed by a marked contractile response. In about half of the preparations the contractile response was followed by a second inhibitory phase. In Fig. 1 the effects of sympathetic (periarterial) and transmural stimulation are compared in a segment of rabbit isolated ileum suspended in Tyrode solution at 37°. Sympathetic stimulation produced a complete inhibition of the pendular movements which outlasted the stimulation period. Complete recovery of the spontaneous movements occurred after several minutes. In contrast, when the same stimulus was applied transmurally, an inhibitory response occurred which changed during the stimulus to a contractile response outlasting the stimulus period by several minutes.

We have attempted to analyse this complex response to transmural stimulation by means of blocking drugs. The inhibitory phase of the response was prolonged, or in those preparations where it was absent initially, it was revealed after the addition of atropine or hyoscine ($10^{-7}$ to $10^{-4}$ g/ml) to the bath (Fig. 2B). These drugs did not affect the excitatory phase and produced either no effect on the responses to sympathetic stimulation, or caused only a slight impairment. The initial inhibitory effect of transmural stimulation was unaffected, or in some preparations partly blocked by guanethidine in concentrations ($10^{-6}$ to $10^{-5}$ g/ml) which completely abolished the responses to sympathetic stimulation (Fig. 2C). The response to transmural stimulation was markedly altered when the bath temperature was lowered, the inhibitory phase being prolonged and the excitatory phase reduced or abolished (Fig. 2D). Both phases of the response to transmural stimulation were unaffected, or occasionally slightly reduced by the anti-adrenaline agents phentolamine and propranolol, added to the bath individually or simultaneously in concentrations ($10^{-7}$ to $5 \times 10^{-7}$ g/ml) which abolished the responses to added catecholamines and to sympathetic nerve stimulation.

From the results obtained with anti-adrenaline agents and with guanethidine we conclude that the inhibitory responses to transmural stimulation are unlikely to be due entirely to activation of sympathetic adrenergic nerve elements within the muscle wall. However, the following preliminary observations suggest to us that the inhibitory responses are nervously mediated.

The local anaesthetic agent cocaine abolished both phases of the response to transmural stimulation in concentrations ($2 \times 10^{-5}$ to $6 \times 10^{-5}$ g/ml) similar to those which abolished the responses to sympathetic nerve stimulation.

All phases of the response to transmural stimulation were present when pulse widths as low as 0·1 msec, which are unlikely to affect smooth muscle directly, were used. Moreover, it was shown that the optimal frequency for the inhibitory component was lower (10 to 20 pulses/sec) than the optimal frequency for sympathetic relaxations (30 to 50 pulses/sec).

The complex responses to transmural stimulation were strikingly similar to the effects of the automatic ganglion stimulants nicotine and tetramethyl ammonium in isolated preparations of rabbit ileum.

The characteristics of the inhibitory responses to transmural stimulation

described here are essentially similar to those recently described in the cat stomach (Martinson, 1965a,b), in the guinea-pig isolated taenia coli (Burnstock, Campbell & Rand, 1966), and in the guinea pig isolated stomach (Campbell, 1966) and suggest the presence of non-adrenergic inhibitory neurons in the gastrointestinal tract.

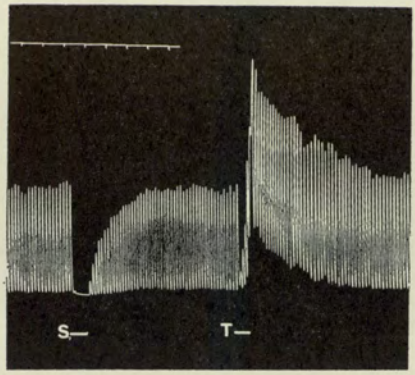


FIG. 1. Longitudinal contractions of rabbit isolated ileum suspended in aerated Tyrode solution at 37°. Sympathetic nerve stimulation (at S) and transmural stimulation (at T) each applied for 20 sec periods with 2 msec 20 V rectangular pulses at a frequency of 50 pulses/sec. Time: 30 sec intervals.

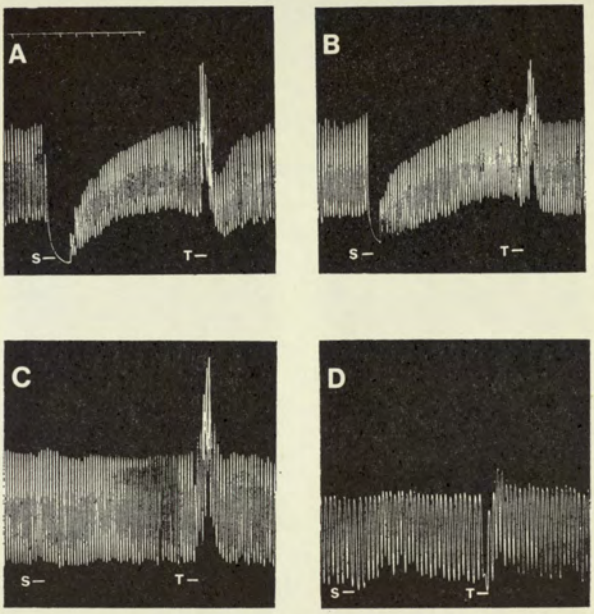


FIG. 2. Rabbit isolated ileum preparations; sympathetic stimulation (at S) and transmural stimulation (at T) applied as in Fig. 1. Control responses in A, 20 min after adding hyoscine ($10^{-6}$ g/ml) in B, 30 min after adding guanethidine ($10^{-6}$ g/ml) in C. In D, in the presence of guanethidine and hyoscine, responses repeated after reducing bath temperature from 37 to 31°.

We have repeated our experiments using transmural stimulation in intestinal preparations taken from duodenum, ileum and colon of the rabbit and cat. In all these preparations, transmural stimulation produced initial inhibitory responses. In some preparations of cat intestine transmural stimulation produced only inhibition which was not abolished by guanethidine. In those preparations of cat intestine showing a mixed response of inhibition and excitation, the excitatory phase was abolished by low concentrations of hyoscine or atropine. The atropine sensitivity of the motor component in cats, and the lack of sensitivity in rabbits, is consistent with the hypothesis that this part of the response is due to activation of parasympathetic nerve elements within the myenteric plexus, since the parasympathetic nerves to rabbit intestine are relatively insensitive to atropine (Ambache, 1951; Ambache & Edwards, 1951) whilst those of the cat are readily susceptible (Gillespie & Mackenna, 1960; Ambache, 1951).

The work of Martinson (1965a,b) and Campbell (1966) using stomach preparations, suggests that the connections of these inhibitory neurons with the central nervous system may be via the vagus nerves. However, since we have obtained inhibitory responses to transmural stimulation in colon preparations it may be that the sacral parasympathetic outflow also contains preganglionic fibres forming connections with non-adrenergic inhibitory fibres within the muscle wall.

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# A PHARMACOLOGICAL ANALYSIS OF THE RESPONSES TO TRANSMURAL STIMULATION IN ISOLATED INTESTINAL PREPARATIONS

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# A PHARMACOLOGICAL ANALYSIS OF THE RESPONSES TO TRANSMURAL STIMULATION IN ISOLATED INTESTINAL PREPARATIONS

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Inhibition of gastro-intestinal motility by stimulation of the vagus nerves was first described towards the end of the nineteenth century by Langley (1898) and by Bayliss & Starling (1899), and has since been reported at intervals (for example, Auer & Meltzer, 1907 ; Veach, 1925 ; McSwiney & Wadge, 1928 ; Harrison & McSwiney, 1936 ; Martinson & Muren, 1960, 1963 ; Paton & Vane, 1963 ; Campbell, 1966a).

Harrison & McSwiney (1936) suggested that the inhibitory responses they recorded were caused by adrenergic fibres in the vagus nerves, and this view was tentatively supported by Greeff, Kasperat & Osswald (1962) and by Paton & Vane (1963). Recent studies, however, both in whole animals (Martinson & Muren, 1963 ; Martinson, 1964, 1965) and in isolated intestinal preparations (Burnstock, Campbell & Rand, 1966 ; Bennett, Burnstock & Holman, 1966 ; Campbell, 1966a) do not support this hypothesis. Thus the inhibitory responses to transmural stimulation in the taenia coli persisted in the presence of guanethidine or bretylium in concentrations which abolished the effects of sympathetic stimulation (Burnstock, Campbell & Rand, 1966). Moreover, the electrophysiological studies of Bennett, Burnstock & Holman (1966) on the taenia suggest that the inhibitory responses to sympathetic and to transmural stimulation are mediated by different sets of nerves.

Recently, Holman & Hughes (1965) have obtained biphasic responses consisting of inhibitory and motor components after transmural stimulation of isolated intestinal preparations taken from mice, rats, guinea-pigs and rabbits. We have obtained similar responses in isolated intestinal preparations taken from rabbits and kittens, and have attempted a pharmacological analysis of the components of the response. In most experiments we have used the isolated ileum of the rabbit because its responses to sympathetic stimulation and its susceptibility to blocking drugs have been well characterized, and the preparation shows regular activity for many hours.

A preliminary account of some of this work has already been published (Day & Warren, 1967).

## METHODS

Rabbits weighing 1–3 kg were killed by a blow on the head and bled. Sections of intestine about 3 cm long were removed together with their mesenteric attachments. The tissues were set up in aerated Tyrode solution and the longitudinal contractions recorded with isotonic frontal writing levers writing on a smoked drum. The bath temperature varied between 28° and 37° C in different experiments; details are given in RESULTS. Preparations of kitten intestine were set up in the same way.

In some experiments with rabbit intestine, Krebs bicarbonate or McEwen (1956) solution gassed with 5% carbon dioxide in oxygen was used, but the results were the same as in aerated Tyrode solution. Preparations of kitten intestine gave poor results in Tyrode solution, however, and McEwen solution with 5% carbon dioxide in oxygen was used for most of these.

The periarterial (sympathetic) nerves were stimulated with an electronic stimulator delivering rectangular pulses through bipolar platinum electrodes of the type described by Burn & Rand (1960). Transmural stimulation was effected with bipolar intraluminal electrodes of the type described by Paton (1955).

In most experiments sympathetic or transmural stimulation was applied for 20 sec periods, repeated at not less than 3 min intervals, with pulses of supramaximal strength (usually 20 V). Details of frequency and pulse width are given in the RESULTS section.

### *Reserpine pretreatment*

Rabbits were pretreated with reserpine by injecting the commercial preparation (Serpasil, CIBA) into the marginal ear vein in a dose of 0.3 mg/kg on each of 3 days and using the animals on the fourth day.

## RESULTS

### *Transmural stimulation of rabbit isolated intestine*

The response to transmural stimulation in segments of rabbit isolated ileum set up at 37° C usually consisted of mixed inhibitory and motor components. There was some variation between different preparations, but in general there were four main types of response. These are illustrated in Fig. 1. The most usual was a rapid and complete inhibition of spontaneous activity which changed, during the stimulation period, into a contraction which subsided at the end of the stimulus and was followed by inhibition of variable extent and duration (Fig. 1a). The second type of response commonly seen was identical, except that the secondary inhibition was absent or very slight (Fig. 1b).

The third type, seen in a few preparations, consisted of an immediate contraction followed, after the stimulation, by inhibition (Fig. 1c). The fourth type, seen in only about ten of more than two hundred preparations, consisted solely of a contraction lasting throughout the stimulation period, followed by the return of normal spontaneous activity (Fig. 1d).

Basically similar responses to transmural stimulation were obtained in preparations taken from all regions of the intestine from duodenum to terminal colon of rabbits, guinea-pigs and kittens. In general, however, the spontaneous movements of rabbit ileum were the most regular and were therefore used in most experiments.

### *Effect of local anaesthetics*

A comparison was made between the action of local anaesthetic agents on the response to transmural and to sympathetic (periarterial) stimulation to determine whether the response to transmural stimulation was likely to be nervously mediated. The local

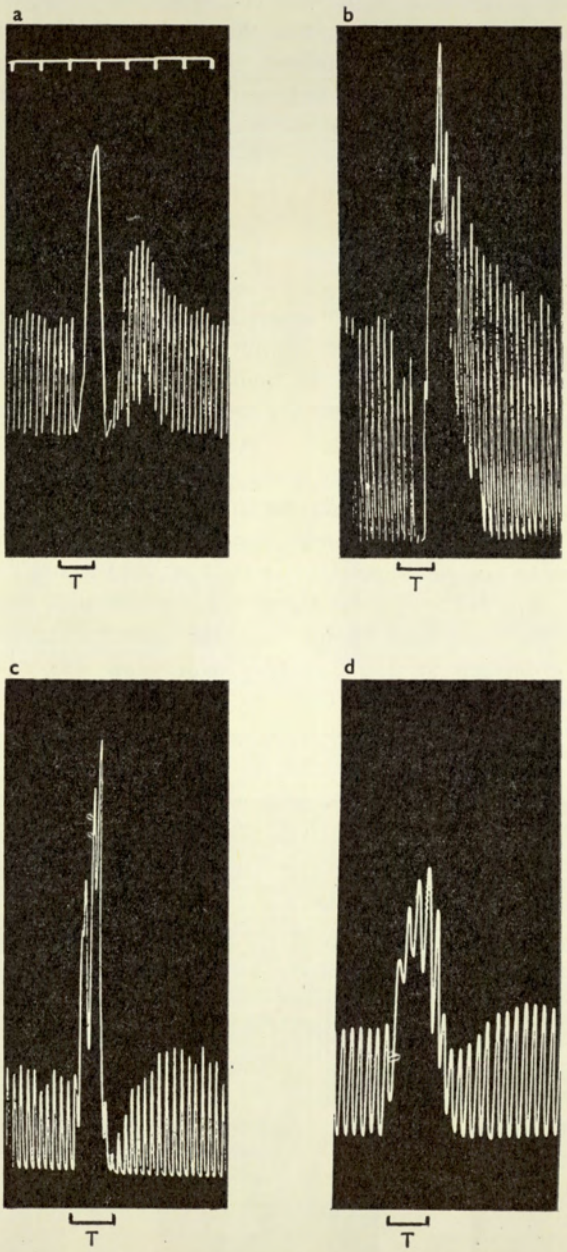


Fig. 1. Four main types of response to transmural stimulation (T) in different segments of rabbit isolated ileum in aerated Tyrode solution at 37° C. Stimulation applied for 20 sec with 1 msec 20 V pulses at a frequency of 20 pulses/sec. Time marker in 30 sec intervals.

anaesthetics used were lignocaine, procaine, cinchocaine and cocaine. These, with the occasional exception of cocaine, depressed the spontaneous activity of the ileum in relatively low concentrations (4 to 50 $\mu$g/ml.) thus making it impossible to establish with certainty whether the responses were impaired. In six experiments out of twelve in which cocaine (50 $\mu$g/ml.) was used there was little impairment of the pendular movements, but the inhibitory and motor components of the transmural response were abolished as was the inhibition to sympathetic stimulation. The impairment of the responses caused by cocaine was partially reversed by washing.

*Effect of altering the bath temperature*

When the bath temperature was progressively lowered from 37° C to 28° C the response to transmural stimulation changed ; the inhibitory phase became more prolonged while the motor phase was greatly reduced. In many experiments at 28° C the response to transmural stimulation was pure inhibition whereas at 37° C the motor component was more marked. This is illustrated in Fig. 2. At 28° C transmural stimulation produced an inhibitory response which outlasted the stimulation period. At 33° C a small motor component appeared in the response and the inhibitory phase was less prolonged, while at 37° C the main response during stimulation was contraction followed by inhibition. The motor responses to acetylcholine (0.01 to 0.04 $\mu$g/ml.) were altered in the same way by lowering the bath temperature as was the motor component of the transmural response. Thus the responses were reduced by lowering the temperature from 37° C and were sometimes almost abolished at 28° C. This depression was completely reversed by returning the bath temperature to 37° C.

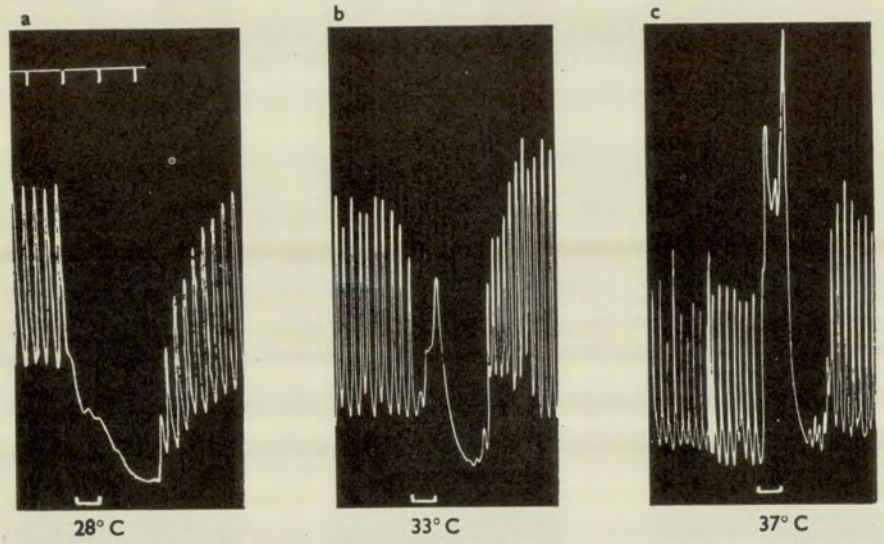


Fig. 2. Effect of raising the bath temperature on the response to transmural stimulation in a segment of rabbit isolated ileum suspended in aerated Tyrode solution. Transmural stimulation (at ⎵ ) applied for 20 sec with 2 msec pulses of supramaximal strength at a frequency of 20 pulses/sec. Time marker in 30 sec intervals.

*Comparison between transmural and sympathetic inhibition*

*Stimulus parameters*

In some preparations the relaxations obtained by stimulating the sympathetic nerves were compared with those obtained by transmural stimulation in order to investigate the possibility of a common origin for these responses. It was found that the optimal frequency of stimulation for transmural inhibition was lower (10 to 20 pulses/sec) than for sympathetic inhibition (50 pulses/sec). It was, however, difficult to obtain a valid comparison between the two responses because the motor component of the transmural response varied in different preparations and at different frequencies and may therefore have influenced the inhibitory response in a variable manner.

In several preparations the relaxation to transmural stimulation was larger at a stimulus frequency of 50 pulses/sec than it was at 20 pulses/sec. In these experiments the addition of guanethidine (1–10 $\mu$g/ml.) to the bath abolished the effects of sympathetic stimulation and reduced the optimal frequency for transmural inhibition to 20 pulses/sec. Thus, in some experiments at least, there may have been a sympathetic component to the transmural inhibition. Submaximal inhibitions to transmural stimulation could be elicited by frequencies of stimulation (1 to 5 pulses/sec) which in most experiments were too low to cause inhibition to sympathetic stimulation.

The motor component of the response to transmural stimulation was fully developed usually at a frequency of 20 pulses/sec. In most experiments increasing the frequency to 50 pulses/sec did not increase this part of the response.

The threshold pulse-width for both sympathetic and transmural inhibition was of the order of 0.1 msec. Pulse-width of 0.5 msec–0.1 msec tended to increase the inhibitory component and decrease the motor component of the transmural response. In most preparations a pulse width of 1 msec was used for transmural stimulation because this was supramaximal for both inhibitory and motor components of the response.

To obtain maximal responses at any given pulse width and frequency for both sympathetic and transmural stimulation the necessary voltage was usually between 5 and 10. A supramaximal voltage (20 V) was used in all experiments for both sympathetic and transmural stimulation.

*Anti-adrenaline substances*

Inhibition of the isolated intestine by sympathetic stimulation and by added noradrenaline is caused by the activation of both $\alpha$ and $\beta$ receptors. Thus, a mixture of both $\alpha$ and $\beta$ receptor blocking substances is necessary to abolish these effects (Furchgott, 1960). An experiment in which a mixture of anti-adrenaline substances was tested on the inhibitory responses to transmural and sympathetic stimulation and to added noradrenaline is shown in Fig. 3. In this experiment the bath temperature was maintained at 32° C and the response to transmural stimulation was predominantly inhibitory. The addition to the bath of a mixture of phentolamine (1 $\mu$g/ml.) and propranolol (2 $\mu$g/ml.) abolished the inhibitory responses to sympathetic stimulation and to added noradrenaline, but the transmural inhibition was unaffected in size and slightly more prolonged in duration than before the blocking drugs.

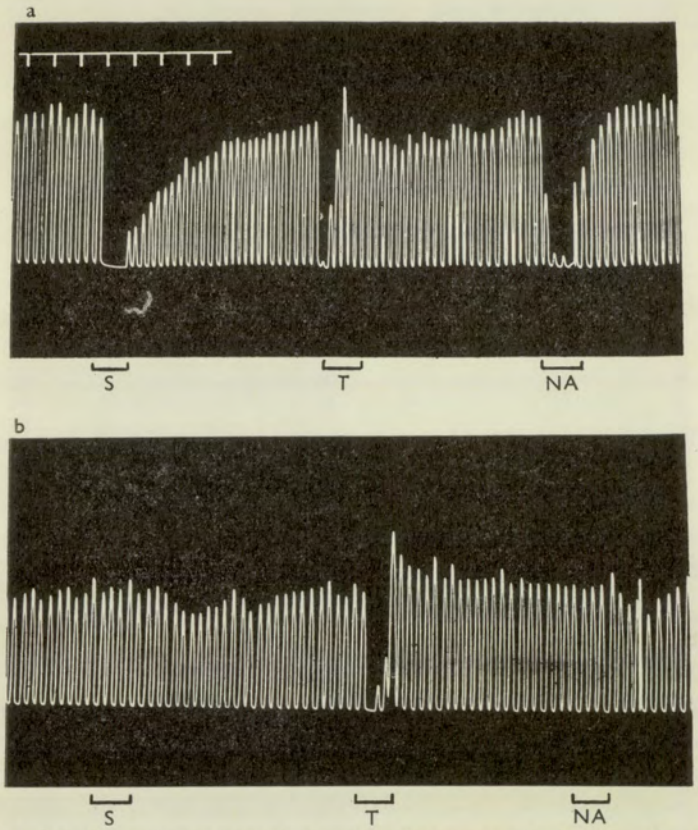


Fig. 3. Rabbit isolated ileum in aerated Tyrode solution at 32° C. a: Control responses to sympathetic stimulation (S) applied for 20 sec with 2 msec 20 V pulses at a frequency of 50 pulses/sec, transmural stimulation (T) with 0.5 msec 20 V pulses at 20 pulses/sec and added noradrenaline (NA) in a concentration of 0.02 μg/ml. left in contact for 30 sec. b: Same responses repeated 30 min after adding a mixture of propranolol (2 μg/ml.) and phentolamine (1 μg/ml.) to the bath. Time marker in 30 sec intervals.

*Adrenergic neurone blocking agents*

Guanethidine (1–10 μg/ml.) or xylocholine (3–20 μg/ml.) when added to the bath abolished the inhibitory responses to sympathetic stimulation while in most preparations producing little or no impairment of the inhibitory component of the transmural response. This is shown in the experiment illustrated in Fig. 4 which is of a preparation maintained at 32° C in order to enhance the inhibitory component and depress the motor component of the transmural response.

Between Fig. 4a and 4b the preparation was left in contact with a high concentration (10 μg/ml.) of guanethidine for 45 min. In Fig. 4b the sympathetic response was abolished while the inhibition to transmural stimulation was only slightly reduced. In preparations maintained at 37° C the motor response to transmural response was more prominent than at lower temperatures and it was not significantly altered by even high concentrations of guanethidine or xylocholine.

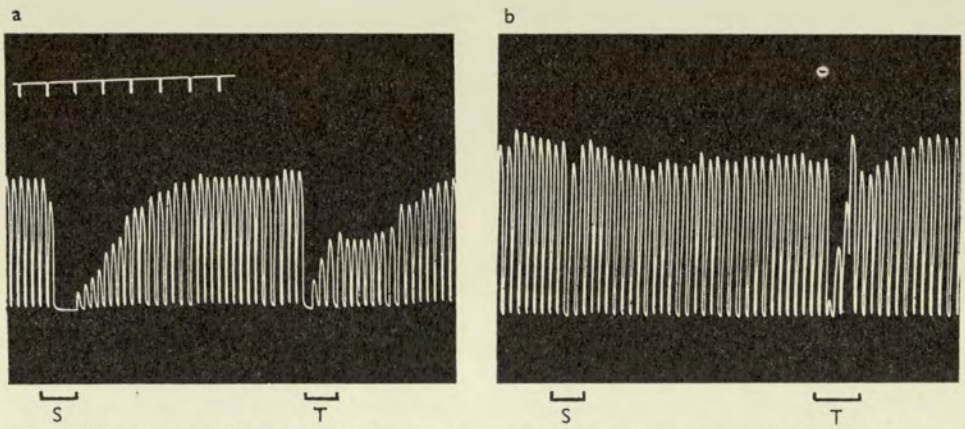


Fig. 4. Rabbit isolated ileum at 32° C. a: Sympathetic stimulation (S) with 2 msec 20 V pulses at a frequency of 50 pulses/sec applied for 20 sec, and transmural stimulation (T) for 20 sec with 0.5 msec 20 V pulses at a frequency of 20 pulses/sec. b: Same responses 45 min after adding guanethidine (10 $\mu$g/ml.) to the bath. Time marker in 30 sec intervals.

In a few preparations at 37° C, guanethidine caused a marked impairment of the inhibitions to transmural stimulation. The block could, however, be distinguished from the sympathetic nerve blockade by the fact that it could be reversed by lowering the bath temperature by 4°–7° C, whereas this did not reverse the sympathetic blockade.

*Reserpine treatment*

Reserpine was either administered intravenously to rabbits for several days before the experiment, or added to the bath containing the tissues.

In twenty preparations of ileum set up at 32° C or 37° C and taken from six rabbits treated with reserpine, the inhibitory responses to sympathetic stimulation were impaired but not abolished. The inhibitory component of the transmural response also seemed to be impaired, but this could have been caused by an enhancement of the motor component masking the inhibition.

Clearer results were obtained in ten other preparations taken from untreated rabbits and set up at 32° C in order to reduce the motor phase of the response. In these experiments reserpine (0.5–1.0 $\mu$g/ml.) was added to the bath and caused a slowly developing impairment of the inhibitory responses to both sympathetic and transmural stimulation which were both usually completely abolished after contact with the drug for 3–4 hr. In these experiments dopamine (50 $\mu$g/ml.) partially reversed the sympathetic nerve block but produced little or no enhancement of the inhibitory component of the transmural response. This observation is illustrated in Fig. 5. In this experiment the inhibitory component of the transmural response and the sympathetic inhibition were abolished after contact for 225 min with reserpine (0.5 $\mu$g/ml.). Dopamine (50 $\mu$g/ml.) was added to bath and left for 45 min after which time the sympathetic inhibition but not the transmural inhibition was largely restored.

The results so far described show that the inhibitory responses to sympathetic and to transmural stimulation are affected differently by varying the conditions of stimulation by $\alpha$- and $\beta$-receptor blocking agents, by adrenergic neurone blocking drugs, and by

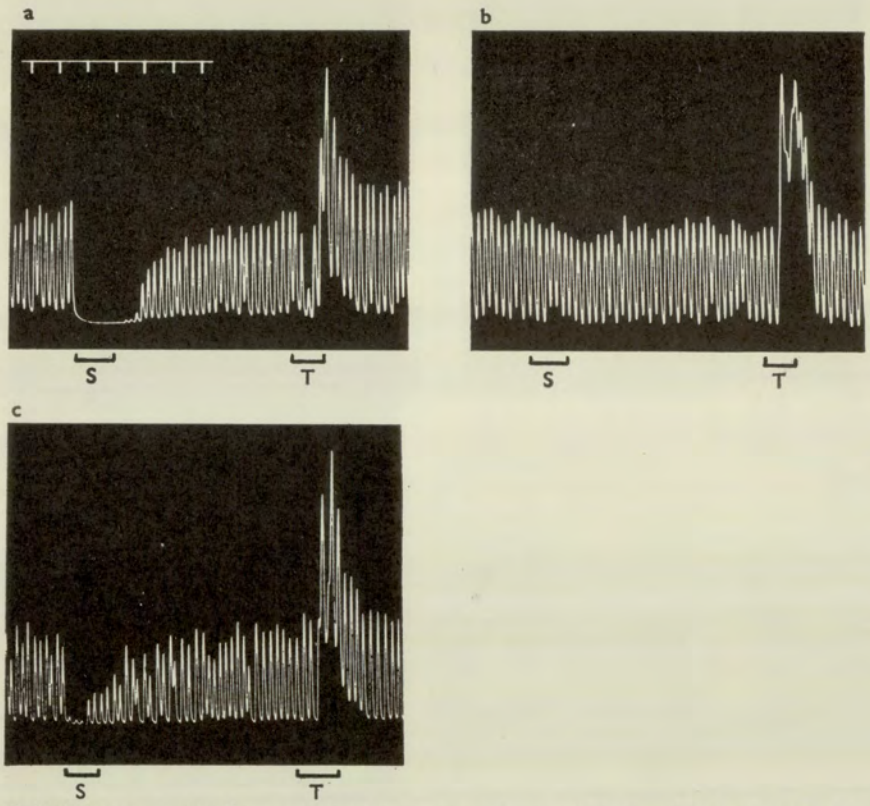


Fig. 5. Rabbit ileum at 32° C. a: Control responses to sympathetic stimulation (S) with 1 msec 20 V pulses at a frequency of 50 pulses/sec and transmural stimulation (T) with 1 msec 20 V pulses at 20 pulses/sec each applied for 20 sec periods. b: The same responses repeated 225 min after the addition of reserpine (0.5 $\mu$g/ml.) to the bath. Between b and c dopamine (50 $\mu$g/ml.) was added to the bath and the responses were repeated 45 min later in c. Hyoscine (1 $\mu$g/ml.) was present in the bath throughout the experiment. Time marker in 30 sec intervals.

depletion of sympathetic transmitter by reserpine. The evidence, therefore, seems to indicate a different origin for the two responses.

The next part of the investigation was designed to determine the nature of the motor component of the response to transmural stimulation.

*Effect of atropine and hyoscine*

The effects of both atropine and hyoscine in concentrations ranging from 0.1 to 100 $\mu$g/ml. were tested on the responses to transmural stimulation. Atropine in concentrations above 0.1 $\mu$g/ml. frequently inhibited spontaneous activity of the gut as noticed by Holman & Hughes (1965). Hyoscine rarely caused this effect and was therefore used in most experiments. In preparations maintained at 37° C in which the motor component of the transmural response was well marked, hyoscine potentiated the initial inhibitory phase of the response but usually had little effect on the motor component. In those preparations in which an initial inhibition was absent (Fig. 1c), or in which the response was entirely motor (Fig. 1d), hyoscine revealed an initial inhibitory component. This

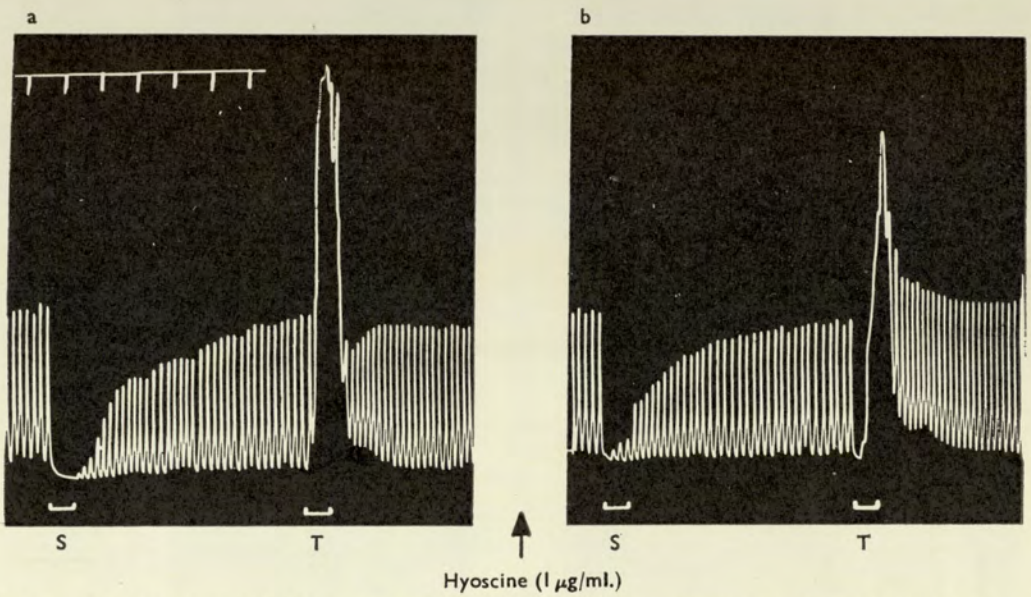


Fig. 6. Rabbit ileum at 37° C. a: Control responses to sympathetic stimulation (S) with 2 msec 20 V pulses at a frequency of 50 pulses/sec and transmural stimulation (T) with 2 msec 20 V pulses at a frequency of 20 pulses/sec each applied for 20 sec. b: Responses repeated 15 min after adding hyoscine (1 μg/ml.) to the bath. Time marker in 30 sec intervals.

observation is illustrated in Fig. 6 where the transmural response was converted by hyoscine (1 μg/ml.) from pure motor to initial inhibition followed by a reduced motor effect. In this experiment there was a slight impairment of the response to sympathetic stimulation after hyoscine.

In preparations maintained below 37° C the motor component of the transmural response was usually much less well marked and was relatively more inhibited by hyoscine. The experiment illustrated in Fig. 7 shows the effect of hyoscine on the response to added acetylcholine and to transmural stimulation in a preparation maintained at 32° C. In Fig. 7a the stimuli for the transmural response were altered in order to get graded motor effects. In Fig. 7b after contact for 15 minutes with hyoscine (1 μg/ml.) the response to added acetylcholine was abolished and transmural stimulation then produced only inhibition. In preparations of ileum taken from kittens, transmural stimulation produced initial motor effects which were followed by long lasting guanethidine-insensitive inhibitions. In preparations at 37° C the motor component of the transmural response in kitten ileum was abolished by low concentrations of hyoscine (0.01 μg/ml.).

*Anticholinesterases*

The addition of physostigmine (eserine) or neostigmine to the bath in concentrations of 0.05 to 0.1 μg/ml. markedly enhanced the motor component of the transmural response with a consequent masking of the inhibitory component. Figure 8 illustrates an experiment in which the response to transmural stimulation was either purely inhibitory or

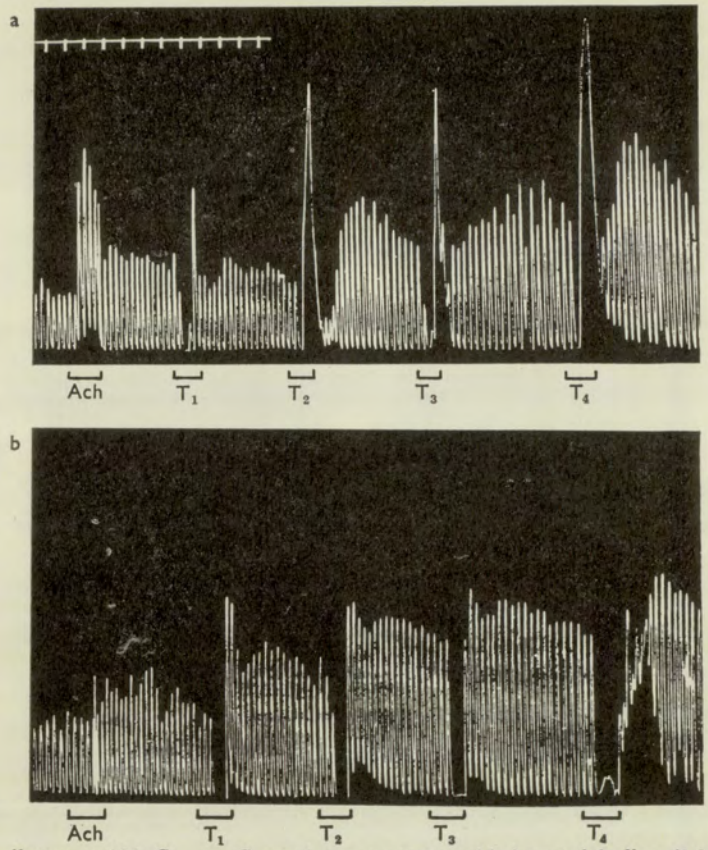


Fig. 7. Rabbit ileum at 32° C. a: Control responses to added acetylcholine (0.02 μg/ml.) (Ach) and transmural stimulations applied for 20 sec periods with supramaximal strength pulses: $T_1$, pulse width of 0.5 msec and a frequency of 20 pulses/sec; $T_2$, 0.5 msec and 50 pulses/sec; $T_3$, 2 msec and 20 pulses/sec; $T_4$, 2 msec and 50 pulses/sec. b: Same responses repeated in the presence of hyoscine (1 μg/ml.). Time marker in 30 sec intervals.

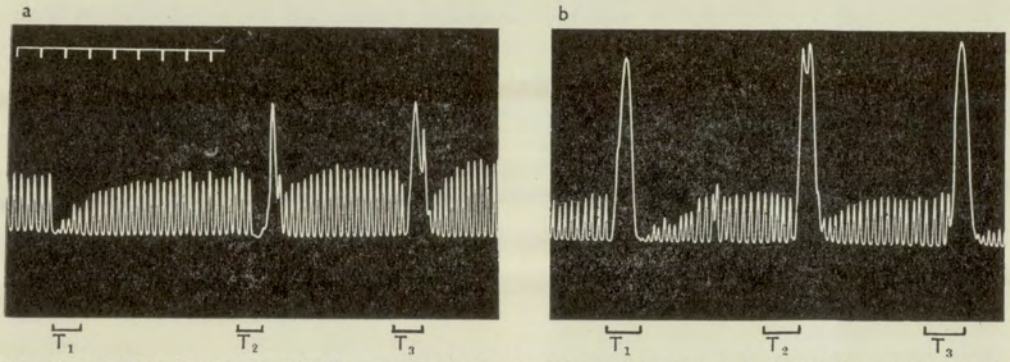


Fig. 8. Rabbit ileum at 32° C. a: Control responses to transmural stimulation with varying stimuli. $T_1$, pulse width 0.5 msec, frequency 50 pulses/sec; $T_2$, 2 msec and 20 pulses/sec; $T_3$, 2 msec and 50 pulses/sec. Each pulse of supramaximal strength and with a stimulus period of 20 sec. b: The same responses are repeated 14 min after the addition of physostigmine (0.1 μg/ml.) to the bath. Time marker in 30 sec intervals.

biphasic according to the intensity of the stimuli used. The addition of physostigmine (0.1 $\mu$g/ml.) to the bath revealed a large motor component to the response which had previously been purely inhibitory and the motor components of the other two responses were enhanced and the inhibitory components inhibited.

## DISCUSSION

Our results suggest that transmural stimulation of segments of rabbit and kitten isolated intestine activates at least three distinct nervous pathways. The transmural response consists of inhibitory and motor components, and the appearance of the response varies according to the relative predominance of one component over the other. The inhibitory component seems to be caused chiefly by activation of non-adrenergic neurones whose presence in various intestinal preparations has recently been described (Burnstock, Campbell, Bennett & Holman, 1964 ; Holman & Hughes, 1965 ; Burnstock, Campbell & Rand, 1966 ; Campbell, 1966a). It seems likely, however, that activation of sympathetic nerve endings within the muscle wall may also contribute to the inhibitory component. Thus the response is usually slightly impaired by guanethidine in concentrations sufficient to cause sympathetic nerve block.

The evidence in favour of a non-adrenergic mechanism to explain the major part of the transmural inhibition is substantial. Thus the inhibitory response to transmural stimulation is slightly enhanced in the presence of a mixture of $\alpha$- and $\beta$-receptor blocking agents in concentrations which abolish the inhibitory effects of sympathetic stimulation and of added noradrenaline. Holman & Hughes (1965) reported that pronethalol depresses the tone of intestinal preparations and reduces the responses to transmural stimulation. We have used propranolol, a more specific and more potent $\beta$-receptor blocking drug than pronethalol, which neither effected intestinal tone nor depressed either component of the transmural response. It may be that the reduction of the transmural responses caused by pronethalol in the experiments of Holman & Hughes (1965) was not specific because, as these authors pointed out, pronethalol is a potent local anaesthetic agent.

In agreement with other workers (Holman & Hughes, 1965 ; Burnstock, Campbell & Rand, 1966 ; Bennett, Burnstock & Holman, 1966) we found the threshold as well as the optimal frequency for transmural inhibition to be lower than that for sympathetic inhibition. Several nervous pathways seem to be activated by transmural stimulation, however, and may therefore alter the characteristics of the non-adrenergic inhibition.

The only substance, apart from cocaine, which convincingly impaired the transmural inhibition was reserpine. The action of reserpine was best demonstrated by adding the drug to the preparation in the bath. The time course of the blocking action of reserpine on the transmural inhibition was similar to that for the sympathetic responses, but could be distinguished from it by the fact that dopamine restored only the sympathetic responses. Thus, the blocking action of reserpine on the transmural inhibitory response seems to be independent of its catecholamine depleting action which causes the sympathetic blockade. Gillespie & Mackenna (1960) found that the inhibitory action of nicotine on rabbit isolated colon persisted in the presence of the adrenergic neurone blocking drug xylocholine, but was reduced or abolished in preparations taken from rabbits which had been pretreated with reserpine. These observations are consistent with our own using guanethidine and reserpine, because nicotine and other ganglion stimulants are known to activate the

non-adrenergic inhibitory neurones in isolated intestinal preparations (Holman & Hughes, 1965 ; Burnstock, Campbell & Rand, 1966). It therefore seems likely that reserpine depletes the stores of neuro-humoral transmitter from both the sympathetic and the non-adrenergic inhibitory nerves in the intestine.

The motor component of the response to transmural stimulation in isolated intestinal preparations has been described by other workers as " rebound " contraction (Holman & Hughes, 1965 ; Campbell, 1966b ; Bennett, 1966). These workers suggest that because the contraction persists in the presence of high concentrations of atropine it is not mediated by cholinergic nerves but occurs as a direct result of the inhibitory phase of the transmural response. The inhibitory response causes hyperpolarization of the smooth muscle membrane which is replaced at the end of stimulation by an increase in rate of firing of action potentials with a consequent increase in muscular tension (Bennett, 1966 ; Campbell, 1966b). This interpretation does not explain the major part of the motor response in our experiments for four main reasons. First, the motor component, like the response to added acetylcholine, was depressed by lowering the bath temperature while the inhibitory component was enhanced. Second, in some experiments the motor component preceded the inhibition or, in a few experiments at 37° C, occurred in the absence of an inhibitory phase. Third, the motor component was partly blocked by hyoscine, particularly at low bath temperatures, and the inhibition was potentiated by hyoscine at all bath temperatures. Finally, the motor component was potentiated by anticholinesterases. These observations taken together strongly suggest that the inhibitory and motor components of the response to transmural stimulation are separate phenomena and are probably mediated through different nervous pathways. Despite the relative insensitivity of the motor response to hyoscine in rabbit intestine, the evidence suggests that the motor response is cholinergic in nature. In the kitten intestine the motor response to transmural stimulation was abolished by low concentrations of hyoscine. The relative insensitivity of the cholinergic nerves in rabbit intestine as compared with those in the kitten intestine to the blocking action of atropine-like drugs has been described previously by Ambache & Edwards (1951). These workers showed that the motor response to nicotine in kitten isolated ileum was converted to inhibition in the presence of atropine while the motor effect of nicotine in rabbit ileum persisted in the presence of high concentrations of atropine. Botulinum toxin, a selective cholinergic nerve blocking drug, however, reverses the nicotine motor response to inhibition in both species (Ambache, 1951).

In our view the most likely mechanism to explain the actions of atropine-like drugs and anticholinesterases on the response to transmural stimulation is that these drugs alter the time-course of the motor component. An anticholinesterase, by preserving the acetylcholine released by transmural stimulation, may reduce the latency of the motor component and consequently obscure the inhibitory phase of the response. A similar mechanism may explain the apparent block of the " rebound " contraction by neostigmine in Campbell's (1966b) experiments, because after inhibition of cholinesterase the motor response may be fully developed during the stimulation period and therefore not persist afterwards. Conversely, atropine-like agents, by partly blocking the effects of acetylcholine released by transmural stimulation, would increase the latency of the contraction thus enhancing the initial inhibition and making most of the motor component occur after the stimulation with an apparent potentiation of the " rebound " contraction.

Our evidence suggests that the motor response to transmural stimulation is mediated by cholinergic fibres—presumably of parasympathetic origin—and that the inhibition results chiefly from activation of non-adrenergic inhibitory neurones within the muscle wall.


## SUMMARY

1. Transmural stimulation of segments of isolated intestine taken from kittens and rabbits and maintained at 37° C produced biphasic responses consisting of initial inhibition of pendular movements followed by a marked increase in tone of the preparations.

2. Progressive reduction of the bath temperature from 37° C to 28° C markedly enhanced the inhibitory component of the transmural response but impaired the motor component and the contractions to added acetylcholine.

3. That the inhibitory responses to transmural stimulation were not of sympathetic origin was shown by their differing optimal stimulus parameters, the resistance of the transmural response to guanethidine and to mixtures of $\alpha$- and $\beta$-receptor blocking drugs.

4. Reserpine treatment impaired both sympathetic and transmural inhibitions but only the former responses were restored by adding dopamine to the bath.

5. Hyoscine and atropine enhanced the inhibitory component of the transmural response in rabbit ileum at 37° C but had little or no effect on the motor component at this temperature. At lower bath temperatures these drugs were relatively more effective at inhibiting the motor component. The motor component of the transmural response in kitten intestine was abolished by a low concentration of hyoscine even at 37° C.

6. The motor component of the transmural response was markedly enhanced by anticholinesterase agents.

7. The evidence suggests that the major part of the inhibitory component of the transmural response is caused by activation of non-adrenergic inhibitory neurones within the muscle wall. On the other hand, the motor component seems chiefly to result from activation of cholinergic nerve endings and is apparently not a " rebound " phenomenon resulting from the initial inhibition as has been suggested by other workers.


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