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A STUDY OF INTESTINAL INNERVATION USING NICOTINE AND RESERPINE

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy in the Faculty of Medicine

by

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I N T R O D U C T I O N

In 1951 Ambache reported that nicotine could, *in vitro*, cause relaxation of the small intestine of mice and rabbits if the animal was first treated with *botulinum* toxin. He suggested that the relaxation was due to the stimulation by nicotine of adrenergic neurones in Auerbach's plexus and was revealed only because the postganglionic parasympathetic neurones had been blocked by *botulinum* toxin. Later, Ambache & Edwards (1951) demonstrated relaxation by nicotine of stomach strips and the small intestine of kittens *in vitro* in the presence of atropine, another cholinergic blocking agent. They were unable to demonstrate relaxation of the rabbit ileum when atropine was used to block the cholinergic nerves. However, Gillespie (unpublished) observed that nicotine could cause relaxation of the rabbit colon without the presence of a cholinergic blocking agent.

The object of the present investigations was to confirm the inhibitory effect of nicotine on the rabbit colon, and if confirmed, to use the colon as a convenient preparation to elucidate the underlying mechanism.

From a study of the literature it appeared that there were at least three possible explanations for the

inhibitory effect of nicotine: first, the stimulation of adrenergic neurones in the wall of the gut, as suggested by Ambache; secondly, discharge of catechol amines from chromaffin cells in the mucosa; and thirdly, activation of an axon reflex. An action on chromaffin cells was suggested by Burn & Rand (1958 a,b), who investigated the adrenaline-like effects of nicotine at several sites in the body and postulated that nicotine released an adrenaline-like substance from chromaffin tissue. That an axon reflex is responsible is the hypothesis of Coon & Rothman (1940) who suggested that nicotine caused contraction of the arrectores pilorum muscles of the cat by stimulating nerve endings, thus initiating an axon reflex in the terminal ramifications of sympathetic nerve fibres.

Since Ambache's original investigation on the intestine, several new sympathetic blocking agents have been developed, for example, reserpine (Serpasil:Ciba) and choline 2:6 xylyl ether bromide (TM 10). It was hoped in the present investigations to confirm or deny the adrenergic nature of the inhibitory response to nicotine by using these drugs. In addition, by section and degeneration of the sympathetic nerves to the colon

it was hoped to show whether or not at this site the inhibition produced by nicotine could be due to an axon reflex. Further, it was decided to examine the colon for cells which give the chromaffin reaction. If such cells liberate the inhibitory agent, as Burn & Rand (1958 a,b) have suggested, it should be possible to identify the cells histologically.

The results of the present work have confirmed the presence of an inhibitory response of the colon to nicotine, and have shown that this inhibition is not due, apparently, either to the presence of adrenergic neurones in Auerbach's plexus, or to discharge of an adrenaline-like substance from chromaffin cells in the gut wall. The inhibitory response is more likely to be due to an axon reflex or to another mechanism to be described later.

In the course of these investigations it was unexpectedly discovered that, after giving reserpine to rabbits for several days, the inhibitory response of the colon to stimulation of its lumbar colonic (sympathetic) nerves was reversed to become a motor response. The mechanism of the motor response after reserpine was investigated and this, together

with the mode of action of reserpine on sympathetic nerves, forms the second part of the thesis.

Both investigations have already been published along with Dr. J.S. Gillespie and reprints are included in the Appendix (Gillespie & Mackenna, 1959, 1960, 1961).

P A R T I

- INTESTINAL INNERVATION - THE ACTION OF NICOTINE

Review of the Literature

It is well known that both nicotine and acetylcholine can stimulate cholinergic and adrenergic autonomic ganglion cells. There have now been several reports of these substances causing adrenaline-like responses when a cholinergic response was expected.

Perhaps the first date of such a report was 1913 when Handovsky & Pick perfused the vessels of the frog's hind legs with a 1% solution of nicotine and surprisingly noted an adrenaline-like constrictor response. They attributed this action to an effect on ganglionic or preganglionic elements. A very obvious objection to this experiment was the high concentration of nicotine that was used, since, with this concentration, the effects of nicotine may not be specific. However, in 1937, Loewi repeated this work on the blood vessels of frogs and used a lower concentration of nicotine, 0.01%, with the same result. The matter was investigated again more recently: in 1946 Haimovici & Pick, again perfusing frogs' blood vessels, reported that an injection of as little as

5 µg of nicotine produced a vasoconstriction and that this effect was unaltered by the removal, immediately beforehand, of the sympathetic chains and spinal nerves. They presumed therefore that the site of action of nicotine in this preparation could not be on the autonomic ganglia: it had to be more peripheral, at the postganglionic nerve endings or at the effector cells themselves.

In 1918 Hunt showed that relatively high concentrations of acetylcholine caused not vasodilatation, as was expected, but vasoconstriction when perfused through the vessels of rabbits' ears. He concluded that acetylcholine exerted an action on blood vessels somewhere 'beyond the ganglia cells'. This constrictor action of acetylcholine was confirmed in 1932 by Hirose who injected acetylcholine into the femoral arteries of cats and showed a diminished outflow.

Reports of adrenaline-like actions of nicotine and of acetylcholine, where a cholinergic response was expected, were not confined to blood vessels. There have been several reports of these substances producing responses on cardiac muscle which would normally be

attributed to an adrenaline-like substance.
Hoffman, Hoffman, Middleton & Talesnik (1945) reported that, in the isolated atropinised hearts of dogs, cats, rabbits and guinea-pigs, nicotine and acetylcholine cause an adrenaline-like effect, namely an increased force and rate of contraction as well as an increase in the coronary flow. This stimulant action of acetylcholine is apparently a 'nicotinic' effect since it is abolished by a large concentration of nicotine and by curare. Also it is mediated by the release of an adrenaline-like substance since it is abolished by ergotamine. The identity of the released substance with adrenaline was further confirmed by parallel assay of the coronary perfusate on the frog heart, on the rectal caecum of the chicken and on the rabbit's gut. The authors concluded that the source of this adrenaline-like substance is either sympathetic ganglia, or chromaffin tissue present in the heart. Hoffman and his coworkers did not speculate on the possible innervation of these ganglia or chromaffin cells. However, in 1949 Middleton, Middleton & Toha reported that vagus stimulation, as well as nicotine and acetylcholine,

could liberate an adrenaline-like substance from the isolated heart after treatment with atropine. They suggested that the action of nicotine and acetylcholine is probably on adrenergic ganglion cells in the heart which lie on the vagus outflow. They made the point that the acceleration therefore occurs after a physiological stimulus, albeit in the presence of atropine, as well as when nicotine or large doses of acetylcholine are applied. They claimed that the effect was not due simply to the presence of sympathetic fibres in the vagal outflow since after section with resultant degeneration of the sympathetic fibres stimulation of the vagus still produced a stimulant action on the heart. However, a later investigation by Benitez, Holmgren & Middleton (1959) showed that these previous results were due to incomplete sympathectomy and to stimulation of sympathetic fibres in the vagus.

Further experiments on the heart were performed by McDowall. In 1946 he demonstrated that although a small dose of acetylcholine produced the expected slowing and weakening of the hearts of cats, rabbits and rats, this was followed by a period of increased

activity. Furthermore, if the muscarinic effects of acetylcholine were blocked by atropine, acetylcholine then produced only an increased activity of the heart, like the action of adrenaline. The stimulant action of acetylcholine was abolished or reversed by ergotoxine but was unaffected by ganglion-cell paralysing doses of nicotine. Consequently McDowall expressed the opinion that the stimulating action of acetylcholine was directly on the cardiac muscle and not on sympathetic ganglia in the heart.

Burn & Dutta in 1948 observed the constrictor effect of acetylcholine on the vessels of the perfused ear of the rabbit and showed that, like the constrictor effect of adrenaline, it was converted to a dilator effect when tolazoline ('Priscol') was added to the perfusion fluid. This was further evidence of an acetylcholine effect being mediated through adrenaline. In 1951 Burn & Robinson again described a constrictor effect of acetylcholine on the vessels of the perfused ear of the rabbit, an effect which could be attributed to the action of adrenaline.

Two years later (1953 a) Kottegoda re-investi-

gated the action of both nicotine and acetylcholine on the vessels of the rabbit's ear. He reported that perfusion of solutions, both of acetylcholine and of nicotine, could cause vasoconstriction, confirming Burn & Robinson's observations with acetylcholine. The constrictor action of acetylcholine and also of nicotine could be blocked by hexamethonium. If the adrenergic blocking agent 'Priscol' was added to the perfusing fluid the vasodilator response to nicotine was restored. This, as the author suggested, was further implication of an adrenaline-like substance in the constrictor response. In a subsequent paper, Kottegoda (1953 b) described the results of his work on isolated rabbit auricles. He reported that nicotine alone caused a mixed inhibitory and stimulant action on this preparation. In the presence of atropine the inhibitory component was blocked and nicotine had a pure stimulatory effect on the auricular beat, an effect which was abolished by hexamethonium.

In another paper, working with Ginzel (Ginzel & Kottegoda, 1953), Kottegoda investigated specifically the possible role of axon reflexes in this phenomenon.

In these investigations, both the cat auricle and perfused ears of rabbits were used. In the cats, the postganglionic sympathetic nerves to the hearts were sectioned and allowed to degenerate: in the rabbit, section with subsequent degeneration of both the postganglionic sympathetic nerves and of the sensory nerves to the ear, was carried out. Such postganglionic sympathetic degeneration and sensory degeneration did not abolish the adrenaline-like effect of nicotine on the heart or perfused rabbits' ears. After such nerve degeneration nicotine still accelerated the heart and still constricted the vessels of the ears. Both effects were blocked by hexamethonium. The authors thus concluded that these anomalous effects of nicotine and acetylcholine were not dependant on the presence of sympathetic or sensory nerve fibres. They postulated that nicotine and acetylcholine were stimulating either peripheral ganglia of adrenergic neurones or chromaffin tissue. They expressed the opinion that more histological evidence was needed before a choice between the two could be made.

A year later in 1954 Hilton reported the

effects of nicotine on the blood vessels of skeletal muscles in the cat. Intra-arterial injection of nicotine could cause either a vasoconstrictor or a vasodilator response. The vasoconstrictor response was unaffected by acute section of either the sciatic nerve or of the lumbar sympathetic chain. However, section plus degeneration of either nerve did abolish the constrictor response. Thus Hilton concluded that the vasoconstrictor response was due to an axon reflex. The response was also abolished by an adrenaline blocking drug, phentolamine (Regitine:Ciba) suggesting that the axon reflex was in the extrinsic adrenergic nerves. The vasodilator response, on the other hand, was only partly abolished by hexamethonium and by botulinum toxin. In the author's view, this incomplete abolition was partly due to an axon reflex in cholinergic neurones and partly to a direct action on the smooth muscle of the blood vessels.

Burn continued his extensive investigations on the effects of nicotine using a new tool, reserpine, which had been recently shown to deplete peripheral stores of noradrenaline (Bertler, Carlsson & Rosengren, 1956; Muscholl & Vogt, 1958). In a first article

with Rand (Burn & Rand, 1958 b), he showed that the stimulant action of nicotine on the isolated heart could no longer be elicited after reserpine treatment. In subsequent work, the isolated perfused rabbit's ear, the perfused hind leg of the dog and aortic strips were used (Burn & Rand, 1958 a). After treatment with reserpine, nicotine no longer caused vasoconstriction of the blood vessels in the isolated perfused ear of the rabbit and in the perfused hind leg of the dog: there was no longer contraction of aortic strips from the rabbit. The authors measured the noradrenaline content of vessel walls and confirmed that it is reduced by reserpine. They therefore attributed the actions of nicotine, which were abolished by reserpine, to the release of noradrenaline by nicotine. Because of the report of Ginzel & Kottegoda (1953) and of Haimovici & Pick (1946) that sympathectomy did not abolish the adrenaline-like effects of nicotine, Burn & Rand looked further than the nerve endings for the stores of adrenaline. With the help of Mr. Leach of the Department of Physiology of the University of Oxford, they stained the skin of rabbits' ears,

using the modified Giemsa method of Sevki described by Adams-Ray & Nordenstam (1956), and described cells which they referred to as chromaffin cells. Few or no cells showed the specific staining reaction after treatment with reserpine. As a consequence, Burn & Rand believe that the stores of noradrenaline in artery walls, described by Schmiterlow (1948), although derived from extrinsic sympathetic nerves, are finally held in some structure, possibly in these chromaffin cells which they describe in the neighbourhood of the blood vessels of the skin.

Burn & Rand (1958 c) showed that some sympathomimetic amines, similar in nature to tyramine, owed their action to their ability to release a noradrenaline-like substance from stores in the tissues. That these stores might also be identical with those from which nicotine and acetylcholine could release adrenaline, was supported by the observation that reserpine abolished the pressor action of tyramine as well as the adrenaline-like effects of nicotine and acetylcholine.

While many of these adrenaline-like effects of nicotine have naturally been demonstrated and investigated in animal experiments, there is evidence that a

similar phenomenon can be demonstrated in man. For example, Strömblad (1959) showed that nicotine, injected into the brachial artery, caused vasoconstriction in the hand. This vasoconstriction was blocked by sympatholytics and ganglion blocking agents, a finding consistent with the hypothesis that nicotine causes the release of an adrenaline-like substance in the human skin. The author, while inclined to regard chromaffin cells as the source of this substance, considered that the substance might be set free by stimulation of adrenergic neurones.

While most investigations of these adrenaline-like actions of nicotine and acetylcholine have referred to the cardiovascular system, there are, in addition, several reports of this phenomenon in other tissues and organs innervated by the autonomic nervous system. For example, the pilomotor muscles normally contract on sympathetic stimulation or on intravenous injection of adrenaline or noradrenaline. However, in 1935 Brücke reported that subcutaneous injection of acetylcholine sometimes caused contraction of the pilomotor muscles in the skin of the cat's tail. He pointed out the similarity between the action of acetylcholine

at this site and its action at the superior cervical ganglia and suggested that its action in the skin was similar to its action in releasing 'sympathin' from the suprarenals. In 1940, Coon & Rothman reinvestigated the effect of nicotine on the skin and confirmed that nicotine caused contraction of the arrectores pilorum muscles. In addition, these authors reported that, after section and degeneration of the sympathetic nerves to the skin, nicotine no longer caused pilo-erection while local nerve block or acute section of the nerve to the skin did not affect the response to nicotine. As a consequence Coon & Rothman concluded that the response was due to an axon reflex involving the terminal ramification of the postganglionic sympathetic fibres supplying the pilomotor muscles. If this is true, then it should be possible to record action potentials in these nerves. Brown & Gray (1948) injected nicotine and acetylcholine intra-arterially into the skin and mesentery of cats and dogs. They showed that these drugs did in fact stimulate sensory nerve endings by recording a centripetal discharge of impulses in the nerves supplying the injected area. The effect was abolished by previous exposure of the

tissue to larger doses of nicotine and acetylcholine. The authors suggested that the impulses probably arose through the direct chemical stimulation of some part of the terminations of the sensory nerves. Later, in 1952, Douglas showed that acetylcholine and nicotine stimulated the receptors in the carotid body: this action of acetylcholine and nicotine was blocked by hexamethonium. In 1953, Douglas & Gray showed that in the skin and mesentery also stimulation of the sensory endings by nicotine and acetylcholine was blocked by hexamethonium.

The more relevant reports of the adrenaline-like effects of nicotine, for the present work, are probably those of Ambache. In 1951 he reported that, after treating the small intestine of mice and rabbits with botulinum toxin to block the cholinergic mechanism, nicotine caused inhibition of the smooth muscle. The response of the smooth muscle to acetylcholine and eserine remained unaltered. The inhibitory process was abolished by large paralysing doses of nicotine and hexamethonium and, like the inhibitor effect of adrenaline, was abolished by large doses of ephedrine. Ambache concluded that the inhibitory response to small

doses of nicotine was due to the stimulation, in the wall of the gut, of ganglion cells which are the origin of short adrenergic fibres. He suggested that there are two functionally distinct ganglion cells in the myenteric plexus. Stimulation of the type which gives rise to cholinergic fibres causes contraction: stimulation of the other type which gives rise to adrenergic fibres, causes inhibition of the intestine. This suggestion was similar to an earlier one of Langley (1922) who believed that there was not only motor, but also inhibitor fibres originating in Auerbach's plexus.

In a joint paper with Edwards, also in 1951, Ambache investigated the action of nicotine after atropine, another cholinergic blocking agent. Prior treatment of the gut with atropine did not reveal an inhibitory response to nicotine in preparations of rabbit ileum. On the other hand, in intestinal preparations from young kittens and in strips from their stomachs, pre-treatment with atropine successfully abolished nicotine contractions and revealed an inhibitory response. Ambache & Edwards repeated the suggestion that the phenomenon was due to the presence

of inhibitory postganglionic neurones in the wall of the gut.

Another tissue where the adrenaline-like effects of nicotine and acetylcholine have been reported is the bronchial smooth muscle. Hawkins & Paton (1958) reported that nicotine produced relaxation of the bronchial smooth muscle in both guinea-pig and cat: this relaxation was abolished by previous treatment with nicotine and hexamethonium. This relaxant action could also be antagonised by ergotoxine, by ergotamine, or by dihydroergotamine, and the antagonism of these alkaloids towards nicotine was comparable to that against adrenaline or isoprenaline. Much higher doses of these alkaloids were needed to block the relaxant action of noradrenaline. The authors suggested that nicotine excites adrenergic ganglion cells in the tracheal wall and that these release a 'sympathin' resembling adrenaline or isoprenaline. They also stated that the possibility existed that nicotine produced its actions by eliciting a local axon reflex in adrenergic nerves, as well as, or in place of, causing ganglionic excitation. They thought, however, that the reaction with

hexamethonium was in favour of an action by nicotine on ganglion cells.

In 1958, Thompson described the effects of nicotine on an isolated *in vitro* preparation of the nictitating membrane of the cat. Here again, nicotine produced effects similar to those produced by adrenaline, namely, contraction of this preparation. As in so many other sites, this response was blocked by higher concentrations of nicotine and also by hexamethonium. In addition, the contractions elicited by nicotine behaved to cocaine and to adrenolytic substances in the same way as contractions evoked by adrenaline and noradrenaline. Thompson concluded that nicotine was acting by releasing adrenaline and/or noradrenaline. Histological investigation of his preparation failed to reveal ganglion cells within the muscle: no report was made in the article of the presence of chromaffin cells. However, he was impressed by the presence of large numbers of small nerve fibres which he assumed to be the terminations of the postganglionic sympathetic supply. Thompson suggested two interpretations of his results.

1) Nicotine might be producing a local axon reflex

in these nerve fibres. 2) Thompson introduced a new possible explanation of these phenomena. If those cells, first described by Cajal (1909) as 'neurones sympathetique interstitiells' and more commonly referred to today as interstitial cells, are in fact modified ganglion cells, then they would constitute a possible site of action of nicotine. Indeed, it has been suggested by Leeuwe (1937) that these cells contain adrenaline.

In this context it is interesting to recall Coon & Rothman's original observation that the site of action of nicotine '.....behaves like an autonomic ganglion towards nicotine.....'

It is clear from the above reports that there are numerous sites in the body at which nicotine produces adrenaline-like effects when cholinergic effects might have been expected. All the reports, except one, are agreed that, where investigated, this adrenaline-like action of nicotine is truly 'nicotinic' in nature, since the action is abolished by hexamethonium, by curare and by paralysing doses of nicotine. The one exception is the report of McDowall (1946) who investigated the action of acetylcholine

on the hearts of cats, of rabbits and of rats and was unable to abolish the stimulatory effect of acetylcholine with large doses of nicotine.

That the adrenaline-like effects of nicotine are, in fact, mediated by an adrenaline-like substance is agreed by all authors who investigated this point. In all instances, where tested, adrenaline blocking agents abolished the response to nicotine and, when the stores of catechol amines were dispersed by reserpine, this response was reduced or abolished. In one instance an adrenaline-like substance was detected in a perfusate (Hoffman, Hoffman, Middleton & Talesnik, 1945).

The actual identity of the transmitter, whether it be adrenaline or some other catechol amine, is obscure. If it is derived from stores in the tissue which are related to the actual nervous elements, then one would expect it to be noradrenaline. But such evidence as exists in fact favours adrenaline. A substance in the lung, which could be more closely identified with adrenaline than with noradrenaline, was detected by parallel assay by Hawkins & Paton (1958). In addition, Burn & Dutta (1948) showed that the

substance released in blood vessels by acetylcholine and causing vasoconstriction could be made to cause vasodilatation by treatment with 'Priscol' - a response to be expected if adrenaline is released but hardly to be expected if noradrenaline is released. If, in fact, the substance released should prove to be adrenaline, then chromaffin cells rather than neurones are likely to be the source of the transmitter.

The possibility that the catechol amine is liberated from extrinsic sympathetic nerve endings by activation of an axon reflex has been investigated by a few workers. Probably the best approach to this problem is to find whether or not section and degeneration of the sympathetic nerves has any effect on the adrenaline-like effects of nicotine. The reports of such experiments are contradictory. Coon & Rothman (1940) and Hilton (1954) agree that the response is abolished by nerve section and degeneration but Ginzel & Kottegoda (1953) take the opposite view, that the adrenaline-like effects of nicotine are not abolished by this procedure. Each of these reports is open to criticism, since in no

case was there an attempt to verify that the sympathectomy was complete. This is especially important in experiments such as Ginzel & Kottegoda's on perfused rabbits' ears. The ear is notoriously difficult to denervate, since nerves reach the ears along anastomotic blood vessels from the vertebral artery. In addition, recent workers have drawn attention to the very rapid and extensive sprouting of sound autonomic nerves in the presence of degenerating nerve. The possibility of reinnervation in the more prolonged degeneration experiments was not considered by any of the above workers.

The suggestion that chromaffin cells, lying within the tissues, act as stores of adrenaline and are responsible for the adrenaline-like effects of nicotine, has been suggested in particular by Burn and his colleagues. This work stemmed from the report of Adams-Ray & Nordenstam (1956) and was confirmed by Burn & Rand (1958 a), that there are in the skin cells which Burn & Rand call chromaffin cells. These cells can be stained by Sevki's method - a modified Giemsa stain. The method of staining is open to objection since the Giemsa stain is not

selective for catechol amines. No other authors have detected these chromaffin cells, while those who support the view that chromaffin cells are responsible for the anomalous effects of nicotine, have not demonstrated these cells by any more specific staining reaction.

It would seem, therefore, that there is no agreed source for the adrenaline which almost certainly is responsible for these reactions. The possibility, of course, exists that the source differs in different tissues: if this is so, it might reconcile some of the observations. It is clear that extrinsic nerve section, with suitable control of the efficacy of the operation, is required to confirm or disprove the participation of an axon reflex in the phenomenon. It is also clear that further histological work is required to discover whether or not chromaffin cells do occur in the periphery, and this work requires to be done by a more specific method than has so far been used. Indeed, in a very recent article, Coupland & Heath (1961) have examined human skin obtained from a variety of sites. Mast cells, melanocytes and

melanophores were identified by the use of various staining procedures, but the 'chromaffin cells' reported by Adams-Ray & Nordenstam (1956) were not observed.

Materials and Methods

For the experiments reported in this thesis two types of isolated *in vitro* preparation of rabbit intestine were used. See Fig. 1.

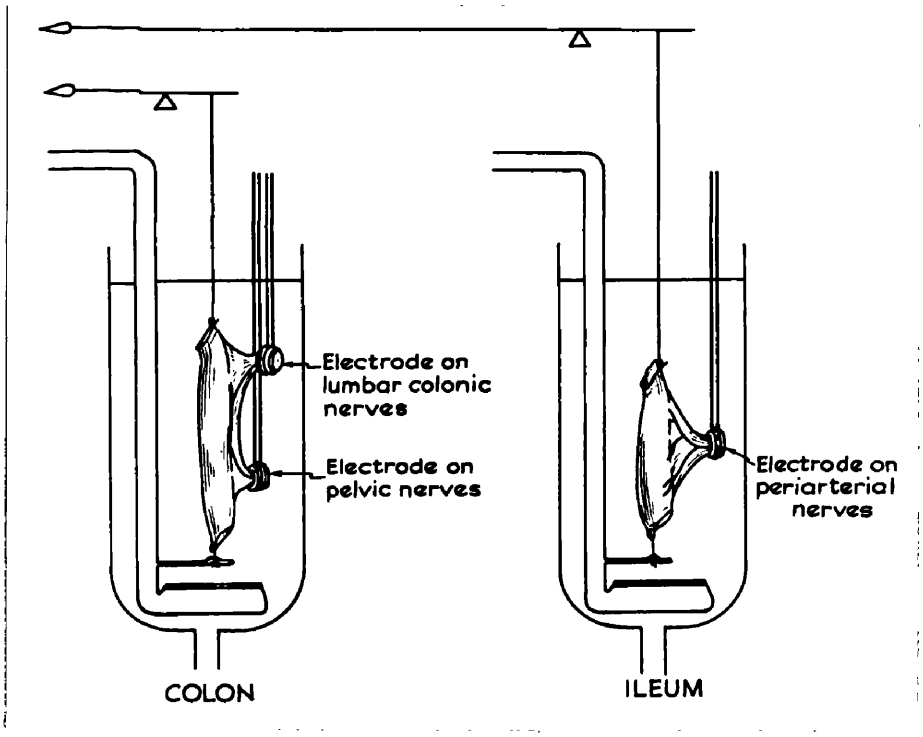


Fig. 1. The two isolated *in vitro* preparations of rabbit intestine used in the present work. O_2 + CO_2 supplied by sintered-glass diffusing cannulae. Outer water bath not shown.

The first was a preparation of rabbit colon, described by Garry & Gillespie (1954, 1955). It consists of about 4 cm of colon with its extrinsic autonomic nerves retained so that they can be stimulated separately.

To set up this preparation a rabbit was killed by a blow on the neck and bled. The abdomen was opened by a midline incision, and the symphysis pubis split and the pelvic bones forcibly parted. The two pelvic (parasympathetic) nerves were dissected out and fine ligatures attached to their ends so that they could be pulled together into one fluid electrode. The lumbar colonic (sympathetic) nerves were similarly dissected out, and ligatured. The portion of colon was then removed from the animal and suspended in Krebs' saline in an isolated organ bath.

In the present investigations the two following minor modifications were made to the technique described by Garry & Gillespie.

First, while the dissection was being carried out, the tissues were kept moist with Krebs' saline, not Ringer's solution. Secondly, no attempt was made to remove the faecal pellets until the dissection was

completed and the preparation was suspended in the organ bath at 37°C. This delay in removal of the pellets avoided trauma to the tissues. In the warm Krebs' solution, pendulum movements and peristalsis soon commenced, and the faecal pellets were quickly extruded into the inner vessel from where they were quickly removed.

The second type of preparation was similar to the preparation described by Finkleman (1930). It consisted of about 4 cm of mid-ileum, together with the attached mesentery, mesenteric nerves and vessels, the whole suspended in Krebs' saline in an isolated organ bath. A fine ligature was tied round the nerves and vessels to pull them into a fluid electrode.

Rabbits of either sex and various species were used. The best weight of animal was about 1.75 kg, because these had little fat. The dissection in male rabbits is a little easier than in female rabbits, since in the latter there is an extensive venous plexus surrounding the vagina and infundibulum, and the inferior haemorrhoidal veins are large. These factors increase the risk of blood escaping and obscuring the dissection of the pelvic nerves.

The preparations were suspended in a 200 ml isolated organ bath in Krebs' saline at 37°C. The composition of the fluid was as follows:- (g/l) NaCl, 6.92; KCl, 0.35; $CaCl_2$, 0.28; KH_2PO_4, 0.16; $MgSO_4 7H_2O$, 0.29; $NaHCO_3$, 2.1; dextrose 2. The method of preparation was that described by Krebs & Henseleit (1932).

The inner vessel was filled from below by gravity, the Krebs' saline being passed through a glass warming coil before it entered the inner vessel. The lower end of the preparation was attached to a small glass hook, just above the sintered-glass disk of a gas distributor which had a pore size of 40-90 μ. The Krebs' saline was gassed with 95% O_2 and 5% CO_2.

The movements of the longitudinal muscle coat of the intestine were recorded by a light isotonic gimbal lever writing sideways on a smoked drum. The lever exerted a tension of approximately 0.5 g and gave a three time magnification.

The fluid electrodes used in these experiments were described by Garry & Wishart (1951) and modified by Garry & Gillespie (1955). See Fig. 2.

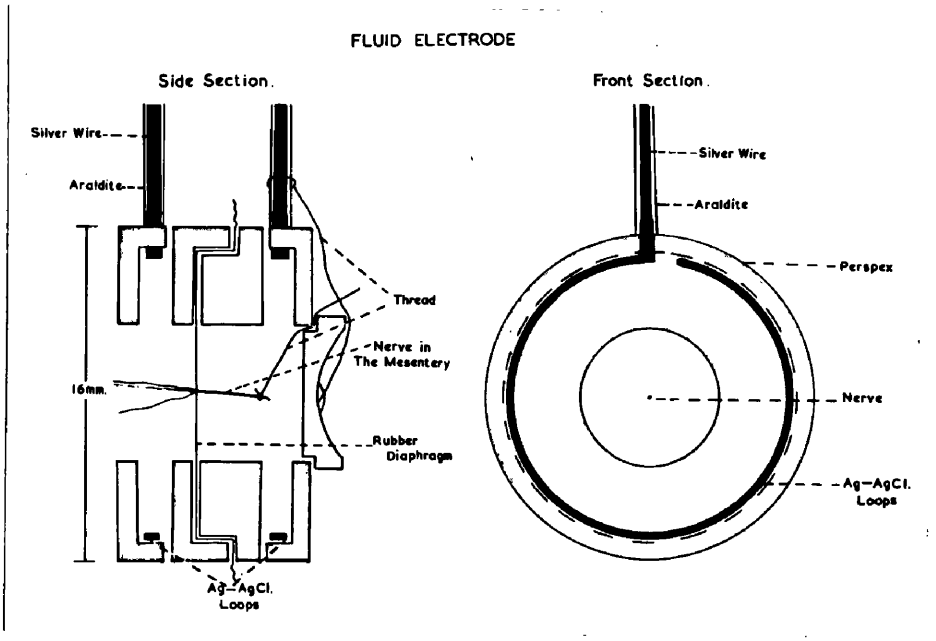


Fig. 2. The fluid electrode used in the present investigations.

One further modification to the electrode was made in the present experiments. The leads to the silver wires of the electrodes, instead of being covered with narrow polyvinyl chloride (P.V.C.) tubing and sealed with perspex cement, were coated with 'Araldite', manufactured by Ciba (A.R.L.) Ltd., which was left overnight to harden. This was a more robust type of seal.

The silver loops in each electrode were coated with silver chloride by immersing them in 0.9% NaCl solution and passing current from a 4 volt battery

through the solution, using each loop in turn as the anode and a large silver plate as the cathode. The electrodes when chlorided were tested for polarisation by measuring their resistance on an Avo 8 testmeter. After chloriding the electrodes, the resistance was between 150Ω and 200Ω, and remained constant.

The electrodes were suspended by clamps from a horizontal bar well above the inner vessel of the bath. These clamps could be swivelled about the bar, moved along the bar, and raised or lowered. Thus each electrode could be easily adjusted to a position which prevented kinking of its nerve.

The gas distributor, gimbal lever and the clamps for the electrodes were all mounted on a common arm which could be raised or lowered by rack and pinion. The nerves were threaded into the electrodes while the preparation was still suspended above the level of the fluid in the inner vessel of the organ bath.

When the electrodes were in position on the nerves, the whole assembly was racked down into the bath. Final adjustments to the position of the electrodes were made when the preparation had been lowered into the inner vessel.

The leads of each electrode were so arranged that the cathode was nearest the preparation. In the colon preparation, usually only one nerve was stimulated at a time. The electrode not in use was short circuited and left 'floating', i.e. without earth connections. In this way the chance of current escape was decreased. As a further safeguard against current escape, the stimulus pulse was isolated by a Muirhead Transformer.

A 100Ω resistance was placed in series with the transformer. Voltage drop across this resistance was recorded on an oscilloscope. Any increase in resistance in the secondary circuit as, for example, polarisation of the stimulating electrodes, would cause an alteration in this voltage.

The nerves were stimulated electrically using square waves whose pulse length, frequency and voltage, could each be varied independently. Pulses of 1 msec were standard in all these experiments. Frequencies of 50 P/sec for the sympathetic nerves and 10 P/sec for the parasympathetic nerves were usually used. These frequencies have been shown to give maximum responses from the nerves (Garry &

Gillespie, 1955). The pulse voltage which was used was twice the threshold voltage. Garry & Gillespie (1955) have shown that this gives supramaximal stimulation. The interval between periods of stimulation was three minutes or longer. This allowed complete recovery from the previous stimulus and avoided fatigue.

In one group of rabbits the inferior mesenteric ganglia were removed at operation and fourteen days allowed for degeneration of the sympathetic nerves. The response of the colon *in vitro* to nicotine was then studied. In another group of rabbits the pelvic nerves were cut at operation and time allowed for their degeneration. The response of the sympathetic nerves after treatment of the rabbits with reserpine was then studied.

The operations were performed under strict aseptic conditions. The rabbits were anaesthetised with pentobarbitone sodium B.P. ('Nembutal'; Veterinary). An average dose of 50 mg/kg was given by injection into an ear vein.

To remove the sympathetic ganglia, a midline abdominal incision was made. The inferior mesenteric

ganglia were located and removed. The nerves were also removed for about 15 mm along the inferior mesenteric artery. The dissection was facilitated by using a Zeiss binocular dissecting microscope providing illumination along the optical axis.

An antibiotic spray, 'Polybactrin', was used as the wound was closed. In the early operations the abdominal wall was closed with interrupted cotton sutures. However, the rabbit usually removed these and, in later operations, the wound was closed with a continuous cotton suture inserted subcutaneously.

All the rabbits survived this operation and were in good condition when the *in vitro* experiment was carried out.

In the operated rabbits in which the pelvic nerves were cut, access was best gained to the nerves by making a midline incision over the sacrum and coccyx and dissecting through the sciatic notches. The nerves were ligatured before cutting so that, at the time of the *in vitro* experiment, the cut ends of the nerves could be identified and pulled into a fluid electrode. In earlier operations, the pelvic nerves were approached through an abdominal incision.

However, with this technique there was much fibrosis round the cut nerves, and damage to the cystic blood vessels caused bladder necrosis in one animal. Hence the reason for approaching the nerves through the sciatic notches. A Zeiss binocular dissecting microscope as used previously was essential for this operation.

Care had to be taken to avoid damage to the sciatic nerve which comes through the sciatic notch. If a little piece of cotton wool was soaked in the local anaesthetic 'Xylocaine' and placed on the sciatic nerve during the operation, it prevented the animal jumping when this nerve was accidentally touched.

The pelvic nerves were allowed to degenerate for fourteen days before the *in vitro* experiment. During the last five days of these fourteen days, reserpine was administered by intravenous injection. If the rabbit seemed unlikely to survive the full course of reserpine, then two injections of 1 mg/kg were given on successive days. This was always sufficient to cause reversal of the sympathetic response in rabbits which had not been operated on.

Rabbits whose pelvic nerves were cut, naturally had difficulty in urinating. We originally tried to overcome this difficulty by inserting self-retaining Foley catheters. This was unsuccessful. Even although the catheter was sewn in, the rabbit managed to pull it out. Emptying the bladder daily by manual abdominal pressure proved more successful.

Mock operations, identical in all respects, were carried out on a series of rabbits with the single omission that the pelvic nerves, after exposure, were neither tied nor cut.

Reserpine ('Serpasil':Ciba) was injected daily into the marginal ear vein for from one to ten days. The technique in most experiments was to give five injections; 0.2 mg/kg for three days, then 1 mg/kg for two days.

Rabbits treated with reserpine were kept in a warm room at 29°C. They were weighed daily, and their food consumption was measured daily. Their weight fell progressively, 50-150 g per day, throughout the course of the injections, probably because food consumption drops to almost nil after one or two injections of the drug. Ciba Ltd. kindly supplied

us with the vehicle in which the reserpine was dissolved. This fluid was administered to a group of rabbits which served as controls.

Other drugs used in these experiments were: acetylcholine chloride, atropine sulphate, hexamethonium bromide, 2:6 xylyl choline ether bromide (TM 10), ergotamine tartrate, dimethylphenylpiperazinium iodide (DMPP), nicotine hydrogen tartrate. Concentrations of these drugs refer to the salt, while concentrations of the following refer to the base: adrenaline hydrochloride, noradrenaline hydrochloride, dopamine, dopa, l-tyrosine and tolazoline ('Priscol'). Drugs were made up in Krebs' saline in such a concentration that it was necessary to add only 0.5 ml. of the solution to the organ bath.

Two methods were used for staining the chromaffin cells in the gut. 1) The unmodified chromaffin reaction was used. The piece of intestine was opened out and pinned with hedgehog quills flat onto a cork board. Cork and tissue were then fixed for 24 hours in Müller's Fluid containing 10% formalin. The material was then 'post-chromed' for three days in a 2.5% aqueous solution of potassium bichromate,

after which it was washed, dehydrated, embedded in paraffin and sectioned at 7 μ. The sections were subsequently lightly counter-stained with haemalum.

2) The second method was that of Sevki, as described by Adams-Ray & Nordenstam (1956). The preliminary treatment and fixation was the same as above: there was no treatment with bichromate. After embedding in paraffin, the tissue was sectioned and the sections stained by a modified Giemsa method.

Results

Effect of nicotine. The response of the smooth muscle of the rabbit intestine to nicotine is generally accepted as being contraction due to the stimulation of cholinergic neurones in the gut wall. However, the response of the rabbit colon to nicotine depends on the concentration used. This is illustrated in Fig. 3.

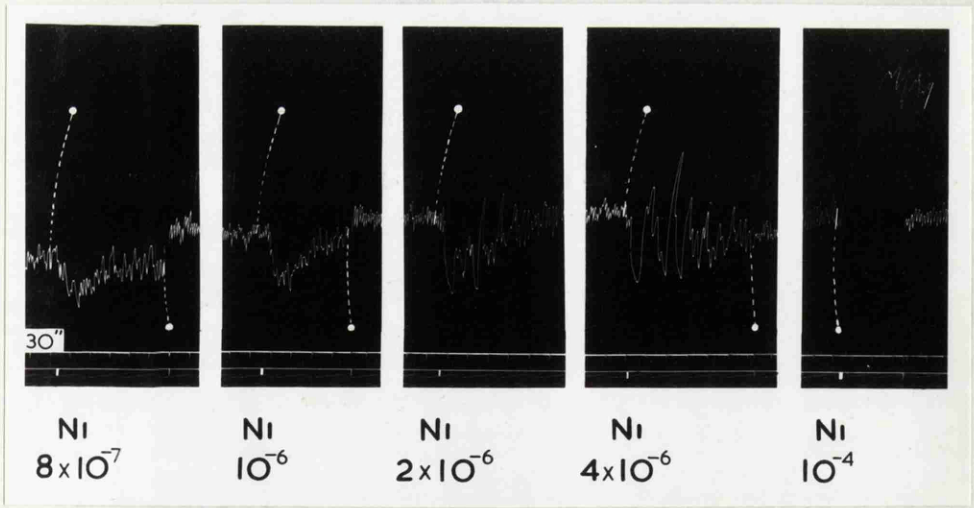


Fig. 3. Rabbit colon preparation. Low concentrations of nicotine elicit an inhibitory and high concentrations a motor response. Nicotine (Ni) added at the first mark, washed out at the second. Time = 30 sec.

Low concentrations of nicotine, usually 10^{-6} to 10^{-5} cause relaxation. Increasing the concentration produces a biphasic response, relaxation followed by contraction. Larger concentrations, usually above 10^{-5}, produce pure contraction. If the concentration of nicotine is increased still further, the paralysing action of nicotine is seen - the initial contraction is followed immediately by relaxation.

Effect of dimethylphenylpiperazinium (DMPP).

Ambache & Lessin (1955) reported that in the botulinum poisoned rabbit ileum the ganglion cell stimulating drug DMPP is as effective as nicotine in causing inhibition. In the rabbit colon DMPP, in low concentrations, can also cause inhibition as is seen in Fig. 4.

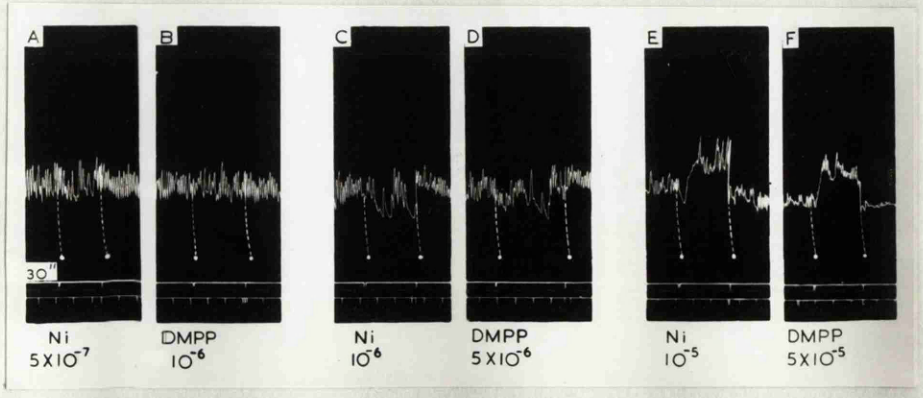


Fig. 4. A comparison of the effects of nicotine (Ni) and DMPP on the isolated rabbit colon preparation. The responses to these two drugs are similar.

In the rabbit colon the inhibitory response to DMPP is neither greater nor more readily elicited than with nicotine. Since DMPP offered no obvious advantage over nicotine, it was not used extensively in these experiments.

Effect of atropine. While low concentrations of nicotine in general produce pure inhibition, the exact concentration needed to give an effective inhibition varies somewhat from one preparation to another. Furthermore, the safety margin between a concentration giving pure inhibition and one giving either a biphasic or pure motor response, is not large. The use of those concentrations producing pure inhibition therefore is not convenient. It seemed possible that concentrations of nicotine, producing a pure motor response by stimulating cholinergic neurones in the wall of the gut, might at the same time be stimulating the inhibitory mechanism, the inhibitory effect being obscured by the dominant motor effect. It should be possible to block the motor response to nicotine by use of atropine with its antimuscarinic action. The true nicotinic effects and the entire

inhibitor mechanism, if it be adrenergic in nature, ought to be unaffected. The effect of nicotine on the colon in the presence of atropine was therefore studied. These experiments confirmed the theoretical expectations. After atropine, in concentrations which could be demonstrated to block cholinergic nerves (the pelvic nerves), concentrations of nicotine which previously produced contraction now produced inhibition. See Fig. 5.

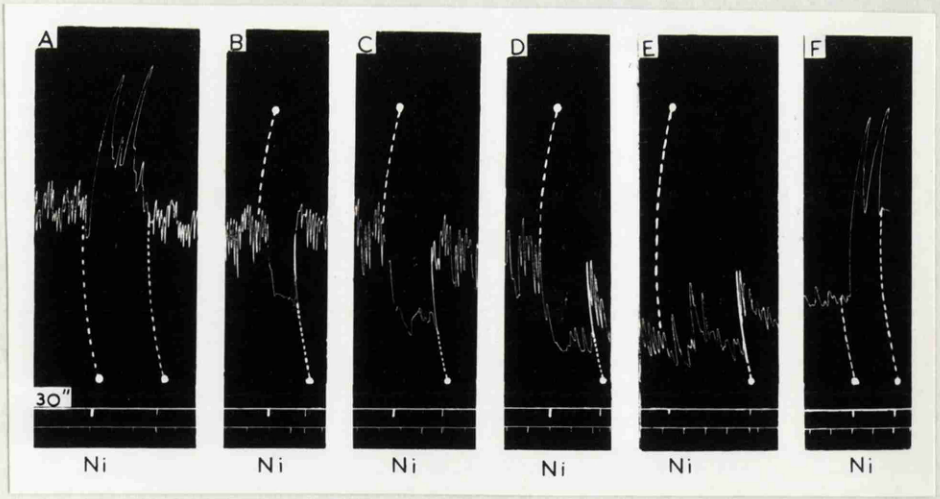


Fig. 5. Reversal by atropine of the nicotine response in the rabbit colon. At Ni, nicotine 10^{-5} was added. A, before atropine; B, in the presence of atropine 10^{-4}, C, D, E, & F, 20 min, 40 min, 2 hours, and 7 hours, respectively, after washing out the atropine.

Nicotine in a concentration of 10^{-5} (salt) before atropine produced a contraction of the rabbit colon. In the presence of atropine, this same concentration of nicotine caused relaxation (5B). Twenty minutes after removing both atropine and nicotine from the bath, the response of the colon to a further similar dose of nicotine was still relaxation, and indeed the relaxation was enhanced (5C). Forty minutes later, this potentiation was still greater (5D). This anti-muscarinic blocking action of atropine was reversible but only with difficulty. This is seen in (5E), when two hours after washing out the atropine the first signs of a motor response to nicotine reappeared and, after seven hours, a pure motor response was once again obtained (5F).

The concentration of atropine used in these experiments (10^{-4}) is high. However, such a concentration is necessary to abolish completely the response to stimulation of the parasympathetic (pelvic) nerves to the colon (Garry & Gillespie, 1955).

Atropine in high concentrations loses its specificity and may reduce both nicotinic and muscarinic effects (Marrazzi, 1939; Konzett, 1949). Evidence

for an anti-nicotinic effect of atropine on the colon was found in the present experiments and is shown in Fig. 6.

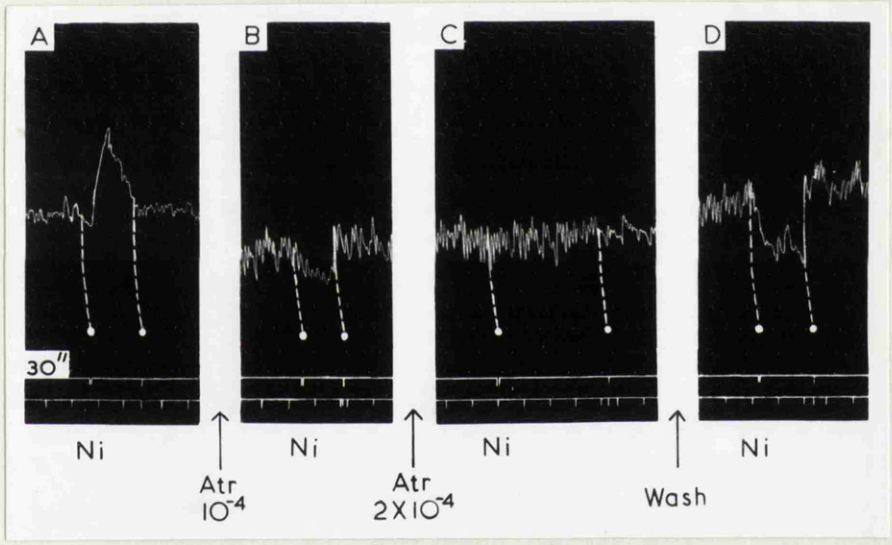


Fig. 6. The anti-nicotinic effect of high concentrations of atropine on the isolated rabbit colon. In all cases, nicotine (Ni) was present between marks in a concentration of 10^{-5}. A, before atropine; B, in the presence of atropine 10^{-4}; C, in the presence of atropine 2×10^{-4}; D, after washing the preparation with consequent reduction in the concentration of atropine.

The contraction produced by nicotine is converted by atropine 10^{-4} to relaxation (6A and 6B). Doubling this concentration of atropine, however, abolishes the inhibitory effect of nicotine (6C). The inhibitory response could still be obtained after reduction in the concentration of atropine by washing (6D). Fortunately, the anti-muscarinic action of atropine persists for some hours, even though the atropine is removed from the bath, whereas the nicotinic blocking action disappears fairly quickly. This difference in the duration of the anti-nicotinic and anti-muscarinic actions of atropine is probably responsible for the deepening of inhibition in Fig. 5.

In most experiments, therefore, atropine sulphate 10^{-4} or 2×10^{-4} was added to the bath fluid and left in contact with the preparation until the response to stimulation of the pelvic nerves was abolished. This was taken to indicate that the postganglionic neurones of Auerbach's plexus were blocked. The atropine was then removed and nicotine in a concentration of 10^{-5} added to produce inhibition. This method reliably demonstrated inhibition and avoided the necessity of determining in each preparation

the exact dose of nicotine which would, acting alone, produce inhibition.

Effect of hexamethonium. The effect of hexamethonium on the response of the rabbit colon to nicotine in the presence of atropine is shown in Fig. 7.

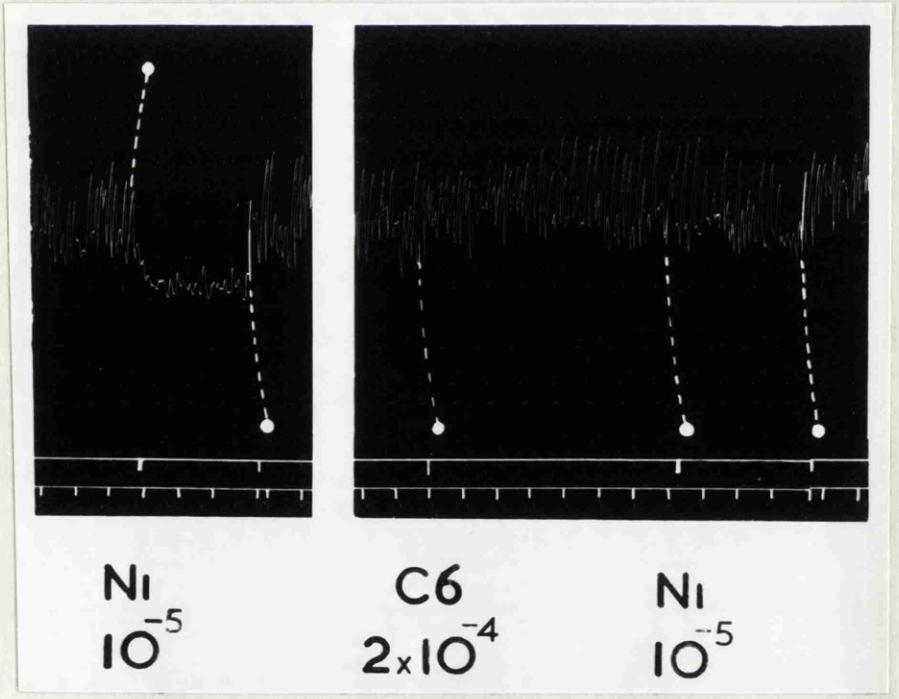


Fig. 7. The action of hexamethonium on the relaxation produced by nicotine on the isolated rabbit colon. Atropine had been added previously to the bath and then washed out (See text). Time = 30 sec.

The inhibitory response to nicotine was abolished by hexamethonium bromide, confirming that the inhibitory response is a true 'nicotinic' effect. This is in accordance with the findings of Ambache (1951) and Ambache & Edwards (1951), who found that hexamethonium blocked the inhibitory action of nicotine on the ileum.

As with atropine, the concentration of hexamethonium required to abolish the response to nicotine is high, but it is the concentration of hexamethonium required to block the response to stimulation of the pelvic nerves, a response involving transmission at a known synapse.

That the action of hexamethonium is specific is shown by the undiminished response to stimulation of the lumbar (sympathetic) nerves (Fig. 12) and to added acetylcholine and noradrenaline.

Effect of large paralysing doses of nicotine. Concentrations of nicotine greatly in excess of those required to give an inhibitory effect, block the effect of a subsequent inhibitory concentration of nicotine. This is shown in Fig. 8.

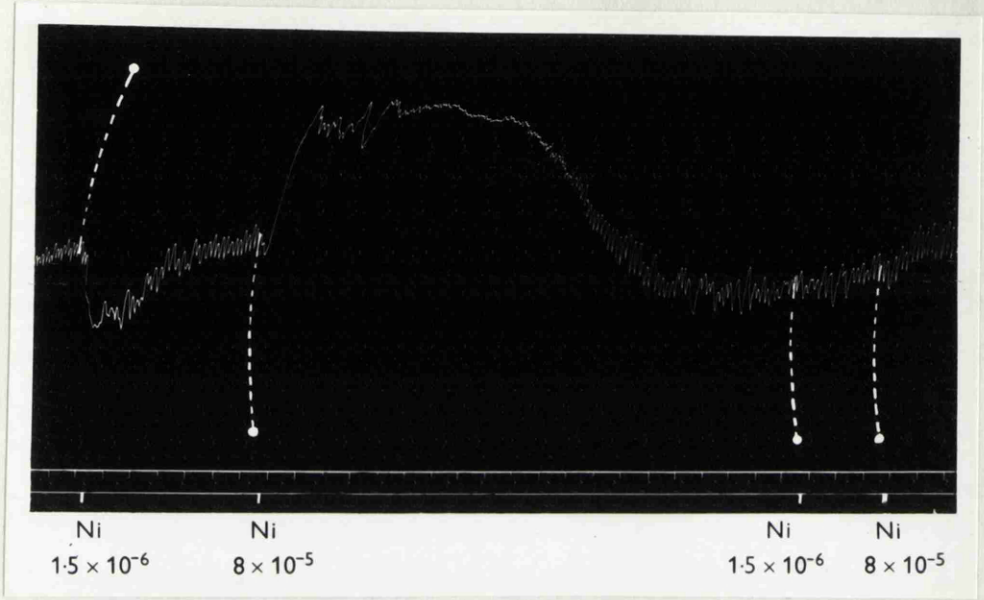


Fig. 8. The effect of large paralysing concentrations of nicotine (Ni) on the inhibitory effect of this drug on the isolated rabbit colon. Nicotine 8 x 10^{-5} left in contact with the preparation abolishes the inhibitory response to a small concentration of nicotine and also the motor effect of a subsequent large dose of nicotine. Time = 30 sec.

Presumably the high concentration of nicotine puts out of action the elements on which an inhibitory concentration of nicotine usually acts, again confirming that the inhibitory action is a true 'nicotinic' effect.

Effect of reserpine. The results so far show that there is a mechanism in the rabbit colon which causes relaxation and inhibits movements. This mechanism is activated by nicotine and is a true 'nicotinic' action since it is blocked by hexamethonium and concentrations of nicotine known to paralyse ganglion cells. The character of the inhibitory response is very similar to the response to stimulation of the sympathetic nerves or to adrenaline. This immediately raises the possibility that this response is, in fact, due to the release of adrenaline or of some other catechol amine from some unknown site. If this is so, then the drug, reserpine, which discharges catechol amines from all sites, should abolish the inhibitory response. This colon preparation has a particular advantage for such an investigation. Stimulation of a recognised sympathetic nerve at once detects failure of reserpine to discharge catechol amines from their sites of storage.

Two groups of four rabbits were used for this investigation. One group was given 0.2 mg/kg of reserpine by single intravenous injections daily for the ten days prior to the in vitro experiment.

The other group, used as control, was given an equivalent quantity of the vehicle in which the reserpine is dissolved. Fig. 9 illustrates the results of these experiments.

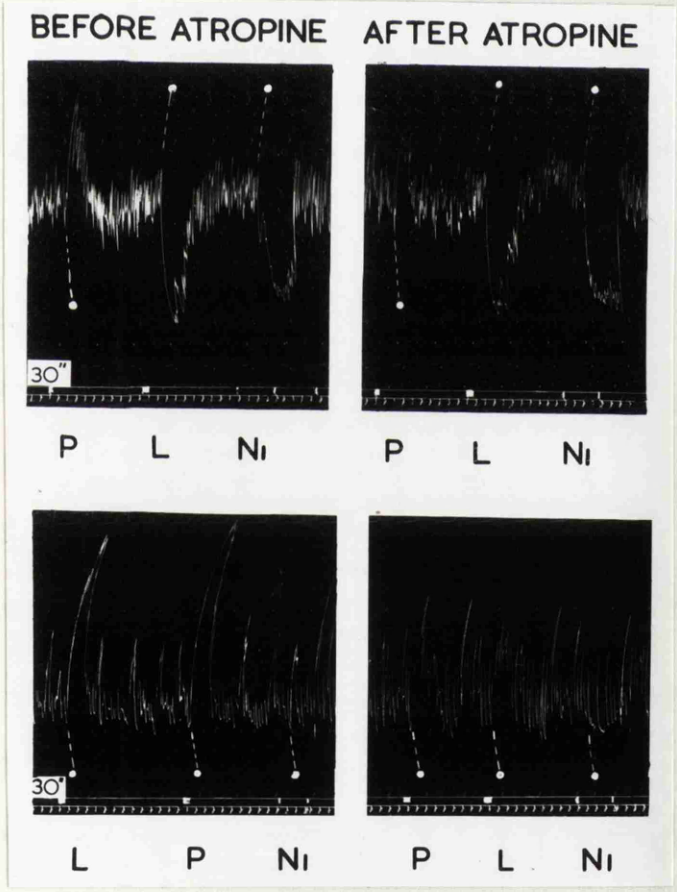


Fig. 9. Effect of reserpine on the response of the rabbit colon preparation to nicotine 10^{-5} (Ni) and to parasympathetic (P) and sympathetic (L) stimulation. Above - preparation from a control animal injected with the reserpine vehicle: below - from an animal given reserpine 0.2 mg/kg daily for ten days. In the control, nicotine causes inhibition and this is enhanced after atropine. In the preparation from the reserpine treated animal, nicotine has little or no inhibitory action either before or after atropine. The response to sympathetic nerve stimulation is reversed to motor and, like the response to stimulation of the pelvic nerve, suffers marked diminution in the presence of atropine.

The inhibitory response of the colon to nicotine is slight or absent both before and after atropine in the reserpine treated animals, whereas, in the control, the response is well marked before and enhanced after atropine.

An unexpected finding was that the response to lumbar nerve stimulation which, as previously mentioned, was expected to be abolished in the reserpine treated animals, was in fact consistently reversed to a motor response. The investigation of this finding makes up the second part of this thesis.

The effect of choline 2:6 xylyl ether bromide (TM 10). The response after reserpine confirms that nicotine, in producing inhibition, acts by releasing catechol amines. As the review of the literature has, I hope, made clear, there are at least three possible sites from which these amines may be released.
1) They may be derived from adrenergic nerve endings of the extrinsic sympathetic nerves. 2) They may be set free from adrenergic neurones whose cell bodies lie in Auerbach's plexus, at which site the ganglion cells can be directly stimulated. 3) The catechol amines may come from stores at some site other than nerve endings; for example, in chromaffin cells or in interstitial cells. Nicotine may be causing release of amines from these stores.

To try to separate these three possibilities the effect of the sympathetic blocking agent TM 10 on the nicotine induced inhibitory response was studied.

TM 10 was shown by Bain & Fielden (1956) to block the inhibitory response of the small intestine to stimulation of the sympathetic nerves. This drug, although possessing numerous other actions, blocks the effects of adrenergic nerve stimulation apparently by interference with the synthesis or release of the transmitter from the nerve endings (Exley, 1957).

If the inhibitory response to nicotine is due to the stimulation of either intrinsic or extrinsic adrenergic neurones, then this response should be blocked by TM 10. Exley (1957) has shown that TM 10 has no effect on the release of catechol amines from the medullary cells of the adrenal gland. If then, the inhibitory response of the gut to nicotine is due to the release of transmitter from chromaffin cells comparable to the cells of the adrenal medulla, then the response should be unaffected by TM 10.

Fig. 10 shows the effect of TM 10 on the inhibitory response to nicotine and on the response

to sympathetic (lumbar colonic) nerve stimulation.

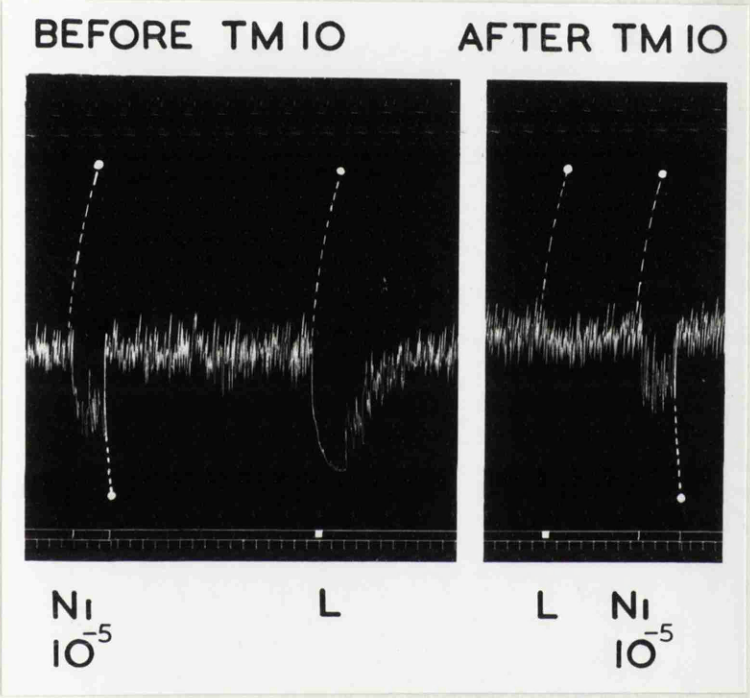


Fig. 10. The isolated rabbit colon preparation showing the action of choline 2:6 xylyl ether bromide (TM 10) on the response to stimulation of the lumbar colonic nerves (L) and to nicotine (Ni). The response to nerve stimulation is blocked but that to nicotine is little affected. Time = 30 sec.

The response to stimulation of the extrinsic adrenergic nerves is completely abolished after 70 minutes exposure of the gut to TM 10, whereas the inhibitory response to nicotine is only slightly reduced.

Histological findings. The results obtained with TM 10 appeared to indicate that the catechol amines were not being liberated from adrenergic neurones,

leaving the third possibility mentioned above that they were released from chromaffin cells in the wall of the intestine. At this juncture, therefore, the experimental gave place to the histological approach and the colons of several rabbits were examined microscopically for chromaffin tissue.

Burn & Rand (1958 a) employed the Sevki staining method described by Adams-Ray & Nordenstam (1956) to identify chromaffin cells. From the nature of this method, a modified Giemsa stain, it is most unlikely that specificity would be high. It is highly probable that other cells would give a positive staining reaction. For this reason, it seemed important to know whether cells staining with Sevki's method would also show a chromaffin reaction using standard methods. Consequently both methods of staining were used under circumstances which made a comparison possible.

As a further check on the histochemical specificity of the staining methods, it was decided to use reserpine-treated tissue as a control. Since reserpine depletes the tissues of both 5-hydroxytryptamine and catechol amines, any cell showing a positive chromaffin reaction in normal tissue ought to give no reaction in

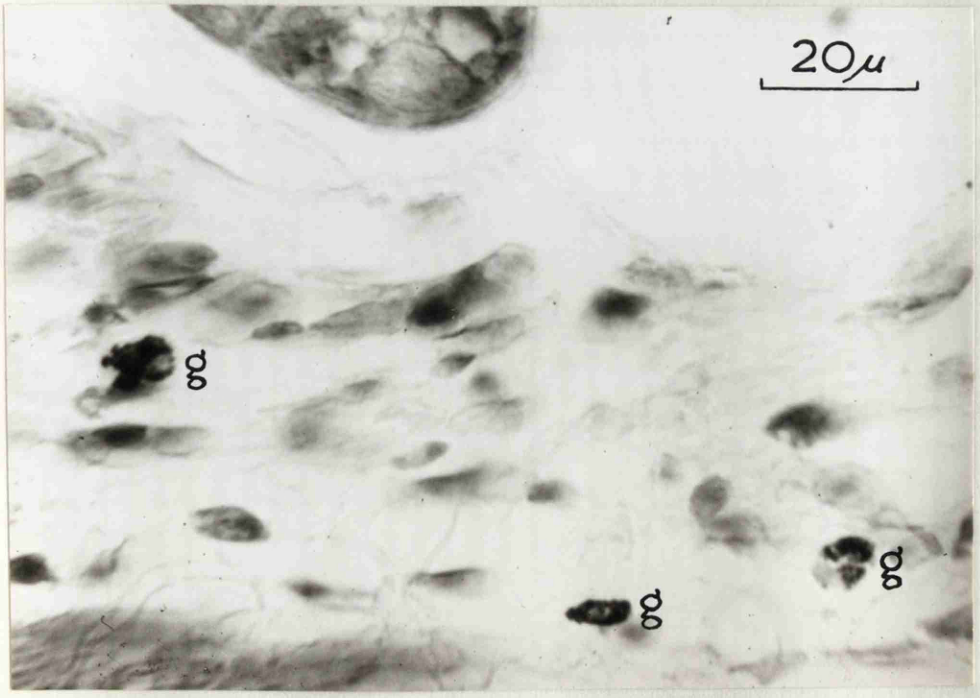
tissue from a reserpine treated animal.

The regions examined were colon, stomach, duodenum and ileum, together with inferior mesenteric ganglia and solar ganglia. For purposes of comparison adjacent pieces from each region of the gut were taken, one stained by Sevki's method and the other by the chromaffin reaction.

In the normal intestine, the only cells which give the chromaffin reaction are the enterochromaffin cells in the mucosa. The region of Auerbach's plexus was examined very carefully in a large number of sections without discovering any chromaffin cells. In the animals which had been given reserpine for five to ten days, the enterochromaffin cells were no longer visible.

Sevki's method also stained the enterochromaffin cells a dark reddish-brown or buff colour, and once again in reserpine treated animals the staining properties were lost. Sevki's method stained another type of cell, not seen in chromaffin stained sections, and this cell was found in the mucosa and submucosa of all parts of the alimentary tract examined. See Fig. 11 (A).

Fig. 11 (A).
Section of normal rabbit colon stained by Sevki's method showing an entero-chromaffin cell (arrowed) in the mucosal glands, and a granular cell (g) lying free in the stroma of the mucosa.



(B).
Section of rabbit colon from a reserpine treated rabbit, stained by Sevki's method. No entero-chromaffin cells are seen, but granular cells (g) are still present lying free in the stroma of the mucosa.

These cells were much commoner in the small intestine than in the large intestine. They contained bright red discrete granules; the cell outline was ill-defined, the granules often appearing to lie almost free; the cytoplasm was unstained and the disposition of the granules was very variable. The nucleus was commonly single, occasionally bi-lobed. These cells resemble closely the cells in the skin which Adams-Ray & Nordenstam (1956) regarded as chromaffin cells. In contrast to the known entero-chromaffin cells, treatment with reserpine left the staining properties unaltered, Fig. 11 (b).

The presence of granules staining brightly red with a Romanowsky stain and the occasional bi-lobed 'spectacle' nucleus make it almost certain that these granular cells are eosinophils, a cell commonly found free in any 'loose connective tissue which underlies epithelium through which there is considerable absorption' (Finerty & Cowdry, 1960).

In the inferior mesenteric and solar ganglia, groups of typical chromaffin cells were found, chiefly on the outside of the ganglia, but occasionally single cells or small groups were found buried among the

ganglion cells, as described by previous authors (Kohn, 1903; Muscholl & Vogt, 1958). Chromaffin cells were not found in the stellate ganglion. In the innervated colon preparation which was used for the nicotine experiments the inferior mesenteric ganglion was retained. It should be emphasised that nicotine could not have produced inhibition by liberating adrenaline or noradrenaline from this ganglion into the bath fluid, since, even when all the mesentery and external nerves had been removed, inhibition was still produced by nicotine. Fig. 3 is from such a preparation.

Effect of mucosal stripping. The only chromaffin cells demonstrable in the gut wall were the enterochromaffin cells in the mucosa. It is almost certain that these cells contain 5-hydroxytryptamine (5 HT), a substance which has a motor effect on the smooth muscle of the colon. It is conceivable, however, that certain of these cells may also produce catechol amines, either alone or along with 5 HT. If such cells are present and liberating catechol amines and thus responsible for the inhibitory action of nicotine, then

removal of the mucosa from the gut ought to abolish the inhibitory effect.

To strip off the mucosa, an innervated preparation of the rabbit's colon was cut open along its antimesenteric border, pinned flat on a cork board with the mucosal side upwards, and the mucosa and submucosa stripped off with fine forceps. The colon was then suspended in Krebs' saline. It displayed the rhythmic activity characteristic of normal preparations and it responded well to electrical stimulation of the pelvic and lumbar colonic nerves. From this ability to respond to nerve stimulation, it can be argued that stripping the mucosa does not derange the terminal innervation of the smooth muscle. This has an important theoretical implication in that, if the inhibitory response to nicotine were abolished by mucosal stripping, this might be due, not to the absence of the mucosa, but to damage to the nerves inflicted by removal of the mucosa.

The effect of removal of the mucosa is shown in Fig. 12.

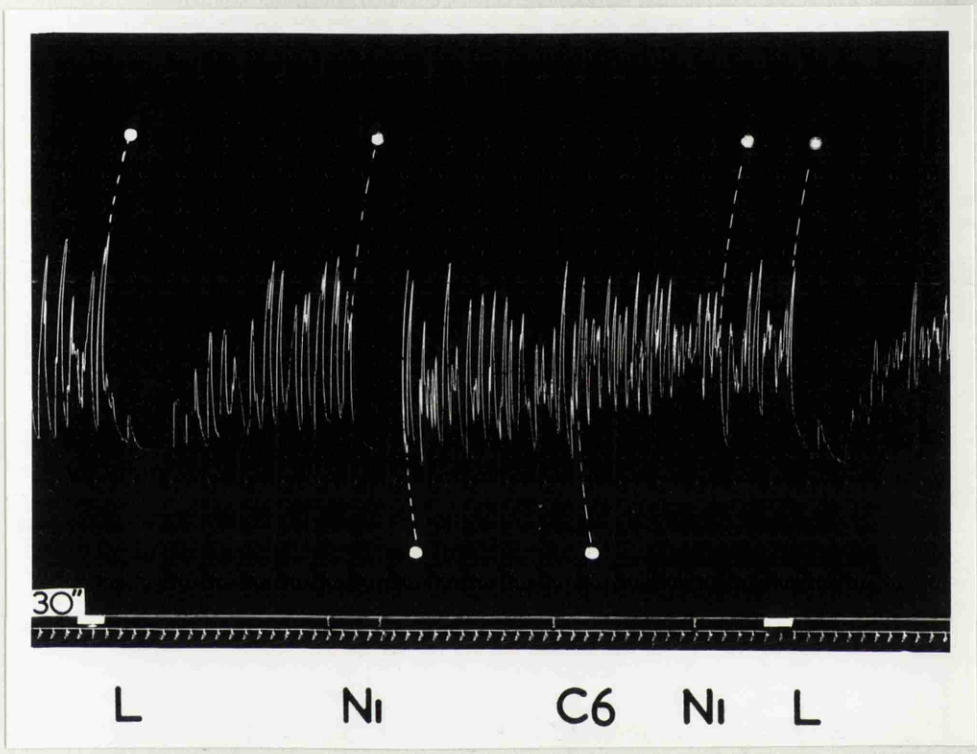


Fig. 12. The response of an isolated rabbit colon preparation, from which the mucosa was removed, to lumbar colonic nerve stimulation (L) and to nicotine 10^{-5} (Ni). Hexamethonium bromide 10^{-4} blocks the response to nicotine, without affecting that due to nerve stimulation.

Nicotine still produced relaxation identical to that in the normal colon and, like it, blocked by hexamethonium.

Effect of prolonged stimulation of the lumbar colonic nerves. The results of the experiments with TM 10 appeared to exclude any participation of extrinsic or intrinsic adrenergic neurones in the inhibitory response to nicotine. However, in the face of this dilemma posed by the inability to demonstrate chromaffin cells, it was felt essential to confirm the conclusions reached from the TM 10 experiments. The possibility that nicotine may release 'sympathin' directly from sympathetic nerve endings was therefore reconsidered and, to test this, an attempt was made to reduce or to eliminate the 'sympathin' in these nerves by two methods - first, by fatigue of the nerves and, secondly, by degeneration of the extrinsic sympathetic nerves.

The nerves were stimulated for long periods at high frequency (50 P/sec) until 'fatigue' set in and the preparation 'escaped' from the initial inhibition; nicotine was then added while the stimulation was continued. Fig. 13 shows the inhibitory effect of nicotine before and during prolonged lumbar nerve stimulation. The inhibition produced by nicotine is unaffected by nerve 'fatigue' produced in this way.

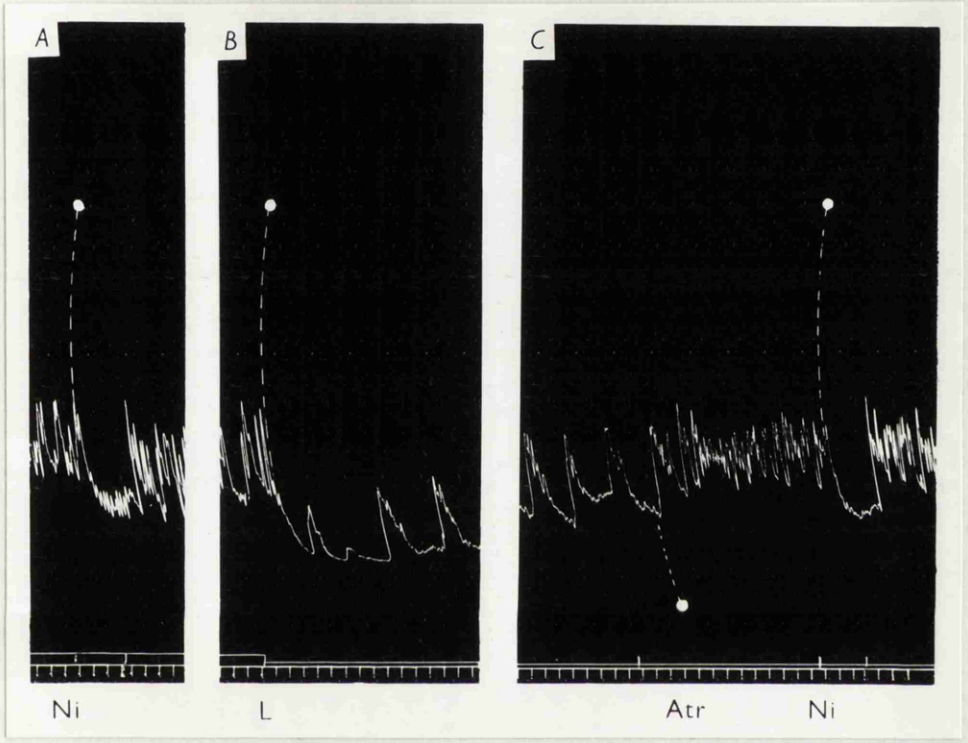


Fig. 13. The effect of prolonged stimulation of the lumbar colonic nerves (L) on the inhibition produced by nicotine (Ni) on the isolated rabbit colon.
A. Response to nicotine 10^{-5} before nerve stimulation.
B. The beginning of nerve stimulation.
C. 100 min later there is some escape from the inhibitory effect; nicotine (after atropine) still produces inhibition of the gut as before.

Effect of nerve section and degeneration. In eight rabbits the postganglionic sympathetic outflow to the colon was cut and 14 days allowed for degeneration to take place. In these operations, the inferior mesenteric ganglia were completely removed, and both the inferior mesenteric artery and the colonic vein apparently stripped clean of nerve tissue with the help of a dissecting microscope. The cut peripheral ends of the nerves were stimulated during the subsequent *in vitro* experiment to test the completeness of the denervation. It was surprising how often some residual inhibitory effect was obtained. This may be due to sprouting of cut preganglionic nerve fibres reinnervating a proportion of postganglionic fibres originating in ganglion cells situated distal to the main ganglionic mass, or to stimulation of these postganglionic fibres directly. In those preparations in which there was a residual response from stimulation of the extrinsic sympathetic nerves, nicotine still elicited an inhibitory response. This appeared to be weaker than would be expected in normal preparations. In those preparations in which stimulation of the extrinsic sympathetic nerves was ineffective, nicotine produced either no inhibition or, more often, only a small inhibitory response.

Discussion

The present experiments demonstrate the suitability of the rabbit colon preparation for the investigation of the inhibitory effect of nicotine. In contrast to the preparation used by Ambache (1951) and Ambache & Edwards (1951), nicotine inhibition of the rabbit colon can easily be demonstrated without the use of a cholinergic blocking agent. The explanation of this is not clear but several possibilities spring to mind. First, the density of the innervation of the colon by extrinsic sympathetic nerves may be higher in the colon than elsewhere in the gut. This would be very relevant since the present results show that nicotine inhibition of the colon is related to the extrinsic sympathetic nerves.

Secondly, it is possible that nicotine may have easier access to the smooth muscle cells of the colon than to those of the ileum. The external smooth muscle coat of the colon is thinner than that of the ileum but it is difficult to imagine that this would play any part in allowing easier access of the drug since the muscle coats throughout the rabbit gut are thin.

There is a third possibility. The site where nicotine acts may be more sensitive to this drug in the colon preparation; in other words, the threshold for stimulation may be lower in the colon preparation. It is certainly true that in the present experiments the inhibition can be obtained with low concentrations of nicotine, while higher concentrations produce a motor response. This is in contrast to the many reports, reviewed by Garry & Gillespie (1955) that low frequencies of stimulation of both the sympathetic and parasympathetic nerves to the intestine favour a motor response, while high frequencies favour an inhibitory response.

Of these three possibilities, it is difficult to choose the most likely. However, it would be interesting to repeat Ambache & Edwards' (1951) investigations on the stomachs of young kittens. If the phenomenon is obtained more easily in the colon because of the high density of sympathetic innervation, then, since it is possible that the stomach may share this high density of sympathetic innervation, it would be interesting to see the effect of low concentrations of nicotine by itself on this region of the gut.

There is now considerable evidence, reviewed

earlier in this thesis, that nicotine causes its adrenaline-like effects by the release of an adrenaline-like substance. The results of the present experiments also suggest that the inhibitory effect of nicotine on the rabbit colon is due to the release of perhaps adrenaline or noradrenaline since the response is abolished by previous treatment of the animal with reserpine which discharges the stores of such catechol amines.

Various sites have been suggested by different authors as the source of this adrenaline-like substance. Briefly, these are:-

(a) Peripheral adrenergic neurones embedded in the various tissues.

(b) Chromaffin cells in the peripheral tissues.

(c) The terminal ramifications of extrinsic sympathetic fibres.

(d) Autonomic interstitial cells.

In addition, the effects of nicotine have been attributed to a direct action on smooth muscle.

It is of interest to consider which of these sites would best fit the results of the present investigations. Pride of place goes to intrinsic adrenergic neurones since this was the first suggested explanation and it was given by Ambache (1951).

Ganglion cells are present in the wall of the gut and this is probably the most important fact in favour of Ambache's hypothesis. Who is to say, in our present state of ignorance, that some of these cells are not adrenergic? Several of the results of the present experiments are, indeed, in accord with Ambache's suggestion. First, the inhibitory effect of nicotine was abolished by concentrations of hexamethonium similar to those required to block the ganglion cells on the parasympathetic pathway. Secondly, large paralysing doses of nicotine likewise blocked the response to small doses of nicotine. Thirdly, after reserpine, the inhibitory effect of nicotine disappeared.

On the other hand, from the literature it is clear that nicotine can produce inhibition of preparations of gut which do not apparently contain ganglion cells. Gasser (1926) studied the response of plexus-free preparations of the small intestine of the cat and his figures show an inhibitory response to nicotine. More recently, Evans & Schild (1953) demonstrated an inhibitory effect of nicotine on ganglion cell free preparations of cat jejunum. In both investigations, the absence of ganglion cells was confirmed by histological examination, although

the absence of ganglion cells does not mean absence of all nervous elements.

Several of the present results are against Ambache's suggestion. In the rabbit colon preparation, TM 10 successfully blocked the response to stimulation of the extrinsic sympathetic nerves. At this time, when stimulation of these nerves produced no response, nicotine could still cause inhibition of the muscle. Although it is unwise to place too great emphasis on the supposed action of TM 10, it does seem reasonable to expect that if TM 10 blocks the extrinsic adrenergic neurones it should, at the same time, block any intrinsic adrenergic neurones. Thus this result is against Ambache's suggestion. Further, section with degeneration and also fatigue of the extrinsic sympathetic nerves abolishes nicotine inhibition. Thus the liberated catechol amines are related to these postganglionic fibres and this makes it unlikely that intrinsic neurones, whose catechol amine content is independent of the extrinsic nerves, are involved.

The second possibility is that the catechol amines come from chromaffin cells. The view that chromaffin cells contribute to the total catechol amines,

particularly adrenaline, in the peripheral tissues has become increasingly fashionable in recent years. For example, the adrenaline in the sheep heart has been attributed to these cells since, unlike noradrenaline, the content of adrenaline is unaffected by sympathetic nerve degeneration (Goodall, 1951). Similarly, it has been suggested that the adrenaline which appears in the perfusate of frogs' hearts, on stimulation of the sympathetic cardiac nerves, may be derived, not from sympathetic nerve endings, but from chromaffin tissue (von Euler, 1961). In the superior and inferior mesenteric sympathetic ganglia, high and variable quantities of catechol amines have been found and correlated with the histologically demonstrable chromaffin cells in these sites (Muscholl & Vogt, 1958).

More relevant to the present investigations are the reports of Adams-Ray & Nordenstam (1956) who, using the modified Giemsa stain of Sevki, claimed to show the presence of chromaffin cells in the human skin. They believed that these cells liberated catechol amines locally and were responsible in part for the tone of blood vessels. Burn & Rand, in a series of subsequent articles, have extended this work and now suggest that

such cells may be the anatomical site for the 'stores' of catechol amines which have been demonstrated pharmacologically to exist in the walls of blood vessels and in other tissues innervated by the autonomic nervous system. Burn & Rand, using the same modified Giemsa method as used by Adams-Ray & Nordenstam, examined both the skin of rabbits' ears (Burn & Rand, 1958 a) and the nictitating membrane of the cat (Burn, Leach, Rand & Thompson, 1959) and identified, in both sites, cells which they called chromaffin cells. Furthermore, after reserpine treatment, the number of such cells in the ears was diminished (Burn & Rand, 1958 a).

Burn and his colleagues attributed the adrenaline-like effects of nicotine and of acetylcholine in various sites, including the rabbit ear, to liberation of catechol amines from pre-formed stores and, by implication, to liberation from these cells which they identified by the modified Giemsa stain of Sevki.

In the rabbit colon, however, it is clear that the inhibitory effect of nicotine is not due to the liberation of catechol amines from chromaffin cells. The only chromaffin cells present in serial sections of the colon are the enterochromaffin cells in the mucosa

and complete removal of the mucosa does not interfere with the inhibitory response.

The practical experience gained using Sevki's method, in addition to the use of the classical chromaffin stain, emphasises the absence of specificity which is likely, on theoretical grounds, with Sevki's stain. Many granule-containing cells, which are not chromaffin cells, stain with Sevki's method and, judging from the resistance of the granules to depletion by reserpine, contain neither catechol nor indole amines. In the present instance, large numbers of cells, almost certainly eosinophils, which are numerous in the sub-mucosa of the gut, were found to stain well with Sevki's method. Such cells, when found outside the vascular system, are not easily identified.

In a recent article, Coupland & Heath (1961) comment on the report by Burn & Rand (1958 a) of the presence of chromaffin cells in the skin of the rabbit. Burn & Rand reported that these cells did not give a positive Schmorl's reaction. Coupland & Heath comment that a positive Schmorl's reaction is one of the most typical of all the properties of chromaffin granules, albeit non-specific, and that this failure of the cells

to react eliminates the possibility that these workers were observing chromaffin elements.

Another, though less likely possibility, that the action of nicotine is a direct one on smooth muscle, must be considered. That such direct actions are possible have been clearly demonstrated. For example, the chick amnion, which consists of a layer of smooth muscle covered with epithelium, has been shown to be free of nervous elements (Peterfi, 1913; Verzar, 1914; Baur, 1928; Pierce, 1933; & Ferguson, 1940). In 1928 Baur found that nicotine at high concentrations 10^{-4} produced pure inhibition of the chick amnion, and in 1946 McDowall postulated a direct action of acetylcholine on heart muscle since its stimulant action was not blocked by high concentrations of nicotine. Evans & Schild (1953) reported inhibition by nicotine of ganglion-free preparations of cat jejunum: this they thought might be a direct action of nicotine on the smooth muscle. It did not occur to them to consider whether or not this action might be on nerve endings. Indeed, the only inhibitory actions of nicotine on truly nerve-free preparations were those on the chick amnion. In these, the concentrations of nicotine were high and were very possibly

non specific.

In marked contrast, in the present experiments, the inhibition is seen with a low dosage of nicotine; inhibition appears before contraction and the action is specific as is shown by its abolition by hexamethonium, reserpine, or by section and degeneration of the sympathetic nerves. For these reasons, it is clear that the direct action of nicotine on smooth muscle cells plays no part in the inhibitory response of the colon.

A final possible source of the transmitter is in the nerve endings of the extrinsic sympathetic nerves and liberation is said to take place as the result of an axon reflex. The evidence which previous authors have usually offered for such a mechanism is that the particular response is unaffected by acute section but abolished by section and degeneration of the sympathetic nerves (Coon & Rothman, 1940; Hilton, 1954).

In the present experiments similar evidence has been obtained. Acute section of the nerves inevitably involved in the removal of the preparation from the animal, obviously does not affect the response: previous operative section and degeneration of the extrinsic sympathetic nerves does abolish the response.

The action of reserpine in abolishing the inhibitory response is consistent with this explanation, since reserpine is known to deplete transmitter stores at sympathetic nerve endings.

An attempt to produce a similar depletion by long, continued stimulation of the sympathetic nerves, failed to abolish the inhibitory response to nicotine. The reason for this is not clear. Brown, Davies & Gillespie (1958) have shown a decrease in transmitter output with prolonged nerve stimulation. On the other hand, Luco & Goñi (1948) have reported that prolonged stimulation does not alter the noradrenaline content of adrenergic nerves. Perhaps the stores of transmitter accessible to the action potential may differ from those available to nicotine. In this context, Hillarp (1960) has produced, from studies of adrenal medullary cells, evidence that might support such a division of catechols. Three amine fractions can be isolated from the adrenal medulla: a) the largest fraction which is stored in granules together with adenosine phosphates, b) a fraction of varying size which is stored in granules without adenosine phosphates, and c) a small fraction ($< 10\%$) which probably exists as free amines in the cytoplasmic sap.

Thus two different amine pools exist - the amines bound to granules (A_B) and free amines (A_F). If the same conditions exist in adrenergic neurones and other stores of sympathomimetic amines, then nerve action potentials may release amines from one of these pools, while nicotine may have access either to the other or both pools of amines. Decrease of transmitter output with nerve stimulation under these circumstances is not necessarily inconsistent with the continuing inhibitory response to nicotine.

The inability to abolish the response by fatigue of the sympathetic nerves is in contrast to a report in the second part of the thesis of the result of prolonged stimulation of cholinergic nerves.

While the results of the present experiments strongly suggest that the source of transmitter is in the nerve endings of the extrinsic sympathetic nerves, the idea that an axon reflex is involved is difficult to believe. For example, TM 10, which blocks the response to sympathetic nerve stimulation, probably by blocking action potentials in the ultimate fibres, does not abolish inhibition by nicotine. If nicotine acted through the agency of an axon reflex, it is difficult

to understand how the action potentials initiated could fail to be blocked by TM 10. Furthermore, from the work of Brown & MacIntosh (1939) and of Bronk (1939) it is clear that acetylcholine is unable to elicit action potentials in preganglionic nerve fibres by action at the endings of these nerves. From the work of Lorente de Nó (1944) and of Hodgkin (1947), nicotine and acetylcholine are similarly ineffective, even in high concentrations, in stimulating nerve fibres along their length.

On the other hand, the site of stimulation by nicotine in producing these adrenaline-like responses, as has been stated by Coon & Rothman (1940) '..... behaves like an autonomic ganglion towards nicotine .....'

For this reason, it is felt that the evidence from the present investigations suggests that the site of action of nicotine may be on some structure interposed between the extrinsic sympathetic nerves and the effector cells.

The nature of the final peripheral autonomic innervation apparatus is still in doubt, and this doubt centres round the nature and function of those cells,

first described by Cajal (1909), as 'neurones sympathiques interstitiels' which form a network throughout all the tissues innervated by the autonomic nervous system.

All histologists who have investigated the peripheral autonomic innervation are agreed that some form of nerve network constitutes the final link with the effector cells. This has been referred to as the 'terminal retinaculum' (Reiser, 1933; Stohr, 1941) 'sympathetic ground plexus' (Boeke, 1940) 'nervous ground plexus' (Hillarp, 1949) 'autonomic interstitial net' (Meyling, 1953) or 'autonomic ground plexus' (Richardson, 1958).

The nature of this nerve plexus, however, is a subject of intense disagreement. According to one group of authors, the nerve net is made up of postganglionic sympathetic and parasympathetic fibres in a Schwann plasmodium and, although the fibres form a complex network, they retain their individuality right to the periphery (Lawrentjew, 1926; Richardson, 1958; Hillarp, 1959).

In sharp contrast to this is the view of those who maintain that the nerve plexus is made up of the anastomosing processes of these 'neurones sympathiques interstitiels' which are true ganglion cells and which

constitute the final link with the effector cells. The postganglionic sympathetic and parasympathetic fibres either lose their identity in this plexus (Boeke, 1940) or end on it (Meyling, 1953).

While it must be admitted that, in the view of most histologists of the present day, the nervous nature of the 'interstitial cells' is doubtful, nonetheless, if such cells should prove to be modified ganglion cells, they would provide a very likely site of action for nicotine. In this respect, it is interesting to recall that Leeuwe (1937) has claimed to demonstrate histochemically the presence of di-phenols in the network composed of these cells and which he suggests might be due to adrenaline. An interstitial network would not give the chromaffin reaction, however, since, as with the extrinsic nerve endings, there is not a sufficiently high local concentration of noradrenaline.

Such a site of action would explain the inability of TM 10 to block the response of nicotine while abolishing the response to stimulation of the sympathetic nerves, since it is possible that TM 10 acts on the sympathetic nerve fibres and not on the interstitial nerve net.

However, acceptance of such a site of action brings its own difficulties. For instance, this work shows that extrinsic sympathetic nerve section and degeneration abolish the inhibitory effect of nicotine. Does such section also cause degeneration of the autonomic interstitial nerve net? We have no evidence one way or the other. One way round this difficulty is to postulate that the stores of transmitter in the nerve net are derived from the extrinsic sympathetic nerves. Burn & Rand (1958 a) have already postulated that the 'stores' of noradrenaline in arterial walls may be separate but derived from the extrinsic sympathetic nerves, which raises the interesting possibility that the sympathetic ground plexus may be the site of these 'stores'.

An alternative to the above explanation is possible if a hypothesis of transmitter release, put forward recently by Koelle (1961) is accepted. Koelle suggests that the release of transmitter from nerve endings may occur in two stages. In the first place, small quantities of acetylcholine are liberated by the action potential at the nerve endings. In addition to its action on the postsynaptic membrane, the acetylcholine

acts on the presynaptic membrane to liberate a further and larger quantity of transmitter. The transmitter liberated in the second phase may be acetylcholine or some other transmitter, for example, noradrenaline, depending on the type of nerve. If the second phase liberation is unaccompanied by changes in membrane potential, then it raises the possibility of direct liberation by acetylcholine or by nicotine, of noradrenaline from the sympathetic nerve endings, without the initiation of an action potential. The direct liberation of noradrenaline from nerve endings by tyramine and other amines has already been demonstrated by Fleckenstein & Burn (1953).

Such a mechanism would explain all the findings in the present investigations, including the paradoxical action of TM 10 in blocking the response to adrenergic nerve stimulation while leaving the inhibitory action of nicotine unaffected. On this theory, the action of TM 10 on the nerve endings would be to block the conducted action potential and so prevent the first phase, the liberation of acetylcholine. The second phase, the direct action of acetylcholine on the nerve endings with the release of noradrenaline would be unaffected.

In the same way, the inhibitory action of nicotine or acetylcholine added to the bath would be unaltered.

In the present experiments it was found most difficult to produce complete sympathetic denervation. The necessity to have some control of this point was clearly demonstrated. The absence of such controls may explain the occasional reference in the literature to the ineffectiveness of denervation in abolishing the adrenaline-like effects of nicotine. For example, in cat auricles and in isolated perfused rabbits' ears, Ginzel & Kottegoda (1953) reported that sympathectomy was ineffective in abolishing this adrenaline-like response.

There are several reasons why sympathectomy may be unsuccessful. First, there may be a number of divergent pathways to the periphery and some of these may be missed at operation. This is likely to occur in the ear, where the sympathetic nerves follow the blood vessels and the vessels anastomose very freely. Secondly, there may be sympathetic ganglion cells peripheral to the well recognised sites. This would leave some postganglionic sympathetic fibres intact. Thirdly, there have been recent reports of re-sprouting of autonomic nerve

fibres (Murray, 1959). Thus, if during sympathectomy any fibres are left uncut, given sufficient time they could effectively restore the peripheral stores of amines. In this context, Dragstedt, Harper, Tovee & Woodward (1947) have reported that, following vagotomy, even a small strand of vagus left intact at operation may eventually be capable of activating the whole of the glandular apparatus of the stomach. Sprouting has now been regarded as the possible cause of recovery (Burge & Vane, 1958).

Summary

1. Nicotine in low concentrations (10^{-6} to 10^{-5}) caused inhibition of preparations of the rabbit colon *in vitro*. Higher concentrations ($> 10^{-5}$) caused contraction. The mechanism of the inhibitory response has been studied.
2. The presence of atropine enhanced the inhibitory effect of nicotine. The concentrations of atropine used were high and such concentrations have some anti-nicotinic as well as anti-muscarinic action. A method is described to overcome the unwanted anti-nicotinic action.
3. The presence of hexamethonium bromide and large ganglion-cell-paralysing concentrations of nicotine abolished the inhibitory action of nicotine.
4. Prior treatment of the rabbit with daily intravenous injections of reserpine for ten days caused virtual disappearance of nicotine inhibition, presumably because of the depletion of the stores of catechol amines by the reserpine. Nicotine therefore appears to produce inhibition by liberating a catechol amine.
5. Choline 2:6 xylyl ether bromide (TM 10) rendered

ineffective stimulation of the lumbar colonic nerves but only slightly reduced the inhibitory effect of nicotine.

6. The only chromaffin material found in the intestine was the enterochromaffin cells in the mucosa. Removal of the mucosa did not abolish the inhibition produced by nicotine. Thus the participation of mucosal chromaffin cells in this effect is excluded.

7. Stimulation of the sympathetic nerves for long periods, in an attempt to reduce the amount of sympathin in the nerve endings, had no effect on nicotine inhibition.

8. After section and degeneration of the extrinsic sympathetic nerves, the inhibitory effect of nicotine was reduced or lost.

9. The inhibitory effect of nicotine, therefore, seems to be due to release of catechol amines either (1) from the extrinsic sympathetic nerves or (2) from some structure associated with them. This response of the colon of the rabbit corresponds to the pilomotor response in the skin of the cat described by Coon & Rothman (1940) and attributed by them to an axon reflex in efferent adrenergic fibres. The possibility that nicotine acts on some form of terminal sympathetic

nerve net intervening between the sympathetic nerves and the smooth muscle is tentatively suggested.

P A R T I I

- INTESTINAL INNERVATION - THE ACTION OF RESERPINE

Introduction

In the course of the investigations reported in Part I of the thesis, an attempt was made to abolish with reserpine the inhibitory effect of the sympathetic nerves to the rabbit colon. This attempt was based on the known ability of reserpine to discharge catechol amines from their stores in the tissues and from adrenergic neurones (Bertler, Carlsson & Rosengren, 1956; Muscholl & Vogt, 1958).

In fact, after reserpine, inhibition was consistently replaced by a large contraction (Gillespie & Mackenna, 1959, 1961). This was unexpected, since Garry & Gillespie when they devised this preparation of rabbit colon, specifically investigated the possibility of 'mixed' responses from either sympathetic or parasympathetic nerves and were unable to demonstrate a motor response to sympathetic nerve stimulation under any circumstances (Garry & Gillespie, 1955).

The origin of the motor response to stimulation of the extrinsic sympathetic nerves after reserpine is the subject of the second part of the thesis.

During these investigations, the colon from a

reserpine-treated rabbit was soaked in noradrenaline or some of its precursors and the inhibitory response to sympathetic nerve stimulation was found to be restored. The mechanism of the restoration is also reported here.

The results of these studies have already been published (Gillespie & Mackenna, 1959, 1961).

Review of the Literature

Since ancient times, extracts from the plant Rauwolfia (genus of the family Apocynaceae) have been recommended by native medicine men and employed in popular medicine for a variety of diseases. Rauwolfia, which grows in the subtropical and tropical parts of India, the East Indies, Africa and Central and South America, was so named by the French botanist Plumier, in honour of the German physician, Leonard Rauwolf, who, in 1582, had published accounts of investigations of medicinal plants he had found on an expedition to the Middle East. Whether Rauwolf was, in fact, acquainted with Rauwolfia, is not certain.

There are now more than fifty species of Rauwolfia known, the most important being R. serpentina. This species of Rauwolfia grows mainly in India and it is described in ancient Indian literature as a febrifuge, as a remedy for snake bites, and as a cure for dysentery. Its use in Europe was known over three hundred years ago when it was stated to be of value in the treatment of 'anxiety states'. It was not until 1933, however, that Chopra, Gupta & Mukherjee reported the hypotensive activity

of material obtained from the plant. This observation, together with its increasing use as a sedative, greatly stimulated interest in this drug.

In the meantime, the plant was being investigated by chemists, and several attempts at isolation of active principles were performed during the first thirty years of this century. However, these attempts were not very successful, until 1931, when the Indian chemists, Siddiqui & Siddiqui isolated the first crystalline alkaloid. Since then, some 14 alkaloids have been identified.

The Western world was plagued by hypertension and, with its abundant wealth, could afford to investigate any new and apparently novel hypotensive agent. The study of R. serpentina was taken up by Ciba, whose excellent pharmacologists, led by Bein, using new isolation techniques such as countercurrent distribution and chromatography, were able to isolate an alkaloid which had pronounced hypotensive and sedative actions. This was reported by Müller, Schlittler & Bein (1952) who gave it the name reserpine.

A host of investigations since have confirmed the initial promise of the drug. It is undoubtedly a novel and important addition to the pharmacological armamentarium

and, as is so common in pharmacological history, not solely in the realm of hypertension which had prompted its investigation.

The major pharmacological investigations of reserpine were carried out by Bein (1953) and by Plummer, Earl, Schneider, Trapold & Barret (1954). From their reports, and those of others, the drug obviously has numerous and varied effects. However, a general plan is discernible. Reserpine tends to produce signs and symptoms of para-sympathetic activity. This is best seen in the peripheral effects - bradycardia, hypotension, gastrointestinal hyperactivity with diarrhoea, dilatation of conjunctival and nasal mucosal blood vessels (snuffles), and pinpoint pupils with relaxation of the nictitating membrane. The central effects are a curious form of sedation. If the animal is left undisturbed, it lies with its eyes closed as if asleep. If disturbed, it comes awake easily but soon lapses again.

If we accept Brodie's concept of two aspects to the central nervous system, the ergotrophic system expending energy with increased activity, increased wakefulness, foraging for food and supported by the sympathetic nerves, and a trophotrophic system concerned with conserving energy,

promoting digestion and absorption, minimising muscular activity associated with the sleeping centre and the parasympathetic centre - then reserpine becomes a drug stimulating the trophotrophic system, and this explains the central and peripheral effects. Some other effects of reserpine are not easily explained in this fashion: for instance, an action on the basal ganglia or reticular formation is presumably responsible for muscle tremors, for ataxia and for muscular weakness.

Originally emphasis was laid on the central origin of all the signs and symptoms produced by reserpine. The first break in this front came in 1956 when Bertler, Carlsson & Rosengren reported that the peripheral stores of catechol amines in the heart disappeared after an injection of reserpine. It was hard to attribute this to the exhaustion of sympathetic nerves, since sympathetic activity is reduced in reserpine treated animals. Their results were confirmed in 1957 by Paasonen & Krayer. The cause of this fall in tissue stores was indicated by Muscholl & Vogt (1958) who showed that reserpine caused losses of up to 80% of noradrenaline from adrenergic nerves. They considered the possibility that the peripheral lack of sympathetic activity was due, not to a reduction in the

central outflow, but to a defect in the peripheral nerve endings. They confirmed this idea by showing that organs with an adrenergic innervation no longer responded to stimulation of their pre- or post-ganglionic fibres when the loss of noradrenaline was severe and had persisted long enough. The final swing of ideas in this direction was made when Iggo & Vogt (1959), recording the electrical activity in the sympathetic fibres of a reserpine-treated animal, showed that the outflow of action potentials in these nerves is, in fact, increased.

The present view is that the predominance of parasympathetic signs and symptoms is due to an imbalance of the autonomic nervous system as a result of peripheral block of the sympathetic system by reserpine.

There have now been several reports that reserpine causes the depletion of adrenaline and noradrenaline from sites in which the amines are stored: for instance, from the adrenals (Holzbauer & Vogt, 1956; Carlsson & Hillarp, 1956), from the brain (Holzbauer & Vogt, 1956; Shore, Olin & Brodie, 1957), from the heart (Bertler, Carlsson & Rosengren, 1956; Paasonen & Krayer, 1957), from peripheral sympathetic tissue (Muscholl & Vogt, 1957 a) and from blood vessels (Burn & Rand, 1958 a).

More recently, von Euler & Lishajko (1960) have shown that reserpine caused release of noradrenaline from transmitter granules in adrenergic nerves.

There are three possible causes for the depletion of catechol amines from peripheral stores. Reserpine could cause a failure of synthesis of transmitter, an increased destruction, or it could cause an inability to retain transmitter. The evidence at present available suggests that depletion is due to an inability to retain transmitter. If reserpine simply caused a failure of synthesis or increased destruction of transmitter, then one would expect that, after reserpine, the level of catechol amines in the plasma and the urine would remain steady for a short time and then fall below the normal level. Alternatively, if reserpine caused an inability to retain transmitter, one would expect a sudden release of catechol amines from their stores, with a consequent initial rise in the plasma levels and an increased output in the urine.

Muscholl & Vogt (1957 b) reported that, after reserpine, the concentration of plasma adrenaline of rabbits was raised. The reports on output of catechol amines in the urine after reserpine are conflicting. De Jongh (1958) and van Proosdij-Hartzema (1959) have observed

a marked increase in the urinary adrenaline excretion in rats after the intramuscular injection of high doses (4 mg/kg) of reserpine, and Carlsson, Rosengren, Bertler & Nilsson (1957) showed that, in rabbits, a single large dose of reserpine was followed by an initial increase in the output of catechol amines in the urine. However, Gaddum, Krivoy & Laverty (1958) have shown that, after a single high dose of reserpine to schizophrenic patients, there was no change in the urinary excretion rate of catechol amines. However, the initial level of urinary catechol amines in these patients was higher than normal and the technique employed by these authors for collecting the urine samples could not exclude the possibility of a brief period of increased secretion.

Indirect evidence for a sudden release of amines by reserpine is provided by several workers. Everett, Toman & Smith (1957) reported that mice passed through a phase of piloerection 30 min after a large dose of reserpine. Kuschke & Frantz (1955) demonstrated a hyperglycaemic effect of reserpine in the rabbit. This effect was sensitive to the ergot alkaloids. Rises in blood pressure in the rat and spinal dog, and contractions of the denervated nictitating membrane of the cat, have been reported to

follow injections of reserpine (de Jongh & van Proosdij-Hartzema, 1955; Maxwell, Ross, Plummer & Sigg, 1957). Thus, depletion of the peripheral stores of catechol amines by reserpine is apparently due to an inability to retain transmitter.

All the effects of reserpine cannot be attributed to its action on peripheral storage depots. Holzbauer & Vogt (1956), Carlsson & Hillarp (1956) and Kroneberg & Schümann (1958) showed that, if the adrenal medulla is denervated, it becomes resistant to the effects of reserpine. This suggests that reserpine's action on the adrenal medulla is mediated centrally and has its effects via the peripheral autonomic nerves. This supports Iggo & Vogt's (1959) report that, after reserpine, there is an increase in sympathetic nervous discharge.

Since Muscholl & Vogt's (1958) report that reserpine caused depletion of catechol amines from sympathetic nerves, most authors have attributed the decrease in assayable catechol amines in tissues to a depletion of the sympathetic fibres. However, Burn & Rand (1958 a) introduced a new idea by attributing 'stores' to some site other than nerve endings. They confirmed that nerve section and degeneration abolished these 'stores' and they interpreted this as

indicating that the 'stores' normally pick up catechol amines from the sympathetic nerve endings. They suggested originally that the 'stores' might be chromaffin cells which were described in the skin by Adams-Ray & Nordenstam (1956) and which Burn & Rand believed they could demonstrate, using the modified Giemsa stain of Sevki. This theory has been modified in process of combining it with the numerous reports showing the ability of nicotine and acetylcholine to liberate catechol amines from a large number of sites. (These reports are reviewed in Part I of this thesis.) Their suggestion that 'stores' of noradrenaline were discharged by nicotine and acetylcholine in turn suggested a cholinergic innervation for these 'stores'. Such a theory, at first sight bizarre, provided an explanation for several interesting and unexplained phenomena. For example, it explained those instances in which stimulation of a sympathetic nerve, after reserpine, produced parasympathetic phenomena (the present results have been used by Burn & Rand to support their theory) the explanation being that the postganglionic sympathetic nerve endings liberate acetylcholine, which in turn liberates catechol amines from their stores. As a consequence, the observed normal

effect is adrenergic. If the adrenergic transmitter is absent, however, after reserpine, then the acetylcholine liberated may itself diffuse to the effector and produce a cholinergic effect. Recently, Burn has further modified his theory (Burn, 1961). He has abandoned the idea of chromaffin cells as the stores and considers that the stores are probably in the nerve endings. His hypothesis now is that all postganglionic sympathetic fibres release acetylcholine, which in turn releases noradrenaline. Such a hypothesis would explain 'stores' of catechol amines, their loss with nerve section and with reserpine, the 'adrenaline-like' effects of nicotine and acetylcholine, and the occasional reports of cholinergic responses from sympathetic nerves after reserpine. Burn & Rand (1958 c, 1959 a) also used this theory to explain the increase in sensitivity of reserpine treated, or denervated, smooth muscle to adrenaline, a phenomenon first described by Bein, Gross, Tripod & Meier (1953). Burn & Rand suggested that there is a continuous small discharge from the 'stores' which diminishes the sensitivity of the smooth muscle to injected noradrenaline. After reserpine treatment, or section and degeneration of the sympathetic fibres, this discharge is abolished and the sensitivity

to added noradrenaline increases.

A hypothesis, similar to Burn & Rand's, which suggests a similar action of acetylcholine at nerve endings, liberating a second transmitter, has been proposed by Koelle (1961) and is reviewed in Part I of this thesis.

Burn & Rand also demonstrated that stores of catechols in tissues could be manipulated by means other than action potentials. The history of this goes back thirty years (Burn, 1932 b). Burn infused adrenaline into the vessels of the dog hindleg and restored the effect of sympathetic nerves which had been depleted of their catechol amines by anoxia. More recently, this phenomenon has been demonstrated, using reserpine to deplete the 'stores'. Infusions of noradrenaline, or of some of its precursors, restores the effect of nerve stimulation and of tyramine and returns the sensitivity of the smooth muscle to noradrenaline back to normal (Burn & Rand, 1958 c, 1959 b, 1960 a; Blaschko & Chruściel, 1960). In addition, it has been indirectly shown by Burn & Rand (1960 a) that the normal 'stores' are less than their maximum and can be increased. Such an uptake in normal tissues has been confirmed by Axelrod, Weil-Malherbe & Tomchick (1959) with radioactive isotopes in the heart and spleen and by

Pennefather & Rand (1960) in kidney and in the horn of uterus. Evidence of a different kind, that the catechol amines in nerve endings are normally less than the maximum, was provided by Brown, Davies & Ferry (1961). They showed that postganglionic sympathetic fibres in a resting state as a result of cutting the preganglionic fibres five days beforehand, gave an increased output of transmitter per stimulus at the subsequent experiment. Thus it would appear that tissue stores can be increased by circulating catechol amines. Such uptake is presumably into the nerve endings, as it is abolished by section and degeneration, and finally it implies a catechol concentration in nerve endings normally less than the maximum possible. The opposite effect - the liberation of catechol amines in the absence of nerve impulses - is also possible. Burn (1932 a) and Burn & Rand (1960 a) have demonstrated that tyramine and associated amines liberate catechol amines directly from nerve endings.

All this evidence appears to indicate that the transmitter in adrenergic nerve endings is not so firmly wedded to the action potential as is acetylcholine in cholinergic nerves. It raises the possibility that adrenergic nerves may occupy a position midway between

the cholinergic nerves, whose transmitter is liberated only by nerve impulses, and the adrenal medulla where the transmitter is liberated from a cell whose role is essentially that of a neurosecreting cell.

The present experiments have a bearing on the theory of Burn & Rand. Although this theory does explain several apparently anomalous aspects of the 'adrenergic mechanism', it does not, as it stands, explain all the aspects of this problem. In particular, the motor response of the rabbit colon to sympathetic nerve stimulation after reserpine cannot be explained simply on the basis of the hypothesis of these authors.

Results

THE ACTION OF RESERPINE IN VIVO

The response of rabbit colon *in vitro* to stimulation of its extrinsic parasympathetic (pelvic) nerves is contraction. The response to stimulation of its sympathetic (lumbar colonic) nerves is relaxation (Garry & Gillespie, 1955). These results were confirmed in the present investigations and are shown in Fig. 14.

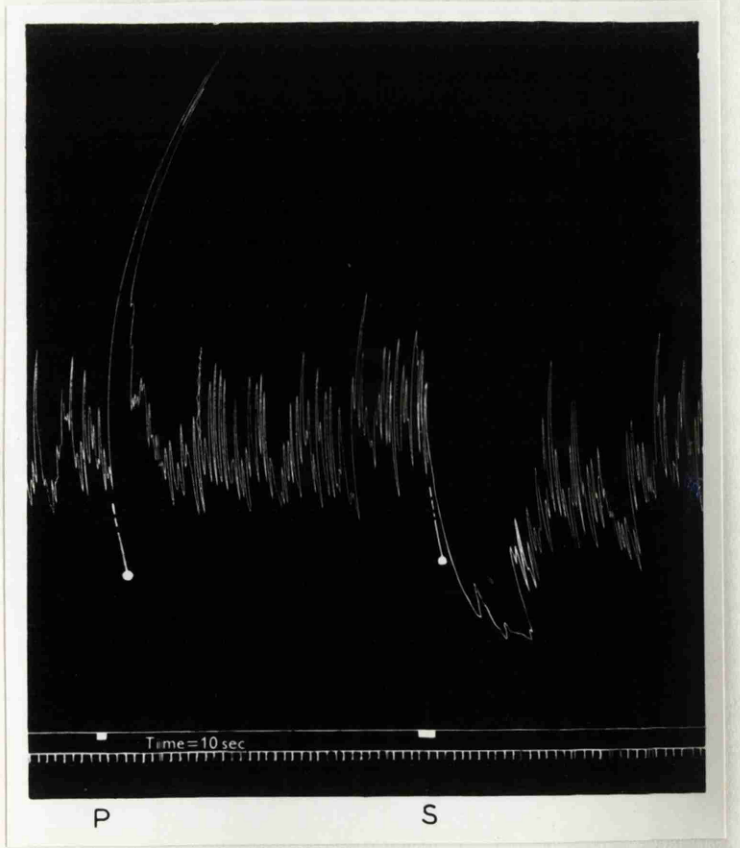


Fig. 14. The response of the colon from a normal rabbit *in vitro* to stimulation of its pelvic (parasympathetic) nerves (P) and lumbar colonic (sympathetic) nerves (S).

The usual response of rabbit ileum to stimulation of its periarterial nerves (mainly sympathetic) in vitro is relaxation (Finkleman, 1930). Such a response, with a modern technique, is shown in Fig. 15.

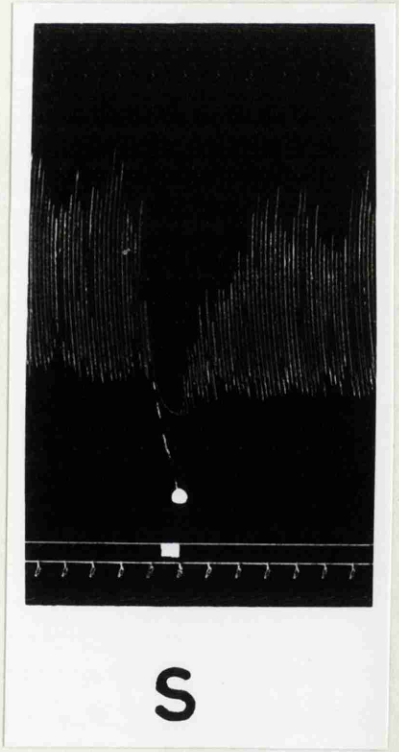


Fig. 15. The response of the ileum from a normal rabbit in vitro to stimulation of its periarterial nerves (S).

Effect of reserpine. As was described in Part I of this thesis, if a rabbit is given reserpine ('Serpasil': Ciba) for several days parenterally, the response of the colon to stimulation of its sympathetic nerves is reversed from relaxation to contraction. This unexpected finding prompted a closer study of the response of the rabbit

intestine to stimulation of its extrinsic autonomic nerves after the administration of intravenous reserpine. In vitro preparations of both colon and ileum were used and the reversal of the normal inhibitory response to contraction after treatment with reserpine is shown for both preparations in Fig. 16.

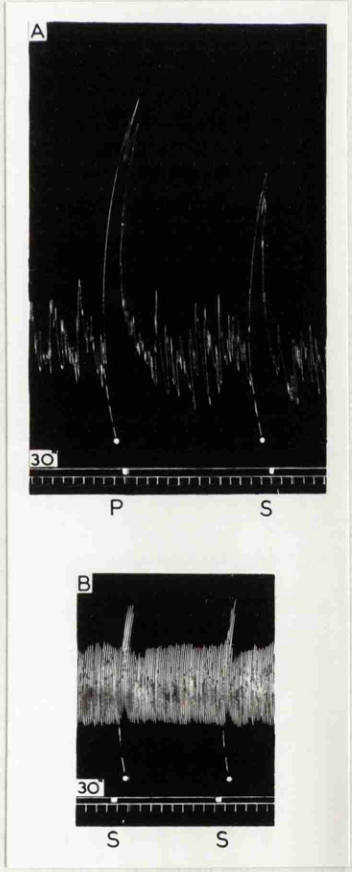


Fig. 16. A. and B. The response of the colon and the ileum respectively from a rabbit treated with reserpine to stimulation of the parasympathetic (P) and sympathetic (S) nerves. After reserpine the response to sympathetic nerve stimulation is contraction.

The action of intravenous reserpine is specifically on the response to sympathetic nerve stimulation.

There is no change in the response to addition of the sympathetic transmitter, noradrenaline, which remains inhibitor. The response to pelvic nerve stimulation is unaltered and there is no obvious difference in the intrinsic tone or rhythmic activity of the smooth muscle.

In the colon, there is a remarkable similarity between the motor sympathetic response after reserpine and the pelvic response (Fig. 16). However, the sympathetic response is not due to an escape of current from the lumbar electrode reaching and stimulating the pelvic (parasympathetic) fibres in the other electrode, since tying the sympathetic nerves as they leave their electrode abolishes the sympathetic motor response without affecting the motor response from pelvic nerve stimulation. Furthermore, stimulation of the sympathetic nerves in a reserpine treated rabbit can produce a motor response even in preparations in which the pelvic nerves are cut away from the preparation.

Effect of atropine. The effect of atropine on the motor response of the colon from a reserpine treated

rabbit to lumbar nerve stimulation is shown in Fig. 17.

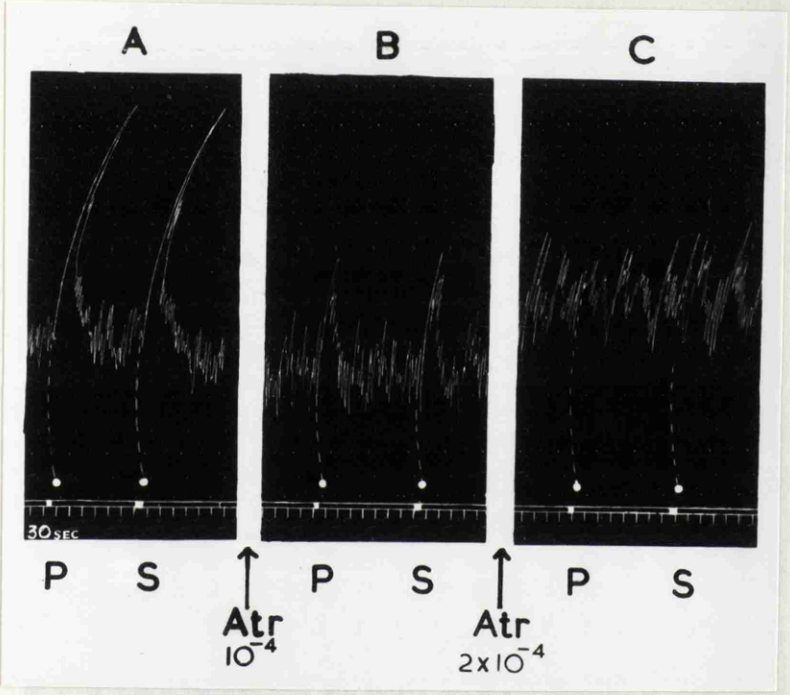


Fig. 17. The action of atropine on the motor response of the rabbit colon to sympathetic nerve stimulation. The rabbit had previously been treated with reserpine. A shows the response to stimulation of the sympathetic (S) and parasympathetic (P) nerves: between A and B atropine sulphate was added to the bath to make a concentration of 10^{-4}. This produced an equal but incomplete block of the two responses. Between B and C a further dose of atropine was added to make a total concentration of 2×10^{-4}. Both responses were completely blocked.

Atropine 10^{-4} produced a similar reduction in the motor responses to the pelvic and lumbar colonic nerves with a similar rate of onset. When the concentration of atropine was increased to 2×10^{-4}, the response to both nerves was almost completely abolished. Recovery of both responses after washing out the atropine occurred, again at the same rate. This result indicates that the motor response to lumbar nerve stimulation after reserpine is due to

stimulation of fibres liberating acetylcholine, since it is affected by atropine in exactly the same way as the pelvic fibres, which are cholinergic.

Effect of hexamethonium. The effect of hexamethonium on the responses of the colon from a reserpine treated rabbit to pelvic and lumbar colonic nerve stimulation is shown in Fig. 18.

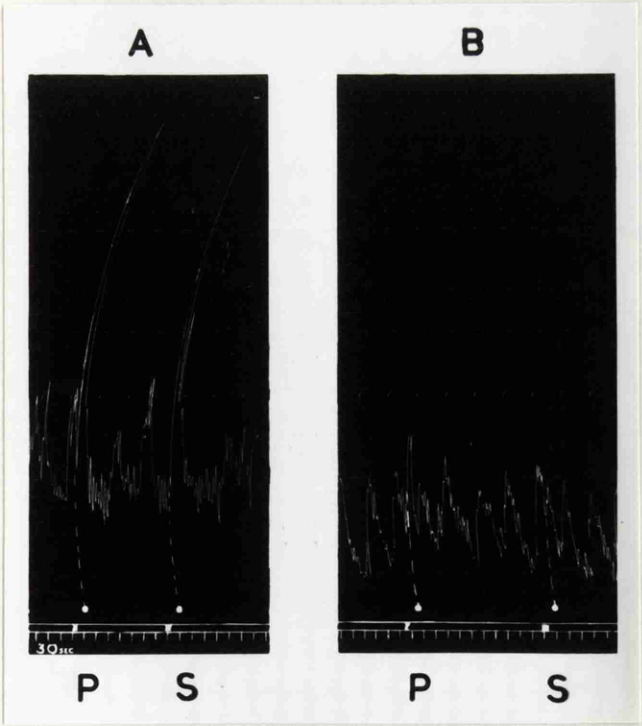


Fig. 18. The effect of hexamethonium bromide on the motor responses to stimulation of the parasympathetic (P) and sympathetic (S) nerves to the colon of a reserpine treated rabbit. A, before the addition of hexamethonium bromide 2×10^{-4}; B, after the addition of hexamethonium. Both responses are blocked.

Once again, both responses were similarly affected by the drug, both in the degree of block achieved and in

the rate of onset and recovery. This indicates that the nerve fibres mediating the motor response to sympathetic nerve stimulation, after reserpine, are preganglionic fibres.

Effect of frequency of stimulation. The effect of varying the frequency of stimulation of the extrinsic autonomic nerves to the normal rabbit colon *in vitro* was described by Garry & Gillespie (1955). The responses showed a characteristic difference between the extrinsic sympathetic and parasympathetic nerves. The maximum response to pelvic (parasympathetic) nerve stimulation was obtained when a frequency of 10 P/sec was used. The maximum response to stimulation of the lumbar colonic (sympathetic) nerves was obtained when a frequency of 50-100 P/sec was used. When a frequency of stimulation below 10 P/sec was used, the response to stimulation of the pelvic nerves was still of considerable magnitude, but the response to stimulation of the lumbar colonic nerves was greatly reduced: at a frequency of one pulse every 2 sec, stimulation of the pelvic nerves was still effective, while stimulation of the lumbar colonic nerves

was rarely effective at a frequency below 5 P/sec.

The upper record in Fig. 19 shows the effect on the colon and the lower record the effect on the ileum of stimulating the lumbar or periarterial nerves at various frequencies. The rabbit had previously been treated with reserpine.

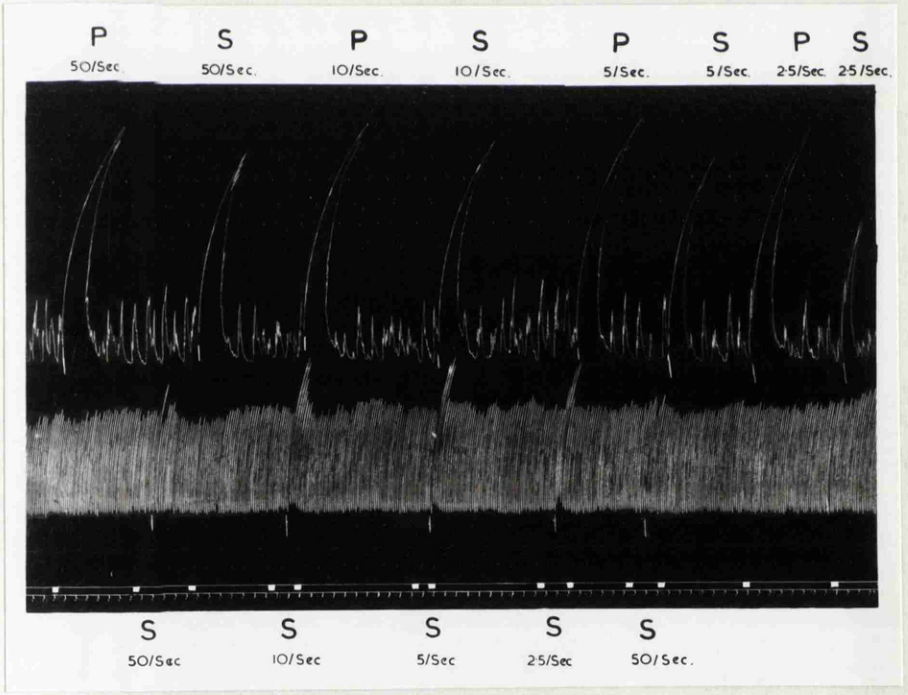


Fig. 19. The effect of various frequencies of stimulation of the parasympathetic (P) and sympathetic (S) nerves to rabbit colon (upper trace) and periarterial (S) nerves to the ileum (lower trace). Both preparations from a rabbit treated with reserpine. The motor response to sympathetic nerve stimulation can still be elicited at frequencies as low as 2.5 P/sec. Time = 30 sec.

The response to pelvic nerve stimulation, as was expected, was highly sensitive to low frequencies and remained near maximal until a frequency of 2.5 P/sec was used, when there was a slight decrease in the response. The response of the colon to lumbar nerve stimulation, however, showed a change in its frequency sensitivity and was now as sensitive as the parasympathetic to low frequencies: for example, the maximum response is seen at 10 P/sec, and the response at 2.5 P/sec is still marked.

A similar change in the range of effective frequencies is seen in the ileum. The frequency spectrum in the ileum from a normal rabbit is shown in Fig. 20. The maximum responses appear at about 50 P/sec. At 10 P/sec, there is a large reduction and at 5 P/sec there is no longer an observable response. In contrast, the motor response, which in the reserpine treated rabbit replaces inhibition, is maximal at 10 P/sec and still well marked at 2.5 P/sec.

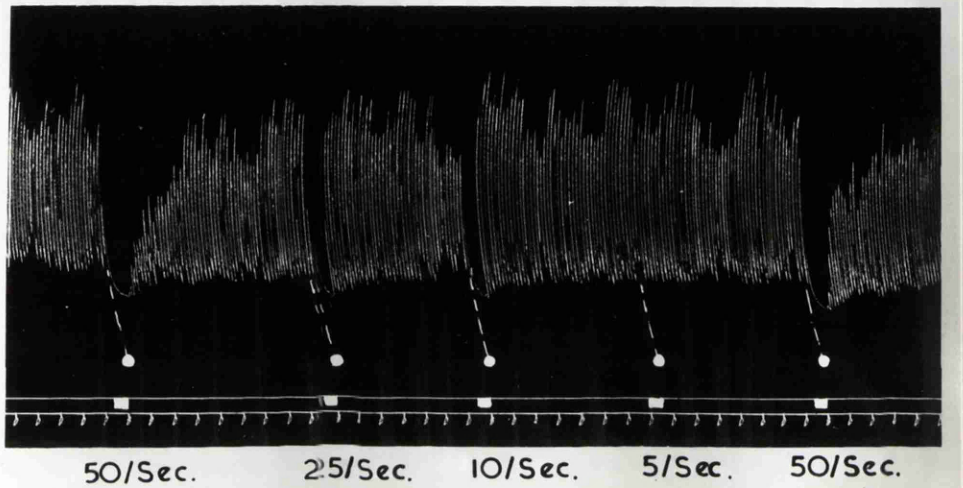


Fig. 20. The response of normal rabbit ileum to stimulation of its periarterial nerves at various frequencies. There is no response at 5 P/sec. Compare with Fig. 19.

These results provide further support for the idea that the fibres mediating the motor response to sympathetic nerve stimulation after reserpine are similar to the normal parasympathetic fibres.

Origin of motor fibres

Having thus established that treatment of rabbits with reserpine uncovers a motor response to sympathetic nerve stimulation both in the ileum and in the colon, and that this motor response is apparently due to stimulation of preganglionic cholinergic fibres, it was decided to try to establish the origin of these fibres.

In the ileum both sympathetic and parasympathetic fibres run in the mesentery and both may be stimulated as periarterial nerves. Motor responses are therefore to be expected and have been reported occasionally without reserpine or other drug treatment (Finkleman, 1930). In the colon, however, previous investigations have failed to produce anything but inhibition on sympathetic nerve stimulation, results which were interpreted as meaning that there were no cholinergic fibres present in the sympathetic outflow

(Garry & Gillespie, 1955). The motor response of the colon, due apparently to cholinergic fibres, was therefore both unexpected and, unlike that in the ileum, inexplicable on existing theories; so this region was chosen for further investigation of the source of these motor fibres.

No further experiments were done on the source and nature of the fibres responsible for the motor response in the ileum since vagal fibres are undoubtedly present in the mesentery.

In the colon, the first line of approach was to see if the known parasympathetic nerves (the pelvic nerves) were the source of the fibres. To this end, the pelvic nerves were cut at a preliminary aseptic operation - see 'Methods' - and fourteen days allowed for degeneration. During the last two days of this period, the rabbit was given daily intravenous injections of reserpine, then killed, and the responses of the in vitro colon preparation examined to see if the motor response to sympathetic nerve stimulation was still present.

The second line of approach was to set up an innervated colon preparation from a reserpine treated rabbit in vitro and to stimulate the pelvic nerves at high frequencies and for long periods. It was hoped in this

way to exhaust the pelvic nerves and, if these fibres were responsible for the motor response to sympathetic nerve stimulation, then this latter response would also be reduced.

Effect of pelvic nerve section. It was found difficult at operation to cut all branches of both pelvic nerves. Access through the sciatic notch is limited and occasionally filaments of origin, especially from lower sacral segments, are missed. Even if only one filament of one pelvic nerve is left uncut, then, at the subsequent *in vitro* experiment, stimulation of the cut peripheral ends of the pelvic nerves still produces a contraction of the colon. This contraction is, in some cases, as big as one would expect in a preparation which had not been operated upon. It was therefore a very necessary control in each experiment to stimulate the cut ends of both pelvic nerves, plus any suspicious filaments in the adjacent mesentery, to prove that degeneration was complete. As a control that the treatment by reserpine was effective and had produced reversal of the response to sympathetic nerve stimulation, an innervated ileal preparation from the same operated animal was set up at the same time as

the colon preparation.

The results of these experiments were as follows. If, after section and degeneration, there was no response to stimulation of the cut ends of the pelvic nerve, then stimulation of the lumbar colonic nerves produced no response. If a small remnant of a pelvic response was present, presumably because of partial section of the pelvic nerves, then a small motor response to sympathetic nerve stimulation was also present. In no instance was this motor response greater than the remnant of the pelvic response; i.e., the normal relationship was preserved.

The impression obtained from these experiments was that reduction or abolition of the response to pelvic nerve stimulation produced a corresponding reduction or abolition of the response to lumbar nerve stimulation.

Effect of prolonged pelvic stimulation. The colon from a rabbit, treated previously with reserpine, was set up *in vitro* with the fluid electrodes on the pelvic and lumbar nerves connected to separate stimulators. The subsequent sequence of events is illustrated in Fig. 21.

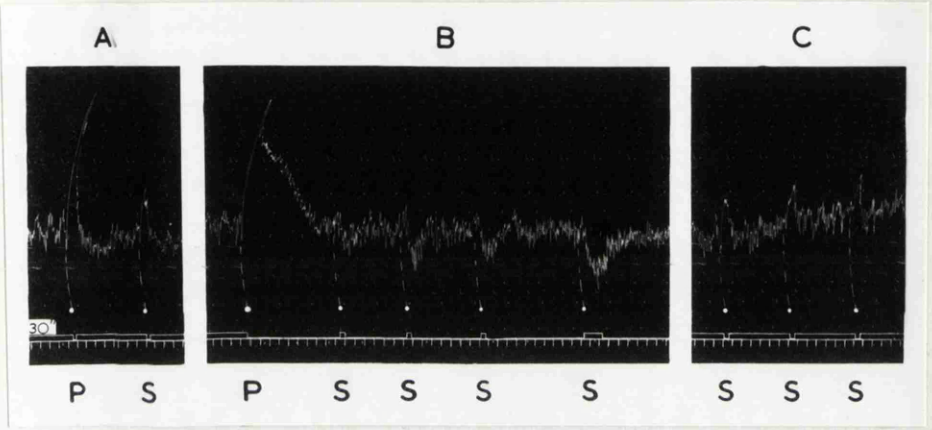


Fig. 21. The effect of fatigue of the parasympathetic nerve on the motor response of the rabbit colon to sympathetic nerve stimulation. The rabbit had been previously treated with reserpine. A shows the motor response to both parasympathetic (P) and sympathetic (S) nerve stimulation. In B, stimulation of the parasympathetic nerve is begun near the beginning of the panel and continued to the end. Interpolation of four short periods of sympathetic nerve stimulation demonstrates the reappearance of an inhibitory response. After an interval of 10 min on recovery from pelvic fatigue the motor effect of sympathetic nerve stimulation is restored (C).

The pelvic and sympathetic nerves were first stimulated independently to demonstrate that reserpine had reversed the sympathetic response to contraction (21 A). The pelvic nerve was then stimulated again (21 B) and stimulation continued until the response had fallen to a low, steady level, just slightly greater than the background spontaneous

activity. At this point repetitive interpolation of short periods of sympathetic nerve stimulation shows that the previous motor response has disappeared and that in fact the normal response - inhibition - is restored. On stopping pelvic nerve stimulation and allowing time for recovery, the motor response to sympathetic nerve stimulation characteristic of the reserpine treated state reappears (21 C).

Thus exhaustion of the pelvic nerves produces exhaustion of the motor fibres in the lumbar nerves, which suggests that the origin of the motor fibres activated by stimulation of the sympathetic nerves after treatment with reserpine is the parasympathetic nerves. It appears, moreover, that there are fibres in the lumbar nerves, even after treatment with reserpine, which can still produce inhibition, and they can be uncovered if the motor component is first exhausted.

Effect of other sympathetic blocking agents. The results of pelvic nerve section and stimulation of the pelvic nerves to fatigue suggest that the pelvic parasympathetic fibres are part of, or the entire, nerve pathway involved in these sympathetic motor responses after reserpine.

The simplest explanation is that some pelvic fibres ascend the hypogastric nerves to join the sympathetic lumbar colonic nerves. If this be so, then the parasympathetic fibres form the entire pathway for the sympathetic motor response. Thus any sympathetic blocking agent which can be shown to block the inhibitory effect of sympathetic nerve stimulation, without affecting the response to pelvic nerve stimulation, ought to uncover the same motor sympathetic response that is so easily demonstrated after reserpine. Experiments were carried out to test this idea.

Three sympathetic blocking agents were used. First, ergotamine, because of the report by Hawkins & Paton (1958) that it blocked the inhibitor action of nicotine on the bronchial smooth muscle. Secondly, tolazoline ('Priscol') because of a report by Varagić (1956) that it blocked the inhibitor response of the rabbit colon to sympathetic nerve stimulation. Thirdly, TM 10, because of our previous experience with the drug, and the report by Bain & Fielden (1956) describing its ability to block the inhibitory effect of sympathetic nerve stimulation in the small intestine. None of these drugs uncovered motor responses.

The effects of TM 10 on the responses of the colon

preparation from a normal rabbit to sympathetic and parasympathetic nerve stimulation, as well as to the appropriate transmitters, noradrenaline and acetylcholine, are shown in Fig. 22.

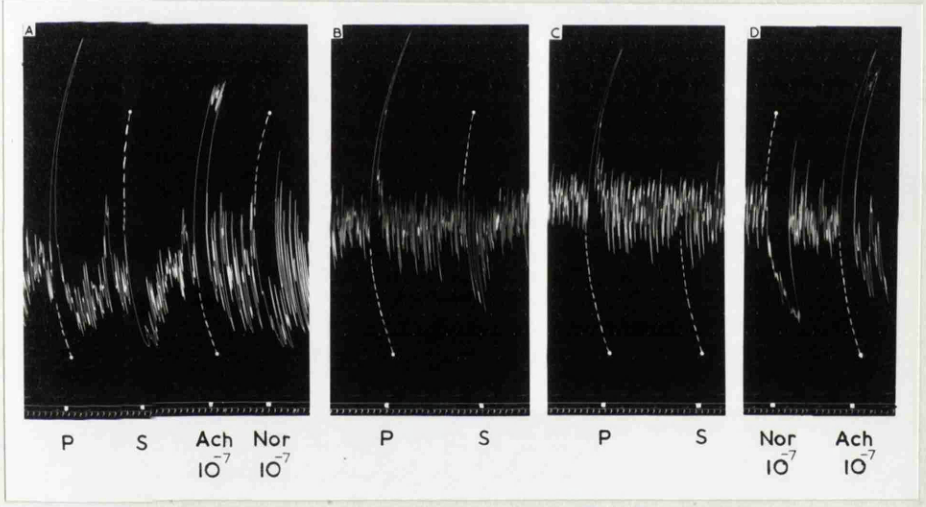


Fig. 22. The effect of TM 10 on nerve and drug stimulation of the normal rabbit colon. A, responses to stimulation of the parasympathetic (P) and sympathetic (S) nerves and to acetylcholine (Ach) and noradrenaline (Nor). Between A and B, TM 10 was added to the bath to produce a concentration of 10^{-5}. B shows the responses to nerve stimulation 20 min and C, 70 min after adding the drug. The inhibitory effect of sympathetic nerve stimulation is abolished with only slight reduction in the parasympathetic motor response. D. The response to acetylcholine is little changed, while that to noradrenaline is enhanced. Time = 30 sec.

TM 10 blocked the inhibitory response to lumbar colonic nerve stimulation without uncovering a motor response (22 C) and without affecting the responses to pelvic nerve stimulation, to acetylcholine or to noradrenaline (22 D).

'Priscol' proved an unsatisfactory drug for the present investigations. Its actions on the innervated rabbit colon preparation are shown in Fig. 23.

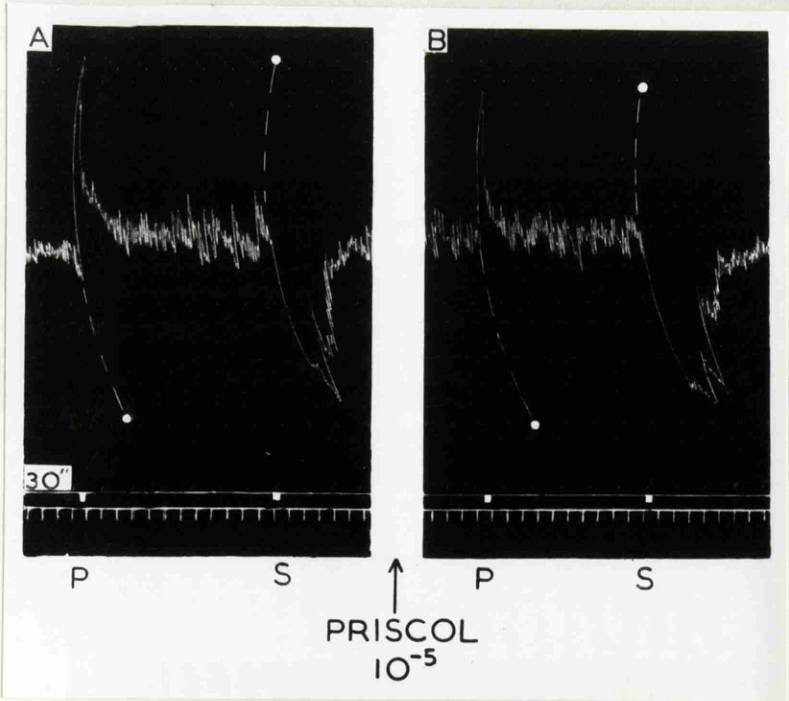


Fig. 23. The effect of 'Priscol' on the response of the normal rabbit colon to stimulation of its extrinsic autonomic nerves. A shows the response to parasympathetic (P) and sympathetic (S) nerve stimulation before the addition of 'Priscol' 10^{-5}. B shows the responses after 'Priscol'. 'Priscol' at this concentration has reduced the parasympathetic response, while the sympathetic inhibitor response is, if anything, enhanced.

A concentration of 10^{-5} tolazoline reduced the pelvic motor response and, if anything, potentiated the inhibitory response to sympathetic nerve stimulation. Higher concentrations

might reduce the inhibitory response, but interpretation would be difficult since the pelvic response would be even more severely affected.

The effect of 'Priscol' on the response of the ileum to stimulation of its periarterial nerves is illustrated in Fig. 24.

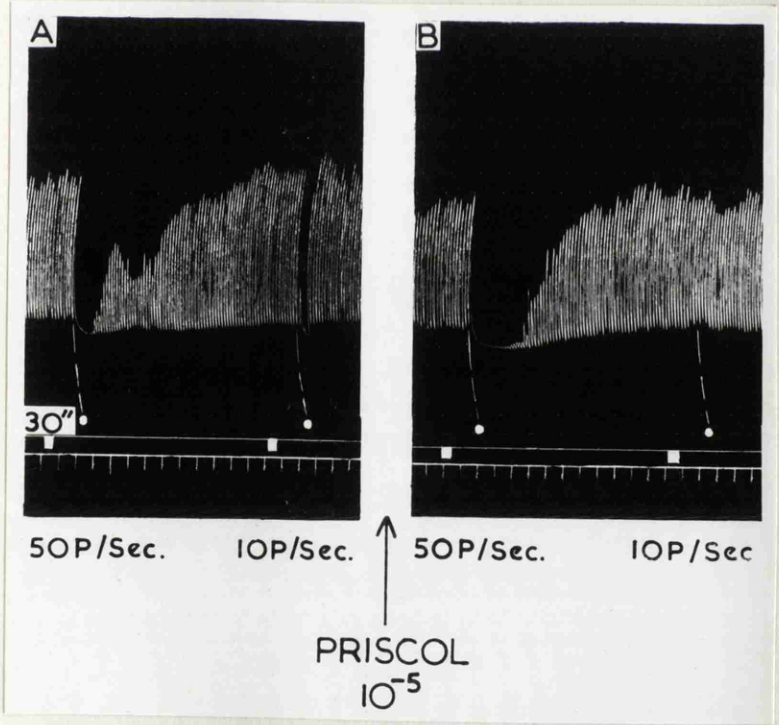


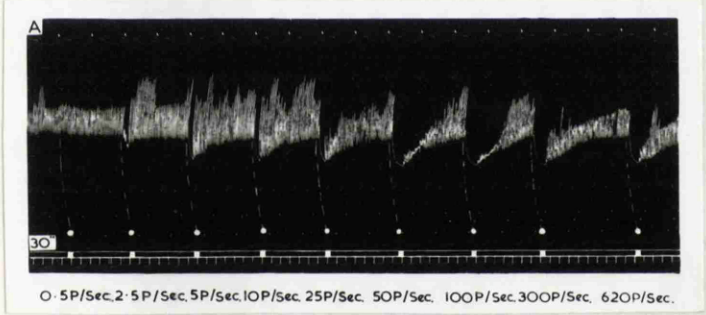
Fig. 24. The effect of 'Priscol' on stimulation of the sympathetic nerves to a normal rabbit ileum. A shows stimulation of the nerves before the addition of 'Priscol' 10^{-5}. B shows the responses after 'Priscol'. No motor response is seen after 'Priscol'.

The effects of this drug on the ileum are somewhat paradoxical. Initially, stimulation at 10 P/sec and 50 P/sec produces inhibition (24 A). After the addition of 'Priscol' 10^{-5} the inhibitory response at 50 P/sec is

greater, but stimulation at 10 P/sec is now ineffective.

The effect of varying the frequency of stimulation of the periarterial nerves on the action of 'Priscol' was then studied, using a wider range of frequency. The results of such an experiment are shown in Fig. 25.

BEFORE 'PRISCOL'



AFTER 'PRISCOL'

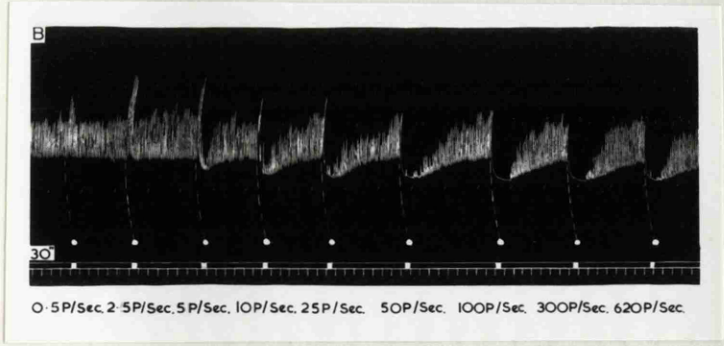


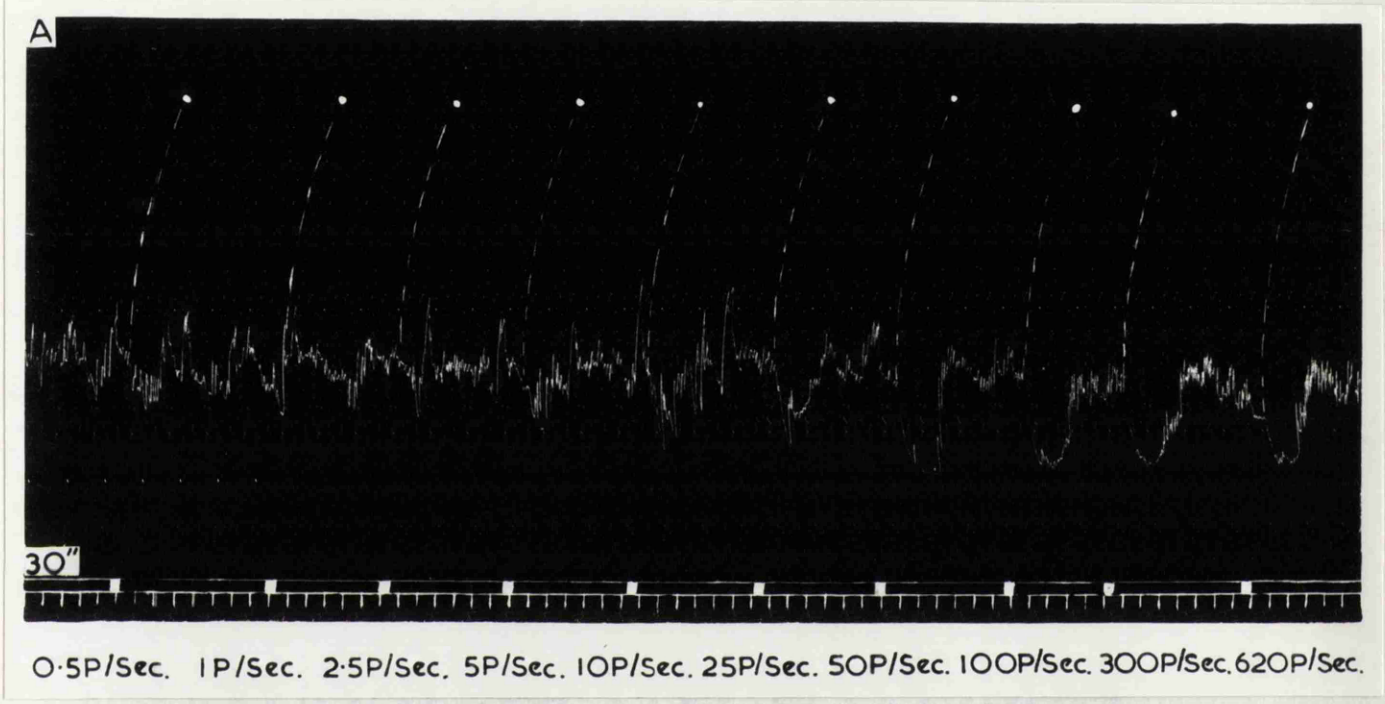
Fig. 25. The effect of 'Priscol' on the response of the periarterial nerves to a normal rabbit ileum. A shows the response of the ileum to various frequencies of stimulation from 0.5 P/sec to 620 P/sec. Between A and B, 'Priscol' was added to the bath fluid to produce a concentration of 10^{-5}. B shows the responses after 'Priscol'. For description see text.

Initially, all frequencies that produced a response produced an inhibitory response. After 'Priscol' 10^{-5} was added, low frequencies of stimulation produced a motor response, intermediate frequencies produced a biphasic

response and the higher frequencies, 50 P/sec and above, produced a purely inhibitory response, usually greater than that before the addition of 'Priscol'.

Since in the ileum the ability of 'Priscol' to unmask the motor effect was very dependent on frequency, the possibility of a similar effect in the colon was investigated by varying the frequency of stimulation of the lumbar colonic nerves before and after the addition of 'Priscol'. The results of one experiment are shown in Fig. 26.

BEFORE 'PRISCOL'



AFTER 'PRISCOL'

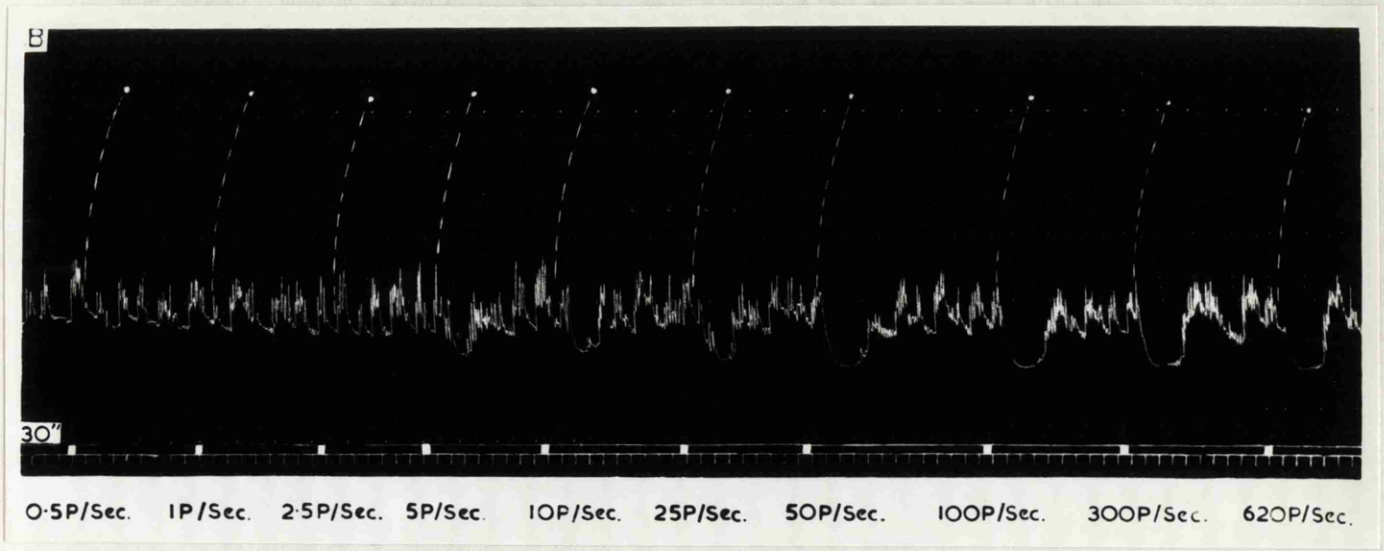


Fig. 26. The effect of 'Priscol' on various frequencies of stimulation of the sympathetic nerves to a normal rabbit colon.
A shows the responses of a rabbit colon to various frequencies of stimulation of the sympathetic nerves before 'Priscol' 10^{-5}.
B shows the responses after 'Priscol'.

No motor response to sympathetic (lumbar colonic) nerve stimulation appeared at any frequency between 0.5 P/sec and 620 P/sec before or after the addition of 'Priscol' 10^{-5}. In fact, after the addition of this drug, all the inhibitory responses appeared prolonged, a result in agreement with those in the ileum at high frequencies of stimulation.

The effect of 'Priscol' on the response to stimulation of the sympathetic nerves to the colon at all frequencies, and of the ileum at high frequencies, was to enhance the inhibitory response. Potentiation by low concentrations of blocking agents has been observed by several workers and for a variety of agents (Jang, 1941; Holzbauer & Vogt, 1954). This phenomenon could be due to a potentiation of the receptor cells to the adrenergic transmitter by the blocking agent. On the other hand, a possible mechanism has been described by Brown & Gillespie (1957) in which the blocking agent prolongs the life of the transmitter in the neighbourhood of the receptors.

The sensitivity of the smooth muscle to adrenaline and its precursors was examined before and after 'Priscol' and the results of one experiment are illustrated in Fig. 27.

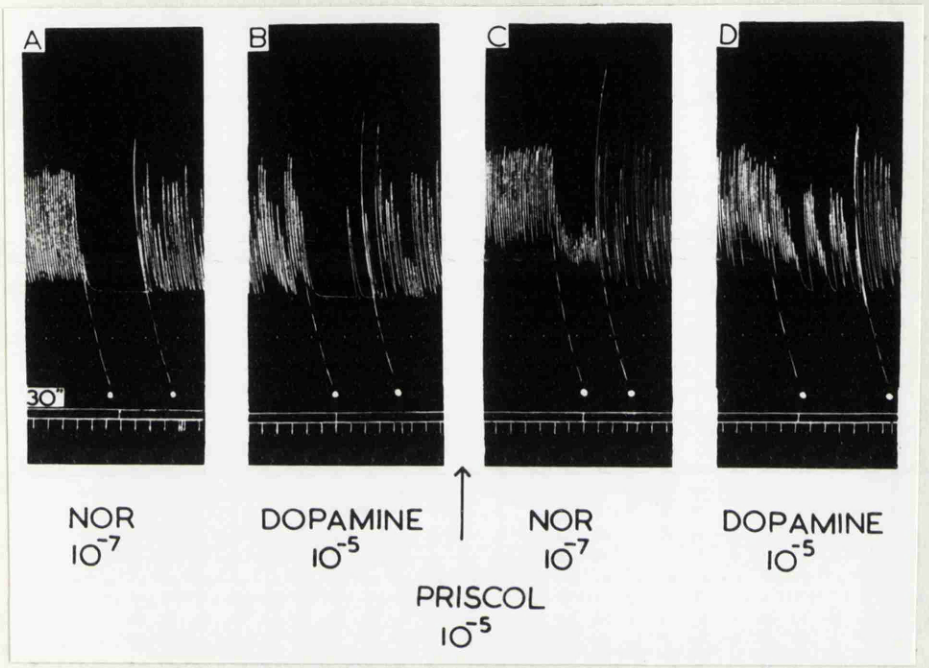


Fig. 27. The effect of 'Priscol' on the response of the normal rabbit ileum to noradrenaline and dopamine. Both responses are reduced by 'Priscol'.

The inhibitory response of both noradrenaline and dopamine was reduced by the presence of 'Priscol'. How this can be reconciled with the results of nerve stimulation in the presence of 'Priscol' is not clear. However, it is a similar phenomenon to that reported by Bacq & Monnier (1935) and Jang (1941).

The effect of ergotamine tartrate on the response of the rabbit colon to stimulation of its extrinsic autonomic nerves is illustrated in Fig. 28.

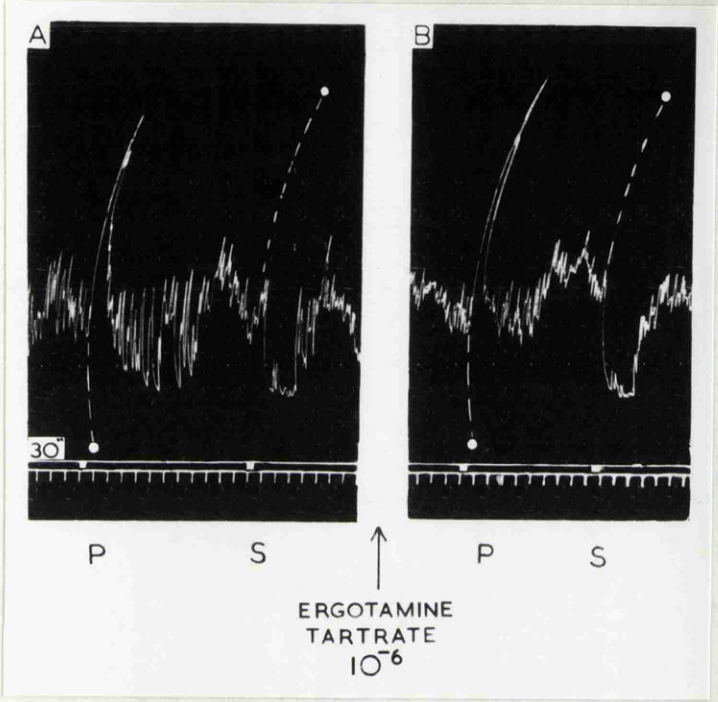


Fig. 28. The effect of ergotamine tartrate on the responses of the colon from a normal rabbit to stimulation of the parasympathetic (P) and sympathetic (S) nerves. There is no motor response to stimulation of the sympathetic nerves after ergotamine.

At low concentrations, this drug potentiated the response to both sympathetic and parasympathetic nerves. Higher concentrations were impossible to get into solution and the effects of this drug were not studied further.

RESTORATION OF INHIBITION

Adrenaline and noradrenaline. At some point in each experiment, noradrenaline was added to the bath to show that, after reserpine treatment, the direct response of the smooth muscle to the adrenergic transmitter remained inhibitory. Since the interesting point was the qualitative nature of the response, the concentration of noradrenaline used was variable and, on occasions, concentrations as high as 10^{-5} were added. It was sometimes noted, after washing out this noradrenaline and restoring rhythmic activity and tone, that, on stimulating the sympathetic nerves, their inhibitory effect was restored. Restoration of this inhibitory effect of sympathetic nerve stimulation could not be demonstrated in every preparation. Sometimes after exposure to, and subsequent removal of, noradrenaline, although the motor response of the colon was abolished, inhibition did not reappear.

Restoration of the inhibitory effect of sympathetic nerve stimulation after soaking in noradrenaline 5×10^{-6} for 15 min is illustrated in Fig. 29. This exposure to noradrenaline had no effect on the motor response to parasympathetic nerve stimulation.

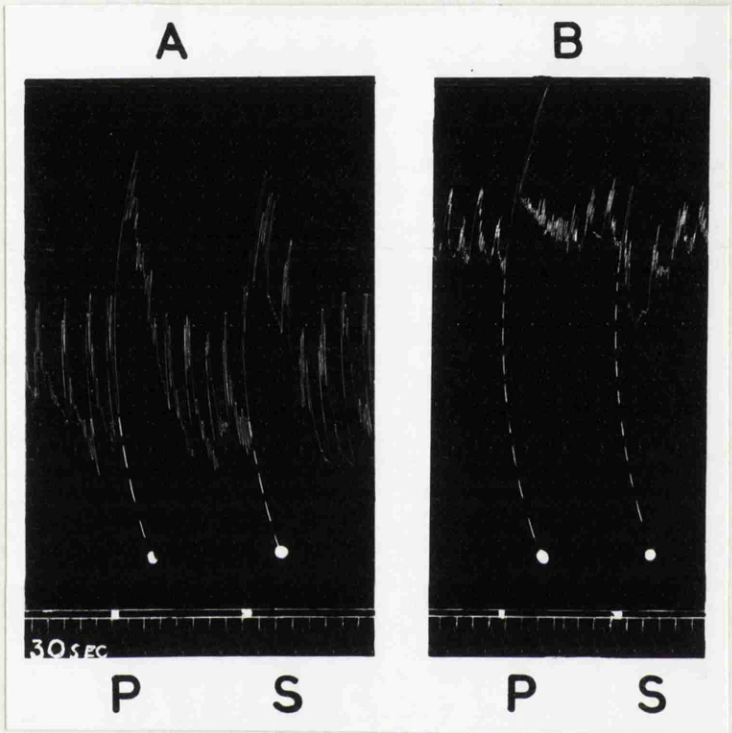


Fig. 29. Preparation of the rabbit colon from an animal previously treated with reserpine.
A shows the motor responses to stimulation of the parasympathetic (P) and sympathetic (S) nerves, before soaking in noradrenaline 5×10^{-6} for 15 mins.
B shows the restoration of the inhibitory effect of sympathetic nerve stimulation.

The response of an ileal preparation to stimulation of the periarterial nerves at two different frequencies before and after soaking in noradrenaline is shown in Fig. 30.

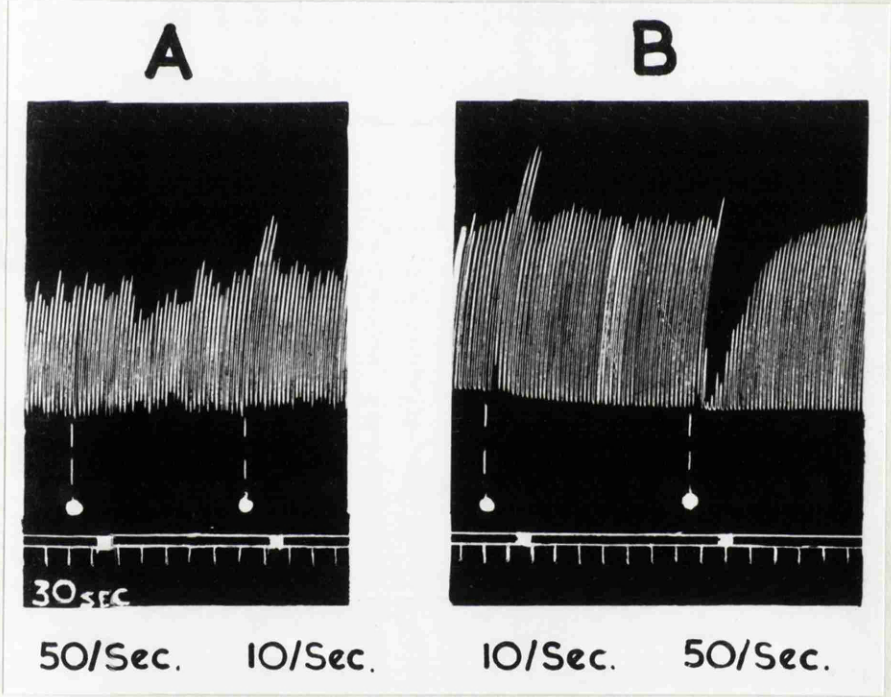


Fig. 30. Preparation of the rabbit ileum from a rabbit treated previously with reserpine. A shows the response to periarterial nerve stimulation at 50 P/sec and 10 P/sec. Between A and B, noradrenaline in a concentration of 5×10^{-6} was added to the bath for 15 min and then removed.
B shows the restoration of the inhibitory effect of nerve stimulation at 50 P/sec.

Initially there was no definite response to stimulation at 50 P/sec and a motor response at 10 P/sec. After soaking the preparation in noradrenaline, a good inhibition was obtained at a frequency of 50 P/sec, but the response to stimulation at 10 P/sec remained motor. Restoration of inhibition, therefore, was seen only at the optimal frequency of stimulation for the sympathetic nerves.

The results obtained with adrenaline were similar to those with noradrenaline. Restoration was obtained as easily with the one amine as with the other. In this respect, these results differ from those of Burn & Rand (1960 a) investigating the restoration of the pressor effect of tyramine in the cat and rat after reserpine. These authors found that noradrenaline, but not adrenaline, would restore the pressor effect of tyramine.

Effect of varying the concentration of noradrenaline. Experiments were carried out to find the optimal concentration of noradrenaline to restore the inhibitory response to lumbar colonic nerve stimulation. Four adjacent lengths of ileum from a rabbit previously treated with reserpine were suspended in separate isolated organ baths. Each length

of ileum was soaked for 30 min in one of the following four concentrations of noradrenaline, 5×10^{-7}, 5×10^{-6}, 10^{-5}, or 5×10^{-5}, and the noradrenaline then washed out. Time was allowed for the intrinsic activity of the preparation to recover from the action of noradrenaline before the periarterial nerves were stimulated.

All four concentrations restored the inhibitory effect of periarterial nerve stimulation. The inhibitory effect following 5×10^{-7} was small; at 5×10^{-6} it was greater; but still higher concentrations were no more effective. As a result, in all subsequent experiments, a concentration of 5×10^{-6} of noradrenaline was used rather than 10^{-5}.

Effect of duration of exposure. A similar experiment was carried out to determine the optimal length of time to soak the preparation in the solution of noradrenaline. Again, four adjacent lengths of ileum were used. Noradrenaline in a concentration of 5×10^{-6} was left in contact with the preparation for 5 min, 15 min, 30 min and 60 min respectively. The reversal of the response to periarterial nerve stimulation was greater after

15 min than after 5 min, but exposure for periods greater than 15 min did not produce any further increase in inhibition. On the basis of these experiments, a minimum time of 15 min was allowed for soaking in all subsequent experiments.

Fatigue of the restored inhibition. The inhibitory effect of sympathetic nerve stimulation, restored by soaking in adrenaline or in noradrenaline, was easily fatigued. This was best seen if the nerves were continuously stimulated at a frequency of 50 P/sec for relatively long periods. In a preparation of normal ileum, periarterial nerve stimulation for 5 min caused complete inhibition for the entire period of stimulation, with no evidence of 'escape'. This is illustrated in the upper trace of Fig. 31.

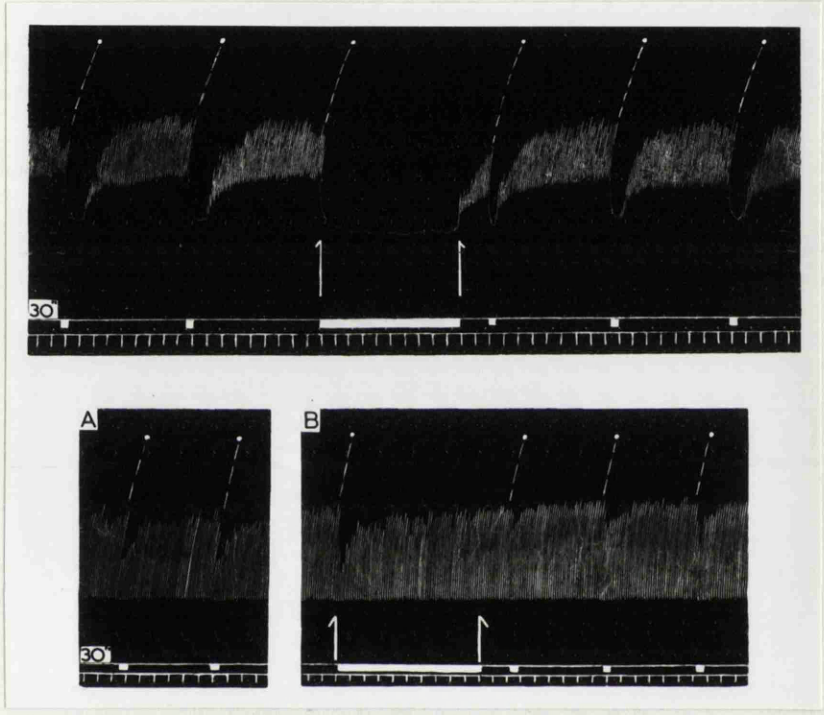


Fig. 31. The upper trace is from a preparation of normal rabbit ileum and shows the responses to short periods of nerve stimulation and to 5 min stimulation at 50 P/sec (Between arrows). There is no escape from inhibition during the 5 min period. The lower traces, A and B, show the responses of the ileum from a rabbit previously treated with reserpine. The inhibitory effect of periarterial nerve stimulation has been restored by soaking in noradrenaline. The ready fatigue during prolonged stimulation at 50 P/sec and the recovery with rest is shown.
For further description see the text.

Some decline in transmitter output probably does occur as the stimulation proceeds. This is shown by giving short test periods of stimulation before, and then at intervals after the prolonged period of stimulation. The inhibitory effect of such short test periods of stimulation, immediately after a period of prolonged stimulation, is diminished but recovery takes place over some minutes (Fig. 31, upper trace). In similar preparations of ileum from a reserpine treated rabbit in which the inhibitory response to sympathetic nerve stimulation has been restored by soaking in noradrenaline, inhibition is no longer maintained throughout prolonged stimulation (Fig. 31, lower trace, A & B). Short test periods of stimulation following the end of the prolonged stimulation show, however, that the nerves regain their inhibitory effect with a time course similar to that of the normal preparation.

From these experiments, it seems that the catechol amine reincorporated into the nerve endings is not all immediately available for release. Fatigue can occur with loss of any visible effect of nerve stimulation at a time when the transmitter is still present in the nerve endings.

Dopamine. Having established that noradrenaline and adrenaline could restore the inhibition produced on stimulating the lumbar nerves to the colon, it seemed of interest to investigate whether or not the precursors of these amines would do likewise. The precursors of adrenaline are illustrated in Fig. 32.

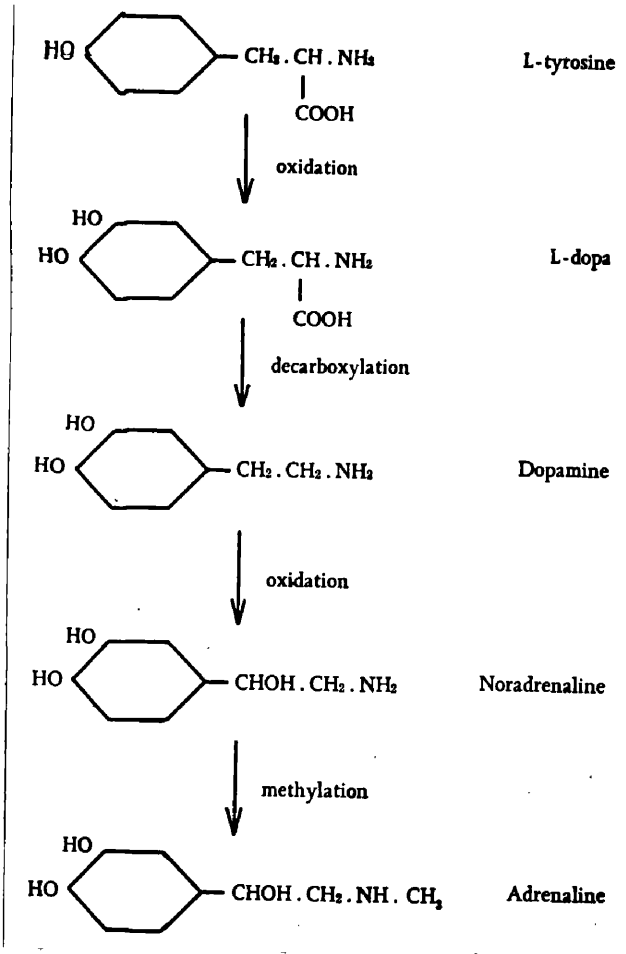


Fig. 32. The relationship of adrenaline to its precursors.

The immediate precursor of noradrenaline - dopamine - was found to be as effective as noradrenaline in restoring the inhibitory effect of the sympathetic nerves. The results of one such experiment are illustrated in Fig. 33.

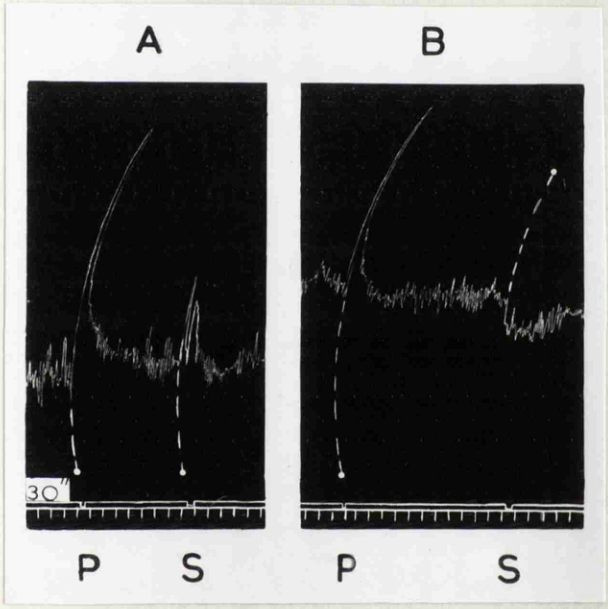


Fig. 33. Restoration in a reserpine treated rabbit of the inhibitory response to stimulation of the sympathetic nerves to the colon.
A, the motor responses to parasympathetic (P) and sympathetic (S) nerve stimulation. Between A and B the preparation was soaked in dopamine 10^{-4} for 90 min. After washing, the response to sympathetic nerve stimulation is inhibition.

Dopa. The effect on pelvic and lumbar nerve stimulation of soaking the colon of a rabbit, previously treated with reserpine, in dopa, is shown in Fig. 34.

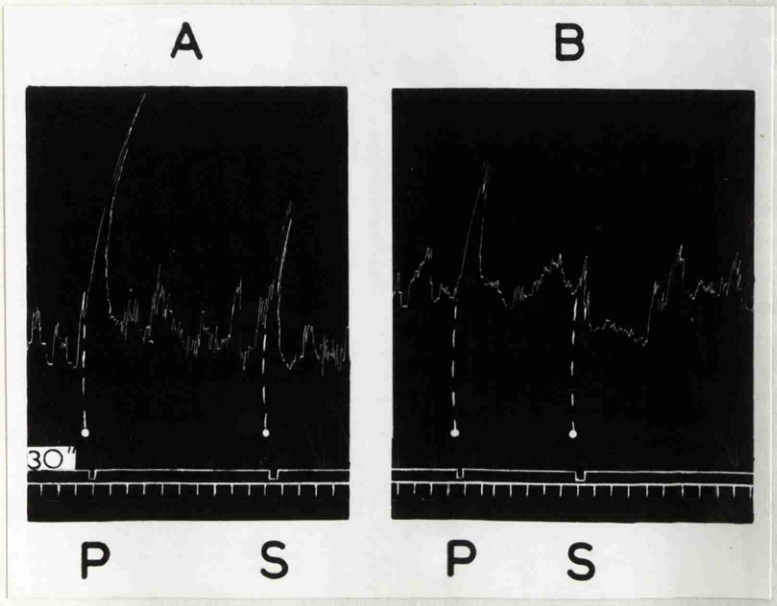


Fig. 34. Restoration of the inhibitory response of the rabbit colon to sympathetic nerve stimulation by soaking in dopa.
A shows the response to stimulation of the parasympathetic (P) and sympathetic (S) nerves. The animal had been treated previously with reserpine. Between A and B, dopa 10^{-5} was added to the bath, left in contact for 60 min and then washed out. After washing, the response to sympathetic nerve stimulation is inhibition.

The motor response to stimulation of the pelvic nerves remained unaltered, while the motor response to stimulation of the lumbar nerves was reversed to relaxation. This happened in several, but not all, experiments, and it was felt that dopa was not as effective as dopamine or noradrenaline in restoring the inhibitory effect.

It is interesting that dopa, in the concentration used in this experiment, has by itself no inhibiting effect on the colon. Consequently the sympathetic nerves, when the inhibition is restored, must be liberating something other than dopa. The most obvious explanation is that the sympathetic nerves are still able to synthesise transmitter if the precursors, dopa or dopamine, are available. If this is so, there has been depletion by reserpine not only of the final transmitter but also of some or of all of the precursors.

Tyrosine. L-tyrosine was used in these experiments. It is difficult to dissolve this drug and it was not possible to make up a concentrated solution, a small volume of which could be added to the 200 ml. inner vessel. Consequently, 10 mg were added to 1 litre of Krebs' saline

and 200 ml. of this solution warmed to 37°C were added. In this way, 10^{-5} l-tyrosine was added to the preparation. However, since 10 mg is very difficult to trace in a litre of solution, it is difficult to know if it all dissolved. Consequently, it is difficult to be sure of the absolute concentration added to the preparation.

Several colon and ileum preparations from reserpine treated rabbits were left in contact, for various times, with l-tyrosine. No reversal of the response to periarterial nerve stimulation or lumbar nerve stimulation was noted in any experiment.

THE ACTION OF RESERPINE IN VITRO

The method used in the above experiments to administer the reserpine i.e. intravenous injection for several days, was inconvenient for several reasons. It was not possible to demonstrate normal inhibitory responses to sympathetic nerve stimulation before the action of the drug. It was never possible to reverse the effect of reserpine other than by soaking in catechol amines. Lastly, the intravenous injections required the use of large quantities of the drug. To attempt to overcome these difficulties, reserpine was added directly to the bath fluid in which a preparation from a normal rabbit was suspended, to see whether the drug under such circumstances would reverse the effect of the sympathetic nerves from inhibition to contraction. Reserpine, pure substance, was used in these experiments. In this form, the reserpine is only sparingly soluble in Krebs' saline. A stock solution of 1 mg/ml. was made up in a 10% solution of ascorbic acid and one or two ml. of this added to the 200 ml. bath to produce the final desired concentration of 5×10^{-6} or 10^{-5}. Care had to be taken in gassing the final solution. If the usual sintered glass oxygenator, producing a large number of small bubbles

was used, then the reserpine came out of solution as a white scum on the surface of the solution. Thus a simple glass tube was used for oxygenation.

The effect of ascorbic acid in the same concentration as would accompany reserpine in later experiments was studied as a control. Ascorbic acid 5×10^{-4} (1 ml. of a 10% solution) left in contact with the <u>in vitro</u> colon preparation for 30 min had no effect on the response to stimulation of either sympathetic or parasympathetic nerves. Usually there was some increase in the spontaneous activity and tone of the preparation. When reserpine, dissolved in a solution containing ascorbic acid, was added to the bath, quite different effects were produced, and these could be divided into two groups - the early effects and the late effects.

After the addition of reserpine, a steady decline in the rhythmic activity and tonus of the preparation occurred. At the same time, the responses to parasympathetic nerve stimulation, to sympathetic nerve stimulation and to the transmitters, acetylcholine and noradrenaline, were greatly reduced. Eventually an atonic, inactive and almost unresponsive preparation was obtained (Fig. 35). These are the early effects of reserpine and are quite

non-specific, affecting both divisions of the autonomic nervous system and their transmitters equally.

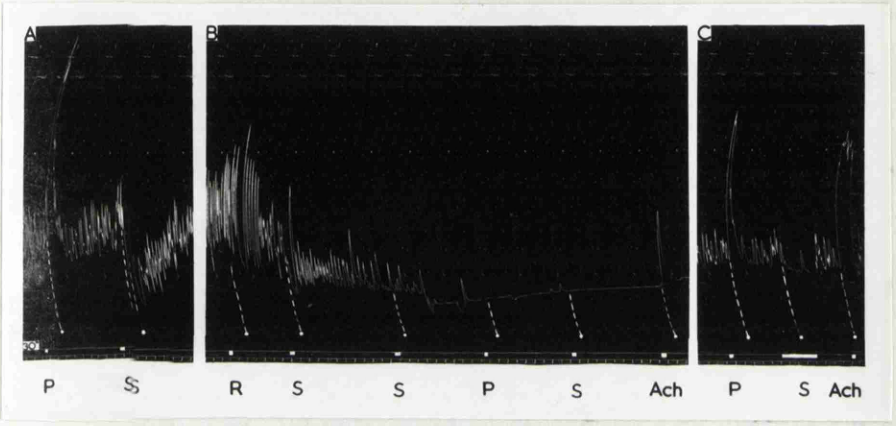


Fig. 35. The early effects of reserpine in vitro on rabbit colon.
A shows the response to stimulation of parasympathetic (P) and sympathetic (S) nerves. B at (R) reserpine in a concentration of 2.5×10^{-5} was added. Sympathetic and parasympathetic nerve stimulation were now ineffective and the response to acetylcholine (Ach) was poor. Between B and C, the preparation was washed. C, the response to stimulation of both autonomic outflows and the response to acetylcholine were restored.

The early effects of reserpine are reminiscent of the effects of anoxia (Garry, 1928; Garry, Gillespie & Pickering, unpublished). The rate of onset of these effects is dependent on the dosage of reserpine. With a concentration of 2.5×10^{-5}, for example, complete inactivity is reached in 12 min (Fig. 35), whereas with

5×10^{-6} it requires 150 min to reach a similar stage (Fig. 36). The early effects of reserpine are completely reversible and, on washing out the drug, the responses to stimulation of both nerve divisions and their chemical transmitters are restored: the action of the sympathetic nerves remains inhibitory (Fig. 36).

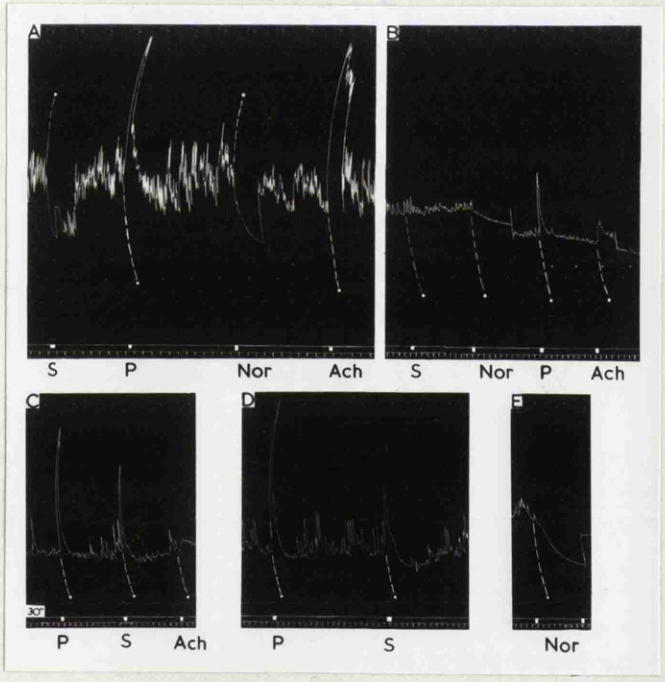


Fig. 36. The early and late effects of reserpine added *in vitro* to a rabbit colon preparation.
A shows the response of the normal colon to parasympathetic (P) and sympathetic (S) nerve stimulation; also the response to noradrenaline (Nor) and to acetylcholine (Ach). Between A and B, reserpine (5×10^{-6}) was added to the bath. B shows the early effects of reserpine - reduction of all responses. C, 4 hours after adding the reserpine the response to sympathetic nerve stimulation is reversed to contraction. D, the reversal remains after washing out the reserpine. E, the response to noradrenaline remains inhibitory.

If reserpine is left in the bath, then the late effects appear. In three to four hours, the rhythmic activity reappears as intermittent groups of spike-like contractions. Coincident with this, the motor response to stimulation of the pelvic nerves increases. At this time, the first sign of a specific effect on the sympathetic nerves appears. Stimulation of the lumbar nerves now causes contraction. This stage is illustrated in Fig. 36(C). A curious feature, for which there is no ready explanation, is that, in spite of the partial recovery of the pelvic nerve response, the smooth muscle at this stage shows no improvement in its response to acetylcholine. If now the reserpine is removed by washing, the rhythmic activity and tone return almost to their previous level; the contraction to pelvic nerve stimulation improves still further; acetylcholine and noradrenaline regain their ability to cause contraction and inhibition respectively, but the reversal of the response to sympathetic stimulation from inhibitor to motor persists for the remainder of the experiment. These late effects of reserpine correspond to the effects produced by reserpine when given by intravenous injections.

Effect of ascorbic acid. Preliminary experiments showed that ascorbic acid added to the bath and left up to 30 min, although increasing activity, did not alter the response to stimulation of either pelvic or lumbar nerves or the response to acetylcholine or noradrenaline.

At that stage in the investigations, only the early responses to reserpine had been detected, and these appear in less than 30 min so that this seemed at the time an adequate control. The effect of leaving ascorbic acid in the bath for several hours was not, unfortunately, tested until after the late effects of reserpine had been found and investigated.

The result of leaving ascorbic acid in the bath for some hours is illustrated in Fig. 37.

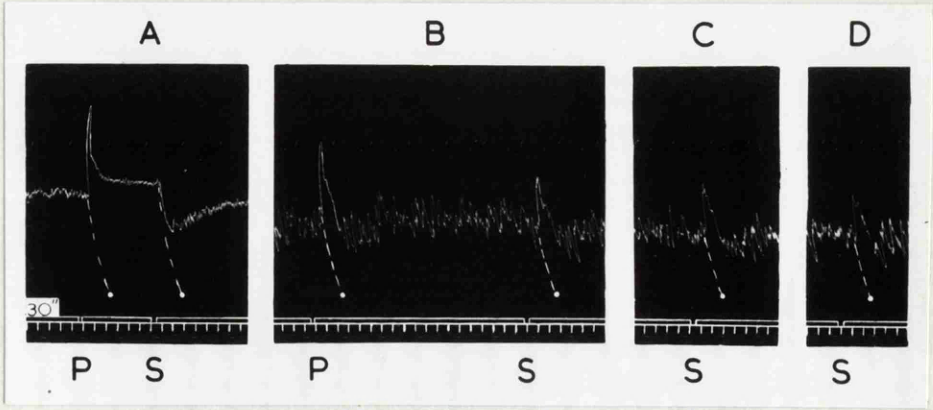


Fig. 37. The effect of ascorbic acid added in vitro to a rabbit colon preparation. A shows the responses of the normal colon to stimulation of its parasympathetic (P) and sympathetic (S) nerves. Between A and B, ascorbic acid was added to produce a concentration of 5×10^{-4} and left in contact for 5 hours. B shows that the response to sympathetic nerve stimulation is now contraction. C and D show that this reversal is stable.

After soaking the preparation in ascorbic acid 5×10^{-4} (1 ml. of 10% solution in 200 ml. Krebs') for 5 hours, the response to pelvic nerve stimulation is unaltered, whereas the response to lumbar colonic nerve stimulation is reversed to motor (Fig. 37B). This reversal is stable even after the ascorbic acid is removed (37C & D).

These results indicate that the reversed response to lumbar nerve stimulation obtained <u>in vitro</u> with reserpine may be due to the ascorbic acid in which the reserpine is dissolved.

This point will be the subject of further investigations: time does not permit inclusion of such work in the present thesis.

The action of reserpine <u>in vivo</u> is not affected by this finding since rabbits injected with the vehicle in which the reserpine is dissolved respond normally to stimulation of their nerves.

Discussion

The conversion by reserpine of the normal inhibitory effect of sympathetic nerve stimulation on the smooth muscle of the rabbit colon to a motor effect, first described in Part I of this thesis, was both an unexpected and a striking finding. The investigation of the underlying mechanism which forms Part II of this thesis has led to the equally unexpected and rather striking explanation that nerve impulses, which start out in sympathetic fibres, can in some unknown way activate the peripheral parasympathetic pathway. Such an unorthodox explanation requires the most careful consideration, first of the possibility of experimental error and, secondly, of alternative explanations.

In the present experiments, the action of reserpine is quite specific in affecting only the response to sympathetic nerve stimulation. The motor response to parasympathetic nerve stimulation and the motor and inhibitory responses to acetylcholine and to noradrenaline respectively, are unaltered. Incidentally, it is worth remarking at this point that there was no evidence of enhanced sensitivity to pelvic nerve stimulation. The implications of this in explaining the predominantly parasympathetic signs and

symptoms in the intact animal, will be considered later.

As the various illustrations in the above results demonstrate, the motor response to sympathetic nerve stimulation showed a remarkable similarity to that from parasympathetic nerve stimulation. From the beginning, some relationship between them was suggested by the observation that, while the response to stimulation of the sympathetic nerves might be almost as large as the response to stimulation of the parasympathetic nerves, it was never larger. Simple pharmacological investigations confirmed this similarity. Both motor responses were equally affected by hexamethonium and by atropine: the relative magnitudes of the responses to the spectrum of frequencies of nerve stimulation were similar. These experiments provided evidence that the fibres in the lumbar nerves, responsible for the motor response, were preganglionic and that the final link in the chain was a cholinergic fibre. They did not necessarily imply that pelvic nerve fibres belonging to the true parasympathetic outflow were involved. However, two further sets of experiments made it clear that the pelvic (parasympathetic) fibres were, in fact, involved and presumably contributed the final cholinergic link in the pathway. The first set of

experiments was that in which the pelvic nerves were cut and allowed to degenerate. Subsequently, after the administration of reserpine, stimulation of the sympathetic nerves failed to produce a motor response. Secondly, in reserpine treated preparations, the motor response to sympathetic nerve stimulation could be abolished by fatiguing the pelvic (parasympathetic) nerves.

How may these results be explained? The first possibility is that the motor response to sympathetic nerve stimulation after reserpine was in all cases due to an escape of current from the electrode on the sympathetic nerves stimulating either the pelvic nerves as they lay in the second electrode or some of the peripheral branches of the pelvic nerves. This would certainly explain the similarity of the two responses, their pharmacological identity and the effect both of nerve section and of fatigue. Such an explanation, however, will not suffice. First, this reversal effect is specific to reserpine. Other drugs, effective in blocking the inhibitory response to sympathetic nerve stimulation, do not uncover a consistent large motor response. TM 10 is one such drug, the results with which are described above. More recent work with two others, guanethidine sulphate and bretylium tosylate, have confirmed

this point (Boyd, Gillespie & Mackenna, unpublished). Secondly, if a tight ligature is tied round the lumbar colonic nerves as they leave the electrode, the motor response after reserpine treatment is abolished, showing that the fibres involved run in the lumbar colonic nerves. Thirdly, preparations dissected out without the pelvic nerves and having only one electrode in the bath, still respond to sympathetic nerve stimulation with contraction. For these reasons, current escape can be excluded as a possible explanation of the motor phenomenon.

Excluding the possibility of experimental error and accepting the results on their face value, what alternative explanations can be offered? The most obvious is that in the sympathetic nerves there are a variable number of cholinergic fibres whose presence is masked normally by the predominantly adrenergic fibres. Such a situation would have many precedents since cholinergic fibres have been demonstrated often in anatomically sympathetic nerves; in the nerves to the tongue (von Euler & Gaddum, 1931); to the sweat glands (Dale & Feldberg, 1934); to the nictitating membrane (Bacq & Fredericq, 1935); to the vessels of the dog's hindleg (Bülbring & Burn, 1935); to the uterus of the bitch

(Sherif, 1935); and to the cat heart (Folkow, Frost, Haeger & Uvnäs, 1948). In the ileum, such an explanation may well apply since there are known cholinergic (vagal) fibres in the mesentery and appropriate stimulation of these, even in the absence of a blocking agent, may produce a motor response (Finkleman, 1930). For this reason, the significance of these motor responses in the ileum after reserpine is difficult to evaluate. While they are considered with the results in the colon, their explanation could be partly or entirely due to the stimulation of normal vagal fibres running in the mesentery.

In the colon, two further possibilities require consideration. First, pelvic parasympathetic fibres may ascend the hypogastric nerve and join the sympathetic nerves. Secondly, there may be true sympathetic cholinergic nerves analagous to those to the sweat glands in the cat which either leave the spinal cord in the sympathetic outflow or, like the spinal parasympathetic fibres described by Kuré, Ichiko & Ishikawa (1931), join the sympathetic outflow later.

There is little evidence in the literature to support either possibility. For example, Langley & Anderson (1896) showed by nerve section and by degeneration that the

pelvic nerve fibres leave the pelvic-hypogastric nerve complex in the sacral colonic nerves without ascending as far as the inferior mesenteric ganglion. In addition, the possibility that cholinergic fibres might be present in the sympathetic nerves to this rabbit colon preparation was specifically investigated by Garry & Gillespie (1955). They showed that cutting the hypogastric nerve just below its origin from the inferior mesenteric ganglion, made no difference to the contraction produced by stimulation of the pelvic nerves, suggesting that few, if any, motor fibres ascend the hypogastric nerves to run finally in the lumbar colonic nerves. Secondly, they examined the effect of different frequencies of stimulation on autonomic nerves. Because of the difference in sensitivity to frequency, it should be possible to detect the presence of cholinergic fibres in mixed nerves. No evidence for such cholinergic fibres in the lumbar colonic nerves was ever found. However, Varagić (1956) has reported occasional motor responses to stimulation of the lumbar colonic nerves in the rabbit colon after 'Priscol'. In contrast to the paucity of evidence in the literature for the existence of cholinergic fibres in the lumbar colonic nerves, the motor response to sympathetic nerve stimulation after reserpine is consistently

present. The response is often almost as great as that to pelvic nerve stimulation and has as short a latency, findings rather difficult to reconcile with the idea of stimulating a few cholinergic fibres. Finally, the positive evidence that pelvic nerve section and degeneration or pelvic nerve fatigue abolishes the motor sympathetic response, at least excludes the possibility that the cholinergic fibres are sympathetic cholinergic fibres running in the sympathetic outflow. The final evidence against cholinergic fibres in the sympathetic nerves being responsible for the motor response is the fact that reserpine is the only blocking agent which produces this consistent reversal of sympathetic inhibition.

It is curious, and probably a reflection on our present understanding of the peripheral autonomic nervous system, to note that the work of the first part of this thesis on 'adrenaline-like' effects, i.e., reversed effects of nicotine, should lead to an investigation of 'acetylcholine-like' reversed effects of sympathetic nerve stimulation. The conventional picture, in effect dividing the autonomic nervous system into watertight compartments labelled 'sympathetic' and 'parasympathetic', each with its appropriate pre- and post-ganglionic fibres, though highly

convenient, all too frequently proves inadequate. This relationship between the adrenaline-like effects of nicotine or acetylcholine after atropine and the frequent appearance of cholinergic responses from 'sympathetic' nerve stimulation has also been remarked on by Burn & Rand (1960 b). They have produced a most ingenious theory which would account for both phenomena. According to their theory, the fibres in the sympathetic nerves are really cholinergic and innervate 'stores' of noradrenaline in the tissues. Stimulation of these cholinergic fibres leads to liberation of noradrenaline from the 'stores' and brings about an adrenergic response. If the 'stores' are depleted, as for example by reserpine, then the adrenergic effect is lost. Under these circumstances, the liberated acetylcholine may diffuse away from the site of liberation around the 'stores' and reach other effectors so as to produce a cholinergic response. Such an explanation cannot be applied to the motor response of the rabbit colon to sympathetic nerve stimulation which is described here, since it has been shown that this response is dependent on an intact and functioning innervation by the pelvic nerves. In the absence of pelvic nerve fibres, or after their stimulation to exhaustion, there is no

motor effect on stimulating the undegenerated or unfatigued sympathetic nerves. In the absence of a satisfactory explanation, the hypothesis which, however unorthodox, would seem best to fit the observations is that, after reserpine treatment, nerve impulses in the sympathetic fibres can in some way unknown, activate the peripheral parasympathetic pathway, and this 'cross-talk' must occur before the synapse in the parasympathetic pathway.

While such a physiological mechanism may seem unlikely in mammalia where the isolation of nerve pathways and freedom from interaction is characteristic of the somatic nervous system, it should be remembered that the autonomic nervous system, like the effector organs it serves, may represent a less specialised situation. Certainly the anatomical arrangement of finely myelinated or non-myelinated C fibres crowded into a single Schwann cell cytoplasm raises the question of how independent such fibres are. In lower animals interaction can occur. For example, the innervation of skeletal muscle in the crayfish is, as in the autonomic nervous system of mammals, a double one, with both inhibitor and motor fibres. As in the autonomic nervous system of mammalia, these fibres are antagonistic. Until recently, this antagonism was believed to be entirely

due to competitive antagonism between the two chemical transmitters. Recently, however, Dudel & Kuffler (1961) have reported that, in addition to the antagonism between the transmitters, there is a pre-synaptic form of inhibition - interaction between the nerve fibres themselves. If some such interaction is possible between the sympathetic and parasympathetic fibres in the gut, then it would provide a basis for this peculiar reversal of the sympathetic activity after reserpine.

Recently Koelle (1961) has proposed a very general hypothesis concerning the mechanism for the release of transmitter substance or neurohormones in a variety of situations. In brief, he suggests that many nerves, not commonly believed to be cholinergic, do in fact liberate acetylcholine at their nerve endings. This acetylcholine, liberated by the action potentials, then acts on the pre-synaptic membrane either to liberate further and more numerous quanta of acetylcholine, if the nerve is cholinergic, or to liberate such other substance as is held in a suitable form in the nerve endings. Such a substance might be noradrenaline. This theory, which is supported by a considerable weight of evidence, even though indirect, would, as already stated in Part I of this thesis, provide

an alternative explanation for the adrenaline-like effects of nicotine. In addition, this theory could be used to explain cholinergic effects of sympathetic nerve stimulation after depletion of noradrenaline by reserpine. In some ways, it resembles the theory of Burn & Rand, which has already been discussed, since both theories require that acetylcholine be liberated by sympathetic nerve fibres as the first stage in the liberation of noradrenaline. Once again, this initial release of acetylcholine suggested by Koelle could be responsible for the cholinergic effects under special circumstances. However, in the rabbit colon, this is clearly not the explanation, because here it is the pelvic cholinergic fibres that are finally responsible for the cholinergic effect. It is, however, quite possible that, if some fibre or synaptic connection exists between the sympathetic and parasympathetic systems, then the preliminary liberation of acetylcholine, postulated at sympathetic nerve endings, might serve as the means of transmission at this junction.

The symptoms of reserpine intoxication are many and varied. There is, however, a certain pattern which corresponds to overactivity of the parasympathetic division of the autonomic nervous system, e.g., bradycardia, miosis,

diarrhoea, etc., together with a peculiar form of sedation not strictly comparable to sleep. The sedation is clearly a central effect but some of the other symptoms could be due either to a central or to a peripheral action. Originally a central origin of the peripheral signs and symptoms of parasympathetic overactivity was favoured (Plummer et al., 1954). With the demonstration first of the depletion of tissue stores of catechol amines (Bertler, Carlsson & Rosengren, 1956) and then the depletion and block of adrenergic nerves (Muscholl & Vogt, 1958), the view has changed and increasing stress is laid on the peripheral block of adrenergic neurones. In this concern with sympathetic block, it is sometimes forgotton that the predominant symptoms are overactivity of the parasympathetic system. This overactivity is usually attributed to a simple imbalance of the autonomic nervous system in favour of the parasympathetic component, the sympathetic component just being ineffective and the parasympathetic being no more than normally active. Such an explanation ignores the evidence that complete surgical sympathectomy does not produce an excess of parasympathetic activity (Cannon, Newton, Bright, Menkin & Moore, 1929). In fact, the only difference between sympathectomised animals and those with

an intact sympathetic system is the inability of sympathectomised animals to withstand stress. Some alternative or additional explanation of the predominantly parasympathetic symptoms in reserpine intoxication is required. As originally suggested, it may be due to a central action of reserpine on parasympathetic centres. An alternative, suggested by the present experiments, is that reserpine has an original and specific action not only in rendering the sympathetic fibres ineffective but in 'passing across' their activity to the parasympathetic system. This would explain the predominant parasympathetic symptoms. Furthermore, it could explain the intensity of these symptoms since the body homeostasis, instead of working on a negative feedback, would be working on a positive feedback. For example, consider the blood pressure. Normally, if the blood pressure falls, then sympathetic activity is reflexly increased, with the result that there is cardiac acceleration, increased peripheral vasoconstriction and a rise in blood pressure. After reserpine, if the present results have any general validity, as the blood pressure falls from the loss of vasoconstrictor tone, the afferent baroreceptor side would be activated. Efferent activity in the sympathetic system would be blocked, activity

transferred to the parasympathetic system, with a consequential slowing of the heart, perhaps some peripheral vasodilation and a further fall in blood pressure.

Some preliminary experiments have been carried out on the eye and heart of a reserpine treated rabbit to test the above idea. In these experiments, the sympathetic nerves to the eye and the heart of a reserpine treated rabbit were exposed and cut under a general anaesthetic. Under these circumstances, if the above idea is correct, and sympathetic impulses are being passed across to the parasympathetic system, then cutting the nerves should reduce or abolish parasympathetic effects. In the case of the eye - section of the sympathetic nerves should cause the pupil to dilate - and in the case of the heart, the rate should be increased. So far, the results of these experiments have been inconclusive.

Both catechol amines and 5-hydroxytryptamine are present in specialised cells at high concentration, probably related to specific storage granules. These cells are responsible for the storage of amine in peripheral tissues. Reserpine clearly depletes these peripheral stores (Bertler, Carlsson & Rosengren, 1956; Burn & Rand, 1958 a; von Euler & Lishajko, 1960). Theoretically, the depletion

might be achieved either by interfering with the synthesis of new transmitter or by interfering with the ability of the stores to retain the transmitter. The evidence favours the latter view. Thus, after reserpine, it can be shown that, temporarily, the blood level of catechol amines rises (Muscholl & Vogt, 1957 b) and the urinary output rises. In individual organs, such as the adrenal glands, reserpine has been shown to increase the concentration in the venous blood from the gland (Kroneberg & Schümann, 1958). Recently, von Euler & Lishajko have gone one step further and shown that reserpine, added to the specific storage granules of the adrenal medulla, isolated by ultracentrifugation, causes the release of the catechols (von Euler & Lishajko, 1960). In contrast to this evidence that reserpine interferes with storage, there is no evidence that reserpine interferes with the synthesis of catechol amines.

The site of action of reserpine in producing depletion of transmitter is not clear and may be different for the two amine groups. Accepting von Euler's work with the adrenal granules, it would appear likely that an action on these granules causing them to leak amine into the cytoplasm and then out of the cell, might account for the effect. Alternatively, reserpine may act on the surface

membrane of the cells to interfere with some carrier there which selectively transports the amines into the cell and which therefore maintains the high intracellular concentration (Hughes & Brodie, 1959). Whatever the site of action, it would seem reasonable to expect that, after reserpine, the cells would lose the ability to retain and concentrate catechol amines.

However, from the ability of all the precursors of noradrenaline subsequent to tyrosine to restore the inhibitory effect of sympathetic nerve stimulation, two conclusions can be drawn. First, these precursors must themselves be depleted by the action of reserpine since, if any store was to remain, it would presumably maintain the inhibitory effect in the same way as the infused precursors. Secondly, the synthetic pathway from dopa onwards is presumably intact. This second deduction depends both on the ability of the precursors to restore the inhibitory effect and on the observation that some of them have little (dopamine) or no (dopa) inhibitory effect themselves in the concentrations used. Consequently, they must have been converted into some more potent substance and the only likely candidate is noradrenaline.

The inability of tyrosine to restore inhibition

supports the suggestion that, normally, the step from tyrosine to dopa in the synthetic pathway is slow. In the reserpine treated animal, failure at this stage in synthesis could be fundamentally responsible for the eventual depletion of all the precursors in the subsequent stages in the synthesis of the transmitter. In view of the evidence that reserpine does not abolish synthesis of catechol amines in the body (the urinary excretion of catechol amines after reserpine is not abolished and most of this is derived from the nerve endings) it is unlikely that reserpine produces any specific effect in preventing the conversion of tyrosine to dopa. A more likely explanation is that this step in normal synthesis is the rate limiting one. The action of reserpine would then be to produce a rate of loss of transmitter which exceeded the maximum normal rate of synthesis, this latter being limited by the conversion of tyrosine to dopa. Some evidence supporting such a theory is available in the literature. Udenfriend & Wyngarten (1956), using radio-active tyrosine and dopa, have shown that the rate of conversion of tyrosine to catechol amines in the rat adrenal gland is much slower than the rate of conversion of dopa. Whether the delay at this stage is due to the chemical

conversion to dopa being slow or to a slow transport of tyrosine into the cell, is difficult to say. Experimental evidence supporting both views could be quoted. For example, in an earlier investigation, Udenfriend, Cooper, Clark, Carrol & Baer (1953) showed that the incorporation of radioactive tyrosine by the liver cells into plasma proteins was much faster than the conversion of tyrosine into catechol amines in the adrenal medulla. From this it might be argued that it was the specific chemical conversion in the adrenal medulla which slowed incorporation since transport through the liver cell membrane at least was more rapid. On the other hand, Burn & Rand (1960 a) found that, although tyrosine was ineffective in restoring function to sympathetic nerves, the more soluble meta-tyrosine was effective, suggesting that transport was the limiting factor rather than chemical conversion. In any case, both reports agree that conversion of l-tyrosine to catechol amines is slow.

Further evidence in favour of the suggestion that an inadequate rate of synthesis of noradrenaline is fundamentally responsible for the depletion of transmitter after reserpine is provided by a study of the output of catechol amines from the adrenal glands. Kroneberg & Schümann (1958) reported

that, after reserpine, the output of adrenaline in the adrenal venous blood of the rat showed an initial increase during the first few hours and then the output fell to a level if anything lower than the resting level. It would seem reasonable to expect that, if the depletion of transmitter after reserpine was due solely to a discharge of amines from their stores, then, having removed the end product, an increase in the rate of synthesis would occur. Hence, after reserpine, the output would show an initial large increase due to the release of preformed stores, followed by a fall to a level which would be above the resting level because of the increased synthesis. The fact that this does not occur suggests that synthesis cannot accelerate to meet the rate of leakage. The present explanation of the action of reserpine does not suggest that this drug in any way interferes with normal synthesis but simply that it causes a 'leak' of the catechols at a rate faster than maximal synthesis. This leads to a depletion not only of the final transmitter but also of all the precursors which follow the step which limits the rate of synthesis. The rate limiting step is the formation of dopa from tyrosine.

It is interesting to speculate whether or not the

ability of nerve endings to take up catechol amines after treatment with reserpine has any physiological counterpart in the absence of this drug. It might be that the ability to reincorporate transmitter is in reality only the converse of the normal effect of the drug in causing a 'leak' of transmitter - both phenomena requiring an increase in permeability to the amines. Certain recent observations, however, suggest that this is not the case and that normal nerve endings can also take up catechol amines. The first report of this was by Burn & Rand (1960 a) who observed that, after infusing noradrenaline into a cat, not only was the pressor and other responses of tyramine enhanced, but the response to sympathetic nerve stimulation was also increased. Recently Burn (1961) has reported several other regions in which a similar potentiation of the effect of sympathetic nerve stimulation is seen after infusing noradrenaline. Hukovič (1961) has reported potentiation of the effect of the sympathetic nerves to the ductus deferens and Waaler (1961), potentiation of the pulmonary vasoconstrictor fibres after exposure to dopamine. Such results suggest that the stores of catechol amines are normally less than the maximum storage capacity of the endings. Further

support for such a view is provided by the observation of Brown, Davies & Ferry (1961) that the output per stimulus of 'sympathin' from the adrenergic nerves in the spleen is increased some 60-90% if the nerve endings have been rested by cutting the preganglionic fibres some days previously.

These two ideas, first that the synthesis of transmitter is barely adequate to maintain the stores and, secondly, that normal nerves may replenish or increase their stores from the blood to a supra-normal level, provide the basis for much interesting speculation. First, the uptake of adrenaline from the blood stream may explain the occasional report of small amounts of adrenaline in the 'sympathin' derived from various nerve endings. (Peart, 1949; West, 1950; Outshoorn, 1952). This adrenaline may be derived, not from synthesis in the nerve endings, but from blood-borne adrenaline from the adrenal glands. It would be interesting to know the nature of 'sympathin' from, say, the uterine and colonic nerves, in which as much as 25% adrenaline has been reported, if the adrenal glands had been removed some time earlier.

This ability to take up transmitter might also play a part in the disposal and dispersal of the transmitter

in the neighbourhood of the effector. If part of the transmitter liberated by each action potential were to be reincorporated into the nerve endings in the interval between nerve impulses, then this mechanism, rather than enzymic destruction, might account for the lowering of transmitter concentration at the receptors which accounts for the termination of the effect. Certainly neither of the enzymes thought to destroy 'sympathin', O-methyl transferase or mono-amine oxidase, has anything like the speed of action of cholinesterase. Such a mechanism might also explain the resistance of adrenergic nerves to fatigue, with repetitive and long continued stimulation, in spite of the fact that synthesis of new transmitter is slow. This mechanism, if true, would beautifully illustrate the body's economy of resources. It is interesting to recall that, while the acetylcholine liberated at nerve endings is probably all hydrolysed by acetylcholinesterase so rapidly that the opportunity for reincorporation does not exist, nevertheless one of the end products of that hydrolysis is reabsorbed and, if this reabsorption is prevented, then failure of synthesis will eventually result (Perry, 1953).

The inhibitory effect restored by offering amines

for reincorporation was easily fatigued by continuous stimulation of the nerves. However, on stopping stimulation, recovery occurred. This could be accounted for if the amines were reincorporated into the endings, in not only a free form which would be available for release, but also in a bound form which was not available for release.

Reserpine dissolved in ascorbic acid and added to the bath fluid produced reversal of the inhibitory response to lumbar (sympathetic) nerve stimulation. However, ascorbic acid alone, in the same concentration, also produced reversal. This made interpretation of the results of these experiments impossible. Nevertheless, this finding in no way invalidates the results of the experiments in which reserpine was given to the rabbits by intravenous injection. Control experiments, in which reserpine vehicle alone was given intravenously to rabbits, produced no reversal of the inhibitory response.

The reversal produced by ascorbic acid is interesting since Eliasson, von Euler & Stajärne (1955) demonstrated the release of noradrenaline from the spleen by ascorbic acid, where the concentration of the ascorbic acid produced only a small change (0.3 units) in pH. It is possible

that ascorbic acid in producing reversal of the inhibitory action of the sympathetic nerves, acts like reserpine by liberating transmitter at a rate exceeding that at which it can be replaced by synthesis.

Summary

1. The effect of reserpine ('Serpasil':Ciba) on the response of the rabbit colon and of the rabbit ileum to stimulation of their extrinsic autonomic nerves *in vitro* has been studied. Reserpine was given daily by intravenous injection for from one to ten days before the experiment.

2. After reserpine, the response of the colon to stimulation of its lumbar colonic (sympathetic) nerves and the response of the ileum to stimulation of its periarterial nerves is reversed from relaxation to contraction. Injection of control animals with appropriate quantities of the vehicle in which the reserpine was dissolved had no effect on the nerve responses.

3. The response of the colon and ileum to noradrenaline, adrenaline and acetylcholine is qualitatively unaltered by reserpine treatment.

4. After reserpine, the motor responses from parasympathetic and from sympathetic nerve stimulation were similar in appearance. Atropine and hexamethonium bromide abolished all these motor responses, both in the colon and in the ileum. The pattern of responses obtained by varying the frequency of stimulation of the lumbar

colonic nerve after reserpine was the same as that obtained from the pelvic (parasympathetic) nerves.

5. Section and degeneration of the pelvic nerves, or stimulation of the pelvic nerves to exhaustion, reduces or abolishes the motor response to stimulation of the lumbar colonic nerves.

6. Three other sympathetic blocking agents, ergotamine, tolazoline ('Priscol') and choline 2:6 xylyl ether bromide (TM 10) were ineffective in producing a motor response to lumbar nerve stimulation. Only TM 10, however, produced a satisfactory selective sympathetic block.

7. It is suggested that, after reserpine, nerve impulses, which start out in sympathetic fibres, can in some unknown way 'cross-over' to activate the peripheral parasympathetic pathway. It is further suggested that this 'cross-talk' is responsible for the peripheral parasympathetic effects of reserpine intoxication.

8. The contraction of the colon on lumbar nerve stimulation after reserpine was restored to relaxation by soaking the preparation <u>in vitro</u> in solutions with either noradrenaline, adrenaline, dopamine or dopa, but not with l-tyrosine.

9. It is suggested that restoration of inhibition is due to reincorporation of transmitter into the nerve endings. It is further suggested that, although reserpine causes a 'leak' of transmitter, the final reason for the depletion of transmitter is that the rate of re-synthesis is too slow to keep up with the 'leak': also, that this slow rate of re-synthesis of transmitter is due to the slow rate of conversion of tyrosine to dopa. The evidence of reincorporation may support the view that sympathetic nerve endings normally reincorporate transmitter after that transmitter has been liberated in the course of activity. This uptake may play an important part in lowering the concentration of 'sympathin' at the receptors.

10. The inhibitory effect restored by reincorporation of amines was easily fatigued by continuous stimulation of the nerves. On stopping stimulation, recovery occurred, suggesting that all the restored transmitter was not equally available for release.

11. If reserpine, held in solution by aid of ascorbic acid, was added <u>in vitro</u> to the bath containing a normal rabbit colon, then the response to stimulation of its lumbar colonic nerves was again reversed from relaxation

to contraction. Ascorbic acid itself in a similar concentration in the bath was found subsequently to be able to produce a similar reversal.

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