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EFFECT OF COOLING, LOCAL ANAESTHETIC COMPOUNDS AND BOTULINUM TOXIN ON THE RESPONSES OF AND THE ACETYLCHOLINE OUTPUT FROM THE ELECTRICALLY TRANSMURALLY STIMULATED ISOLATED GUINEA-PIG ILEUM

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Electrical transmural stimulation of the isolated guinea-pig ileum preparation which was distended by an intraluminal pressure insufficient to elicit peristalsis produced two effects, one a longitudinal contraction and the other an emptying response brought about by a co-ordinated propagated contraction of the circular muscle. The parameters of the electrical stimulus used were such that these effects were probably produced by stimulation of the nervous plexuses in the wall of the ileum. In the presence of the anticholinesterase *NN*-diisopropylphosphodiamidic fluoride (Mipafox), acetylcholine was detected in the fluid passing through the lumen and also in the fluid in the organ bath. The amount was increased after a period of electrical stimulation. As variation of the frequency of stimulation from 5/min to 25/sec did not alter statistically significantly the acetylcholine output, a frequency of 5/min was used. Cooling to 13° C, the effect of local anaesthetic compounds and *botulinum* toxin reduced both the emptying response and longitudinal contractions of the ileum which followed transmural stimulation, with a concurrent reduction in acetylcholine output. It was concluded that the acetylcholine was released by electrical transmural stimulation from the intramural nerve plexuses in the wall of the guinea-pig ileum.

Electrical transmural stimulation of the guinea-pig ileum has been shown by Paton (1956, 1957) to cause vigorous twitch-like responses of the longitudinal muscle of the gut. As the responses were antagonized by atropine, potentiated by anticholinesterases, and resistant to ganglionic block and as the acetylcholine content of the bath fluid rose after stimulation of the gut, he suggested that the twitches arose in response to stimulation of postganglionic cholinergic nerve fibres in the gut wall which released the acetylcholine into the bath fluid.

The idea that acetylcholine might be continuously released from the isolated mammalian intestine is not new, arising perhaps from the suggestion by Le Heux (1919) that choline was liberated into Locke solution in which the rabbit gut had been suspended for several hours, and from the finding of Dikshit (1938) that acetylcholine could be synthesized by the isolated dog intestine. Goffart (1939) and Bacq & Goffart (1939) first gave direct evidence of release of acetylcholine from denervated dog intestine and suggested the myenteric plexuses as the source. More recently Feldberg & Lin (1949b, 1950) have brought forward evidence that the

source of the continuous acetylcholine release might be from non-nervous structures in the gut wall.

The results of experiments reported here support the view of Paton (1957) that acetylcholine may be released from postganglionic nerve fibre terminals. But this may not be the only site of acetylcholine release, since the emptying response which follows transmural stimulation could be abolished selectively by hexamethonium in the same manner as ganglionic blocking agents abolish the emptying phase of the peristaltic reflex (Paton & Zaimis, 1949 ; Feldberg & Lin, 1949a), so implicating the preganglionic nerve terminals.

METHODS

Segments 6 to 8 cm in length were taken from the ileum 10 cm from the ileo-caecal junction because Munro (1951) found that terminal ileum differs from other parts of the gut in its responses. A segment was cannulated at both ends, the inflow cannula being in the oral end. Krebs solution at 37° C containing Mipafox 10 μg/ml. and bubbled with 95% oxygen and 5% carbon dioxide was passed through the lumen and also filled the bath in which the preparation was set up. Krebs solution was allowed to transfuse for 30 min so that inactivation of the tissue cholinesterase might be complete before any stimulation was attempted. The rate of passage of fluid from the outflow cannula was restricted to 5 ml./10 min by maintaining the pressure of the transfusing fluid at 3 to 5 cm of water above the mid-point of the length of the preparation. Under these conditions a substance appeared in the transfusing fluid and in the bath fluid which had a depressor effect on the blood pressure of the rat previously treated with the anticholinesterase Mipafox 1 mg/kg. The amount of this substance increased after a 10 min period of stimulation. The stimulating electrodes were applied as described by Paton (1957). Stimulation was usually at a rate of 5/min by rectangular pulses of duration of 0.3 msec at 20 to 30 V.

The transfusing and the bath fluids were collected separately, weighed and stored at −20° C ; they were usually assayed on the same day and never later than the next day. A 10-min period of stimulation and collection was found to give a convenient output of acetylcholine for assay purposes.

In each experiment in which the ileum was exposed to either cooling, or drugs, or toxin, there were six collection periods, each of 10 min. The first, during which the ileum was not stimulated, provided the amount of acetylcholine released from the resting ileum. The second was a period in which the ileum was stimulated. The amount of acetylcholine found in the second period minus the amount found in the first period represented the quantity set free solely by stimulation. The third period was a resting release period during cooling or in the presence of drugs or of toxin, and the fourth period was a period of stimulation under the same conditions. Once again the quantity of acetylcholine set free by stimulation during cold or the presence of drugs or toxin was found by subtracting the third period resting release amounts from those of the fourth period. Similarly, the fifth period was a resting release period for the sixth period in which the amount of acetylcholine was measured after the temperature was again returned to 37° C, or the drugs were washed out.

The amounts of acetylcholine released by different ileum preparations by cooling, or drugs, or toxin were expressed as the % of the original quantity set free solely by stimulation.

Identification and estimation of depressor substances as acetylcholine

The depressor activity of samples of the transfusing and bath fluids was matched against the responses to standard concentrations of pure acetylcholine on the rat blood pressure preparation as described by Straughan (1958, 1960). The standard acetylcholine concentration was prepared in Krebs solution containing Mipafox 10 μg/ml. The rat was injected with Mipafox (1 mg/kg) intravenously 30 min before the assay.

The identification of the depressor substance as acetylcholine was based on the following criteria. The depressor activity of the samples and of the standard acetylcholine solution was abolished by a previous injection of 50 μg of atropine which left the depressor activity of 5-hydroxytryptamine and of histamine unaltered.

The depressor activity of both the samples and of the standard acetylcholine solution was abolished after boiling with 5 N-sodium hydroxide for 3 min, but was not affected after boiling with 5 N-hydrochloric acid for 3 min.

The preparation was relatively insensitive to other biological substances likely to be present in the samples. Thus the fall in blood pressure produced by 2 ng of acetylcholine was equivalent to that produced by 2 μg of 5-hydroxytryptamine, 2 μg of histamine or 20 μg of choline. The threshold dose of pure bradykinin on the blood pressure of the rat has been reported by Elliott, Horton & Lewis (1960) and G. P. Lewis (personal communication) to be 0.3 μg.

Acetylcholine outputs are expressed as nanograms of acetylcholine base.

Drugs

The drugs used were: *NN*-diisopropylphosphodiamidic fluoride (Mipafox), acetylcholine chloride, atropine sulphate, 5-hydroxytryptamine creatinine sulphate, histamine phosphate, cocaine hydrochloride, procaine hydrochloride, *botulinum* toxin (type A) and papaverine hydrochloride. All drugs, with the exception of *botulinum* toxin and Mipafox, are expressed as base. Drug solutions were prepared in Krebs solution. The dried *botulinum* toxin was suspended in Krebs solution.

Choice of anticholinesterase

Concentrations of eserine (1 μg/ml.) and of neostigmine (1 μg/ml.), which were considered to inhibit cholinesterase completely, induced strong rhythmical contractions of both circular and longitudinal muscle of the ileum. The irreversible organophosphorus anticholinesterase Mipafox in a concentration of 10 μg/ml., shown by the Warburg technique to inactivate both true and pseudo-cholinesterase completely in the isolated guinea-pig ileum, caused little spontaneous activity. The direct muscarinic effect of neostigmine and, to a lesser extent that of eserine, appeared to be lacking in Mipafox, so making it the anticholinesterase of choice.

RESULTS

The effect of changing the frequency of stimulation on the output of acetylcholine from the ileum

In thirteen experiments the frequency of stimulation was varied from 5/min to 25/sec in four stages of 5/min, 30/min, 3/sec, and 25/sec, each period of stimulation being for 10 min. Usually the ascending order of 5/min to 25/sec was followed, but occasionally the reverse order was adopted. The total output of acetylcholine caused by stimulation was not altered significantly statistically by frequency change, whichever order of change was used. Mean output is plotted against log. of stimulating frequency in Fig. 1.

The effect of three successive periods of stimulation on the output of acetylcholine from the ileum

The responses of both the circular and longitudinal muscle to transmural stimulation at 5/min did not vary during the experiments (Fig. 2). Whether the changes seen after cooling, drugs or toxin are statistically significant depends upon the variations observed between successive periods of stimulation when no agents are used.

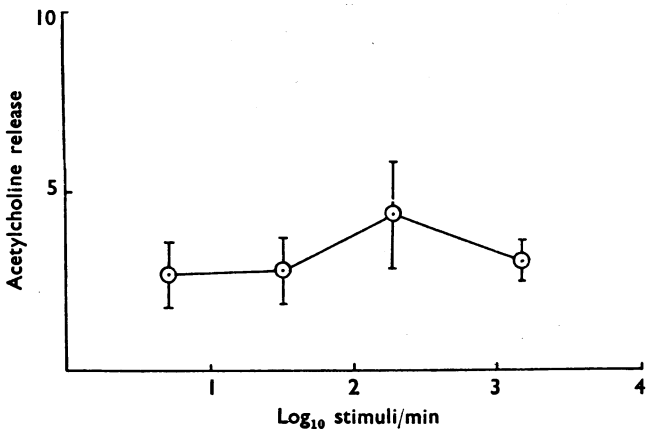

Fig. 1. The relation between the output of acetylcholine and the frequency of stimulation. Each point represents the mean value, with standard errors, of thirteen different experiments. The acetylcholine output is expressed as ng/g of guinea-pig ileum per min of stimulation.

The actual amounts of acetylcholine released from ileum preparation from four animals, for each of three successive periods of stimulation, are shown in Table 1. The difference between the estimates is large and ranges from 2.44 to 8.90 ng/g.

By expressing the output of acetylcholine as % output of the initial period of stimulation it becomes possible to compare the range of variation between-periods

TABLE 1

ACETYLCHOLINE RELEASE BY STIMULATION DURING SUCCESSIVE PERIODS OF TRANSMURAL STIMULATION OF THE GUINEA-PIG ILEUM AT 5/MIN

The amount of acetylcholine base in ng/g of guinea-pig ileum/min of stimulation from 4 different experiments each with 3 successive periods of stimulation. The amount of acetylcholine released during the resting period has been subtracted

Period of stimulation	Acetylcholine released after stimulation (ng/g)	% of output of first period of stimulation
1	8·90	100
2	7·20	81
3	6·50	73
1	5·40	100
2	5·45	101
3	3·30	63
1	3·02	100
2	2·89	96
3	3·71	123
1	2·44	100
2	3·00	122
3	3·66	150

and between-experiments. Inspection shows that the between-experiments variation is larger than the between-periods. The mean of the outputs for the second periods from four experiments is 100% ± s.e. 9% and that for the third period is 102% ± s.e. 21%. These means are evidence of the reliability of outputs in three successive periods of stimulation provided four experiments are made.

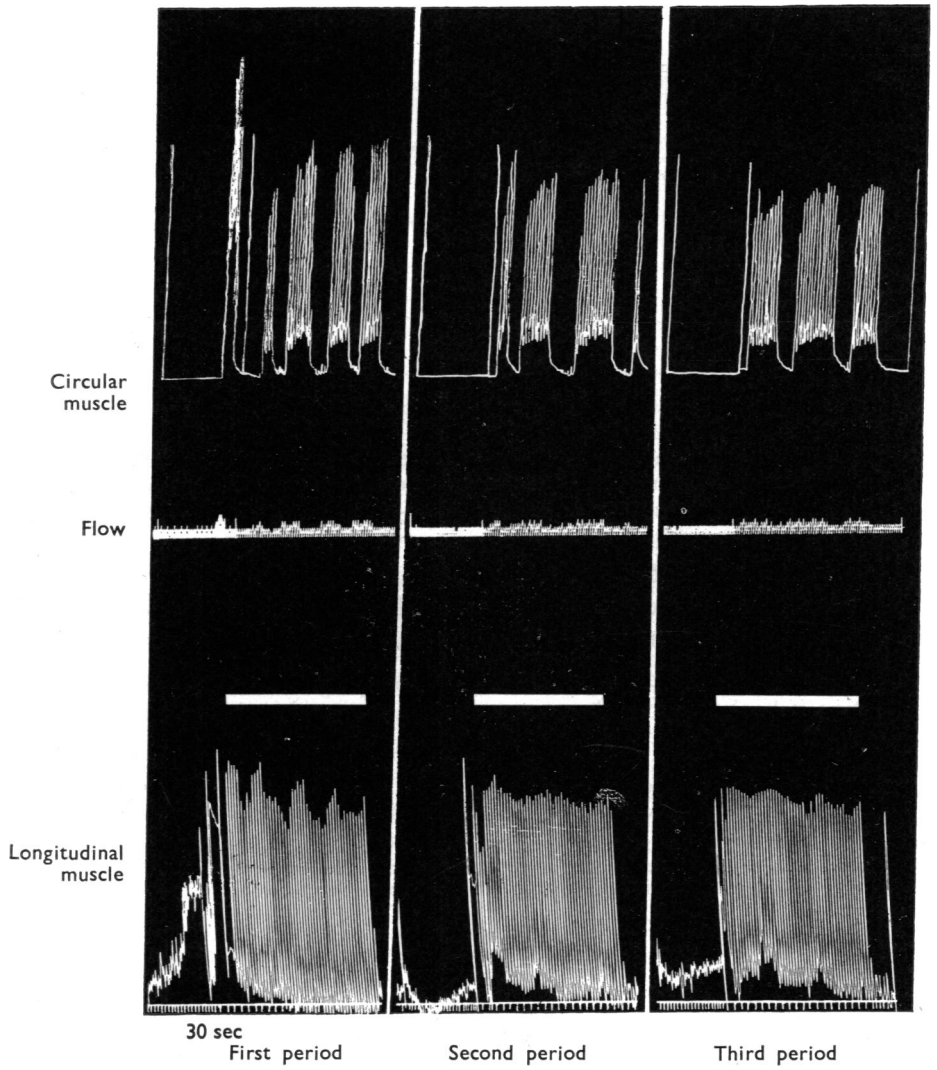

Fig. 2. Three successive periods of electrical transmural stimulation, each of 10 min duration, of the ileum for the period denoted by the horizontal white bars. Between each stimulation period a rest period of 10 min. The drum speed was increased during stimulation. The upper record shows changes in intraluminal pressure and represents circular muscle activity. The middle record shows the volume of the outflow from the gut after passing through a narrow-bore glass tube. The lower record is of longitudinal muscle activity. Time, 30 sec.

The effect of cooling to 13° C on the responses of and the acetylcholine output from the stimulated ileum

After cooling for 30 min at 13° C, the responses of the circular and longitudinal muscle of the ileum to transmural stimulation were abolished, but the response to a test dose of 1 μg/ml. of histamine was slightly potentiated (Fig. 3). After rewarm-

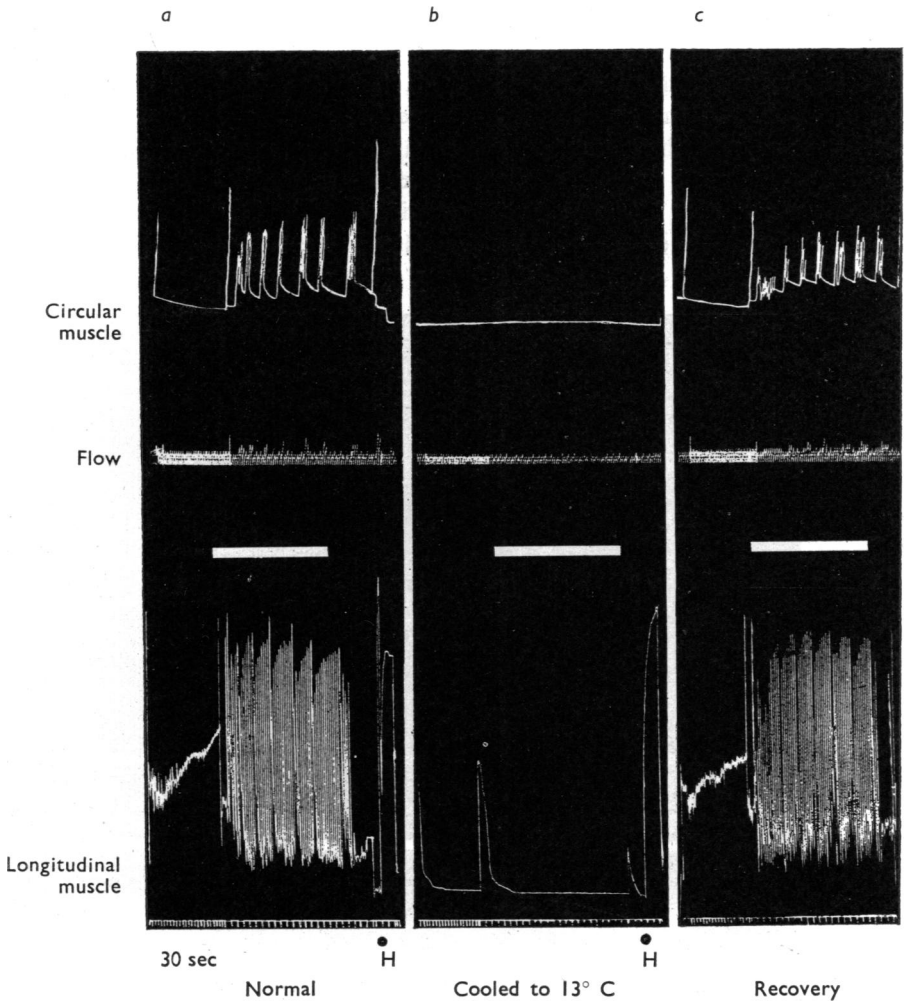

Fig. 3. Records as in Fig. 2. The effect of cooling to 13° C on the responses of the ileum to transmural stimulation denoted by the horizontal white bars. (*a*) Control record at 37° C. (*b*) After cooling to 13° C for 30 min. (*c*) After rewarming to 37° C for 30 min. The response to a test dose of 1 μg/ml. of histamine (H) added to the bath was slightly potentiated at 13° C whereas the muscle responses to transmural electrical stimulation were abolished.

ing the tissue to 37° C for 30 min the responses to electrical stimulation recovered. In five experiments in which the ileum was cooled to 13° C for the second period of stimulation, the output fell to a mean value of 16% ± s.e. 9% of the output for the first period, and recovered to 85% ± s.e. 17% after rewarming to 37° C.

The effect of local anaesthetic compounds on the responses of and release of acetylcholine from the stimulated ileum

Feldberg & Lin (1949a) first used cocaine to paralyse the nervous structures of the intestinal wall of guinea-pigs and rabbits. In seven experiments, I found cocaine in

a transfusing and bath concentration of 100 μg/ml. reduced both longitudinal and circular muscle responses of the guinea-pig ileum to transmural stimulation. The same concentration of cocaine also reduced the sensitivity of the blood pressure of the rat to acetylcholine, possibly because of the central stimulating action of cocaine, a property which made cocaine unsatisfactory for use in these experiments. Procaine in the same concentration produced the same effects on the contractions of the ileum produced by transmural stimulation, but it did not reduce the sensitivity of the blood pressure of the rat to acetylcholine. Fig. 4 shows a typical experiment

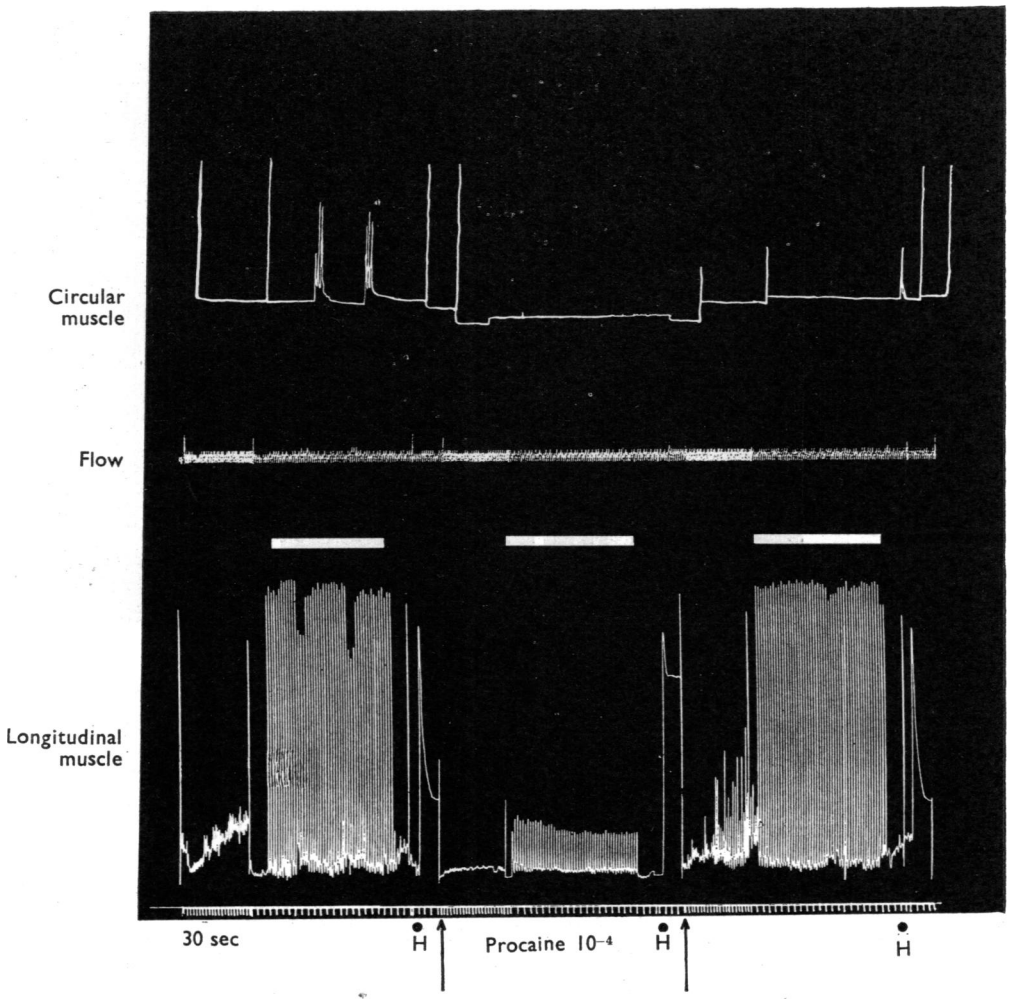

Fig. 4. Records as in Fig. 2. The effect of procaine (between the arrows) on the responses of the ileum to transmural stimulation. A control period of stimulation is shown on the left and after recovery on the right. Duration of transmural stimulation is indicated by horizontal bars. The bath and transfusing fluid concentrations of procaine were 100 μg/ml. The response to a test dose of histamine (bath concentration of 0.5 μg/ml.) is shown at H, and this was not antagonized by procaine which greatly reduced the responses to transmural stimulation.

in which 100 μg/ml. of procaine was added to the fluids for the 2nd period of stimulation. The response of the longitudinal muscle to a test dose of 0.5 μg/ml. of histamine (H) was not reduced in the presence of procaine, indeed it was sometimes slightly augmented. Washing with procaine-free fluids restored the responses to transmural electrical stimulation in 30 min.

In six experiments, the output of acetylcholine during the second period of stimulation in the presence of 100 μg/ml. of procaine fell to a mean value of 20% ±

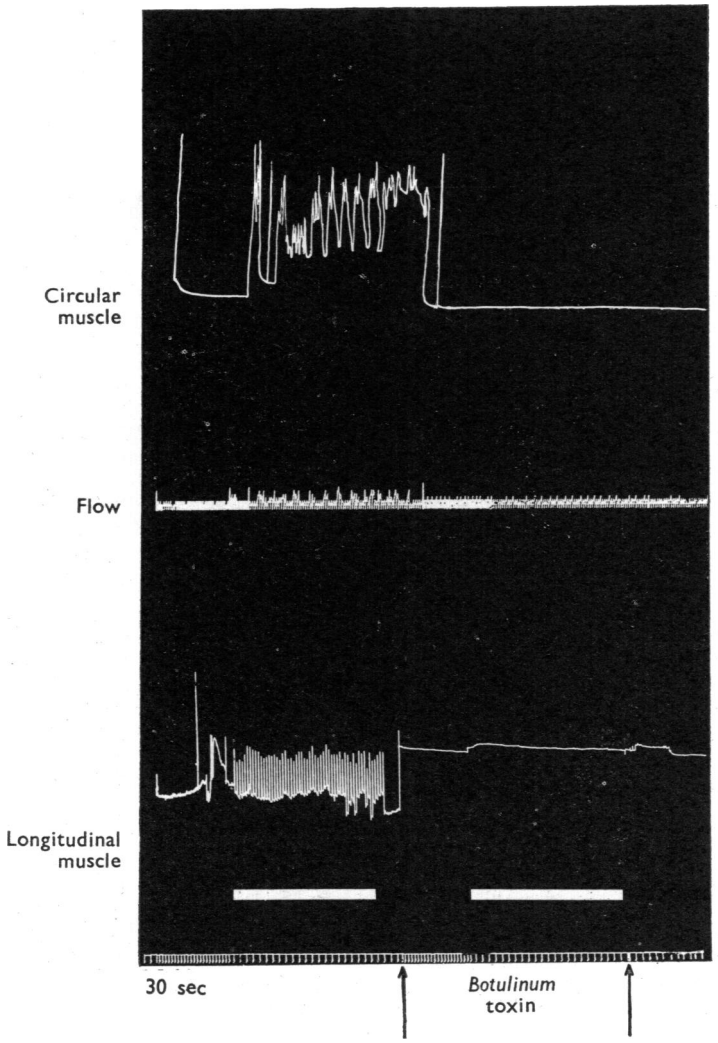

Fig. 5. Records as in Fig. 2. The effect of *botulinum* toxin (type A) on the response of the ileum to transmural stimulation denoted by the white bars. The first period of stimulation was the control. Then the tissue was exposed to a bath concentration per ml. of 8×10^5 M.L.D. mouse of the toxin for 1 hr. After washing, the ileum was stimulated transmurally: no responses were obtained. No subsequent recovery of responses was obtained.

s.e. 8% of the original value measured in the first period of stimulation. The output partially recovered after washing to 64% ± s.e. 9% of the original.

The effect of botulinum *toxin (type A) on the responses of and the output of acetylcholine from the stimulated ileum*

As the paralysing effect of *botulinum* toxin was irreversible, a third period of transmural electrical stimulation such as was employed in the foregoing experiments could not be made. After the first period of stimulation, *botulinum* toxin was added to the bath fluid to make a final bath concentration of 8×10^5 M.L.D. (mouse) to which the ileum was exposed for 1 hr. The tissue was then washed for 30 min. Samples were then collected for assay after transmural electrical stimulation for 10 min.

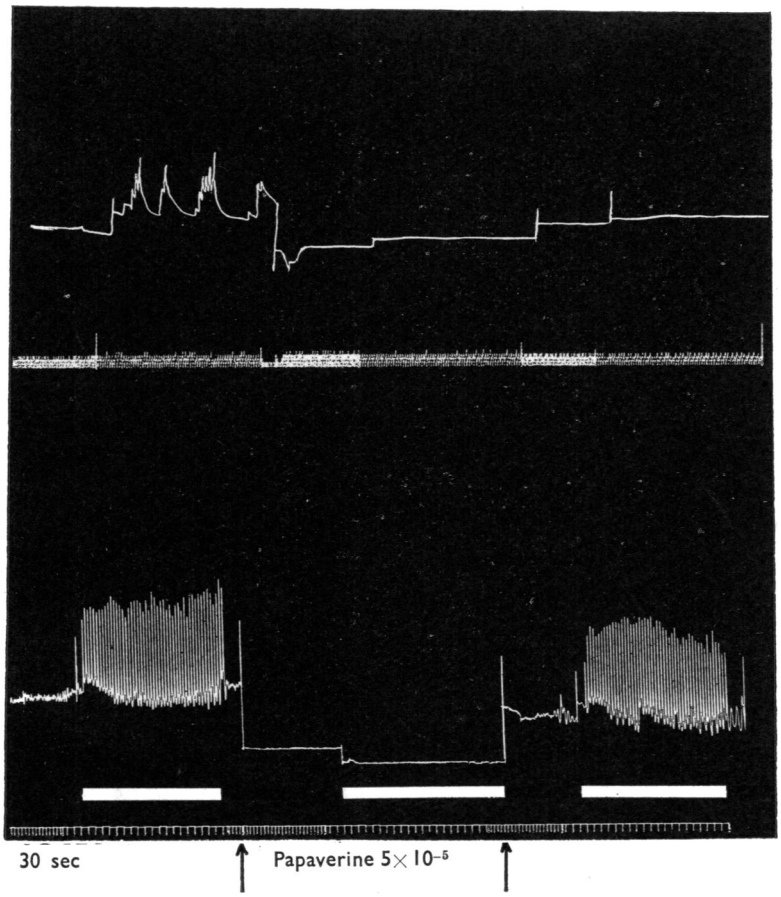

Fig. 6. Records as in Fig. 2. The effect of papaverine (applied between the arrows) on the response of the ileum to transmural stimulation. A control period of stimulation is shown on the left and a recovery period on the right. Duration of transmural stimulation is indicated by the horizontal bars. The bath and transfusing fluid concentrations of papaverine were 50 μg/ml.

The longitudinal and circular contractions of the ileum elicited by transmural stimulation were abolished by *botulinum* toxin (Fig. 5) and, at the same time, the amount of acetylcholine released from the ileum was reduced In five experiments, the output of acetylcholine fell to a mean value of 16% ± s.e. 9% of the original value during the first period of stimulation. The output never recovered.

The effect of papaverine on the responses of and the output of acetylcholine from the stimulated ileum

Hartfelder, Kingschinsky & Mosler (1958) showed that smooth-muscle relaxants can inhibit the longitudinal contractions of the ileum produced by transmural stimulation. Papaverine, in a concentration of 50 μg/ml. in the transfusing and the bath fluids, inhibited both circular and longitudinal muscle contractions (Fig. 6). In two experiments, the output of acetylcholine from the second period of stimulation in the presence of papaverine showed a mean value of 97% of the output from the first period of stimulation, which is in contrast to the other methods used to produce inhibition. The muscle responses returned after washing.

DISCUSSION

Cooling the intestine to free the intestinal smooth muscle from the influence of nerves within it seems to have been exploited first by Ambache, Dixon & Wright (1945) on the crop and gizzard of the earthworm. Later Ambache (1946) used the same technique on the guinea-pig ileum to separate substances with a "direct" action on the smooth muscle from those with an "indirect" action on the intrinsic nerve plexuses. Innes, Kosterlitz & Robinson (1957) found that cooling to 13° C for 3 to 30 min inactivated the nervous components of the guinea-pig ileum but did not affect the responses to histamine and carbachol acting directly on the smooth muscle until cooling had been extended to 1 hr. I found that, after cooling the guinea-pig ileum to 13° C for 30 min, the responses of both the circular and longitudinal layers to transmural electrical stimulation were abolished. This is consistent with the view that these responses are mediated through nervous plexus activation.

Further support for this view stems from results of experiments with local anaesthetic agents. The first suggestion that cocaine could affect the neuronal elements of the intestine arose from the work of von Anrep (1880) and of Bayliss & Starling (1899), who both described inhibition by cocaine of the peristaltic reflex on the dog intestine *in vivo*. Trendelenburg (1917) first showed that cocaine inhibited the activity of the isolated intestine of the rabbit and dog. But it was not until recently that Feldberg & Lin (1949a) distinguished that the inhibitory effect of the local anaesthetic drugs arose from an action on the nervous plexuses rather than an effect directly on the smooth muscle. They showed that these drugs inhibited, not only the emptying phase of the peristaltic reflex, but also the nicotine-induced contraction of the longitudinal muscle of the guinea-pig ileum. In the present experiments the concentration of cocaine (100 μg/ml.) required to abolish the activity of the nerve plexuses in response to electrical stimulation was higher than that used by Feldberg & Lin (1949a) to abolish the nicotine-induced contractions. These discrepancies are hardly surprising when the differences in the two techniques are

recalled. In the present experiments the ileum had been treated with an anticholinesterase, so prolonging the action of acetylcholine, a condition which would be expected to require higher concentrations of cocaine to abolish the effects of electrical stimulation of the nerve plexuses. Further, Feldberg & Lin (1949a) did not obtain an inhibition of the emptying phase of the peristaltic reflex until the tissue had been soaked for several hours in the bath fluid, while in my experiments the ileum would not have been in the organ bath fluid for more than 1.5 hr.

With procaine, as with cocaine, I had to use higher concentrations to obtain inhibition of the responses of the ileum to transmural stimulation than did Feldberg & Lin (1949a). As the response to a test dose of histamine was unaffected, procaine appeared to have no direct action in decreasing the smooth muscle response to this drug.

The paralysis arising from botulism has been attributed to the interference with release of acetylcholine at cholinergic nerve endings (Ambache, 1949; Burgen, Dickens & Zatman, 1949). Ambache (1951) showed that *botulinum* toxin selectively paralysed cholinergic nerves in the intestine. In experiments on the isolated intestine of the kitten and the rabbit, he demonstrated that after *botulinum* toxin the longitudinal contractions produced by the action of nicotine before the toxin were converted to a relaxation. In one of my experiments a similar conversion of the contraction to relaxation in response to electrical transmural stimulation was observed. Usually when nicotine stimulates the postganglionic soma of the gut nerve plexus, the cholinergic action masks adrenergic effects but, after the selective paralysis of the cholinergic fibres by *botulinum* toxin, the response to nicotine stimulation of the adrenergic fibres is revealed. Ambache & Lessin (1955) showed that the neural (indirect) actions of nicotine and dimethylphenylpiperazinium in eliciting longitudinal contractions of the ileum were abolished by *botulinum* toxin whereas the myogenic (direct) actions of acetylcholine or of histamine were but little affected. I found that *botulinum* toxin abolished the responses of both the circular and longitudinal muscle of the ileum to electrical transmural stimulation.

Thus there is an abundance of experimental evidence that cooling to 13° C, the use of cocaine and procaine and *botulinum* toxin depress the neuronal activity of the guinea-pig ileum, without affecting the myogenic contractability of this tissue. Each of these three procedures also reduced the output of acetylcholine from the ileum after a 10-min period of electrical transmural stimulation. It seems, then, that the release of acetylcholine after transmural stimulation is dependent upon the normal functioning of the nervous elements in the ileum wall, and that its source is the cholinergic nerve endings when stimulated transmurally . The experimental results reported here accord well with this view but do not eliminate the possibility that the acetylcholine could be released from stores in the wall of the ileum by pressure changes on its wall such as might occur during contraction of the circular and longitudinal muscle. Hartfelder, Kingschinsky & Mosler (1958) showed that contraction of the longitudinal muscle by electrical transmural stimulation could be antagonized by agents relaxing smooth muscle; therefore some experiments were made with papaverine which relaxed smooth muscle and which was without an action on nervous elements in the gut wall. By this means pressure

changes in the ileal wall due to muscular contraction were eliminated. Papaverine abolished the responses to transmural stimulation, but the outputs of acetylcholine during the 10-min periods of stimulation in its presence hardly differed from those in its absence. Thus it is most unlikely that acetylcholine released by electrical stimulation was extruded from hypothetical stores by mechanical pressures due to muscular contraction.

Atropine (0.1 μg/ml.) abolished both the circular and longitudinal muscle responses to electrical transmural stimulation. This action of atropine was by block of the postganglionic parasympathetic neuro-effector cell junction, antagonizing the transmitter released from the postganglionic nerve terminals. Since atropine at this concentration is a specific antagonist to acetylcholine, it must mean that acetylcholine is released from the nerve endings. Also as cooling, local anaesthetic compounds and *botulinum* toxin depressed the response of neuronal stimulation in this tissue, they must interfere with the release of acetylcholine.

Thus the evidence presented here together with that of previous workers leaves little doubt that the acetylcholine released from the ileum after a 10-min period of electrical transmural stimulation had its origin in the nervous plexuses. Further, it is highly probable that this acetylcholine was responsible for the contraction of both the circular and longitudinal muscle coats of the guinea-pig ileum.

It is of interest to speculate whether the reduction of acetylcholine achieved in these experiments was of sufficient magnitude to produce a failure of postganglionic nerve smooth muscle transmission in the ileum. Paton (1957) showed that, at a stimulation rate of 20/min in the guinea-pig ileum in the absence of an anti-cholinesterase, the height of the response of the longitudinal muscle was not maintained. At this frequency he found that the output of acetylcholine was 0.2 ng per shock. He thus inferred that if the output per shock fell to this value then transmission might be regarded as failing. Paton (1957) also found that, with a stimulation rate of 3.5/min, the output of acetylcholine was 0.4 ng per shock and that, at this frequency, the height of contractions was maintained. As the rate of stimulation in my experiments was 5/min, it could be assumed that reduction in the release of acetylcholine in the presence of a drug by half or more represented transmission failure and reduced muscular response to transmural electrical stimulation. The reduction of acetylcholine outputs in my experiments to about 16% on cooling, to 20% in the presence of procaine and to 16% in the presence of *botulinum* toxin may then be regarded as causing a marked degree of block in the neuromuscular transmission in the ileum.

The results relating the output of acetylcholine to increasing frequency of stimulation in the ileum show no change ; this may be taken to mean a decrease in output per stimulus with increase in frequency of stimulation. This is in agreement with the work of Paton (1957), who showed that the release of acetylcholine per stimulus from the isolated transmurally stimulated guinea-pig ileum fell with increasing frequency of the stimulation between 3.5/min to 27/sec. Straughan (1960) showed that, over a range of frequencies of 6/sec to 100/sec, the release of acetylcholine from the electrically stimulated isolated rat or guinea-pig phrenic nerve diaphragm preparations increased to a maximum at 25/sec and above which frequency the

output fell off. Perry (1953) showed that the total output of acetylcholine from the perfused superior cervical ganglion of the cat changed but little at frequencies ranging from 5/sec to 100/sec, but no measurements of release at lower rates of stimulation were made. So also, in both the rat and guinea-pig diaphragm and in the superior cervical ganglion of the cat, acetylcholine output per stimulus falls rapidly at the higher frequencies of stimulation.

Four possible sites of release of acetylcholine from the intrinsic plexus may be postulated; the cholinergic nerve endings in the longitudinal muscle layer (Paton, 1956, 1957); the possible cholinergic nerve endings in the circular muscle layer (the emptying phase of the peristaltic reflex can be blocked with atropine (Trendelenburg, 1917; Schaumann, 1955; Kosterlitz *et al.*, 1957); at ganglionic synapses in the intrinsic plexuses of the ileum wall (the emptying phase of the peristaltic reflex can be blocked by ganglionic blocking agents); the glands which secrete mucus into the lumen of the isolated guinea-pig ileum; these have been seen in these experiments to be active on electrical stimulation and that this is more prominent in the presence of an anticholinesterase.

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