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ORIGIN OF THE CHOLINERGIC RESPONSE OF THE RABBIT INTESTINE TO STIMULATION OF ITS EXTRINSIC SYMPATHETIC NERVES AFTER EXPOSURE TO SYMPATHETIC BLOCKING AGENTS

BY

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The effect of guanethidine and of bretylium on the response to nerve stimulation has been studied on two types of isolated innervated preparations of rabbit intestine. One preparation was that of the rabbit ileum in which the periarterial (mainly sympathetic) nerves were stimulated: the other was the doubly innervated rabbit colon where either parasympathetic (pelvic) or sympathetic (lumbar colonic) nerves were stimulated. In both preparations guanethidine and bretylium in appropriate dosage specifically blocked the inhibitory effect of sympathetic nerve stimulation while leaving the response to parasympathetic nerve stimulation and to acetylcholine unaltered: the response to noradrenaline was unaltered or potentiated. In the ileum, after the addition of guanethidine or of bretylium, the inhibitory response to periarterial nerve stimulation was replaced in every preparation by a motor response which had the same frequency sensitivity as parasympathetic nerves. In the colon a motor response to sympathetic nerve stimulation was rarely obtained after blocking the inhibitory response. When such a motor response was uncovered it had similar characteristics to the motor response in the ileum. Furthermore, if the parasympathetic nerves were stimulated for prolonged periods both the parasympathetic and sympathetic motor responses were reduced. These results do not support the idea that post-ganglionic sympathetic nerves to the intestine are generally cholinergic and are themselves responsible for the motor responses. The experimental results are more conveniently explained by assuming a mixture of cholinergic and adrenergic fibres in the nerves stimulated.

In the isolated colon preparation from a normal rabbit, stimulation of the extrinsic parasympathetic nerves causes contraction of the smooth muscle and stimulation of the sympathetic nerves produces relaxation. If, however, the animal has previously been treated with daily intravenous injections of reserpine for 1 to 4 days the response to stimulation of the sympathetic nerves is reversed to contraction whereas the effect of parasympathetic nerve stimulation is unaltered (Gillespie & Mackenna, 1961). The response of the smooth muscle to acetylcholine, to noradrenaline and to adrenaline is not affected by previous treatment with reserpine. Similar reversal by reserpine of the response to stimulation of sympathetic nerves has been observed at several other sites (Burn, 1961; Day & Rand, 1961). While there is general agreement that these reversed responses to sympathetic nerve stimulation following reserpine are caused by the release of acetylcholine, there is no corresponding agreement concerning the source of the acetylcholine.

Perhaps the simplest explanation is that the response is caused by the presence of a proportion of cholinergic fibres in the sympathetic nerves stimulated. These cholinergic fibres in some instances may be parasympathetic in anatomical origin, for example in the periarterial nerves in the mesentery as suggested by Finkleman (1930), or sympathetic in anatomical origin as in the nerve fibres to the sweat glands of the cat's paw (Dale & Feldberg, 1934). Such autonomic nerves have also been shown to supply the blood vessels of the cat's hind limb (Bülbring & Burn, 1935), the nictitating membrane (Bacq & Fredericq, 1935) and the uterus (Sherif, 1935). More recently the presence of cholinergic fibres in the sympathetic nerves to the blood vessels of the cat's limb has been confirmed and they have also been found in the nerve supply to the coronary vessels (Folkow, Haeger & Uvnäs, 1948 ; Folkow, Frost, Haeger & Uvnäs, 1948) and to the spleen (Brandon & Rand, 1961).

An alternative source of the acetylcholine has been suggested by Burn & Rand (1960), who claim that the post-ganglionic sympathetic nerves may themselves be cholinergic and that, in the vicinity of the nerve endings, there are stores of noradrenaline. Nerve impulses, on reaching the nerve endings, liberate acetylcholine which, in turn, releases noradrenaline from these stores. The final response of the effector is, therefore, " adrenergic." Reserpine depletes these tissue stores of noradrenaline, so making an adrenergic response impossible ; the acetylcholine liberated by the post-ganglionic sympathetic nerve endings under these circumstances may itself diffuse to the effector cells and produce a cholinergic response. Recently Burn (1961) and Burn & Rand (1962) have suggested that the stores of noradrenaline may indeed be in the post-ganglionic nerve endings. Gillespie & Mackenna (1961) have advanced a further theory. They found that, in the rabbit colon, the cholinergic response to sympathetic nerve stimulation after reserpine was dependent upon the presence of an intact and functioning parasympathetic nerve supply, the pelvic nerves. They postulated, therefore, that reserpine had a specific effect whereby sympathetic nerve stimulation could activate the peripheral parasympathetic pathway and thus produce a cholinergic response. Of these three theories, the first two imply the presence of cholinergic fibres in the anatomical sympathetic pathway. Therefore, in either case, any agent which blocks the actions of liberated noradrenaline ought to uncover a motor effect similar to that seen after reserpine. Gillespie & Mackenna (1961) examined the action of three such blocking agents on the rabbit colon ; none uncovered a cholinergic effect on sympathetic nerve stimulation. However, only one of the three substances, choline 2:6 xylyl ether bromide (TM10), produced a specific sympathetic block.

Recently, other specific sympathetic blocking agents have been introduced, notably guanethidine and bretylium, and it was felt that these, too, should be examined to determine if they would uncover a motor response on sympathetic nerve stimulation. In addition, our earlier results with TM10 were open to the criticism that the effect on the response to sympathetic nerve stimulation was not followed for a long enough period after the appearance of the block. It could be argued that, since the action of TM10 is slow in onset, a stage of partial block of the inhibitory nerves would be reached at which the reduction of the inhibitory effect was just sufficient to nullify the effect of the cholinergic fibres present, a condition giving the appearance of

complete block. If the response was followed for a longer period until block of the inhibitory fibres was complete, the emergence of a motor effect might be demonstrated. The present investigations were therefore designed to determine, first, whether guanethidine and bretylium would block selectively the inhibitory fibres to the gut and, if so, whether a motor response would then be uncovered and, secondly, to see if the time factor after apparent block was of importance. A brief account of these experiments was given to the Pharmacological Society (Boyd, Gillespie & Mackenna, 1961).

METHODS

Twenty-four rabbits of either sex in the weight range 1.2 to 1.8 kg were killed by a blow on the head and bled. Two types of innervated intestinal preparation were taken from each animal. The first was the doubly innervated preparation of the rabbit colon in which the extrinsic sympathetic (lumbar colonic) and parasympathetic (pelvic) nerves can be separately stimulated. The technique of dissection and the details of the electrodes have been described (Garry & Gillespie, 1955). The second preparation was a length of about 3 cm of mid-ileum together with the mesentery and mesenteric vessels supplying the region. The mesenteric vessels plus the adjacent mesentery were drawn into a fluid electrode and the periarterial nerves stimulated.

The preparations were suspended in separate 200 ml. organ baths filled with Krebs solution at 37° C and gassed with a mixture of 95% oxygen and 5% carbon dioxide. The composition of the Krebs solution was (g/l.): sodium chloride 6.92, potassium chloride 0.35, calcium chloride 0.28, potassium dihydrogen phosphate 0.16, sodium bicarbonate 2.1, magnesium sulphate heptahydrate 0.29, glucose 2.0. The contractions of the longitudinal smooth muscle were recorded with a light isotonic gimbal lever writing sideways on a smoked drum. The nerves were stimulated with 1 msec rectangular pulses whose voltage was adjusted to give maximal responses. The duration of stimulation was usually 15 sec for all nerves; the frequency was 10/sec for the pelvic nerves, 50/sec for the lumbar colonic nerve and either 10/sec or 50/sec for the periarterial nerves to the ileum. In addition to those *in vitro* studies in which guanethidine or bretylium were added to the bath, four animals received single daily intravenous injections of 10 mg/kg of either bretylium (2 animals) or of guanethidine (2 animals) for a period of four days. The last dose was given some 22 hr before the animal was killed.

The doses of guanethidine sulphate and bretylium tosylate are expressed as the salt, that of noradrenaline as the base.

RESULTS

The blocking action of guanethidine and bretylium. Both guanethidine and bretylium selectively blocked the inhibitor response of both colon and ileum to stimulation of the sympathetic nerves without interfering with the response of the smooth muscle to noradrenaline added to the bath. These effects can be seen in Figs. 1 and 2.

The action of bretylium was easily reversed by washing whereas the action of guanethidine was long-lasting and reversal was not achieved. One great advantage of the colon preparation is that stimulation of the pelvic nerves serves as a convenient control of drug specificity for the sympathetic nerves. Using this preparation it could be shown that concentrations of 10^{-7} of guanethidine or bretylium had little or no effect on the response to stimulation of either nervous outflow. In concentrations of 10^{-6}, guanethidine blocked completely the sympathetic inhibitor

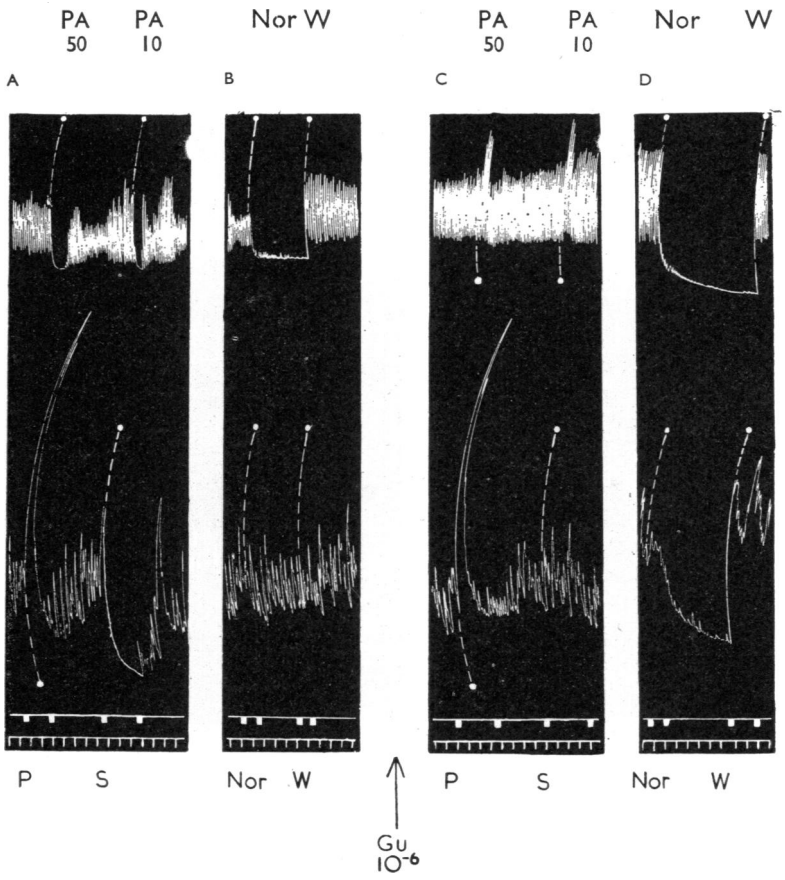

Fig. 1. The responses of the isolated rabbit ileum (upper trace) and colon (lower trace) to stimulation of their extrinsic nerves and to noradrenaline 10^{-6} (Nor). The periarterial nerves were stimulated at 50/sec and 10/sec (PA), the pelvic nerves at 10/sec (P), and the lumbar colonic nerves at 50/sec (S). Panels A and B show the responses before, and C and D after, the addition of guanethidine (Gu) to the bath to produce a concentration of 10^{-6}. The sympathetic inhibitory response is blocked in the colon but reversed in the ileum; the parasympathetic response is unaltered. The response to noradrenaline is increased. Time = 30 sec. W = wash.

response with no reduction in the response to parasympathetic stimulation, that is, a specific sympathetic blockade. Bretylium, in a concentration of 10^{-6}, also produced a complete sympathetic blockade, but a longer exposure was necessary than with guanethidine: at the same time bretylium 10^{-6} caused a slight reduction in the parasympathetic response. Concentrations of 10^{-5} of guanethidine caused a small reduction in the parasympathetic response; bretylium in this concentration almost abolished the response to parasympathetic nerve stimulation. Thus specificity of guanethidine for sympathetic nerves in these preparations appears to be greater than that of bretylium. The effects of varying concentrations of guanethidine on the response of the colon preparation are shown in Fig. 3.

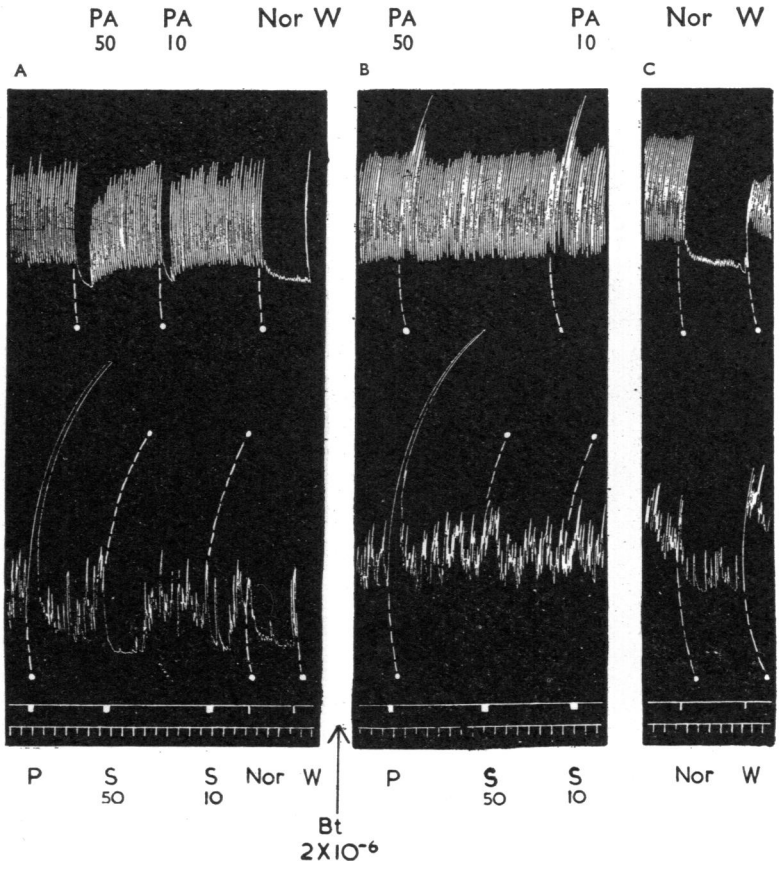

Fig. 2. The responses of the isolated rabbit ileum (upper trace) and colon (lower trace) to stimulation of their extrinsic nerves and to noradrenaline 10^{-6} (Nor). The periarterial nerves were stimulated at 50/sec and 10/sec (PA), the pelvic nerves at 10/sec (P) and the lumbar colonic nerves at 50/sec and 10/sec (S). A shows the response before, and B and C in the presence of, bretylium 2×10^{-6} (Bt). The sympathetic inhibitory response is abolished in the colon but reversed to a motor response in the ileum. The response to parasympathetic nerve stimulation and that to noradrenaline is unaltered. Time=30 sec.

Motor responses to sympathetic nerve stimulation after guanethidine and bretylium. In the ileum, both guanethidine and bretylium consistently reversed the response to periarterial nerve stimulation from inhibition to contraction. This motor response was more readily demonstrated at low frequencies of stimulation (10/sec) than at high frequencies (50/sec) as is shown in Fig. 4.

In contrast to these results in the ileum, motor responses to sympathetic nerve stimulation were rarely seen in the colon after either drug. Of the 24 colon preparations investigated, 12 with bretylium and 12 with guanethidine, only 5 showed motor responses, 2 after bretylium and 3 after guanethidine. These motor responses were, in all cases, small, and the largest seen after either drug is shown in Fig. 5.

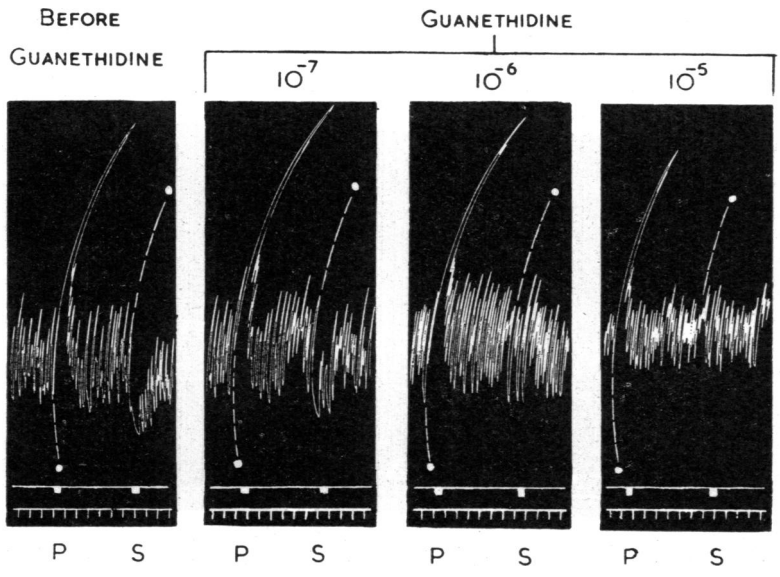

Fig. 3. The responses of an isolated rabbit colon preparation to stimulation of its extrinsic pelvic nerves at 10/sec (P) and lumbar colonic nerves at 50/sec (S) before and in the presence of guanethidine, 10^{-7}, 10^{-6} and 10^{-5}. The sympathetic response is slightly reduced by guanethidine 10^{-7}, and blocked by either 10^{-6} or 10^{-5}. The parasympathetic response is only slightly reduced at a concentration of 10^{-5}. Time=30 sec.

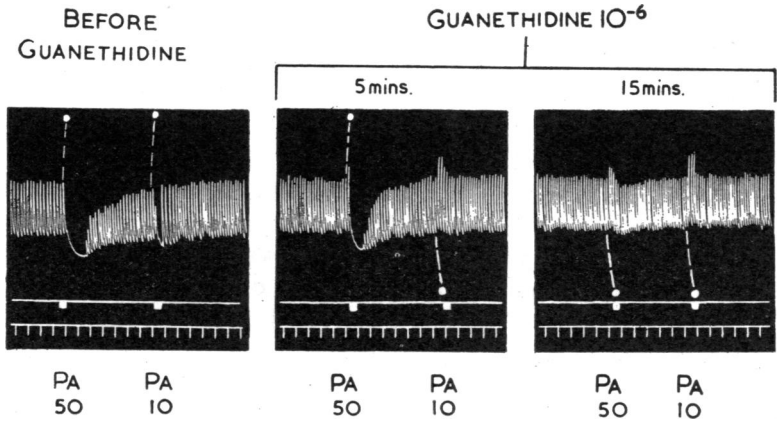

Fig. 4. The responses of the isolated rabbit ileum to stimulation of the periarterial nerves (PA) at 50/sec and 10/sec before and after the addition of guanethidine 10^{-6}. After 5 min, a motor response replaces inhibition at low frequencies of stimulation and at 15 min at both frequencies. After 15 min, when the block of inhibition is complete, low frequencies of stimulation give the bigger response. Time=30 sec.

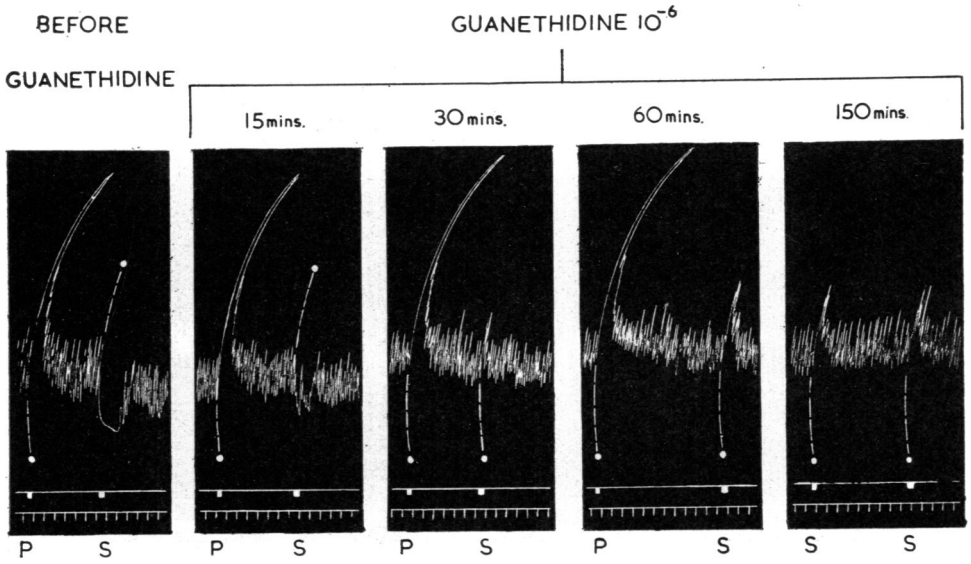

Fig. 5. The responses of the isolated rabbit colon to stimulation of the pelvic (P) and lumbar colonic nerves (S) before and at varying times after adding guanethidine 10^{-6} to the bath. These records show the gradual appearance of a motor response to stimulation of the lumbar colonic nerves —the largest seen in these experiments. Time=30 sec.

In the remaining 19 experiments, complete blockade of the inhibitory response was obtained without the appearance of a motor response. In order to exclude the possibility of such a motor component being missed because of the limited duration of drug action, intermittent sympathetic nerve stimulation was continued in the presence of each drug for periods ranging from 3 to 24 hr. A motor response was never obtained. The effects of these prolonged exposures to guanethidine and bretylium are illustrated in Fig. 6.

The action of guanethidine and bretylium in vivo. In the experiments reported by Gillespie & Mackenna (1961) the reversal of the inhibitory response to sympathetic nerve stimulation was achieved by daily intravenous injections of reserpine for several days before setting up the preparation. It was decided therefore to investigate whether this mode of administration or the long duration of exposure to the drug before the experiment was the cause of the reversal of the response. Four animals (2 guanethidine, 2 bretylium) received 10 mg/kg of either guanethidine or bretylium intravenously as a single daily dose for a period of 4 days ; the final dose being given 22 hr before killing the animal. None of the colon preparations from animals treated in this way showed a motor response on stimulating the lumbar colonic (sympathetic) nerves although, in the ileum, stimulation of the periarterial nerves at the optimum frequency for motor responses (10/sec) did produce a contraction (Fig. 7).

Unlike the effect produced by reserpine, however, the sympathetic nerve blockade produced by intravenous administration of bretylium or guanethidine was incomplete.

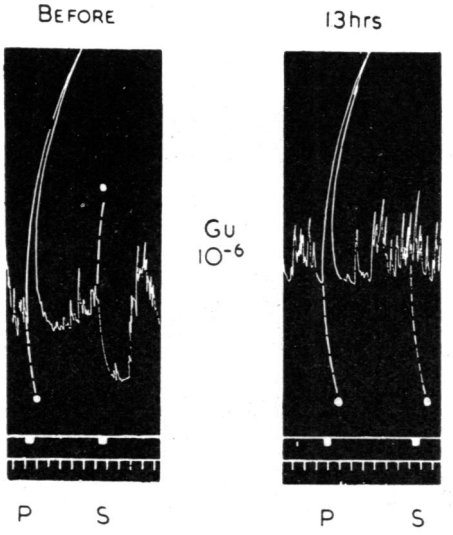

FIG. 6a

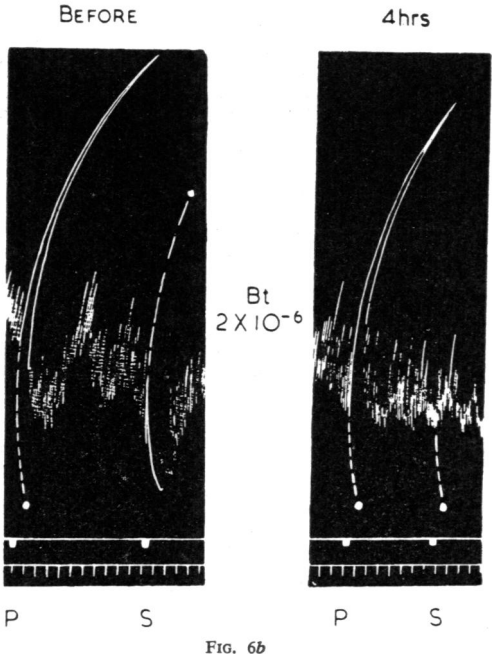

FIG. 6b

Fig. 6. The responses of two isolated rabbit colon preparations to stimulation of their pelvic (P) and lumbar colonic (S) nerves. The upper trace shows the responses before and 13 hr after exposure to guanethidine 10^{-6}. The lower trace shows the responses before and 4 hr after exposure to bretylium 2×10^{-6}. Time=30 sec.

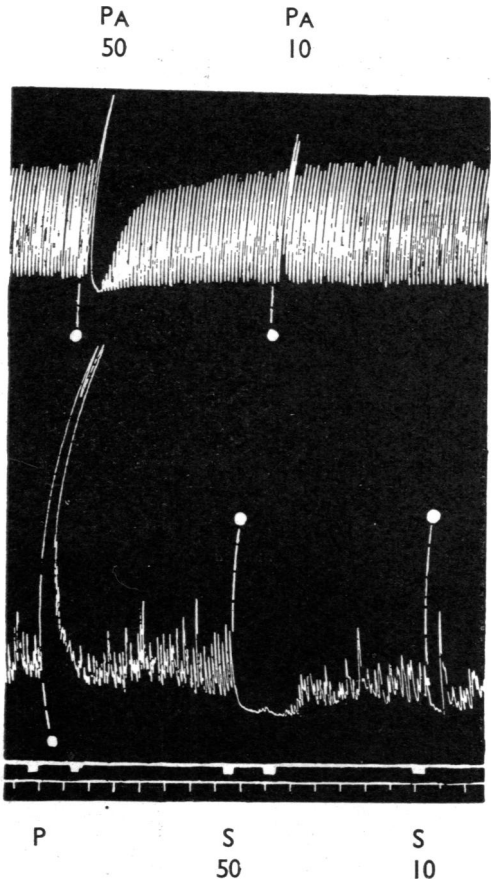

Fig. 7. Preparations of ileum (upper trace) and colon (lower trace) from a rabbit previously treated with intravenous guanethidine, showing the responses to stimulation of the periarterial nerves (PA) and lumbar colonic nerves (S) at 50/sec and 10/sec, and the pelvic nerves at 10/sec (P). Time=30 sec.

In the colon all effective frequencies of stimulation of the lumbar colonic nerves still produced inhibition as did stimulation of the periarterial nerves to the ileum at 50/sec. This incomplete block was not caused by a resistance of these particular animals to sympathetic blockade as shown by subsequent addition of bretylium or guanethidine to the isolated tissue in the bath when the nerves were easily blocked.

The origin of the cholinergic fibres. The cholinergic responses seen after bretylium or guanethidine are presumably due to the excitation of cholinergic fibres. In the ileum such cholinergic responses are well documented and have been obtained without the use of sympathetic blocking agents (Finkleman, 1930). They have usually been attributed to the stimulation of vagal parasympathetic fibres running in the mesentery. Such an explanation would fit the observed greater sensitivity of these motor (cholinergic) responses to low frequencies of stimulation compared

with the initial inhibitory response. In the colon the occasional inclusion in the stimulating electrodes of a few parasympathetic fibres running with the sympathetic nerves might also explain the infrequent motor response after sympathetic blockade. Such motor responses were obtained so infrequently and were usually so inconspicuous that their further investigation was difficult. In one experiment, however, the effect of prolonged stimulation of the parasympathetic nerves on the motor response to sympathetic nerve stimulation after guanethidine was studied. The results are illustrated in Fig. 8.

Fatigue of the motor response to parasympathetic nerve stimulation was accompanied by the disappearance of the small motor response to sympathetic nerve stimulation. Recovery from fatigue of the parasympathetic response was accompanied by a reappearance of the small motor response to sympathetic nerve stimulation. This result would support the suggestion that, in the colon as in the ileum, the motor response to sympathetic nerve stimulation was mediated by parasympathetic nerve fibres.

Restoration of inhibitory effect by noradrenaline. After reversal of the inhibitory effect of sympathetic nerve stimulation by reserpine the original inhibitory effect can be restored by adding noradrenaline or certain of its precursors to the bath fluid and subsequently washing (Gillespie & Mackenna, 1961). Similar experiments

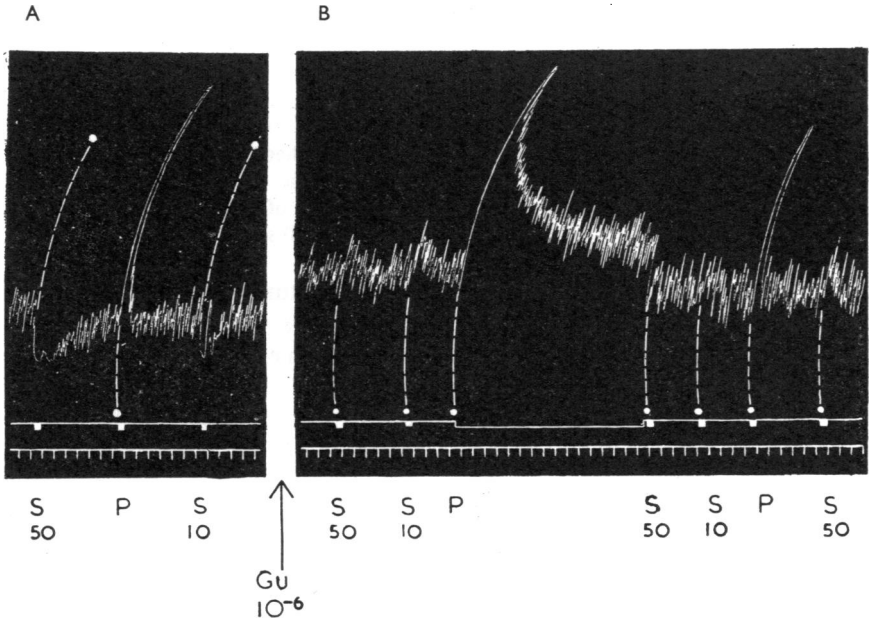

Fig. 8. The response of the isolated rabbit colon preparation to stimulation of its extrinsic nerves before and in the presence of guanethidine 10^{-6}. Panel A shows the responses to pelvic (P) and lumbar colonic (S) nerve stimulation before guanethidine. Panel B, in the presence of guanethidine, shows the appearance of a small motor response to lumbar colonic nerve stimulation at the frequencies shown. Prolonged stimulation of the pelvic nerve leads to fatigue of the response and, at this time, the sympathetic motor response also disappears. Recovery of the pelvic response is accompanied by the reappearance of the sympathetic motor response. Time=30 sec.

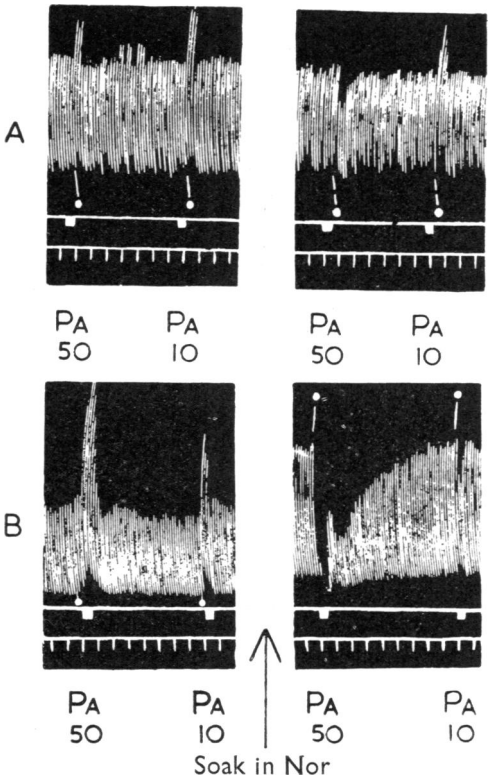

Fig. 9. The responses of rabbit ileum previously treated with either guanethidine (A) or bretylium (B) showing the effect of the addition of noradrenaline to the bath fluid and then washing. In each instance, exposure to noradrenaline in a concentration of 10^{-5} for 15 min leads to a restoration of inhibition on stimulating the periarterial nerves at 50/sec. Time = 30 sec.

were carried out using ileal preparations after guanethidine reversal. Exposing the tissue to noradrenaline in a concentration of 10^{-5} and then washing resulted in the reappearance of an inhibitory effect on periarterial nerve stimulation. This effect is shown in Fig. 9.

DISCUSSION

Day & Rand (1961), in similar experiments on the ileum to those presented in the present paper, have reported that, after blockade with guanethidine, the inhibitory response to sympathetic nerve stimulation is replaced in some 70% of preparations by a motor response. This observation supports their hypothesis, first put forward by Burn & Rand (1959), that the sympathetic post-ganglionic fibres are themselves cholinergic and that the catecholamines mediating the normal inhibitory effect are derived from some source other than the nerve endings. These results of Day & Rand in the ileum we have confirmed; a motor response being obtained in 100% of ileal preparations following either guanethidine or bretylium induced blockade of inhibition. One would need to be cautious, however, in drawing general conclusions from these results. In the colon the inhibitory effect of sympathetic nerve stimulation can be completely abolished without affecting the response to the para-

sympathetic (cholinergic) nerve stimulation and without the appearance of a motor sympathetic response in the great majority of the preparations. Another minor inconsistency in the interpretation placed on these results by Day & Rand is the difference in frequency sensitivity in the ileum between the original inhibitory response and the motor response after guanethidine (a change noticed in both the present experiments and in those of Day & Rand). If both inhibition and contraction are caused by two separate groups of fibres " in parallel," then the difference in sensitivity could easily be explained. If, however, the motor response is produced by cholinergic fibres " in series " with a store of noradrenaline, it is surprising that the optimum frequency for the first link in this chain (the cholinergic post-ganglionic neurone) should differ markedly from that of the second link (the tissue stores of noradrenaline).

The origin of the consistently obtained motor response in the ileum and the infrequent motor response in the colon is not clear. In both regions the optimum frequency of stimulation was similar to that of the known parasympathetic nerves. In one preparation of the colon, the motor response to sympathetic nerve stimulation after guanethidine was abolished by stimulating the pelvic nerve to fatigue. On these, perhaps slender, grounds we would suggest that the motor responses obtained after blockade of the sympathetic inhibitory fibres are caused by the presence of a few parasympathetic fibres running with the sympathetic nerves within the stimulating electrodes. The regular appearance of motor responses in the ileum compared with the infrequent motor responses in the colon would be a reflection of the extent and frequency of inclusion of these parasympathetic nerves.

It is interesting to compare these results with guanethidine and bretylium with those previously reported using reserpine (Gillespie & Mackenna, 1961). After reserpine the rabbit colon consistently responded to sympathetic nerve stimulation with a large contraction. The absence of a similar consistent reversal with guanethidine or bretylium is not due to these drugs blocking transmission in the terminal sympathetic nerve fibres so that neither motor nor inhibitor responses are possible. This is shown by the failure of guanethidine to block the sympathetic motor responses in preparations from reserpine treated animals (Allison & Gillespie, unpublished). It is, therefore, difficult to attribute the action of reserpine in reversing the response to sympathetic nerve stimulation simply to its ability to block the sympathetic inhibitory nerves. Rather, it suggests that this action of reserpine is peculiar to the drug. This unique action may explain the marked difference in appearance of animals treated intravenously with reserpine and guanethidine. The reserpine treated animals all showed signs of parasympathetic over-activity whereas those given guanethidine appeared normal.

These results do not support the concept of the peripheral sympathetic pathway suggested by Burn & Rand (1959, 1960), that is, a pre- and post-ganglionic cholinergic fibre with the post-ganglionic element liberating noradrenaline from some store in the vicinity of the nerve ending. However, they are not inconsistent with the view that acetylcholine may play some part in the process of release of noradrenaline from the post-ganglionic adrenergic nerve terminals as suggested by Koelle (1961) and Burn (1961).

REFERENCES

BACQ, Z. M. & FREDERICQ, H. (1935). Essai d'identification du médiateur clinique libéré dans la membrane nictitante du chat par l'excitation sympathique. *Arch. int. Physiol.*, **40**, 297–310.

BOYD, G., GILLESPIE, J. S. & MACKENNA, B. R. (1961). The action of guanethidine and bretylium on the response of the small and large intestine of the rabbit to stimulation of the extrinsic autonomic nerves. *Brit. J. Pharmacol.*, **17**, 2.

BRANDON, K. W. & RAND, M. J. (1961). Acetylcholine and the sympathetic innervation of the spleen. *J. Physiol. (Lond.)*, **157**, 18–32.

BÜLBRING, E. & BURN, J. H. (1935). The sympathetic dilator fibres in the muscles of the cat and dog. *J. Physiol. (Lond.)*, **83**, 483–501.

BURN, J. H. (1961). A new view of adrenergic nerve fibres, explaining the action of reserpine, bretylium and guanethidine. *Brit. med. J.*, **i**, 1623–1627.

BURN, J. H. & RAND, M. J. (1959). Sympathetic postganglionic mechanism. *Nature (Lond.)*, **184**, 163–165.

BURN, J. H. & RAND, M. J. (1960). Sympathetic postganglionic cholinergic fibres. *Brit. J. Pharmacol.*, **15**, 56–66.

BURN, J. H. & RAND, M. J. (1962). A new interpretation of the adrenergic nerve fibre. *Advances in Pharmacol.*, **1**, 1–30.

DALE, H. H. & FELDBERG, W. (1934). The chemical transmission of secretory impulses to the sweat glands of the cat. *J. Physiol. (Lond.)*, **82**, 121–128.

DAY, M. D. & RAND, M. J. (1961). Effect of guanethidine in revealing cholinergic sympathetic fibres. *Brit. J. Pharmacol.*, **17**, 245–260.

FINKLEMAN, B. (1930). On the nature of inhibition in the intestine. *J. Physiol. (Lond.)*, **70**, 145–157.

FOLKOW, B., FROST, J., HAEGER, K. & UVNÄS, B. (1948). Cholinergic fibres in the sympathetic outflow to the heart in the dog and cat. *Acta physiol. scand.*, **15**, 421–426.

FOLKOW, B., HAEGER, K. & UVNÄS, B. (1948). Cholinergic vasodilator nerves in the sympathetic outflow to the muscles of the hind limbs. *Acta physiol. scand.*, **15**, 401–411.

GARRY, R. C. & GILLESPIE, J. S. (1955). The response of the musculature of the colon of the rabbit to stimulation, *in vitro*, of the parasympathetic and of the sympathetic outflows. *J. Physiol. (Lond.)*, **128**, 557–576.

GILLESPIE, J. S. & MACKENNA, B. R. (1961). The inhibitory action of the sympathetic nerves on the smooth muscle of the rabbit gut, its reversal by reserpine and restoration by catechol amines and by Dopa. *J. Physiol. (Lond.)*, **156**, 17–34.

KOELLE, G. B. (1961). A proposed dual neurohumoral role of acetylcholine: its function at the pre- and post-synaptic sites. *Nature (Lond.)*, **190**, 208–211.

SHERIF, M. A. F. (1935). The chemical transmitter of the sympathetic nerve to the uterus. *J. Physiol. (Lond.)*, **85**, 298–308.