J. Physiol. (1968), **199**, *pp.* 705–727
With 1 *plate and* 11 *text-figures*
Printed in Great Britain

ATROPINE-RESISTANT LONGITUDINAL MUSCLE SPASMS DUE TO EXCITATION OF NON-CHOLINERGIC NEURONES IN AUERBACH'S PLEXUS

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(*Received* 31 *July* 1968)

SUMMARY

1. In accordance with the dual histology of Auerbach's plexus (Dogiel, 1899; Hill, 1927) two types of neurone can be shown to be humorally active in plexus-containing preparations of longitudinal muscle from guinea-pig ileum, taken at measured distances up to 95 cm above the ileocaecal valve, when such preparations are stimulated electrically under different conditions.

2. The rapid twitch, lasting 3–8 sec, which is elicited by single shocks of 0·1 or 0·2 msec pulse width, and the effect of 5–15 ng doses of acetylcholine which matched this twitch, were both extinguished equally effectively and completely by atropine sulphate ($0{\cdot}4$–1×10^{-8} g/ml.) or by hyoscine hydrobromide. This twitch-response is therefore caused by an excitation of cholinergic motor neurones of normal susceptibility to atropine. These are believed to be the Dogiel (1899) Type II cells of Auerbach's plexus, as suggested by Hill (1927).

3. After extinction of the twitch by the invariably effective atropine-block, a second type of muscle response was revealed by tetanic stimulation with 1 sec trains of 50 pulses of the same voltage and of pulse width preferably 0·2 msec. The tetanic responses consisted of spasms of longer delay and duration (20–60 sec). These spasms could be matched by doses of acetylcholine of the order of 200 ng. However, if the atropine concentration was now raised to 10^{-7}, or even 10^{-6} g/ml., the effect of 200–1000 ng of acetylcholine was abolished, but the tetanic spasms persisted without decrease in amplitude. In other experiments the height of the spasms remained constant as the concentration of atropine sulphate was raised from 10^{-8} to 10^{-6} g/ml. and was only slightly decreased by 10^{-5} g/ml. Hence, these tetanic contractions are not due to a surmounting of the atropine-block by the increased release of acetylcholine following the 50 pulses.

4. The tetanic spasms originate from excitation of non-cholinergic

neurones, perhaps the associative Dogiel Type I cells of Auerbach's plexus (Hill, 1927), since the spasms were abolished reversibly by tetrodotoxin 2×10^{-7} g/ml. and were absent from plexus-free, nicotine-insensitive preparations of the longitudinal muscle, both before and after atropinization.

5. The tetanic spasms were not reduced by ganglion-block with paralysing doses of nicotine, with (+)-tubocurarine or with hexamethonium.

6. The tetanic spasms are not mediated by a release of 5-hydroxytryptamine (5-HT) or of histamine, since they persisted in concentrations of methysergide and mepyramine adequate to block matching doses of histamine or 5-HT, or multiples thereof. Catecholamines were also excluded.

7. The tetanic spasms are not mediated by a release of a prostaglandin, because they were not reduced by $0{\cdot}5$–2×10^{-6} g/ml. of patulin (Ambache, 1957), which blocked the contractions evoked by matching doses of prostaglandins PGE_2 or $PGF_{2\alpha}$; even after this block, PGE_2 still potentiated subsequent tetanic responses.

8. The tetanic spasms were reduced or virtually abolished by strychnine in concentrations which did not depress the twitch.

INTRODUCTION

This paper describes the effect of stimulating the nervous elements in Auerbach's plexus. The plexus contains the cholinergic post-ganglionic neurones of the vagus; according to Catherine Hill (1927) these are the Dogiel Type II cells (Dogiel, 1899), with long dendrites and an axon which she was often able to trace towards the musculature. Hill (1927) found that the preganglionic fibres of the vagus link with these cells both in Auerbach's and in Meissner's plexus. The neurones in Meissner's plexus are all of one kind and correspond to these Type II cells (Hill, 1927, p. 359). In Auerbach's plexus, on the other hand, another kind of nerve cell, of characteristic appearance and staining properties, is found, often in profusion (Dogiel, 1899, Figs. 11–12; Hill, 1927). These are the Dogiel Type I cells, or a violet staining variant thereof known as the Type III cells, both of which are intercalated neurones with numerous short dendrites establishing synaptic connexion with other ganglion-cells, but without axonal outflow towards the musculature (Hill, 1927, p. 361).

The neurones in Auerbach's plexus can be excited either with nicotine or by electrical stimulation. The twitch due to single shocks and part of the motor effect of nicotine are both susceptible to botulinic paralysis and to atropine-block and therefore are certainly due to stimulation of the cholinergic ganglion-cells. Yet, after atropine, a motor component can

still be detected with nicotine (Ambache & Edwards, 1951; Ambache & Robertson, 1953; and this paper). These observations constituted the starting-point of the present research, since they indicated the possibility that nicotine was, in fact, exciting other neurones that are not cholinergic.

The subject of the present investigation is a comparable atropine-resistant spasm induced by tetanic stimulation of plexus-containing sheets of longitudinal muscle from guinea-pig ileum. This 'tetanic spasm' is extinguished by tetrodotoxin and is absent from plexus-free preparations. It is therefore due to a repetitive excitation of non-cholinergic neurones in Auerbach's plexus, perhaps the intercalated Dogiel Type I cells. The spasm indicates that the tetanic excitation is followed by a release of an active substance, possibly the synaptic transmitter of such neurones, which happens also to excite the longitudinal muscle sheet directly, when the sheet is eventually reached by slow diffusion some 2–3 sec later. The reasons for accepting the resistance to atropine as decisive evidence that this humoral effect is non-cholinergic are given more fully in the Results and the Discussion. It is also shown not to be due to histamine, 5-hydroxytryptamine or prostaglandins.

A preliminary account of these experiments has been given to the Physiological Society (Ambache & Freeman, 1968).

METHODS

In order to obtain robust preparations the albino guinea-pigs chosen were large males, preferably weighing 800 g or more. They were stunned by a blow on the head and bled out. The total length of the excised, uncoiled small intestine was measured (130–180 cm); the lumen was then flushed with Locke's solution. Selected segments of the ileum, taken at measured distances from the ileocaecal valve, were drawn over a horizontal glass rod, and all mesenteric tags were either torn off by pulling with forceps or cut away with curved scissors as close to the gut as possible.

Sheets of the longitudinal muscle, usually with Auerbach's plexus adherent, were obtained, as in rabbits, by the method of Ambache (1954). The initial detachment of the longitudinal sheet at one end of the segment before the sheet could be pulled, was facilitated by tangential stroking, starting at the mesenteric border, with a damp wisp of cotton wool, until some 2–3 cm of longitudinal muscle were completely free circumferentially at one end. This free end was now pulled vertically to obtain the sheet, as illustrated in Plate 1*A*; occasionally, strands of circular muscle adhered to the starting end but were soon left behind as the rest of the sheet was detached by pulling. The presence of Auerbach's plexus was detected in oblique light (Plate 1) and the widest 4–5 cm of the sheet were chosen for the preparation, which was transferred to a Petr. dish after ligation. At one end, a length of fine black silk was tied for eventual suspension to Kavanagh's (1962) lever; at the other end, a small (2–3 mm) loop of the same thread. A number of plexus-free preparations (Paton & Zar, 1968) were also obtained but as these were narrower than the plexus-containing strips, some 8–10 cm were taken and folded to make a doubled preparation.

Usually two preparations from the same animal were set up in twin baths. The organ baths were of the jointed type, with two detachable sections centred round a B_7 ground

glass cone-and-socket joint, as described by Ambache, Kavanagh & Whiting (1965, Fig. 1) but with the following modifications: (*a*) their capacity was slightly greater, 1·8–2·0 ml.; (*b*) the platinum-iridium side-tube (*I* in that figure) was dispensed with; (*c*) the glass dome at the top of the cone was replaced by a tight fitting bung of silicone rubber, through which were threaded the platinum bubbler and hook (*M* and *L*); (*d*) the glass hooks (*N*) on the stem carrying the cone were replaced by a ring-like thickening of the glass on the stem providing a shelf for the spring clip securing the joint (see below); (*e*) the bath was fitted with a pair of vertical 4–5 cm platinum electrodes on the inner wall; these diametrically opposite parallel wires (0·34 mm diameter) were bent at the top to emerge through glass side-tubes fused onto the top end of the bath, and were connected by small crocodile clips to the leads of an electronic stimulator capable of delivering high currents (Bell, 1968). Two such stimulators were used in different experiments: one with a maximum output of 27 V at 500 mA; the other of 60 V at 800 mA, but with a safety device which cut out the output when overloading occurred, which was at 20–22 V in our experimental conditions. References to maximum voltage in the text therefore mean either 27 V at 500 mA or 20–22 V at 800 mA.

Electrical stimulation could be delivered to either bath or to both simultaneously. In any given experiment the pulse width was kept constant at 0·1 or 0·2 msec; such brief pulses failed to excite the muscle fibres in plexus-free preparations. Individual stimuli consisted either of single shocks or of tetani lasting 1 sec at a frequency of usually 50 pulses/sec; these were repeated at set intervals, which are stated in Results. Responses were recorded on a smoked drum at a lever magnification of × 12, and a load of 0·3–0·4 g.

Before setting up the muscle preparation the organ bath was dismantled and a doubled length of a white thread was passed through the upper section and looped onto the platinum hook (*L*). The ground glass joint, previously lightly smeared with silicone stopcock grease (Edwards High Vacuum Ltd.), was then closed and secured with a tight spring clip (Quickfit type JC 13) grasping, above, the lip of the socket and, below, the ring-shelf on the cone-stem. After admitting oxygenated fluid to the organ bath, one end of the white thread was tied to the black loop on the preparation in its Petri dish; the muscle was then lowered into the organ bath by gentle traction on the other end of the white thread. When the muscle preparation reached its desired position in the bath, the anchoring end of the white thread was secured by a rubber ring which was slipped over the outer wall of the organ bath at its top end; the ring was of tight fit and had a slit of 2–3 mm for ease of fixation and removal.

The bath fluid was Krebs–Henseleit solution of the following composition (g/l.): NaCl, 6·9; KCl, 0·35; $CaCl_2.2H_2O$, 0·37; $MgSO_4.7H_2O$, 0·29; $NaHCO_3$, 2·1; KH_2PO_4, 0·14; glucose, 1. This was gassed with a 95 % O_2–5 % CO_2 mixture both in the bath and in the reservoir. A small constant flow of the solution in the organ bath was maintained throughout the experiment, except during drug-contacts, when the tap was closed. The experiments were performed at 35° C, the water tank being maintained at constant temperature by an electric microthermostat with built-in stirrer (Rotax Type MTH/F, A. Balzer, Basle).

Drug doses were injected with syringes of guaranteed ± 0·05 % accuracy (Fritz Kuhn, Frankfurt-am-Main). Contact times varied from 15 to 30 sec. Dosages refer to the salts which were: acetylcholine chloride, atropine sulphate, dimethyltubocurarine bromide, dopamine HCl, hexamethonium bromide, histamine acid phosphate, hyoscine HBr, mepyramine maleate, methysergide bimaleate, morphine sulphate, nicotine hydrogen tartrate, L-noradrenaline bitartrate, strychnine HCl dihydrate, (+)-tubocurarine chloride pentahydrate. The tetrodotoxin was supplied by Sankyo, Japan.

The reserpinized guinea-pigs were pre-treated for 24–48 hr with a total of 10–20 mg/kg I.P. subdivided into 3 or 4 doses.

RESULTS

Total block by atropine of twitches elicited by single shocks

The cholinergic post-ganglionic neurones of the myenteric plexus can be excited selectively with brief pulses of 0·1 or 0·2 msec width (Paton, 1955). Such a single shock evokes a rapid contraction, which will be referred to as the 'twitch-response': it has a relatively short delay and duration; the delay, timed by stopwatch, is of the order of 1–1·2 sec and records on a fast drum show that the base line is regained in 3–8 sec when the contraction is over.

In the present experiments these twitch-responses to single shocks were matched by a dose of acetylcholine of the order of 5–20 ng left in the 1·8 ml. organ bath for 15 or 30 sec. This will be referred to as the 'twitch-matching dose' of acetylcholine. Under normal conditions the response to tetanic stimulation with 1 sec trains of 50 pulses/sec at the same voltage and pulse width was usually off scale and therefore could not be matched until the preparations were lightly atropinized.

In all preparations except a few taken from the anomalous terminal region of the ileum (Munro, 1953), the twitch was abolished promptly by atropine or by hyoscine. The usual dose of atropine for total block was 10^{-8} g/ml., as in Text-fig. 1, but in the experiment of Text-fig. 9 the lower concentration of 4×10^{-9} g/ml. virtually abolished this response. At this stage tetanic stimulation at various frequencies revealed the appearance of an atropine-resistant spasm; this second type of motor response, the 'tetanic spasm', was obtained even from the middle portion of the ileum, and as far away as 95 cm from the ileocaecal valve.

The atropine-resistant 'tetanic spasm'

In most of the experiments the spasms were elicited by delivering 1 sec trains of 50 pulses/sec, each of 0·1 or preferably 0·2 msec pulse width and maximum voltage; but they were also recorded with other frequencies of stimulation (10–100/sec). As this response to tetanic stimulation was now on scale, it could be matched in these lightly atropinized preparations with doses of acetylcholine of the order of 150–200 ng. If the atropine concentration was now raised to 10^{-7} g/ml., and even sometimes to 10^{-6} g/ml., the effect of these higher matching doses of acetylcholine, or of several multiples of these doses, was either abolished or greatly reduced, but there was no corresponding extinction of the tetanic spasms. Hence these contractions cannot be attributed to a surmounting of the atropine-block by the larger amounts of acetylcholine released by the fifty stimuli in each tetanic burst.

That the tetanic response was not due to acetylcholine was shown again, more conclusively, in other experiments. Paton & Zar (1968, Fig. 7) have found that the volley output of acetylcholine in such plexus-containing preparations declines sharply as the rate of stimulation is increased. At a frequency of 10 pulses/sec the volley output is already down to a quarter of the value obtained at low rates of stimulation (1 shock in 20 sec). Hence, when fifty stimuli have been delivered at the even higher rate of stimulation most frequently used by us (50/sec for 1 sec), the total acetylcholine release expected will not be fifty times greater than the release after a single shock, but probably much less than 12·5 times greater. In some of our experiments in which concentrations of atropine of 10^{-7} or 10^{-6} g/ml. were used, the preparations were insensitive to as much as 50–200 times the dose of acetylcholine that originally matched the twitch elicited by single shocks. For example, in the experiment of Text-fig. 1, the initial twitch-response to single shocks was matched by 5 ng of acetylcholine in Panel *A*; but when the atropine concentration was raised to 10^{-7} g/ml. tetanic spasms were still present, as shown in panel *E*, although the preparation was virtually insensitive to 200 times the original matching dose of acetylcholine (ACh), i.e. to 1 μg. Similar results were obtained with hyoscine 10^{-7} g/ml.: when the preparation, taken 70–75 cm from the ileocaecal valve, became completely insensitive to as much as 2 μg of acetylcholine, 200 × the original twitch-matching dose, spasms could still be evoked by trains of 10 or of 50 stimuli (1 sec bursts at 10/sec and 50/sec, respectively).

Legend to Text-fig. 1.

Atropine-resistant tetanic spasms in a plexus-containing preparation of longitudinal muscle from guinea-pig ileum, taken 70–75 cm above the ileocaecal valve. Drum speed as shown in *C* and *E*, except for panel *B* 1. Drug contacts, 30 sec.

Electrical stimulation with 0·2 msec pulses at 27 V, delivered either singly (S) or in 1 sec trains of 50 pulses/sec (T).

Panel *A*: closest match to single shock twitches, obtained with acetylcholine (ACh) 5 ng.

Panel *B* 1: extinction of responses to single shocks by atropine sulphate 10^{-8} g/ml.; drum at half speed.

Panel *B* 2: (after a 4 min gap) normal speed; atropine-resistant spasm at T.

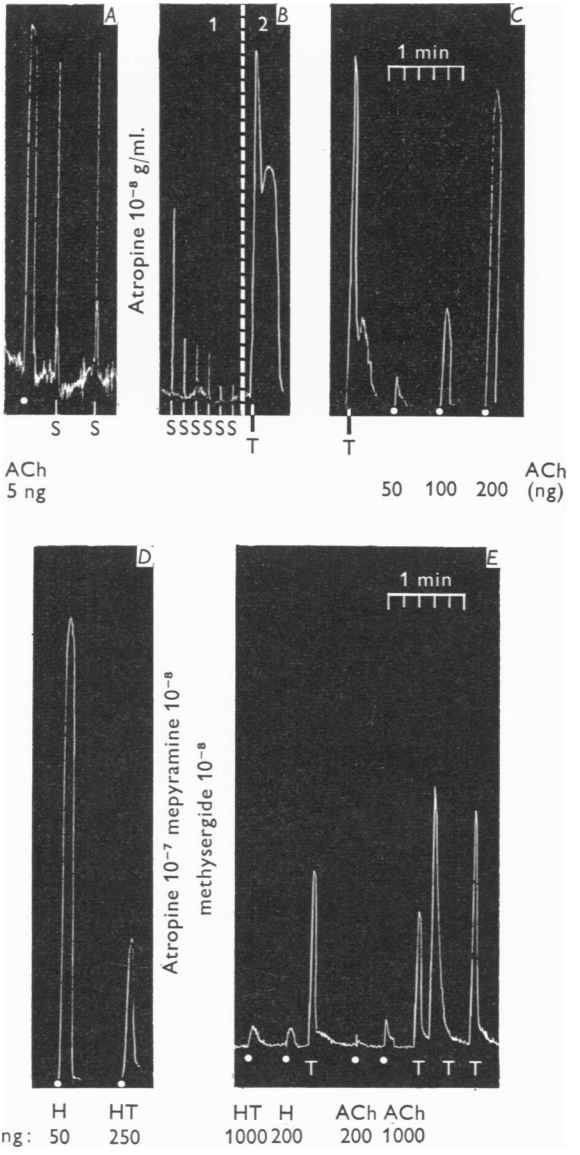
Panel *C*: (after an 8 min gap) closest match to tetanic spasm, obtained with 200 ng acetylcholine.

Panel *D*: 50 ng histamine (H) and 250 ng 5-hydroxytryptamine (HT). Atropine concentration then raised to 10^{-7} g/ml., and mepyramine and methysergide added to reservoir, both 10^{-8} g/ml.

Panel *E*: persistence of tetanic spasms, although preparation has become insensitive to 1 μg 5-HT, to 0·2 μg histamine, and to 0·2 and 1 μg acetylcholine (200 × original twitch-matching dose).

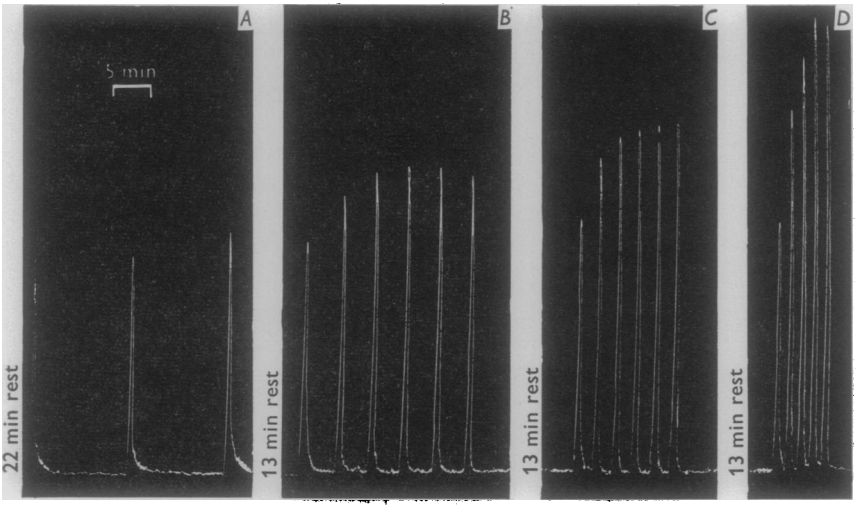
The staircase phenomenon

An important feature of the tetanic responses, and one which influenced the temporal design of subsequent experiments, was the fact that the size of the atropine-resistant tetanic spasms was markedly affected by the



Text-fig. 1. For legend see opposite page.

spacing of the tetani. Since the muscles did relax fully between successive spasms while this sensitization effect was taking place, it was apparently some kind of staircase phenomenon, as shown in Text-fig. 2. The experiment illustrates the growth of the staircase with closer tetani. Thus, with the very long intertetanic intervals of 782 sec (13 min) in Group *A*, the height of successive spasms remained nearly constant. But when, after a further 13 min of rest, stimulation was resumed in Group *B*, with a shorter



Text-fig. 2. Effect of spacing of tetani upon the amplitude of atropine-resistant spasms: appearance of staircase phenomenon in *B*, *C* and *D* with closer tetani, and growth in response amplitude.

Plexus-containing preparation 30–35 cm above ileocaecal valve, rendered virtually insensitive to 50 times the original twitch-matching dose of acetylcholine by atropine sulphate 10^{-7} g/ml. Each 1 sec tetanus consists of 50 pulses of 0·1 msec width at 27 V.

Each of the four groups of tetanic responses was preceded by a period of adequate rest: 22 min before *A*; and 13 min before *B*, *C* and *D*. Intertetanic intervals: Group *A*, 782 sec; Group *B*, 260 sec; Group *C*, 156 sec: Group *D*, 97 sec.

intertetanic interval of 260 sec, a distinct staircase phenomenon developed. This was even more pronounced in Groups *C* and *D*, with intertetanic intervals of 156 sec and 97 sec, respectively; the closer the tetani the steeper the staircase and the higher the final steady level of contraction-heights reached. In other words, the amplitude of a tetanic spasm at any time is clearly governed not only by the prevailing rhythm of stimulation, but also by past events. That is why, before recording each of these four groups of tetanic spasms, a suitable period of rest was allowed, to ensure the full subsidence of any build-up due to previous stimulation. In a total

of some ninety experiments this staircase phenomenon never failed to manifest itself.

Further characteristics of the atropine-resistant tetanic spasm

Tetanic spasms differed from acetylcholine-mediated responses in another important respect, namely in their slower onset. Whereas the twitch-delay in unatropinized preparations was of the order of 1 sec, which appears to be the time taken for released acetylcholine to diffuse into the longitudinal muscle, the tetanic spasms did not begin until some 2–3 sec had elapsed after the end of the 1 sec period of stimulation.

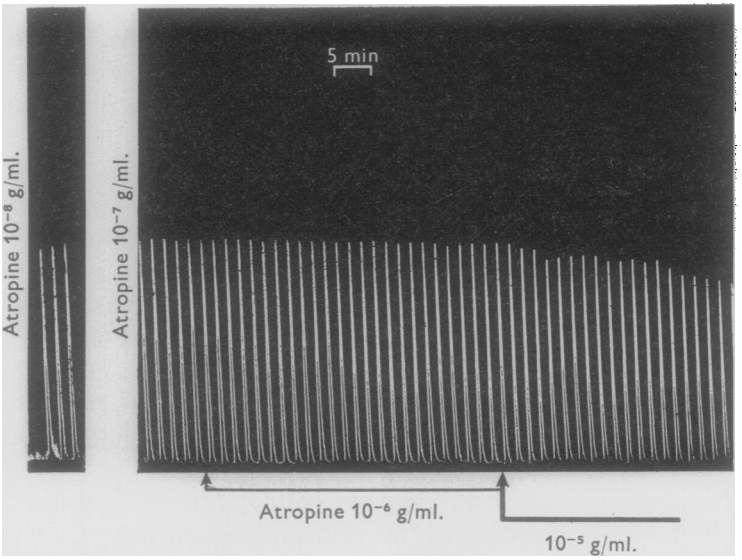
Fatigue. In prolonged experiments the height of the tetanic spasms, after reaching a steady level for a time, began to decline gradually. When this occurred in an experiment in which the pulse width had been fixed at 0·1 msec, the decline could be completely offset by increasing the pulse width to 0·2 msec. This observation suggests that 0·1 msec may be on the borderline of effectiveness for some of the excitable units concerned in the tetanic response and that better recruitment may be ensured with 0·2 msec stimuli. Even with 0·2 msec pulse widths, however, fatigue often occurred.

Re-examination of the atropine-resistance of the tetanic spasms, taking into account the staircase phenomenon

If the tetanic spasms were due to some kind of escape from the atropine-block, i.e. due to the action of unblocked acetylcholine, they should show a progessive decrease as the concentration of atropine is raised. This can be deduced from our knowledge of atropine–acetylcholine antagonism. Thus, it is well established that the antagonism of acetylcholine-contractions by low concentrations of atropine can be overcome if the dose of the agonist, acetylcholine, is raised sufficiently; but if the concentration of atropine is then raised further, the contractions due to this higher dose of acetylcholine are again abolished owing to increased occupancy of acetylcholine-receptors by the antagonist.

With these considerations in mind, the atropine-resistance of the tetanic spasms was re-examined under more rigorous conditions in experiments in which variations due to the staircase phenomenon were eliminated by maintaining a constant rhythm of stimulation at a set intertetanic interval throughout the experiment, without interruption for any tests with acetylcholine or other agonists, in order to avoid any loss of the build-up. To reduce fatigue, the pulse width was set at 0·2 msec. Such an experiment is illustrated in Text-fig. 3. When the tetanic spasms reached a steady post-staircase level in the presence of atropine sulphate 10^{-8} g/ml., there was no noticeable change in the height of the spasms as the atropine concentration was raised in steps of ten, first to 10^{-7} and then to 10^{-6} g/ml.

With atropine 10^{-5} g/ml. there was, in some experiments, a gradual decline in the amplitude, e.g. 17 % over the next 30 min in this experiment, 9 % in another, and no decline in a third.

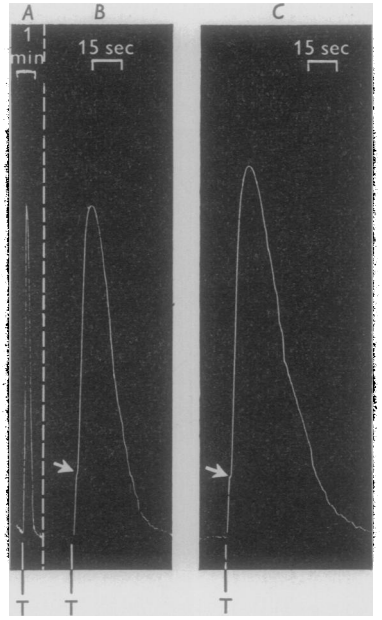


Text-fig. 3. Constancy of tetanic spasms during stepwise increases in atropine concentration from 10^{-8} to 10^{-5} g/ml. Plexus-containing preparation taken 32–44 cm above ileocaecal valve. Uninterrupted electrical stimulation with 0·2 msec pulses in 1 sec trains of 50 pulses/sec at intervals of 100 sec. The height of the tetanic spasms is shown initially after 47 min in atropine sulphate 10^{-8} g/ml. The amplitude of the spasms did not change as the concentration of atropine sulphate was increased after 1 hr from 10^{-8} to 10^{-7} and after 0·5 hr to 10^{-6} g/ml. A further increase to 10^{-5} g/ml. (to the end of the tracing) eventually decreased the amplitude by 17 % after 30 min (see text).

Other components in the response to tetanic stimulation

The atropine-resistant response consisted usually of two, but occasionally of three, components which could be clearly separated by recording at faster drum speeds (Text-fig. 4). The main component, always present, was the 'tetanic spasm', which lasted 20–60 sec. This will be referred to as Component 2, because in distal preparations it was often preceded by a small, brief twitch (see Text-figs. 4 and 5), separated from Component 2 by a distinct notch. In more proximal preparations this small twitch appeared to be absent, but in some experiments observation suggested that the absence of a notch was only due to a merging of Component 1 into Component 2; this occurred occasionally in the experiment of Text-fig. 4. Lastly, the relaxation phase of Component 2 was sometimes interrupted

by a prolonged contraction which may be called Component 3; this is seen in Text-figs. 1 (panel *B* 2) and 9(*B*). When tetani were delivered at regular intervals this Component 3 was sometimes present only in response to the first tetanus and disappeared subsequently.



Text-fig. 4. Separation of tetanic spasms into Components 1 and 2 at faster drum speeds; see text. Plexus-containing preparation 25–30 cm above ileocaecal valve; atropine sulphate 10^{-7} g/ml. T, tetanic spasms elicited by 1 sec trains of 50 pulses/sec at a pulse width of 0·2 msec.

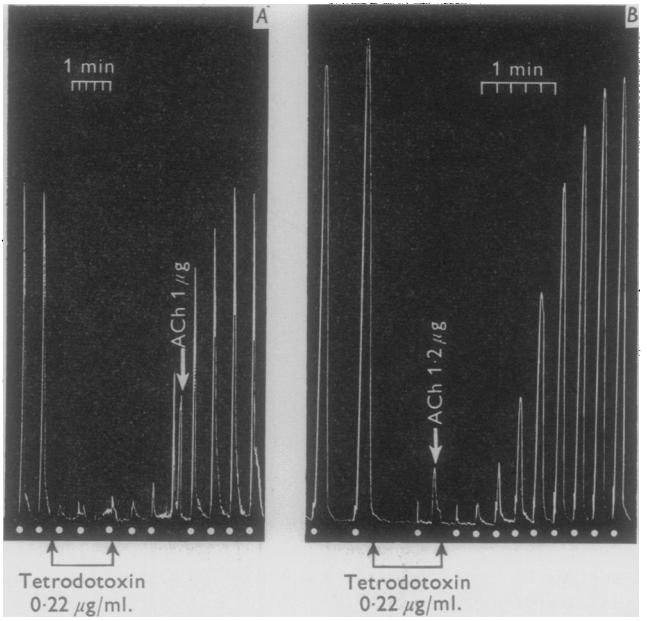
Component 1 appears to be absent in Record *A*, taken on a slow drum. Records *B* and *C* reveal the separation between Component 1 (below the arrow) and Component 2 (above the arrow) by a notch, which is distinct in *C* but indistinct because of mergence in *B*, recorded earlier in the same experiment.

Nervous origin of the main component of the atropine-resistant tetanic response

The following tests show conclusively that the main component of these tetanic responses is the result of excitation of non-cholinergic neurones in Auerbach's plexus.

Extinction by tetrodotoxin. In atropinized, plexus-containing preparations the main component of the response to tetanic stimulation (Component 2) was reversibly abolished or reduced by the application of tetrodotoxin 1–2 $\times 10^{-7}$ g/ml. (Text-fig. 5). All that remained of the tetanic response in tetrodotoxin was a small rapid contraction, often identifiable as a residual

Component 1 and more marked in distal or terminal preparations; it is well seen in Panel *B* of Text-fig. 5. A tenfold increase in tetrodotoxin concentration to 2×10^{-6} g/ml. failed to extinguish this small remnant, the origin of which needs further elucidation. The fairly rapid recovery of the Component 2 spasms, after the tetrodotoxin was washed out, is evident in Text-fig. 5. A distinct notch, between this large component and the small Component 1 preceding it, is discernible in some of the responses before and after the tetrodotoxin.



Text-fig. 5. Atropine-resistant tetanic spasms in plexus-containing preparations, reversibly abolished by tetrodotoxin 0·22 μg/ml. between the lower arrows. Preparation *A* taken 45–50 cm, and *B* 25–30 cm, above ileocaecal valve; both in atropine sulphate 10^{-7} g/ml.

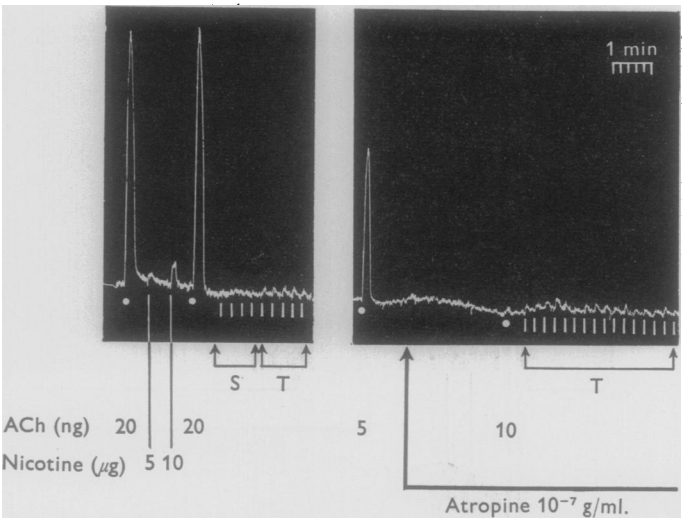
At the dots, 1 sec tetani of 50 pulses/sec, at 27 V and pulse width 0·1 msec in *A* or 0·2 msec in *B*. Intertetanic intervals: in *A*, 156 sec and in *B*, after the tetrodotoxin, 80 sec. ACh: acetylcholine 1 μg for 15 sec in *A* and 1·2 μg for 30 sec in *B*.

In *B* each large tetanic spasm was preceded by a small twitch, with an intervening notch; these small twitches persisted in tetrodotoxin. Details in text.

Absence of tetanic spasms from plexus-free preparations. If the tetanic responses owe their origin to neuronal excitation, they should be absent from plexus-free preparations of the longitudinal muscle. That this is so is illustrated in Text-fig. 6.

Normal, innervated preparations responded by contraction to 1–2 μg of nicotine. This virtually plexus-free preparation failed to respond to 5 μg,

but there was a minute response to 10 μg, of nicotine. However, though not totally denervated, this preparation was unresponsive to electrical stimulation. Thus, although before atropine the preparation was sensitive to 5–20 ng of acetylcholine, there was no response to single shocks of 0·1 msec width and maximal voltage at *S*, and virtually none to 1 sec tetani of 50 pulses at *T*. After administration of atropine sulphate 10^{-7} g/ml. the tetanic spasms could still not be obtained. In one or two other plexus-free muscles tetanic stimulation elicited only minute rapid contractions resembling the tetrodotoxin-resistant component described above, but the broader Components 2 and 3 were absent.



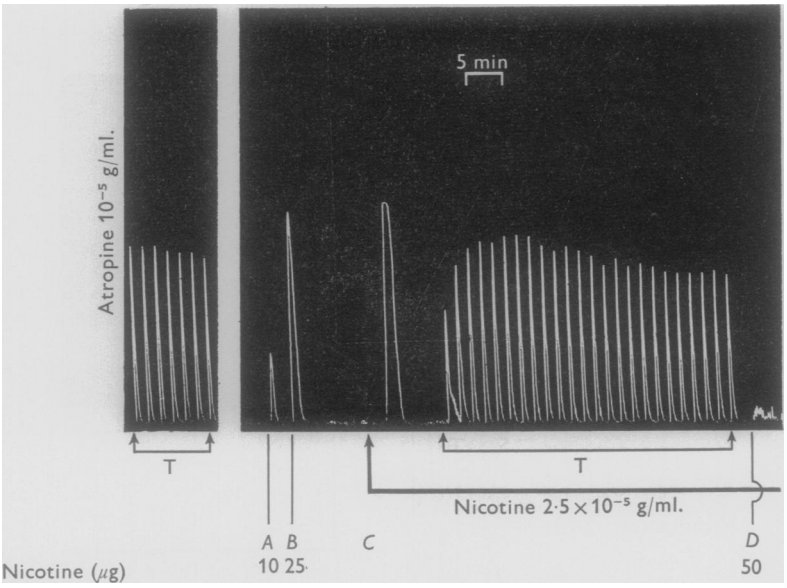
Text-fig. 6. Electrical inexcitability and absence of tetanic spasms in a nicotine-insensitive, virtually plexus-free preparation of longitudinal muscle from guinea-pig ileum, taken 17–25 cm above ileocaecal valve.

Between the arrows electrical stimulation at 27 V and 0·1 msec pulse width, delivered either singly (S) or in 1 sec trains of 50 pulses/sec (T) at 80 sec intervals. There is no response to S or T before atropine, or to tetani in the presence of atropine 10^{-7} g/ml. At the dots, acetylcholine and at the lines, nicotine in the doses indicated; 30 sec contacts; bath volume, 1·8 ml.

Persistence of tetanic spasms after ganglionic paralysis by large doses of nicotine, by (+)-tubocurarine and by hexamethonium. The motor response of unatropinized, plexus-containing preparations to small doses of nicotine (0·55–2·8 μg/ml.) is due to excitation of the cholinergic post-ganglionic neurones; its delay is usually 1 sec. After atropine the effect of these near-threshold doses may often be completely blocked, as reported by Ambache & Robertson (1953); but the preparation is not wholly insensitive to nicotine and contractions can be recorded again if the dose of nicotine is

raised slightly, but with a delay of 3–4 sec. Thus, in the experiment of Text-fig. 7, contractions were obtained at *A* with 5 μg/ml. and at *B* with 12·5 μg/ml. of nicotine, administered for 30 sec, although the preparation was in atropine 10^{-5} g/ml. These atropine-resistant contractions appear to be due to stimulation by small doses of nicotine of non-cholinergic neurones, possibly at their ganglion-cells and nerve-endings.

By contrast, the tetanic spasms of atropinized preparations appear to originate mainly from an excitation of nerve fibres rather than specifically



Text-fig. 7. Persistence of tetanic spasms in the presence of a ganglion-paralysing concentration of nicotine. Plexus-containing preparation taken 38–44 cm above ileocaecal valve; in atropine sulphate 10^{-5} g/ml.

Between arrows (T), electrical stimulation with 0·2 msec pulses delivered in 1 sec trains of 50/sec at 100 sec intervals. Drug contacts, 30 sec; 2 ml. bath.

Contractions to 10 and 25 μg nicotine at *A* and *B*. Nicotine 2×10^{-5} g/ml., introduced to reservoir at *C*, produced a contraction lasting some 2 min, followed by ganglion-paralysis as confirmed by the absence of response to a dose of 50 μg nicotine at *D*. When tetanic stimulation was resumed for 38 min after *C* there was no decrease in amplitude of the spasms, although the ganglion-cells were paralysed.

of ganglion-cells, because they persisted after ganglionic paralysis by larger doses of nicotine. For example, when nicotine $2{\cdot}5 \times 10^{-5}$ g/ml. was introduced into the reservoir (Text-fig. 7, from *C* onwards), it produced the contraction shown in *C*, which was not maintained, indicating that ganglion-cell paralysis had taken place. When tetanic stimulation was then resumed, there was no significant reduction in the height of the spasms

during the following 38 min. A test was carried out at the end of the experiment with a 50 μg dose of nicotine, administered by syringe for 30 sec at *D*, which showed by its ineffectiveness that the ganglion-cells were still paralysed.

In other experiments much larger paralysing doses of nicotine, 0·2–1·0 mg, were administered by syringe into the organ bath at intervals over a period of several minutes. In one of these experiments tetanic spasms persisted and were even potentiated during an 11 min exposure to 1 mg nicotine, although the ganglia were paralysed within 1–2 min of the first 1 mg dose of nicotine, as shown by the fact that renewal of this 1 mg dose of nicotine after 3 min was ineffective. But in other experiments these large doses of nicotine gave rise to unsteadiness of the base line.

The tetanic spasms of atropinized preparations were also unaffected by other ganglion-blocking agents, such as (+)-tubocurarine, 6–12 μg/ml., or its dimethyl ester 6–30 μg/ml., both adequate to block nicotine-stimulation in the atropinized preparation, and hexamethonium, 14–56 μg/ml.

Exclusion of other known humoral agents as possible mediators of the tetanic spasms

(*a*) 5-*Hydroxytryptamine.* That atropine, by eliminating a cholinergic nervous component in the response to 5-hydroxytryptamine (5-HT), reduces the sensitivity of guinea-pig ileum preparations to 5-HT was first reported by Robertson (1953) and confirmed by Cambridge & Holgate (1955). In the present experiments on the plexus-containing longitudinal muscle preparations, after block of response to the cholinergic nerves by atropine 10^{-8} or 10^{-7} g/ml., contractions could still be elicited by relatively high doses of 5-HT, acting directly upon the smooth muscle. But this residual effect of 5-HT was easily abolished by a suitable antagonist such as methysergide, which is capable of blocking the receptors concerned in the smooth muscle, i.e. the so-called *D* receptors of Gaddum & Picarelli (1957). In several experiments, when this methysergide-block was well established, there was, however, no corresponding extinction of the tetanic spasms.

One such experiment is illustrated in Text-fig. 1. The small contraction of the lightly atropinized preparation evoked by 0·25 μg of 5-HT is shown in Panel *D*. But, as illustrated in Panel *E*, when methysergide 10^{-8} g/ml. was present there was virtually no response even to 1 μg 5-HT; yet tetanic spasms were present as usual.

These results indicate quite clearly that the atropine-resistant tetanic spasms are not mediated by a release of 5-HT.

(*b*) *Histamine.* Although histamine is said to be present in some nervous tissues, its role as a synaptic transmitter has never been established. A

release of histamine from guinea-pig intestines was reported by Ambache & Barsoum (1939). Because the longitudinal muscle of the atropinized guinea-pig ileum is extremely sensitive to histamine, it was necessary to exclude this substance by the use of a suitable antagonist, such as mepyramine. As shown in Text-fig. 1, the amplitude of the response to 50 ng of histamine in Panel *D* exceeded that of any previous tetanic spasm. When the block produced by mepyramine maleate 10^{-8} g/ml. was well established, in Panel *E*, histamine was virtually inactive even in 200 ng doses, but the tetanic spasms were not extinguished.

It was noticed in several experiments that mepyramine maleate 10^{-8} g/ml. reduced, sometimes substantially, the amplitude of the tetanic spasms. But since bradykinin contractions were also somewhat reduced, this seems to be due to a non-specific effect of mepyramine, resembling perhaps its slight antagonism of other agonists, such as acetylcholine and 5-HT (Cambridge & Holgate, 1955). The tetanic spasms persisted, though somewhat reduced, after a tenfold increase in mepyramine maleate concentration to 10^{-7} g/ml.

In conclusion, histamine does not appear to be the transmitter responsible for these atropine-resistant tetanic spasms.

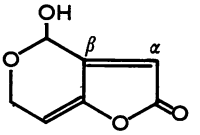
(*c*) *Catecholamines.* It is only in the terminal portion of the guinea-pig ileum that catecholamines, in high doses, exert a motor effect (Munro, 1951); elsewhere they are inhibitory. Since the tetanic spasms could be obtained even as far away as 95 cm from the ileocaecal valve, it seemed unlikely that catecholamines were directly involved in the mediation of this response. In such non-terminal preparations contractions could not be elicited with 50–500 ng of noradrenaline or 1 μg of dopamine; and, before atropine, these amines inhibited the twitch response to single shocks.

Reserpine is known to produce a catecholamine depletion. Tetanic spasms were present as usual in preparations taken from previously reserpinized guinea-pigs.

(*d*) *Prostaglandins.* Recent work from this laboratory (Ambache, Brummer, Whiting & Wood, 1966) has shown that prostaglandins $F_{2\alpha}$ and E_2 are present in extracts of plexus-containing longitudinal muscle of the guinea-pig ileum. Moreover, this muscle is contracted by prostaglandins even in the presence of atropine. There was therefore a distinct possibility that the atropine-resistant tetanic spasms in our plexus-containing preparations might be due to a release of prostaglandins. This has been excluded by the use of patulin.

The spasmolytic action of the $\alpha\beta$-unsaturated lactone patulin (formula in Text-fig. 8), and of some other lactones, was described by Ambache (1957, 1959); use was made of this property to block contractions produced

by irin and by other unsaturated hydroxy-acids. This effect of patulin was explored further by Eliasson (1958), who reported that patulin could antagonize most of the substances which contract smooth muscles, including PGE_1. In the present investigation patulin was used to block the even more active PGE_2. Care was taken to use the least effective concentration of patulin ($0{\cdot}5–2 \times 10^{-6}$ g/ml.), in order not to paralyse the response of the muscle to all agonists. With these precautions it was possible to abolish PGE_2 contractions without significantly affecting the tetanic response of atropinized preparations.



Text-fig. 8. Patulin.

Such an experiment is illustrated in Text-fig. 9. The response to 40 ng PGE_2 in Panel *C* exceeded in height any of the preceding tetanic spasms. After the dose of PGE_2 was washed out there was a marked potentiation of subsequent tetanic spasms; this potentiation was noted regularly in all our experiments. The decline of PGE_2-response in panel *D* was quite slow in the presence of patulin 5×10^{-7} g/ml. When, however, the concentration of patulin was doubled to 10^{-6} g/ml. at P, the response to 40 ng PGE_2 was virtually extinguished, but the tetanic spasms remained potentiated.

Similar results were obtained with $PGF_{2\alpha}$. On atropinized preparations the action of this prostaglandin was some twenty times weaker than that of PGE_2; it was also blocked by patulin.

These experiments strongly suggest that prostaglandins cannot be directly implicated in the humoral transmission of the atropine-resistant tetanic spasms.

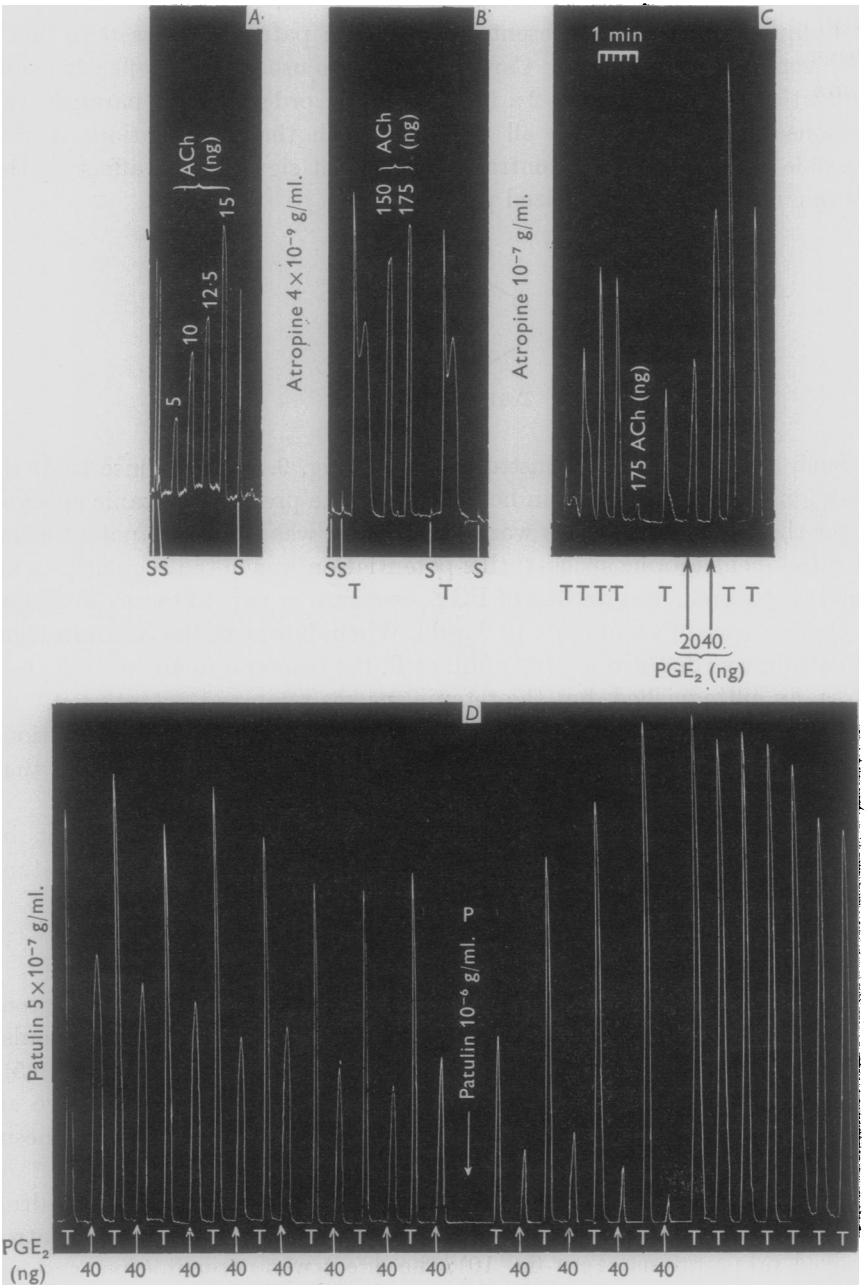
Effect of other drugs or reagents on the tetanic spasms

Tetanic spasms could not be blocked with, and were in fact slightly potentiated by, sodium thioglycollate, 1·25–2·25 mg/ml., a specific antagonist of active substances containing an –S–S– group (Martin & Schild, 1965).

Tetanic spasms were unaffected by various drugs known to have an action on the central nervous system: morphine 1–5 μg/ml., mephenesin 5 μg/ml., diphenylhydantoin 0·5–2·5 μg/ml., and picrotoxin, 1 μg/ml.

Strychnine HCl. In concentrations of $2{\cdot}2–5{\cdot}5 \times 10^{-5}$ g/ml. this drug promptly reduced or virtually abolished the tetanic spasms in the atropinized preparations (Text-fig. 10); the effect was reversible on washing out the drug. In these concentrations strychnine did not depress the

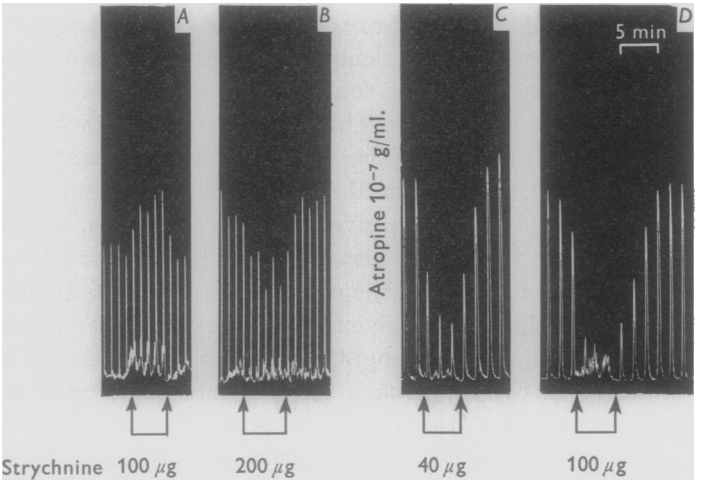
twitch response of the same preparations before atropine, but $1{\cdot}1 \times 10^{-4}$ g/ml. had a slight depressing effect.



Text-fig. 9. For legend see opposite page.

DISCUSSION

In agreement with the histological observations of Dogiel (1899) and of Hill (1927), the above results can be taken as evidence for the presence in Auerbach's plexus of two electrically excitable structures, differing in humoral activity: (*a*) the cholinergic post-ganglionic neurones, probably



Text-fig. 10. Reversible block of tetanic spasms by strychnine.

Plexus-containing preparation, 70–75 cm above ileocaecal valve; in 1·8 ml. bath. Electrically stimulated with 0·1 msec pulses, delivered either singly at 1 min intervals in *A* and *B*, or in 1 sec trains of 50/sec at 100 sec intervals in *C* and *D*, after atropine sulphate 10^{-7} g/ml.

Strychnine HCl in the doses indicated reduced or virtually abolished the atropine-resistant tetanic spasms, but did not have a corresponding effect upon the twitch-response before atropine.

Legend to Text-fig. 9.

Persistence of atropine-resistant tetanic spasms after prostaglandin-block by patulin. Preparation taken 70–75 cm above ileocaecal valve. Stimulation with 0·1 msec pulses at 27 V, delivered either singly (S) or in 1 sec trains of 50 pulses/sec (T). Drug contacts, 30 sec.

Panel *A*: S, single shock twitches matched by 12·5–15 ng acetylcholine.

Panel *B*: Virtual extinction of S by atropine sulphate 4×10^{-9} g/ml., introduced 11 min before *B*. Tetanic spasms now matched by 150–175 ng acetylcholine.

Panel *C*: Atropine raised to 10^{-7} g/ml. 11 min before *C*; 175 ng acetylcholine now blocked, but tetanic spasms persist. The contraction to 40 ng PGE_2 exceeds any previous tetanic spasm and is followed by potentiation of T.

Panel *D*: Effect of patulin 5×10^{-7} g/ml. introduced 7 min before *D* and increased to 10^{-6} g/ml. at P. Although there is a decline and virtual extinction of PGE_2 contractions (40 ng at arrows), the tetanic spasms persist and are still potentiated by the PGE_2.

Dogiel Type II cells, which release acetylcholine in a manner such that it is here easily blocked by atropine; and (*b*) other neurones that release a second active substance, which can diffuse slowly out of the plexus and then contract the longitudinal muscle, even in the presence of very high concentrations of atropine.

There is a well known discrepancy in the humoral physiology of some cholinergic endings, which must be considered here as it has a great bearing upon the significance of our results, namely, that atropine, though usually capable of blocking acetylcholine receptors in minute amounts, may fail to block transmission at some nerve-endings which from other evidence appear to be cholinergic. Thus, cholinergic fibres fall into two distinct categories: (*a*) those that are easily blocked by atropine, such as the pupilloconstrictor fibres from the ciliary ganglion and the cardio-inhibitory fibres of the vagus, to give only two examples, and (*b*) those that are not, such as the motor nerves to the bladder and intestine in some species. Possible reasons for the unusual refractoriness to atropine in this second category have been given elsewhere in a fuller treatment of this subject (Ambache, 1955), where reference is made to Dale & Gaddum's (1930) 'proximity theory' that such refractoriness may arise when the liberation of acetylcholine 'takes place...within the (atropine) barrier'.

In the longitudinal muscle of the guinea-pig intestine, the terminal portion apart (Munro, 1953), there is evidence that the vagal cholinergic post-ganglionic endings fall into the first category. Thus, *in vivo*, Straub & Stefánsson (1937) showed convincingly that intestinal contractions elicited by vagal stimulation were easily blocked by atropine in low concentrations (10 μg/kg). Paton's (1955) results on non-terminal isolated ileum preparations *in vitro* fully bear this out: direct, coaxial, electrical stimulation with brief pulses was shown to excite the cholinergic post-ganglionic neurones in this preparation, and the resulting twitch was completely abolished by atropine 10^{-8} g/ml. In the present investigation on nearly a hundred plexus-containing longitudinal muscle sheets peeled away from guinea-pig ilea we have, in confirmation of Paton & Zar's (1968) observations, never failed to extinguish this twitch promptly with atropine, sometimes even in concentrations as low as 4×10^{-9} g/ml., or with hyoscine. It is clear, therefore, that the anatomical arrangement of the cholinergic nerve-endings in this case allows atropine and hyoscine to exert their usual action and permits a full block not only of administered, but also of nervously released, acetylcholine. This orthodox atropine-susceptibility is entirely consistent with the microscopical observations of Paton (1964), reported by Paton & Zar (1968) as follows: 'Auerbach's plexus, together with the nervous ramifications originating in it, appears to form a layer on the inner surface of the longitudinal muscle and to send

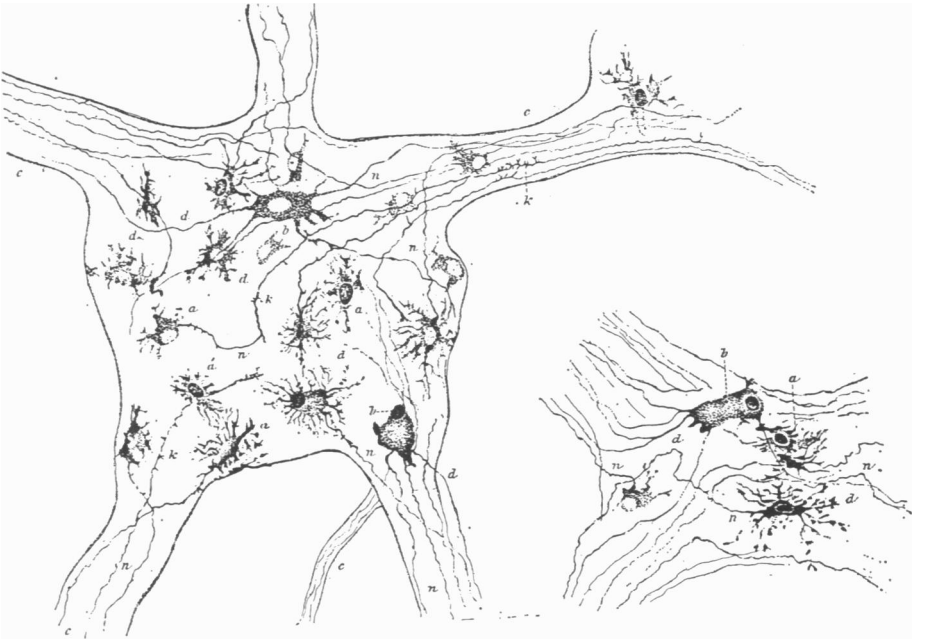
no nerve fibres which penetrate the muscle layer.' Thus, we have a situation in this preparation where Dale & Gaddum's (1930) explanation is inapplicable, since the nervously released acetylcholine has to diffuse across from the plexus to the longitudinal muscle fibres and would therefore be as susceptible to atropine-block as is administered acetylcholine itself. Hence, the atropine-resistance of the tetanic spasms can, in this case, be taken to indicate the operation of a non-cholinergic humoral mechanism. Lastly, in the present experiments the most usual form of stimulation has consisted of tetani of fifty volleys. According to Paton & Zar (1968, Fig. 7), the volley output of acetylcholine declines sharply as the rate of stimulation increases. Therefore, a tetanus of fifty volleys would release much less than fifty times the amount of acetylcholine released by a single volley; yet we found persistence of the tetanic spasms even after > 200 times the twitch-matching dose of acetylcholine had been blocked by atropine. Moreover, the height of the spasms remained constant as the atropine concentration was raised stepwise from 10^{-8} to 10^{-6} g/ml. and declined only slightly in 10^{-5} g/ml.

All these facts suggest strongly that the 'tetanic spasmogen' is not acetylcholine but some atropine-resistant substance. Although this second active substance happens to be spasmogenic to the smooth muscle in the longitudinal layer, its primary role may be as a synaptic transmitter, within the plexus, of non-cholinergic neurones. It could be released either by the endings of afferent fibres in the peristaltic reflex arc or quite possibly from the Dogiel Type I cells. These neurones appear to have a purely local, 'associative' function (Hill, 1927) in Auerbach's plexus, as they are in synaptic relation with other nerve cells within the ganglia of the plexus but do not have an axonal outflow towards the musculature (Hill, 1927). They were found by Dogiel (1899) and by Hill (1927) in several species, including the guinea-pig; the illustration in Text-fig. 11, taken from Dogiel's (1899) paper, is of two Auerbach ganglia from a human ileum, showing a preponderance of the Type I with a few Type II cells.

The available histological evidence suggests, then, that these Dogiel Type I cells do not constitute an alternative, non-cholinergic vagal postganglionic outflow from Auerbach's plexus to the longitudinal muscle. The long delay of the tetanic spasms, too, is consistent with the view that the spasms are secondary to humoral events occurring primarily within the plexus. The nicotinic contractions after atropine are also delayed by 3–4 sec and would appear to have a similar origin.

If this view is correct, it may also explain the dependence of these spasms on the frequency of stimulation; although they can be obtained with 10 pulses/sec they are more marked at 50/sec. Maybe it is necessary to

release a gross excess of the active substance in order to swamp transmitter-destroying mechanisms within the plexus and permit enough of the substance to overflow and to escape, undestroyed, towards the muscle. In considering the chemical nature of this active substance, our results exclude histamine, 5–HT, catecholamines and prostaglandins, though a local release of prostaglandin(s) might account for the staircase phenomenon, since the tetanic spasms were potentiated by PGE_2 even in the



Text-fig. 11. Dogiel's Type I (a) and Type II (*b*) cells in two ganglia of Auerbach's plexus from human ileum; methylene blue (from Dogiel, 1899; Figs. 11 & 12).

presence of patulin. Adenosine derivatives are also probably not involved. Although present in some extracts which we have made of guinea-pig plexus-containing longitudinal muscle sheets, adenosine and its derivatives fail to contract this particular muscle.

Our thanks are due to Mr J. Verney and Mr D. Rose for skilful assistance throughout the work, and to our colleague Dr G. C. R. Morris for textual clarifications.

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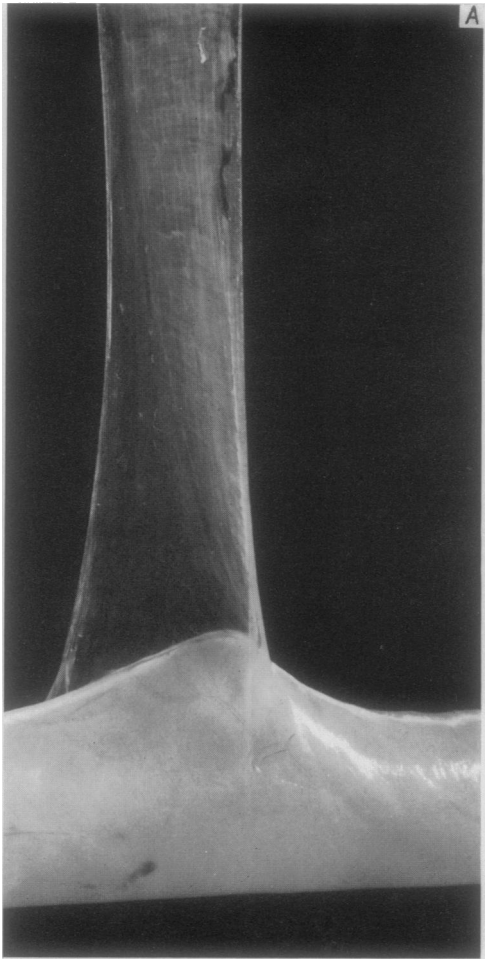
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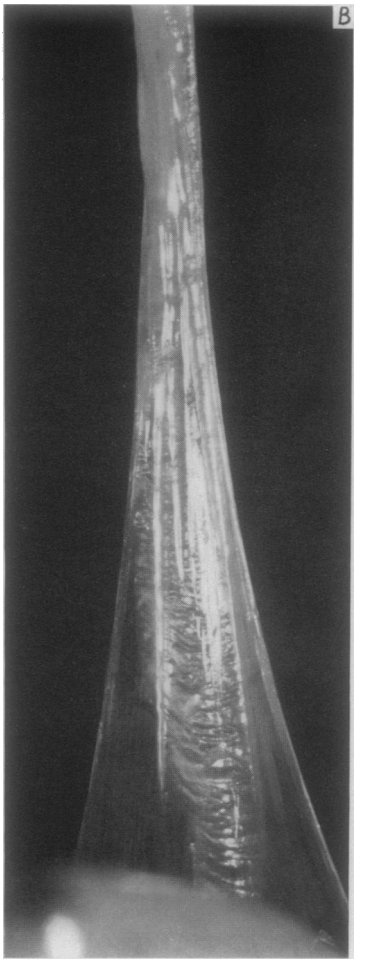
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EXPLANATION OF PLATE

A. Method of peeling off longitudinal muscle sheet by traction (Ambache, 1954). Rabbit ileum; the pearly appearance is due to Auerbach's plexus which was present throughout.

B. Separated sheet from guinea-pig ileum; at the lower end, the plexus is present in the middle of the sheet but absent from the triangular areas at the edges.