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UNMASKING, AFTER CHOLINERGIC PARALYSIS BY BOTULINUM TOXIN, OF A REVERSED ACTION OF NICOTINE ON THE MAMMALIAN INTESTINE, REVEALING THE PROBABLE PRESENCE OF LOCAL INHIBITORY GANGLION CELLS IN THE ENTERIC PLEXUSES

BY

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There are several indications that the motor nerves to the intestine are susceptible to the paralysing action of botulinum toxin. Dickson and Shevky (1923) noticed a distinct interference with the intestino-motor function of the vagus in their experiments on cats; and most accounts refer to the obstinate constipation which is a prominent feature of human botulism.

The present experiments, conducted on mice and rabbits, aim at demonstrating this condition in isolated intestinal preparations. In mice systemic botulism was produced, and peristaltic activity was studied in surviving portions of the intestine. In rabbits the toxin was injected locally into the wall of an intestinal segment, which was examined later after the death of the animal from a spread of intoxication. Further information was gained by studying the response of these poisoned segments to nicotine; with this drug it is possible to stimulate the same ganglion cells in the intestine as are involved in the mediation of peristaltic reflex activity, and the extinction of the motor response to nicotine can therefore be used equally well as an index of the paralysis in the myenteric plexus.

Botulinum toxin has a selective affinity for cholinergic nerve endings, and produces effects resembling denervation (Guyton and MacDonald, 1947; Ambache, 1949, 1951). It is generally assumed that the nerve fibres originating from the cells of the myenteric plexus are cholinergic. These motor neurones should therefore also succumb to the paralytic action of this toxin, and should cease to respond to nicotine-stimulation of their ganglion cells. It has been found that, when the action of nicotine on these motor neurones is paralysed by botulinum toxin, an opposite action of nicotine is unmasked at the same time, revealing a stimulation of ganglion cells which are *inhibitory*, and appear to be the cell bodies of short adrenergic neurones.

METHODS

Botulinum toxin.—For the experiments on mice, which were performed in 1949 in Guy's Hospital Medical School, a type A toxin broth was used. It was kindly supplied by Dr.

C. R. Amies, then at the Lister Institute. It contained 200,000 minimum lethal doses per ml. For the later experiments on rabbits two powder preparations of toxin A were available. The first preparation, toxin A_1, was identical with that used two years ago (Ambache, 1949); its LD50 by intravenous injection was then 0.1 μg./kg. of mouse. The second toxin, A_2, was from a more recent batch, available to me through the kindness of Drs. D. W. Henderson and J. Keppie, of the Microbiological Research Station, Porton. This batch had the further advantage of greater purity. Since its toxicity (LD50 by intraperitoneal injection, 0.035 μg./kg. mouse) was three times as great as that of A_1, the dosage was scaled down accordingly. Both powders were taken up in sterile saline (0.9 g. NaCl per 100 ml.), but only some of the toxin went into solution, the rest remaining in suspension. After breaking up the larger visible particles in this suspension, the toxin was injected with the minimum delay; freshly made toxin solutions were used for each experiment on rabbits.

Experiments on mice (*systemic botulism*).—Four mice were injected subcutaneously, or intraperitoneally, with suitable dilutions (in unbuffered saline) of the culture-broth. These dilutions lost their toxicity in 3–4 weeks, presumably owing to contamination with alkali from the glass container; in the absence of a buffer, the resulting change in *p*H would naturally lead to destruction of the toxin. For this reason some of the mice had to be reinjected with a fresh dilution of toxin. The animals were either allowed to die of the intoxication or had to be killed before its full development. Immediately after death a portion of the small intestine (ileum) was excised, and was examined for peristaltic activity in a 5-ml. organ bath. A liquid bolus of Tyrode solution was entrapped in the piece of intestine, which was ligated at both ends; the bolus was intended to act as a distending stimulus, which in the normal intestine produces peristalsis (Ambache, 1946).

Experiments on rabbits (*local injections of toxin into the intestinal wall*).—The rabbits were of mixed stock and weighed 1.4 to 3.35 kg. The intestinal injection was performed under ether anaesthesia, with the usual precautions. The abdomen was shaved and the gut delivered on to a sterile towel through a long midline incision. The period of exposure of the viscera during the injection was kept as brief as possible (*ca.* 5–10 min.).

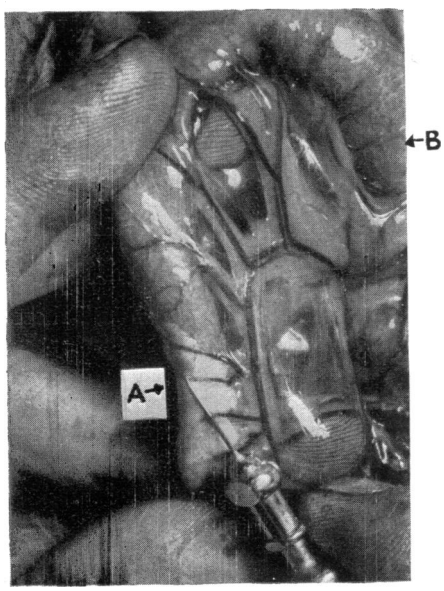

FIG. 1.—Method of local injection of toxin into intestinal wall. The whitish appearance round the syringe needle (at A) is due to a thin sheet of air injected in advance of the toxin and spreading between the longitudinal muscle-layer and its peritoneal covering. Small air-bubbles have spread along the gut and can be seen as far as B. Subsequently the intestine is similarly injected, first with air and then with toxin, on its reverse side, from the other end of the segment (i.e., at B) with the needle pointing towards A (for details see text).

The aim was to place the toxin subperitoneally, i.e., in between the longitudinal muscle-layer and its peritoneal investment. This was done by creating an artificial separation between these two layers, preparatory to the injection of toxin, by injecting air as follows. The syringe was loaded with the appropriate volume of the toxin-solution and 0.3–0.5 ml. of air also drawn into it; thus, from the same syringe, air and toxin could be injected into the gut in succession, the air as the forerunner of the toxin. When the preliminary injection was in the desired anatomical plane, air could be seen spreading out, sometimes for a considerable distance along the intestine, in several rows of bubbles, or as a sheet of air producing the whitish appearance shown at A in Fig. 1. This appearance was always taken as a criterion of success before proceeding with the introduction of toxin, which was then observed entering the artificial space so created and displacing the bubbles of air.

At first toxin was injected at one end of the intestine, but a more satisfactory procedure was evolved for the last thirteen experiments; this consisted of injecting air and toxin at two points, 5–10 cm. apart, in such a way as to produce a high concentration of toxin in the intervening stretch of intestine, which was later used for examination. The dose of toxin was divided into equal halves. The first half was injected in a caudal direction at a point marked A in Fig. 1, on one side of the gut, at about 42–130 cm. from the pylorus. The intestine was then turned over, and the remainder of the toxin was injected at a point (marked B in Fig. 1) on its under side, 5–10 cm. below A, with the needle pointing, this time, in an oral direction. This left a segment of gut AB, injected with toxin on both sides, marked by two small haemorrhagic puncta at A and B, and easily identifiable after death. The distances from the pylorus were measured *post mortem* in nearly all the experiments, as well as, sometimes, the distance from B to the ileo-caecal valve.

After the injection the intestines were replaced and the abdominal wall was sewn up in two layers, the muscular layer with an autoclaved cotton-thread and the skin with catgut. Recovery from the anaesthetic was prompt.

The total amounts of toxin injected into an intestinal segment were: of toxin A_1, 1–2.2 mg. when one end was injected (4 experiments), and 8 mg. when both ends were injected (1 experiment); of toxin A_2, 0.4–0.8 mg. when one end was injected (2 experiments), and 0.35–2 mg. when both ends were injected (8 experiments).

The rabbits died later on the same day, in $3\frac{2}{3}$–$7\frac{1}{2}$ hr. An indication of the impending death of the animal was obtained from its pulse rate, which fell progressively after a time and to very low values just before death. The phenomenon was recorded electrocardiographically, but its nature has not been further investigated. One rabbit was killed two hours after the injection when showing signs of intoxication.

The moment the animals were dead the abdomen was reopened and the appropriate piece of gut (usually of 4 to 5 cm., sometimes of 6 to 7.5 cm. length) was excised between ligatures. The preparation was then tied at one end on to a stainless steel-wire strut and transferred at once to the warm, oxygenated, Tyrode's solution in the organ bath (approximately 10 ml.), into which the strut fitted tightly. The preparations were made from the middle of the injected segment so as to exclude the traumatized, slightly haemorrhagic region in the vicinity of the puncta. Intestinal movements and responses to drugs were recorded with a gimbal lever writing sideways.

The Tyrode's solution was made without magnesium and with 0.11 g. $NaHCO_3$ per 100 ml. It was aerated with a gas mixture of 95 per cent O_2 and 5 per cent CO_2, which was calculated to produce a *p*H of 7.2 in the bath. For some experiments the Tyrode was made in glass-distilled water, for others in copper-distilled water; there was no difference in the results.

Drugs were usually left in the bath for a period of 30 sec. (timed by a stopwatch) unless otherwise stated.

Two experiments (Figs. 4 and 10) were performed at the Instituto Biologico, São Paulo, Brazil, in the department of Biochemistry and Pharmacodynamics, through the courtesy of Dr. M. Rocha e Silva. In these experiments the poisoned intestinal segment was suspended, with its lumen *open*, in a 7-ml. bath, and its movements were recorded with a compensated, linear, frontal-writing lever (Schild, 1946). The Tyrode solution used was made with glass-distilled water and contained magnesium; it was oxygenated with air.

Control experiments were performed not only on intestinal preparations from a normal rabbit killed by concussion but also on four preparations previously injected (under ether) with " boiled toxin " (0.35–1 mg. of toxin A_2). The toxin solution was sealed in ampoules which were heated in boiling water for 45–90 min.; the ampoules were shaken from time to time during the heating in order to re-disperse the coagulum. The injection technique was exactly as described above. These rabbits recovered rapidly from the operation, and were not displaying any abnormality when they had to be killed by concussion 6½ to 23 hours later.

In addition, a preparation was examined from a rabbit which died 1 hr. 35 min. after an intravenous injection of 4 mg. of A_1 (dissolved in a 50 per cent (v/v) glycerinated buffer).

Results

Loss of peristalsis in mice

Mice were chosen for these experiments because of the ease with which peristaltic contractions can be demonstrated *in vitro*, in normal preparations from these animals, if the precaution is taken of filling the lumen with a fluid which acts as a " liquid bolus " and provides a suitable stimulus for peristaltic activity.

Of the four mice injected with suitable dilutions of the toxic broth, only two (Nos. 3 and 4 in Table I) exhibited severe terminal symptoms of a generalized locomotor paralysis. No. 1 died, and No. 2 was killed, before the paralysis in the limbs was fully developed; the presence of peristaltic activity in these two animals (weak in No. 2), and of a response to 25 and 50 μg. of nicotine, was therefore consistent with their general condition. Their inclusion in Table I is intended both as a control and to show that it is possible for various organs to escape complete nervous paralysis in systemic botulism.

In mice Nos. 3 and 4, which were severely paralysed, there was no peristaltic activity. In the absence, or presence, of eserine the response to the customary doses of nicotine was either minute or absent, but the gut still contracted powerfully in response to acetylcholine (0.1–1 μg.).

Experiments on rabbit's small intestine

The response to nicotine.—The addition of nicotine to the bath for 30 seconds, in doses ranging between 5 and 300 μg., produces a contraction of the rabbit's intestine; after the nicotine has been washed out the preparation relaxes again. The effect of a given dose is repeatable at 3 to 5 minute intervals and the preparation remains responsive to the stimulating action of nicotine for 2½–5 hours; it may even become more sensitive to nicotine in the course of an experiment. In some instances, after the nicotine has been washed out, relaxation proceeds below the original base line, and this state of relaxation persists for several minutes despite frequent washing. During this period the amplitude of pendulum movements is often reduced; an example of this is given in Fig. 2. Since this post-nicotine phase of inhibition was never seen in the absence of a preceding nicotine contraction,

TABLE I

LOSS OF PERISTALSIS AND OF THE RESPONSE TO NICOTINE IN MICE POISONED WITH BOTULINUM TOXIN (BROTH)

MLD = Minimum lethal dose

Mouse No.	Dose of toxin (subcutaneous except in expt. 1); and age of toxin dilution	Terminal symptoms and progress	Peristalsis	Response to nicotine
1	200 MLD (fresh) intraperitoneally	Immobile at 17 hr., but able to hang on with hind limbs. *Died* 18 *hr.*	Present	Responds to 50 μg.
2	10 MLD (fresh)	Slow sliding gait. Able to grasp with its hind limbs. *Killed* 29½ *hr.*	Present (but weak and infrequent)	Responds to 25 and 50 μg.
3	5 MLD (fresh)	Advanced locomotor paralysis in all four limbs. Unable to support its weight. When lifted, performs abortive movements of hind limbs. Dribbling urine. *Died* 50 *hr.*	None	1. Minute response at first to 25 and 50 μg. 2. 30 min. later, no response to 50 μg. in the presence of 1 μg. eserine
4	5 MLD (toxin 7 days old)	Unable to right itself or support its weight. Weak abortive movements when lifted. Lachrymating. *Died* 102 *hr.*	None	1. No response to 5, 15, and 75 μg. 2. Small response to 100 μg. in the presence of eserine. Followed by a small response to 75 μg.

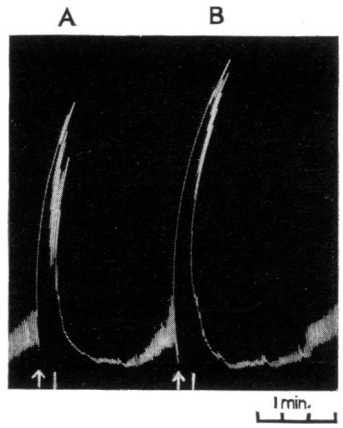

FIG. 2.—Rabbit's ileum (63 cm. below pylorus) of animal killed 6½ hours after local injection of 0.35 mg. inactivated toxin A_1. Contractions to 100 μg. (at A) and 200 μg. (at B) of nicotine. Note the after-effect, i.e., relaxation and reduced pendular movements.

i.e., in the poisoned preparations (see below), its development seems to depend on whether the nicotine has induced a contraction or not. In this it bears a resemblance to a phenomenon of depression, observed by Cantoni and Eastman (1946), subsequent to powerful contractions elicited by other drugs.

The same kind of response to nicotine as on the normal intestine was obtained (*a*) on four preparations subjected to previous local injections of " boiled toxin "; the response of one of these is shown in Fig. 2; (*b*) on the duodenum of an animal injected with active toxin at the other extremity of the gut (Fig. 3); and (*c*) in a

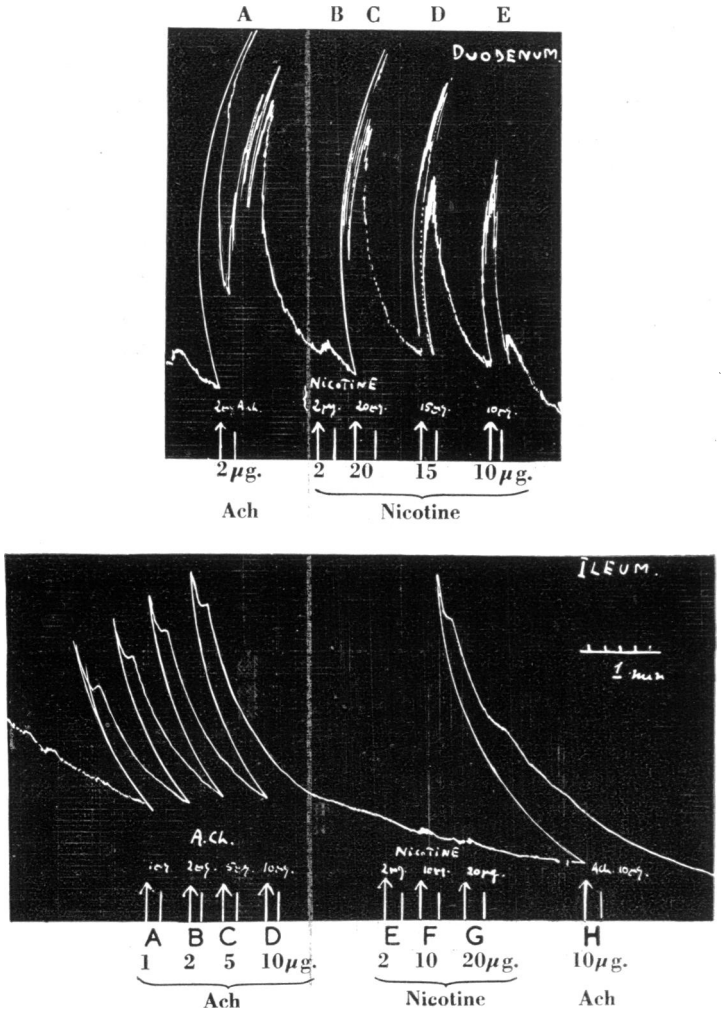

FIG. 3. — Duodenum (upper tracing) and lower ileum (bottom tracing) of rabbit (2.2 kg.). Death 5 hours after injection of 1 mg. botulinum toxin A_1 into ileal segment, from which lower tracing was obtained. Both preparations responded to acetylcholine, but only the duodenum to nicotine. Acetylcholine in upper tracing at A, in lower tracing at A, B, C, D, H. Nicotine in upper tracing at B, C, D, E, and in lower tracing at E, F, G.

preparation from an animal which died after an intravenous injection of a large dose of toxin.

An initial relaxation, instead of the usual contraction, was only seen once after the addition of nicotine to the bath; this was in one of the preparations which had been subjected to previous local injection of " boiled toxin." It occurred when the preparation had been in the bath for $4\frac{1}{4}$ hours. During the first $1\frac{1}{2}$ hours nicotine elicited only motor responses. Thereafter the preparation was left at rest

for about 1½ hours, and then the first administration of 5 μg. nicotine caused pure relaxation, but this effect could not be repeated with subsequent doses of nicotine. This exceptional relaxation was of the same kind as that described later for poisoned pieces of intestine.

The experiment in which toxin was injected into the wall of the intestine, at some distance away from the piece subsequently used for pharmacological examination, showed that the injection of toxin into one end of the gut may leave the other end unparalysed. Direct spread of toxin over such a distance, which is more than 2 m., was not to be expected. But, in addition, the experiment showed that, after systemic absorption of what proved to be a lethal amount of toxin, the local concentration of toxin in the gut at a distance from the site of injection was inadequate to abolish the motor response to nicotine, i.e., to produce paralysis of cholinergic nerves. This was also true in the other control experiment in which the animal was killed by a large dose of toxin injected intravenously. Concurrent observations on the pupil, in both experiments, showed that the paralysis of the sphincter pupillae was also incomplete in these animals. When the pupillary reaction to light was taken as another index of cholinergic paralysis, it was found in several experiments that, even just before death, the iris would still respond to light, though at times sluggishly.

These results showed the necessity of achieving a high local concentration of toxin in an organ, if complete paralysis of its cholinergic nerve supply was desired.

The response to nicotine after local injection of active toxin.—When a segment locally injected with active toxin was suspended in the organ bath after the death of the animal from general intoxication, a difference in the response to nicotine from the normal was observed either straightaway or during the further course of the experiment. A total of fifteen botulinized preparations from different animals was examined. In all of them there was a loss of the normal intestino-motor action of nicotine (dose : 5–300 μg.). In eight this extinction of the motor response to nicotine was present *ab initio*; in six others weak contractions to nicotine, and in a seventh a slight augmentation of pendulum movements, were present at first but were soon lost. In twelve out of these fifteen experiments nicotine produced inhibitory responses, in six of them *ab initio*.

Two preparations demonstrated strikingly the change-over in the nicotine response which is produced by the toxin, because this reversal phenomenon occurred *post mortem*, some time after suspension of the poisoned intestinal segment. For instance, in the experiment of Fig. 4, 50 and 100 μg. of nicotine could still elicit powerful contractions from the poisoned gut 62–67 minutes after death; but after 147 minutes this was no longer possible. Both doses now produced relaxation (Fig. 4, C and D); and the same was true at 196 minutes (F). The rapidity with which this reversal in the action of nicotine may occur is shown in another experiment in which the rabbit had died three and a half hours after the local injection of 8 mg. of toxin A_1. Nineteen minutes after death 100 μg. of nicotine was still motor; but five minutes later 50 μg. produced relaxation, and after another hour a 100–μg. dose was inhibitory as well. In yet a third experiment of a slightly different type the rabbit was *killed* during the development of generalized symptoms, two hours after the local injection of 2 mg. of toxin A_2. The first response to nicotine of the

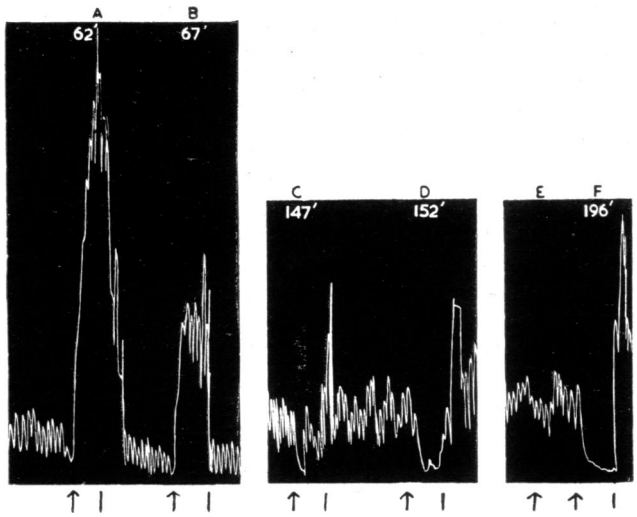

FIG. 4.—Ileum of rabbit (1.5 kg.) previously injected at both ends with 0.8 mg. botulinum toxin A_2, showing the change-over from a motor to an inhibitory response to nicotine. Death of animal 4½ hours after injection. The minutes on top of tracing refer to time after death. B and C, 50 μg., A, D, and F, 100 μg. nicotine; at E, 10 μg. benadryl.

suspended segment, when tested thirty minutes after death, i.e., only two and a half hours from the time when the toxin was injected, was already inhibitory.

Details of the twelve experiments in which nicotine produced the inhibitory response are as follows. In the first six experiments, four with toxin A_1 and two with toxin A_2, the portion of intestine destined for later use was injected on one side only and just at one end. In all there was a loss of the motor response to nicotine, either immediately or soon after suspension. It was in the course of these experiments that the appearance of distinct inhibitory responses to nicotine

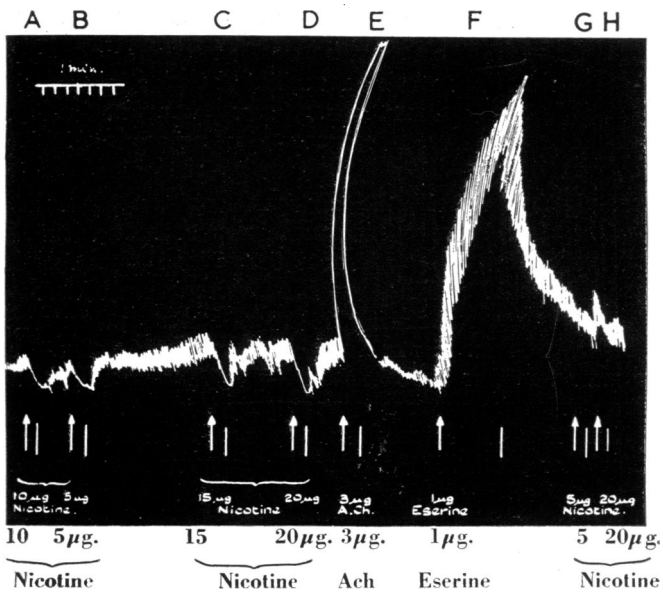

FIG. 5.—Jejunum of rabbit (3 kg.) with 2.2 mg. of botulinum toxin A_1. Injection at one end only of intestinal segment. Death after 6⅓ hours. Tracing begins 65 minutes after death. Effects of nicotine (A, B, C, D, G, and H), acetylcholine (E), and eserine (F) (for details see text).

was first noted; one of them is illustrated in Fig. 5. Even when the motor response to nicotine was lost, the otherwise normal behaviour of the intestine was indicated by the presence of pendulum movements and of strong motor responses to acetylcholine and to eserine (see Figs. 3 and 5); but after eserine there was no outbreak of large peristaltic contractions of the type seen in normal preparations (Ambache, 1946).

In the experiment of Fig. 3, the loss of motor response to nicotine in a segment of ileum injected with toxin is contrasted with the normal response of a duodenal segment about 2 m. away from the site of injection.

In the last nine experiments (one with toxin A_1 and eight with toxin A_2) toxin was injected at both ends and on reverse sides of the chosen segment of intestine. This improvement in technique, combined with the institution of a higher dosage of the A_2 toxin, appeared to achieve a complete cholinergic paralysis with greater regularity. In four out of the nine experiments contractions could not once be evoked by nicotine, even early in the experiment; instead, inhibitions were recorded. The response to the whole gamut of nicotine doses (5–300 μg.) in this normally stimulating range was tested in three of these four experiments, and was inhibitory from start to finish; such an experiment is illustrated in Fig. 6.

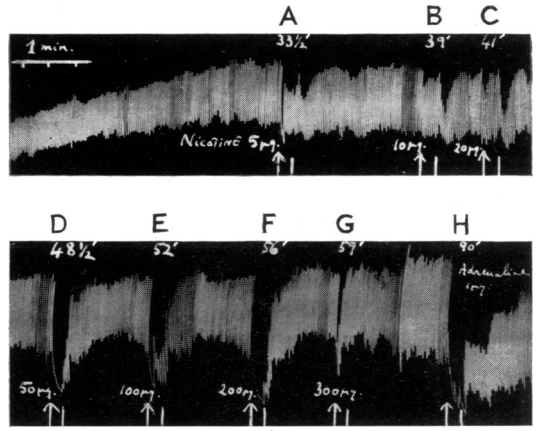

FIG. 6.—Small intestine of rabbit (2 kg.), 85 cm. below pylorus, injected with 0.8 mg. botulinum toxin A_2. Death after 5 hours. Minutes on top of tracing refer to time after death. A to G, effects of increasing doses (5, 10, 20, 50, 100, 200, and 300 μg. respectively) of nicotine; H, 1 μg. adrenaline (for details see text).

When higher doses of nicotine (2 mg.) were tested, traces of a motor action could be detected only once in three experiments; this was the brief contraction seen in Fig. 7 at C. Even this remnant of a motor action was lost when the same dose was tested again 3–4 hours later, when it was replaced by a distinct relaxation (at I) which in the other experiments was seen from the start.

The inhibitory reaction to nicotine in the poisoned intestinal segments has to be distinguished from the Cantoni and Eastman effect mentioned earlier (page 55). The latter is seen only *after* a large stimulating dose of nicotine is washed out of the organ bath. It lasts for several minutes and appears to be consequential upon the contraction preceding it; it is also seen after contractions produced by other drugs, e.g., by acetylcholine, and may originate in a temporary exhaustion of some energetic metabolite in the gut (Cantoni and Eastman, 1946).

On the other hand, the inhibitory phenomenon observed in the botulinized gut occurred regularly in the absence of any preceding contraction. It could be

elicited by small doses of nicotine (5–20 μg.); it developed at once when the nicotine was introduced into the bath and began to vanish as soon as the bath fluid was renewed. In no instance was a prolonged refractory state observed thereafter, whatever the previous dose of nicotine.

Analysis of the inhibitory action of nicotine on botulinized intestines

The inhibitory action of nicotine on the botulinized gut could be shown to be due to stimulation of nerve cells in the wall of the intestine, because it no longer occurred when these were paralysed either by large doses of nicotine or by hexamethonium.

Large doses of nicotine.—It was found that when the dose of nicotine was stepped up in a sequence, from 100–200 μg. to 300 μg. or 2 mg., the resulting inhibition decreased both in magnitude and duration, instead of showing further deepening (Figs. 6, G, and 7, I). This phenomenon may be taken to indicate the onset of ganglion-cell paralysis at the higher dose-level.

In untreated normal intestine it is easy to show that large doses of nicotine leave behind them a state of paralysis of the ganglion cells; when the intestine is in that condition small stimulating doses of nicotine are no longer able to contract it. This phenomenon of paralysis is known to persist for some time after a large dose of nicotine is washed out. If the inhibitory action of nicotine on the poisoned gut were also due to stimulation of ganglion cells, it too should be abolished after a paralysing dose of nicotine and then show gradual recovery in the course of time. Such an effect could, in fact, be observed in several experiments and an instance thereof is illustrated in Fig. 7.

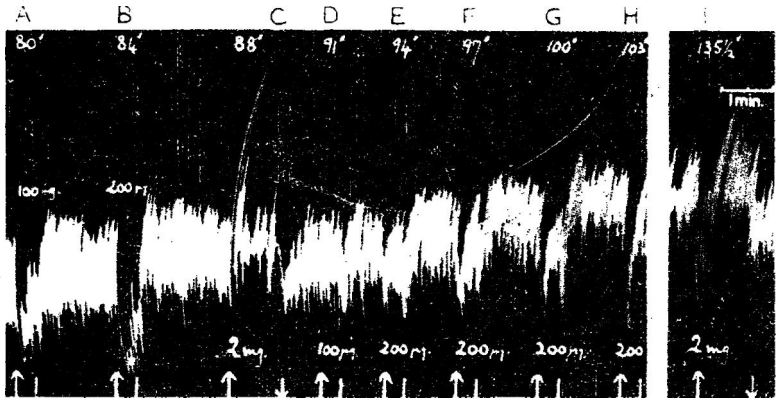

FIG. 7.—Small intestine of rabbit (2.6 kg.), 90 cm. below pylorus, injected with 0.75 mg. botulinum toxin A_2. Injections at both ends of the segment. Death in 5½ hours. The minutes on top of the tracing refer to time after death. Abolition of inhibitory effect of small doses of nicotine (100 μg. at A and D; 200 μg. at B, E, F, G, and H) by a large dose, 2 mg. at C. At I, a further 2 mg. nicotine (for details see text).

Against this interpretation of a nicotine-paralysis of " inhibitory ganglion cells " the argument may be put forward that after large doses of nicotine the sensitivity of normal preparations is reduced not only to ganglion-stimulating substances but

to others as well, and that in this condition even tone and spontaneous activity are diminished. Therefore the removal of the inhibitory effect of small doses of nicotine by large doses need not necessarily be a sign of ganglion paralysis. This argument does not invalidate the interpretation given above. It is true that, on the untreated preparation, paralysing doses of nicotine leave in their wake a condition of reduced responsiveness, but these doses of nicotine first produce contraction in the normal intestine, and strong contractions are generally followed by a period of reduced responsiveness of the intestine, a condition which has been referred to as the Cantoni-Eastman effect. In the poisoned preparation, however, nicotine has lost its motor action, and consequently the after-effect, whereby rhythmic activity and tone are depressed after large doses of nicotine, is absent; nevertheless, the inhibitory action of small doses is abolished. The deduction that this result is the outcome of ganglion paralysis is borne out by the experiments in which hexamethonium was used to paralyse the ganglia instead of large doses of nicotine.

The fact that there was no " depression " of spontaneous movement and tone after large doses of nicotine in botulinized preparations is interesting in another context. Since nicotine *per se* does not appear to exert a depressant action on the muscle fibres in the poisoned gut, it seems unlikely that it does so in normal preparations, where the possibility of such a depressant action on the fibres themselves may have been envisaged in the light of Bernheim's (1933) work, and was not clearly excluded more recently by Feldberg (1950a, b).

Hexamethonium.—The strong ganglion-blocking action of hexamethonium was discovered by Paton and Zaimis (1949). Its ability to abolish reversibly the motor effect of small doses of nicotine on the normal intestine has recently been described by Feldberg (1950b). The experiment of Fig. 8 shows that it can also abolish the other, inhibitory, action of nicotine which is seen on the poisoned preparation. This effect of hexamethonium is also reversible and can be obtained repeatedly on

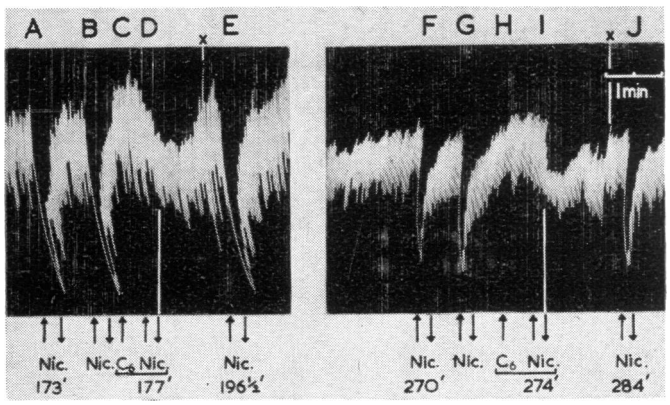

FIG. 8.—Small intestine of rabbit (2.5 kg.), 61 cm. below pylorus, injected with 2 mg. of botulinum toxin A_2. Injections at both ends of intestinal segment. Killed after 2 hours. The minutes at the bottom of the tracing refer to time after death. Abolition of inhibitory response to 100 μg. nicotine (at A, B, D, E, F, G, I, and J), by 2 mg. hexamethonium iodide (C6) (at C and H). Nicotine given at D and I in the presence of C6; both washed out together at the white line. At × drum stopped for several minutes (for details see text).

the same preparation. In the experiment of Fig. 8, the preparation was removed from the bath between E and F and slit open longitudinally from its mesenteric surface in order to exclude the possibility that the nicotine inhibitions result from passive lengthening of the longitudinal muscle by contractions of the circular layer.

Antagonism of the nicotine-inhibitions by ephedrine.—The inhibitory action of small doses of nicotine on the poisoned intestine resembles an adrenaline effect (Fig. 9), and the possibility that the ganglia activated by the nicotine are the cell

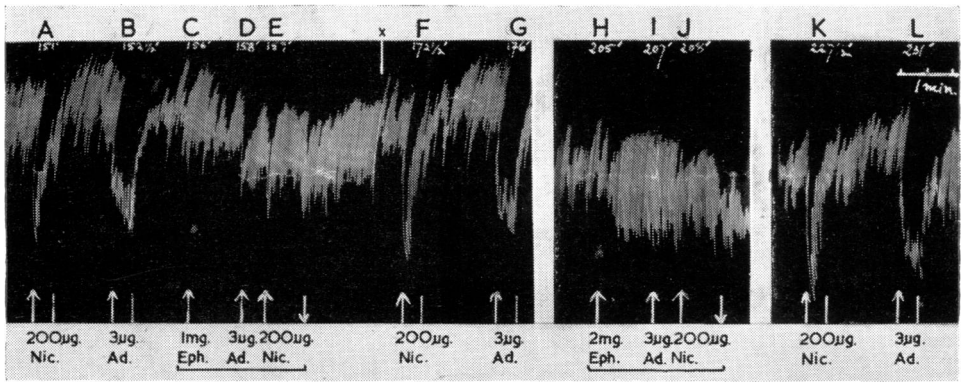

FIG. 9.—Continuation of Fig. 7. Effect of large doses of ephedrine (at C and H) on the responses to 200 μg. nicotine and to 3 μg. adrenaline respectively. At C to E, and at H to J, ephedrine, adrenaline, and nicotine were given in succession without washing out in between (for details see text). Drum stopped at X.

bodies of short adrenergic neurones suggests itself. This possibility could be tested by examining whether the inhibitory effect of these small doses of nicotine is, like the adrenaline effect, abolished by high concentrations of ephedrine. The antagonism of adrenaline actions by ephedrine was described by Schaumann in 1928 and also by Curtis in 1929. Finkleman (1930) observed that, in concentrations of $0.5–1 \times 10^{-4}$, ephedrine had a parallel action in abolishing the effect of splanchnic-nerve stimulation on the rabbit's intestine.

In five experiments the effect of ephedrine on the botulinized gut was examined in concentrations varying between 0.5 and 2×10^{-4}. In all five the inhibitory response to small doses of nicotine was abolished, provided the ephedrine was left in the bath for over 60 seconds; after shorter exposures to ephedrine the extinction of the inhibitory effect of nicotine was sometimes incomplete. In the experiment of Fig. 9, a concentration of 1×10^{-4} of ephedrine reduced considerably, but did not completely abolish, the inhibitory effects of adrenaline and of nicotine (at C to E), but later in the same experiment complete extinction of the two parallel effects was achieved (at H to J) by doubling the concentration of ephedrine. In another experiment (Fig. 10) the higher concentration (2×10^{-4}) of ephedrine itself caused inhibition, which, however, showed no further increase when nicotine was added to the bath in its presence (Fig. 10 at C). When the ephedrine was washed out and its own inhibitory effect had passed off, the intestine, which was again exhibiting strong

pendulum movements, remained for some time insensitive to the inhibitory action of nicotine (Fig. 10 at D).

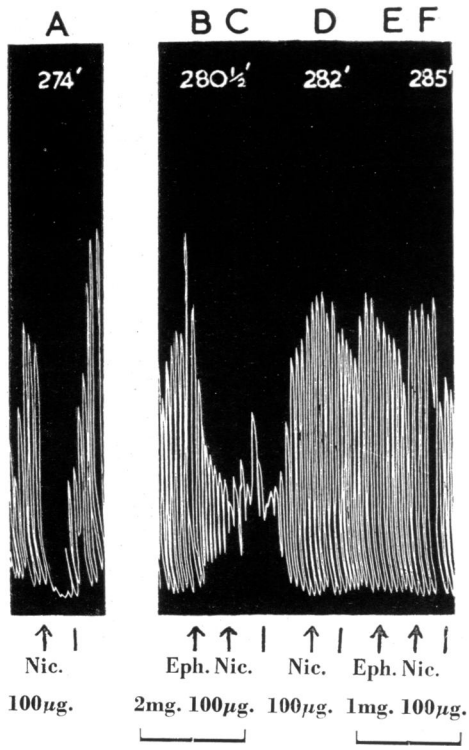

FIG. 10.—Ileum of rabbit (1.4 kg.) injected with 8 mg. of botulinum toxin A_1. Injection at both ends of intestinal segment. Death after 3¼ hours. The minutes on top of the tracing refer to time after death. Effect of 2 mg. (at B) and 1 mg. (at E) of ephedrine on the responses to 100 μg. nicotine (at A, C, D, and F). At C and F, nicotine given before washing out the ephedrine (for details see text.)

DISCUSSION

The results demonstrate the selective action of botulinum toxin upon certain elements within the nerve plexuses of the intestine. The disappearance of the motor response to nicotine in the poisoned gut serves to locate the paralysis in those post-ganglionic fibres which probably constitute a final common path not only for augmentor vagal impulses but also for the motor component of the myenteric reflex. These post-ganglionic neurones are generally considered as cholinergic, and their susceptibility to botulinum toxin is therefore entirely according to expectation (see Ambache, 1951).

The centre of interest shifted at once when inhibitory responses to nicotine began to appear in the poisoned preparations, because they seemed to reveal a new aspect of the complex neural organization of the enteric plexuses. It is believed that the present findings may fall into line with certain other inhibitory phenomena which have been observed previously by various workers, both in physiological and in pharmacological experiments on mammalian intestines, and that the explanation of them may have a common neurological basis.

In 1899 Bayliss and Starling showed that the peristaltic reflex comprises two distinct components: a motor component above the bolus, and an inhibitory component, consisting of active relaxation of the intestine, below the bolus. This preceding wave of inhibition is abolished by paralysing doses of nicotine and is therefore also of nervous origin. Thus there seems to be little doubt that the organization of the peristaltic reflex centres provides yet another example of reciprocal innervation. That being so, it is not unnatural to suppose that inhibitory neurones are present in one or both enteric plexuses. It is also reasonable to assume that these neurones are accessible both to nervous impulses impinging upon them from the afferent limb of the peristaltic reflex arc (or from the vagus) and to the influence of drugs which stimulate the ganglion cells of the autonomic nervous system.

These conclusions, arrived at on purely theoretical grounds, receive some support from histological findings. Certain observations published by Kuntz (1922) are

consistent with such a concept of a functional duality within the myenteric plexuses. Kuntz drew attention to the existence of two separate types of ganglion cell which were found together, within the same ganglion, either in the myenteric or the submucous plexus. The cells appeared to lie in close spatial proximity to each other, and were distinctive both in their staining properties and in their mutual connexions. These cell-pairs are shown in Fig. 11, which is a reproduction from Kuntz's paper. One member of the pair is stated to stain intensely with methylene blue, and sends out a short process ending in numerous synaptic terminations surrounding the other ganglion cell, which stains lightly or not at all. In attempting to correlate function with structure, it is worth speculating whether this anatomical arrangement typifies the duality of these nerve-centres and is at the basis of the reciprocal phenomena in the intestine.

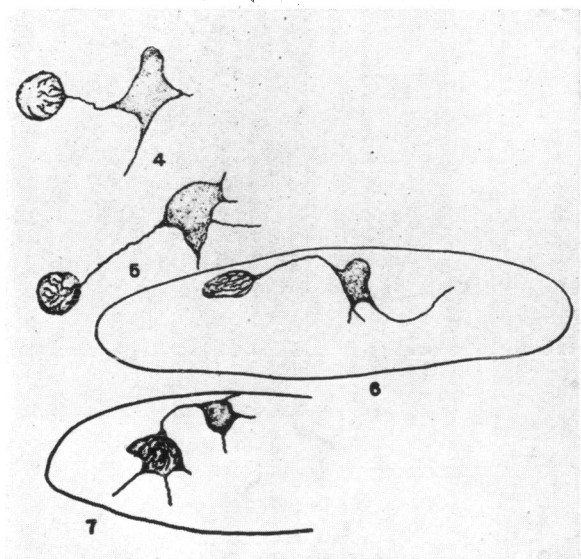

FIG. 11.—Paired arrangement of nerve-cells in enteric ganglia, reproduced from Kuntz (1922), *Anat. Rec.*, **24**, 199.

The present experiments provide physiological evidence for the existence in these nerve-centres of ganglion cells which, when stimulated by nicotine, are capable of producing inhibition in the intestine, and the histological fiindings of Kuntz may provide the structural basis for this function. Moreover, these ganglion cells appear to be the cell bodies of short adrenergic neurones. This conclusion is based on the finding that the inhibitory function is abolished by ephedrine, which suggests that the nerve fibres arising from these cells act on the smooth muscle by releasing adrenaline, or a mixture of adrenaline and *nor*adrenaline, i.e. sympathin. On this interpretation it is necessary to assume that both types of ganglion cell found by Kuntz send fibres to the smooth muscle, apart from the fact shown in Fig. 12 that one may be internuncial to the other.

Next we must consider whether these inhibitory neurones behave as isolated units subserving a purely local reflex function or whether they are linked up in any way with the extrinsic innervation of the gut. The latter seems more probable as there are good experimental grounds for supposing that this inhibitory pathway is also accessible to vagal impulses in certain species, for instance in cats and dogs. Even under normal conditions, in which the motor effect is predominant, signs of underlying inhibition on vagal stimulation can be detected according to Bayliss and Starling. In addition, after atropine, stimulation of the vagus can produce pure inhibition in the stomach and intestines of cats and dogs (Bayliss and Starling,

1899, 1901; McSwiney and Robson, 1929), because atropine abolishes the cholinergic motor effect, and so allows the stimulation of the inhibitory nerve-cells to be revealed.

The presence of both motor and inhibitory neurones in the gut was clearly envisaged by Langley in 1922 when he published his interesting diagram featuring two vagal pathways, one motor and the other inhibitory, each of which, in the intestine, is distinct throughout. His conception was that the vagus contains two sets of preganglionic nerve fibres, each impinging upon a distinct group of intermediate ganglion cells (his " vagus cells "), and that each of these in turn innervates a distinct set of terminal ganglion cells (his " local cells "), one set supplying motor and the other inhibitory fibres to the peripheral musculature. Langley thus postulates a succession of three neurones in both peripheral pathways of the vagus, the last two neurones being situated in the intestinal wall. The terminal neurones are of two types, either motor or inhibitory, and are in turn innervated by penultimate neurones of the appropriate type.

Since the present experiments do not throw any light on the problem of the connexion of the vagal fibres with the different ganglion cells, the " vagus cells " of Langley have been omitted from the diagram in Fig. 12 for the sake of clarity, the two types of " local cells " postulated by Langley being represented as the cell bodies of cholinergic and adrenergic neurones respectively. The diagram further

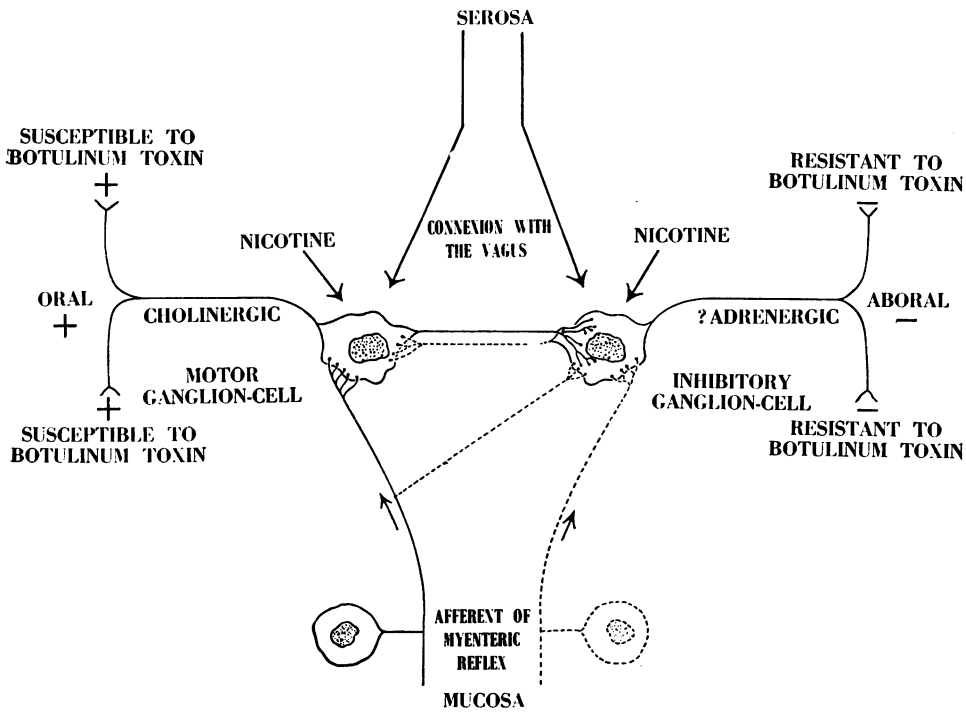

FIG. 12.—Diagram of the suggested connexions, within the enteric plexus, which would account for the reciprocal antagonism in the peristaltic reflex, for the dual effect of the vagus in certain species, and for the pharmacological actions of nicotine. (For explanation see text.)

indicates that nicotine is able to stimulate both types of " local cells." Usually, the predominant effect is that of the motor cells, whether they be excited by nicotine or by vagal impulses, although there are occasional signs that the inhibitory cells are stimulated as well. In order, however, to unmask the function of these inhibitory neurones, the motor set of neurones has to be inactivated; this is achieved with botulinum toxin, which eliminates the response of the final motor neurones by paralysing their cholinergic terminations. Previous experiments on the iris and on the nictitating membrane (Ambache, 1949, 1951) have proved that botulinum toxin has a selective affinity for nerve-endings of the cholinergic variety, but that it spares other types of nerve-endings in the same tissue, for instance those that are adrenergic. The appearance in the poisoned preparation of inhibitory responses to nicotine, which are the result of ganglionic stimulation of what are probably adrenergic neurones, is easily understood if we assume that botulinum toxin produces the same kind of differential denervation in the intestine as in the iris. In the diagram the susceptibility of the cholinergic nerve terminals to botulinum toxin and the resistance of the adrenergic terminals are indicated. It will be clear from this diagram why nicotine, acting on both types of ganglion cell, can exert only an inhibitory effect on the botulinized gut once the cholinergic neurones have succumbed. This unmasking action illustrates the kind of use to which botulinum toxin may be put in the analysis of nervous phenomena involving a mixture of cholinergic and non-cholinergic neurones.

The diagram also illustrates the participation of the two types of terminal neurones in the peristaltic reflex and includes another feature which may help the reader to visualize the unidirectional polarity of this reflex. It is only necessary to assume that the two sets of terminal neurones are orientated in the axis of the intestine in such a way that the cholinergic motor axons are directed orally, and the adrenergic inhibitory axons aborally, to account for the contraction above the bolus and for the phenomenon of " descending inhibition " described by Bayliss and Starling. Lastly, an attempt has been made to represent also the sensory side of the peristaltic reflex arc. It is not certain whether each type of ganglion cell commands a separate sensory innervation. In view of the existence of a collateral connexion between the two terminal neurones, a connexion to one type is all that is needed to complete the arc. But the possibility of a separate sensory connexion to the inhibitory ganglion cell is shown by dotted lines.

The presence, locally, of adrenergic neurones makes it necessary, in the analysis of the effects of certain pharmacologically active substances on the intestine, to take into consideration the possibility that stimulation of this type of ganglion cell contributes a component to the final response of the intestine. For it is possible to imagine that various known ganglion-cell stimulating agents (e.g., KCl, $BaCl_2$, etc.) can activate the two antagonistic types of local nerve cells and that a different balance may be struck between the two possible (motor and inhibitory) effects according to the substance or the dose thereof.

Summary

1. Loss of peristaltic activity and of the motor response to nicotine has been observed in isolated intestinal preparations taken from mice injected with botulinum toxin and showing symptoms of systemic botulism.

2. Botulinum toxin has been injected into the wall of the small intestine of rabbits in order to produce a high local concentration of the toxin in the intestinal wall ; a few hours later the poisoned intestinal preparation was examined in an organ bath. It showed its normal response to acetylcholine or eserine, but a gradual loss of the stimulating action to small doses of nicotine; these produced instead inhibition.

3. The inhibitory nicotine response of the intestinal preparation subjected to botulinum toxin is, like the stimulating response of the normal preparation, due to stimulation of ganglion cells, as it disappears after large, paralysing doses of nicotine and after hexamethonium; i.e., after paralysis of the ganglion cells of the myenteric plexus.

4. The inhibitory response to small doses of nicotine is abolished by large doses of ephedrine, which abolish the inhibitory action of adrenaline. It is therefore concluded that the inhibitory response to small doses of nicotine results from stimulation of ganglion cells which are the origin of short adrenergic neurones.

5. The change in the nicotine response of the rabbit's intestine after local intoxication with botulinum toxin is explained by the selective affinity of the toxin for cholinergic nerve-endings; this leads to abolition of the stimulating action of small doses of nicotine; at the same time it unmasks the response of ganglion cells which give rise to adrenergic fibres, since the latter are spared by the toxin.

6. Evidence is thus presented that there are two kinds of functionally distinct ganglion cells in the myenteric plexus. Stimulation of the one, which gives rise to cholinergic fibres, causes contraction; stimulation of the other, which gives rise to adrenergic fibres, causes inhibition of the intestine. The role of the two different types of ganglion cells in the peristaltic reflex is discussed.

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