

## EFFECTS OF TETRODOTOXIN ON INNERVATED SMOOTH MUSCLE PREPARATIONS

BY

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The puffer fish poison, tetrodotoxin, abolishes action potentials in nerve and striated muscle by preventing the rapid entry of sodium through the depolarized cell membrane (Narahashi, Moore & Scott, 1964). By contrast, at the neuromuscular junction, tetrodotoxin has no effect on the frequency of spontaneous miniature end-plate potentials (Elmqvist & Feldman, 1965), and does not abolish the end-plate potentials elicited by local depolarization of the terminal axon (Katz & Miledi, 1966). Tetrodotoxin also fails to abolish the spontaneous electrical activity of vertebrate smooth muscle or that evoked by direct stimulation (Toida & Osa, 1965; Bülbring & Tomita, 1966; Kuriyama, Osa & Toida, 1966). In the guinea-pig taenia coli concentrations of tetrodotoxin up to  $5 \times 10^{-7}$  g/ml. have no effect on the spike amplitude, or the rate of rise and fall of the action potential, and either do not affect or increase the frequency of spontaneous discharge. On the basis of this electrical evidence it would seem that tetrodotoxin could be used to study smooth muscle responses in the absence of nervous influences. The action of tetrodotoxin was therefore studied on the responses, *in vitro*, of a variety of smooth muscle preparations to drugs and to stimulation *via* their nerve supply. In addition, the effect of tetrodotoxin was determined on the electrically recorded compound action potential of the vagus nerve.

Some of the results have been demonstrated at the meeting of the Physiological Society in Oxford (Gershon, 1966). An important review of the actions of tetrodotoxin (Kao, 1966) has recently appeared and contains references to the relevant Japanese literature, much of which is not readily available outside Japan.

### METHODS

Seven different types of smooth muscle preparations were used in these experiments. They were prepared from animals of either sex, stunned and bled.

The guinea-pig stomach was removed from the animals with the oesophagus, thoracic vagus nerves and coeliac axis intact and attached. The vagi were dissected from the oesophagus, which was then tied off and removed. The branches of the coeliac axis running to the stomach were cleaned and prepared for perivascular nerve stimulation. The pylorus was cannulated, and the contents of the stomach were washed out with saline. The stomach was then filled with 25 ml. saline and set up in a 100 ml. organ bath. Internal pressure changes were measured by a water manometer as described by Paton & Vane (1963). The vagus nerves and perivascular nerves were drawn through shielded platinum ring electrodes and stimulated separately. The possibility of current spread was tested, with maximal stimulator output (150 V or five times the usual stimulation voltage), by placing the

electrodes in various positions in the bath. No response could be elicited from the stomach by stimulation, unless the nerves were drawn through the electrodes.

The mouse stomach was prepared in essentially the same way except that it was cannulated through the duodenum and filled with 0.7 ml. saline. The cannulated mouse stomach was connected through saline-filled tubing to a vertical glass tube, 2 mm in diameter, containing a black Perspex float which moved with the surface of the column of saline. The glass tube was enclosed in a slit within a black box and a light was focused through it on to a photosensitive element. The movements of the float determined the amount of light passing through the slit, and thus, by measuring the light-induced change in resistance across the photo-sensitive element, a record of the intraluminal pressure of the mouse stomach could be obtained.

Movements of the longitudinal muscle of the rabbit jejunum were recorded from 3 cm lengths of intestine suspended in a 25 ml. or 100 ml. organ bath. Preparations were stimulated through the perivascular nerves (Finkleman, 1930) or transmurally (between an Ag-AgCl coil within the lumen of the intestine and a large Ag-AgCl electrode in the bath).

Lengths of non-terminal guinea-pig ileum were suspended in a 10 ml. organ bath, either as the whole gut, or only the longitudinal muscle (Ambache, 1954; Paton & Rang, 1965) was used. The results were the same with both. These preparations were stimulated between two large Ag-AgCl electrodes placed at opposite ends of the organ bath.

These four preparations were stimulated by a Grass Model S4 stimulator delivering monophasic square-wave pulses at various frequencies. For stimulation *via* the nerves, pulse duration was set at 1 msec throughout and the nerves were stimulated in 10 sec bursts, generally at intervals of 3-4 min. For transmural stimulation the pulse duration was 0.1 or 0.2 msec unless otherwise stated. Both single shocks and repetitive stimulation were tested. In all experiments the intensity of stimulation was supramaximal. Pulse repetition frequency ranged from 1 to 30 cps, and is indicated in the legends of figures.

The remaining three preparations were used only to test the responses to drugs. The fundus of the rat stomach was prepared as described by Vane (1957) and was suspended in a 25 ml. organ bath. The rat duodenum was suspended longitudinally in a 10 ml. organ bath. The guinea-pig taenia coli was suspended in a 10 ml. or 25 ml. organ bath.

In all, except the isolated whole stomach preparations, movements of the longitudinal muscle were recorded. In about half of the experiments on each preparation tension was recorded isometrically with a mechano-electric transducer valve (RCA 5734) mounted in the manner described by Bülbring (1955). In the remainder of the experiments contractions were recorded with a frontal-writing auxotonic lever on a smoked drum.

Krebs solution was used in all the experiments and had the following composition (mM): NaCl 113, KCl 4.7, CaCl<sub>2</sub> 2.5, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, dextrose 11.5. It was bubbled with 95% O<sub>2</sub>+5%CO<sub>2</sub> and was kept at 37° C.

Drugs used were acetylcholine bromide, angiotensin amide (Hypertensin, Ciba) synthetic bradykinin, choline phenyl ether bromide, dibenamine hydrochloride, dimethylphenylpiperazinium iodide (DMPP), eserine (physostigmine) sulphate, histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate (5-HT), hyoscine hydrobromide, methysergide maleate, nicotine hydrogen tartrate, 1-noradrenaline bitartrate, and tetrodotoxin citrate (Sankyo). All doses except those for bradykinin refer to the salts.

The effect of tetrodotoxin on the conducted action potentials in the guinea-pig thoracic vagus nerve was recorded by Dr. Edith Bülbring using the sucrose-gap technique (Bülbring & Burnstock, 1960).

## RESULTS

### *Responses to electrical stimulation*

#### *Response of isolated stomach to vagal and perivascular nerve stimulation*

When the vagus nerves to the isolated mouse or guinea-pig stomachs were stimulated, a diphasic or triphasic response (Paton & Vane, 1963) was obtained. In the mouse

stomach the response, which was quite constant, consisted of, in order, a relaxation, a contraction, and a long-lasting relaxation. In the guinea-pig the response was more variable. In about 30%, the response was as described, but more frequently only a contraction preceded the long-lasting relaxation. The vagus nerve has been shown to contain both excitatory and inhibitory fibres (Paton & Vane, 1963; Martinson, 1965), and the complex responses are the result of the simultaneous stimulation of both kinds of fibres. In the presence of atropine or hyoscine the cholinergic excitatory component was blocked and the vagal response was converted to a relaxation only (Paton & Vane, 1963). Eserine markedly accentuated the contractile component of the vagal response, but only occasionally abolished the relaxation which followed the contraction.

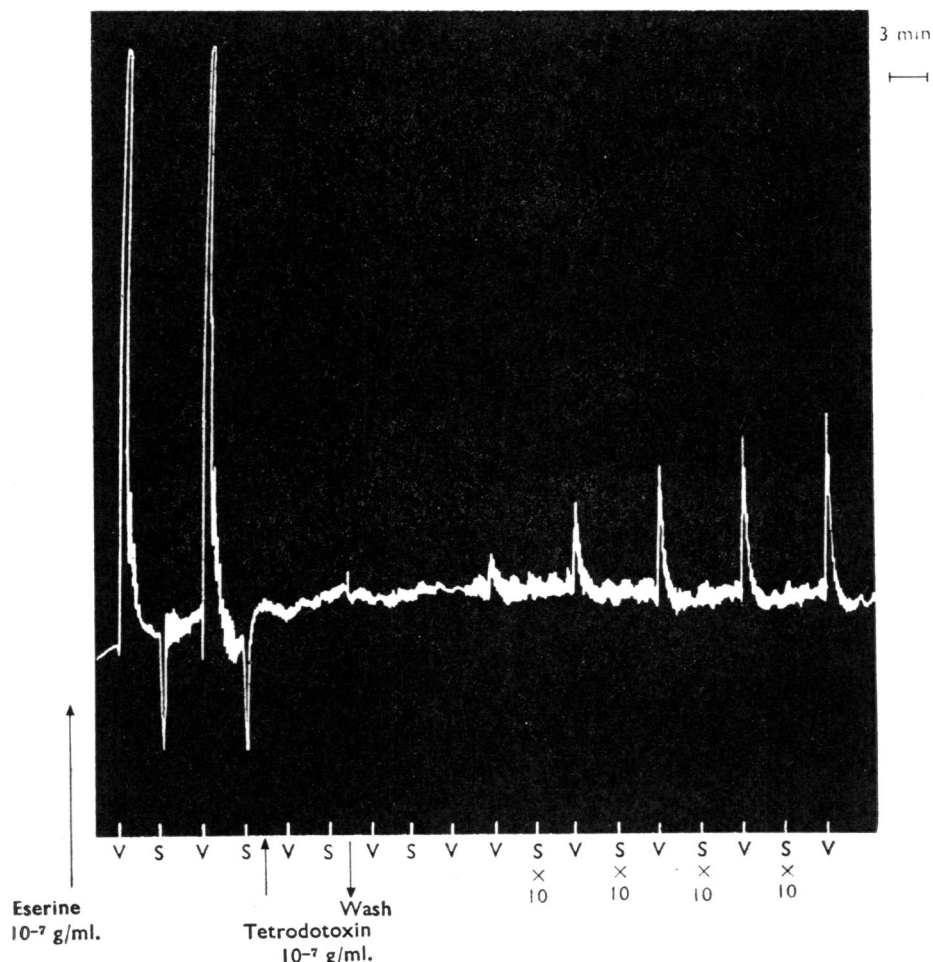


Fig. 1. Guinea-pig stomach. Intraluminal pressure. Tetrodotoxin ( $10^{-7}$  g/ml.) abolishes both the contraction produced by vagal stimulation and the relaxation by perivascular nerve stimulation. The response to vagal stimulation recovered partially 36 min after removing tetrodotoxin. (Wash.) The sympathetic nerves took 2 hr to recover (not shown). Eserine ( $10^{-7}$  g/ml.) is present throughout. V=vagal stimulation, 10 1.0 msec pulses at 1/sec. S=sympathetic (perivascular) nerve stimulation, 30 1 msec pulses at 3/sec; S×10=300 pulses at 30/sec.

Stimulation of the perivascular nerves always produced a relaxation of both the guinea-pig and mouse stomachs. These responses were not affected either by hyoscine or by eserine.

Tetrodotoxin  $10^{-7}$  g/ml. rapidly and reversibly abolished both vagal relaxation and contraction, and abolished the perivascular response as well. On washing out, all responses to nerve stimulation recovered. These results, noted in both guinea-pig and mouse stomachs are illustrated in Fig. 1. Neither hyoscine nor eserine influenced tetrodotoxin blockade of the nervous effects.

*Response of small intestine to transmural and perivascular nerve stimulation*

The relaxation of the rabbit small intestine in response to perivascular nerve stimulation is well known (Finkleman, 1930). The rabbit jejunum, used in these experiments, responded similarly. With increasing frequency of stimulation, from 5 to 50/sec, the height of the spontaneous contractions was first reduced, then spontaneous activity was interrupted and finally a relaxation of the muscle was produced. The response of the rabbit jejunum to transmural stimulation was more complex. When single shocks of 0.1 msec duration were delivered the result was to increase the size of the spontaneous contraction coinciding with the stimulus. With repetitive transmural stimulation a maintained contraction of the muscle was produced, the peak tension of which increased with increasing frequency of stimulation up to 10 pulses/sec (Fig. 2a). These contractions were followed by an after-relaxation which was long-lasting (up to 1 min after the end of stimulation). As the stimulus frequency was raised beyond 10 pulses/sec, the tension produced during contraction decreased, the relaxation began already during stimulation, and the after-relaxation increased (Fig. 2a). In the presence of hyoscine ( $10^{-7}$  g/ml.) only the inhibitory response to transmural stimulation was seen (followed by a rebound contraction). Repetitive stimulation was required to demonstrate the inhibition, the magnitude of which increased with increasing frequency of stimulation, reaching a maximum at 30 pulses/sec (Fig. 2c & d). Eserine ( $10^{-8}$  g/ml.) increased the size of the spontaneous contractions, potentiated the contractions produced by transmural stimulation and tended, in the absence of hyoscine, to obscure the after-relaxation (Fig. 2f & g). Eserine in higher concentration,  $10^{-7}$  to  $10^{-6}$  g/ml. caused a slowly developing sustained contraction of the intestine (see Fig. 3).

Tetrodotoxin,  $10^{-7}$  g/ml. had no effect on the normal spontaneous activity of the rabbit jejunum, affecting neither the regularity, nor the frequency or magnitude of contractions. In contrast to the results of Ishihara (1918; quoted by Kao, 1966) no stimulation of the intestine was noted. However, all response to transmural or perivascular nerve stimulation was quickly abolished. Neither eserine nor hyoscine prevented this action of tetrodotoxin. In fact, if tetrodotoxin in the above concentration was applied to an eserinated ( $10^{-7}$  g/ml.) preparation, the increased spontaneous contractions returned to their pre-eserine size (Fig. 2h & i). After washing out tetrodotoxin, all responses to nerve stimulation recovered.

The longitudinal muscle strip of the guinea-pig ileum responded with a contraction to a single electrical shock of 0.1–0.2 msec (Paton, 1955). In Fig. 3 responses to single shocks of constant intensity and to constant doses of acetylcholine are shown. The contraction in response to transmural stimulation was reduced by hyoscine  $10^{-7}$  g/ml.

and increased by eserine ( $10^{-7}$  g/ml.) which produced, in addition, a slowly developing maintained contraction of the muscle. Tetrodotoxin  $10^{-7}$  g/ml. abolished the response to transmural stimulation but not that to acetylcholine (Fig. 3a). If given at the peak of an eserine-induced contraction, the muscle was promptly relaxed. If the concentration of eserine did not exceed  $10^{-7}$  g/ml. relaxation by tetrodotoxin was complete. However, if the concentration of eserine were raised to  $10^{-6}$  g/ml. the muscle contracted even in the presence of tetrodotoxin. This contraction rose to only about 1/10 of the size

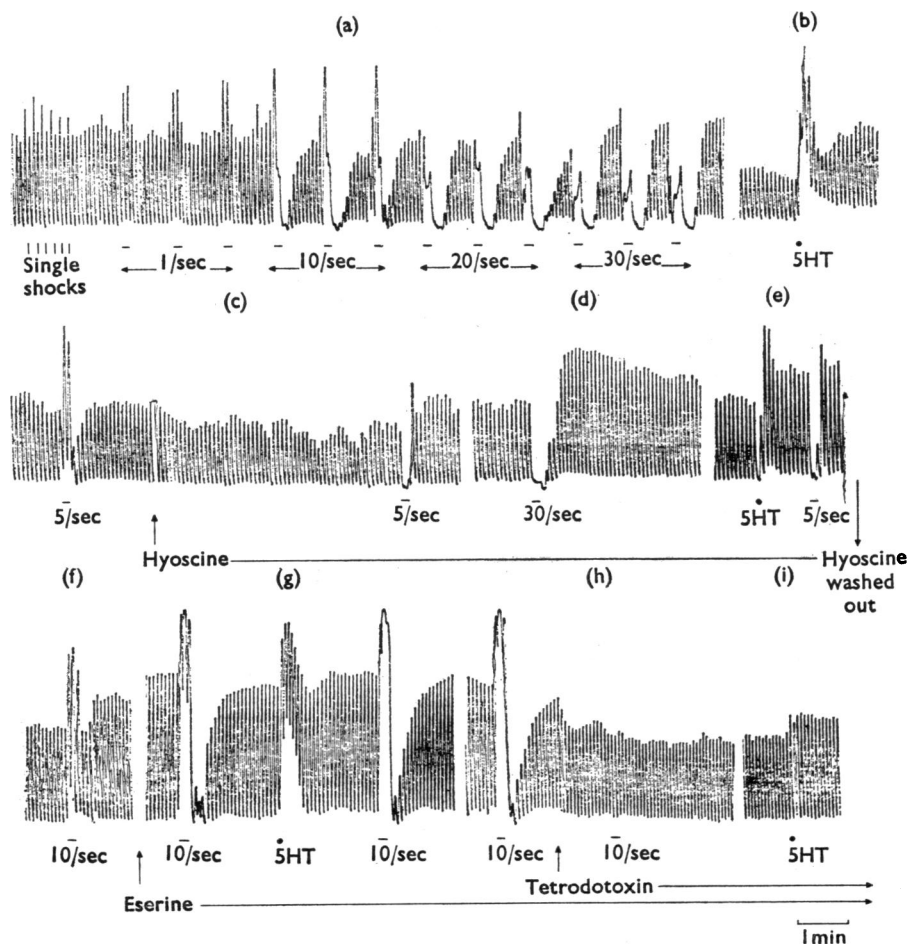


Fig. 2. Rabbit jejunum, transmural stimulation. (a) Single pulses (1) (0.1 msec) or 10 sec trains of pulses (—) at low frequency evoke contractions, higher frequencies evoke diphasic responses and relaxation becomes more prominent. (b) 5-hydroxytryptamine ( $10^{-5}$  g/ml.) produces contraction. (c) Hyoscine ( $10^{-7}$  g/ml.) abolishes the contractile component and unmasks the inhibitory component of the response to transmural stimulation and to 5-HT (e). (d) Relaxation is maximal in response to 30 pulses/sec. (f) After removal of hyoscine the contractile component is restored. (g) Eserine ( $10^{-8}$  g/ml.) potentiates the spontaneous activity and the responses to transmural stimulation and to 5-HT. (h) Tetrodotoxin ( $10^{-7}$  g/ml.) reduces spontaneous activity to the pre-eserine level and abolishes both the contraction and the inhibition produced by transmural stimulation. (i) Tetrodotoxin reduces the response to 5-HT to a small remnant.

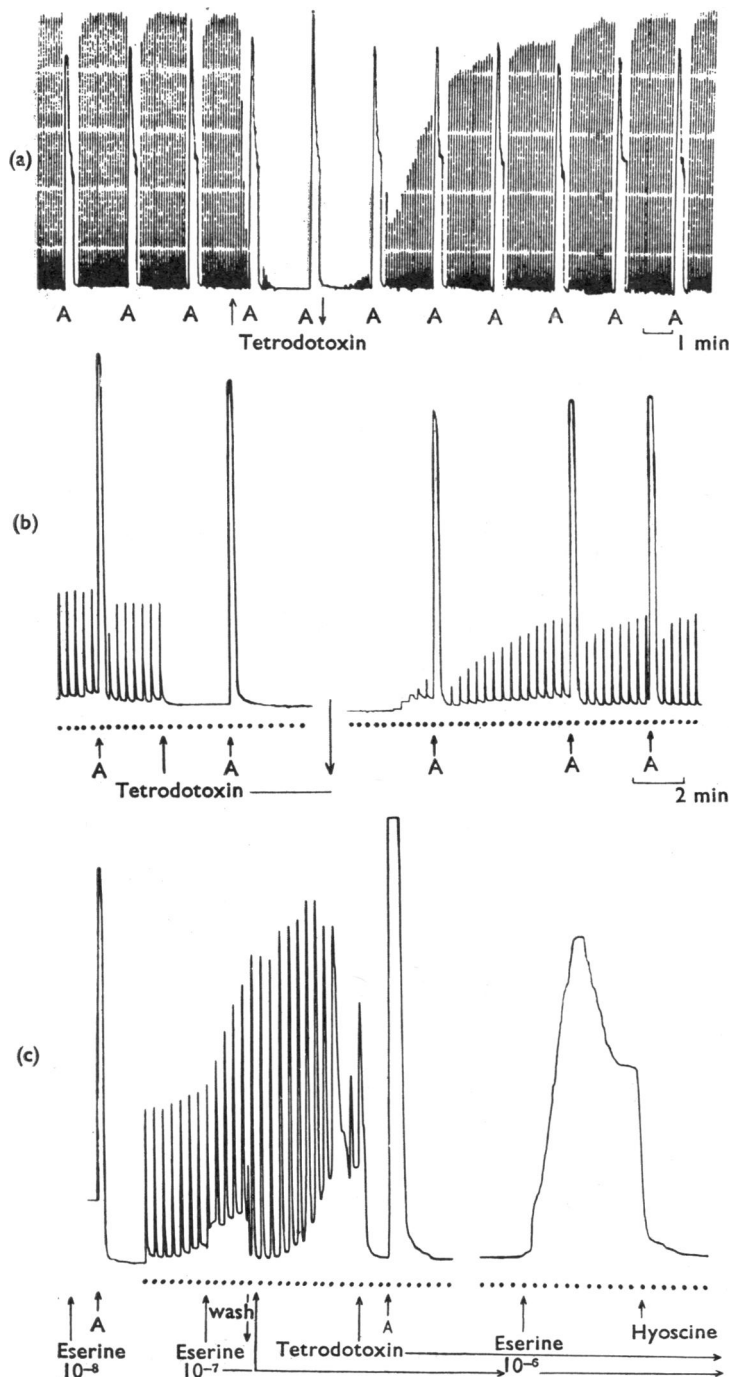


Fig. 3. Guinea-pig ileum. Tetrodotoxin ( $10^{-7}$  g/ml.) abolishes the contraction in response to transmural stimulation (a) single shocks of  $200 \mu\text{sec}$ ; (b)  $50 \mu\text{sec}$  duration, marked by dots), but does not affect the contraction in response to acetylcholine ((a)  $10^{-8}$  g/ml.; (b)  $5 \times 10^{-8}$  g/ml.). The effect is reversible. (c) Same preparation as (b), response to transmural stimulation (dots) is potentiated by eserine ( $10^{-8}$  g/ml.). A tenfold increase in the eserine concentration causes further potentiation and a contracture of the muscle. Tetrodotoxin ( $10^{-7}$  g/ml.) abolishes the transmural twitch and also relaxes the eserine contracture. The response to acetylcholine remains potentiated. Despite the presence of tetrodotoxin, raising the eserine concentration to  $10^{-6}$  g/ml. causes a contraction which is relaxed by hyoscine ( $10^{-6}$  g/ml.). There is no response to continued transmural stimulation. These results indicate that tetrodotoxin completely abolishes acetylcholine release related to conduction of nervous impulses. It does not appear to interfere with the spontaneous leakage of acetylcholine from the nerve endings.

of the contraction noted with eserine  $10^{-6}$  in the absence of tetrodotoxin and it was relaxed by hyoscine ( $10^{-7}$  g/ml.). Once more the responses to transmural stimulation recovered when tetrodotoxin was washed out.

In none of the nerve stimulated preparations described above could the action of tetrodotoxin be overcome by increasing the stimulus frequency, duration or amplitude. In the transmurally stimulated preparations, however, effects could be obtained by increasing the pulse duration. In the rabbit jejunum long-lasting disruption of the regular spontaneous activity and a decrease in the size of spontaneous contractions could be produced by pulses of long duration (1–10 msec). Since these effects often outlasted stimulation by several minutes and required a relatively long pulse duration they were thought to be most probably due to direct electrical effects on the smooth muscle itself. Similarly, in the presence of tetrodotoxin  $10^{-7}$  g/ml. small contractions of unequal size could be produced by stimulating guinea-pig ileum transmurally with a relatively long pulse duration (longer than 0.3–0.5 msec). These contractions were unaffected either by hyoscine  $10^{-6}$  g/ml. or eserine  $10^{-7}$  g/ml. and were probably, therefore, non-cholinergic and also due to an effect on the smooth muscle itself. Such small contractions have also been observed by Paton & Zar (1966) when mechanically denervated guinea-pig ileum was stimulated electrically with pulses of long duration.

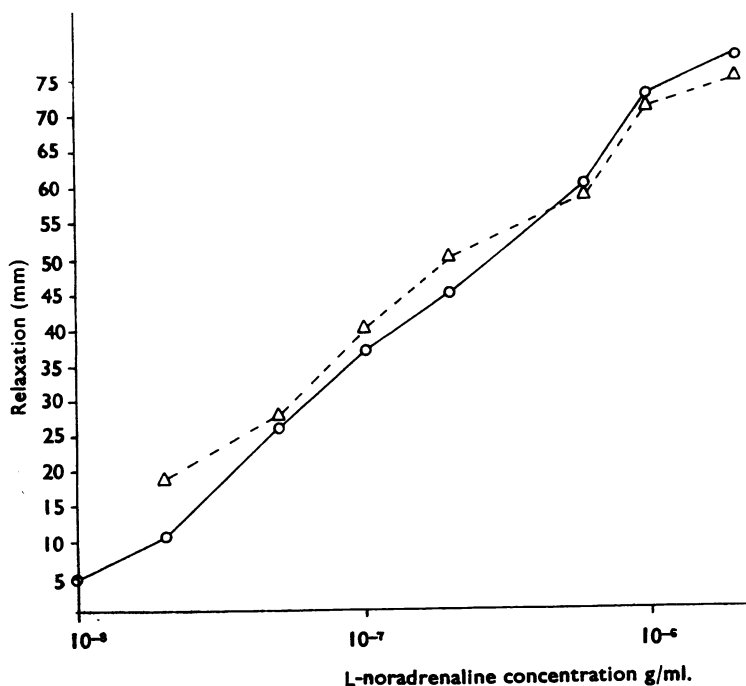


Fig. 4. Guinea-pig stomach. Intraluminal pressure. Ordinate=change from resting pressure in mm on kymograph record (about  $2 \times$  magnification of the change in mm H<sub>2</sub>O). Tetrodotoxin ( $5 \times 10^{-7}$  g/ml.) has no effect on the relaxation caused by 1-noradrenaline. Dose-response curves, ○—○=control, Δ—Δ=in the presence of tetrodotoxin.

*Drug responses**Noradrenaline and histamine*

In all preparations a relaxant effect of noradrenaline could be demonstrated. In the guinea-pig and mouse stomachs the resting tone of the muscle was high, and the log-dose response relationship for noradrenaline could readily be determined (Fig. 4). Threshold was about  $10^{-8}$  g/ml. and the maximal response was reached at about  $10^{-6}$  g/ml. The rabbit jejunum was also relaxed by noradrenaline, the threshold dose being about  $10^{-8}$  g/ml. In the guinea-pig ileum and the fundus of the rat stomach noradrenaline produced no demonstrable effect unless the tone of these preparations had previously been raised. Histamine ( $10^{-8}$  g/ml.) was used for this purpose for guinea-pig ileum and 5-HT ( $10^{-9}$  g/ml.) (Armitage & Vane, 1964) was used for rat fundus. The response of the guinea-pig taenia coli to noradrenaline was very variable and depended on the degree of resting tone (Burnstock, Campbell & Rand, 1966). The responses of all preparations to noradrenaline were unaffected by tetrodotoxin  $10^{-7}$  to  $10^{-6}$  g/ml.

All preparations were contracted by histamine. The guinea-pig ileum and taenia coli were most sensitive,  $10^{-9}$  g/ml. being the threshold concentration. The fundus of the rat stomach and the rabbit jejunum were least sensitive,  $10^{-6}$  and  $10^{-5}$  g/ml. being necessary to elicit a response. Tetrodotoxin ( $10^{-7}$  g/ml.) almost never affected the log-dose response relationship for histamine. (Results for guinea-pig ileum are shown in Fig. 5.) Rarely, however, the response to high doses ( $10^{-6}$  to  $10^{-5}$  g/ml.) of histamine was reduced in some guinea-pig stomachs. In these, the responses recovered after the tetrodotoxin had been washed out and they could then be similarly reduced by hyoscine  $10^{-7}$  g/ml. This finding, therefore, is probably due to the ability of high concentrations of histamine to stimulate cholinergic nerve endings in the stomach (Paton & Vane, 1963).

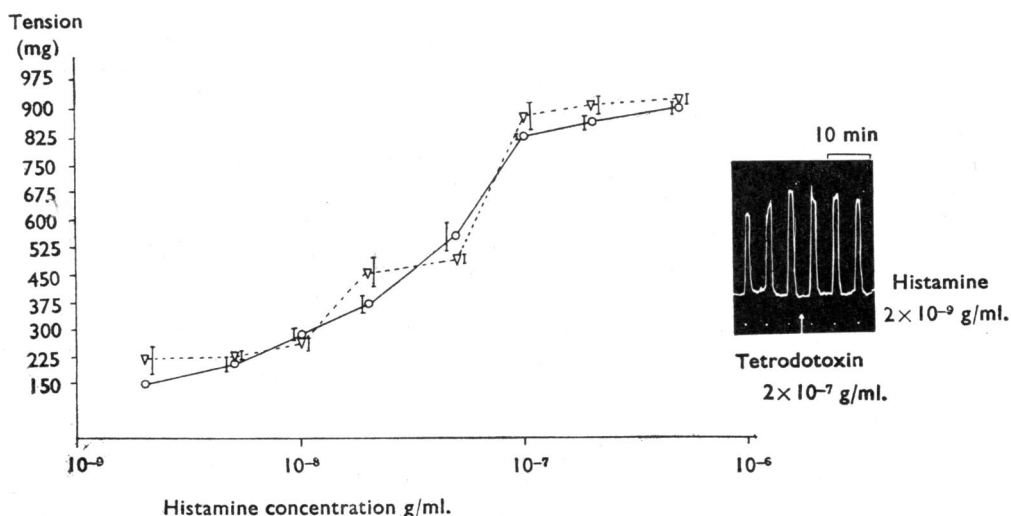


Fig. 5. Guinea-pig ileum. Tetrodotoxin ( $2 \times 10^{-7}$  g/ml.) has no effect on the contractions in response to histamine. Left. Dose-response curves to histamine;  $\circ$ — $\circ$  = control,  $\nabla$ — $\nabla$  = in the presence of tetrodotoxin ( $10^{-7}$  g/ml.). Vertical bars indicate standard errors of the mean of five determinations. Right. Constant doses of histamine ( $2 \times 10^{-9}$  g/ml.).



The histamine response of the rabbit jejunum was so irregular that tetrodotoxin could not be tested accurately.

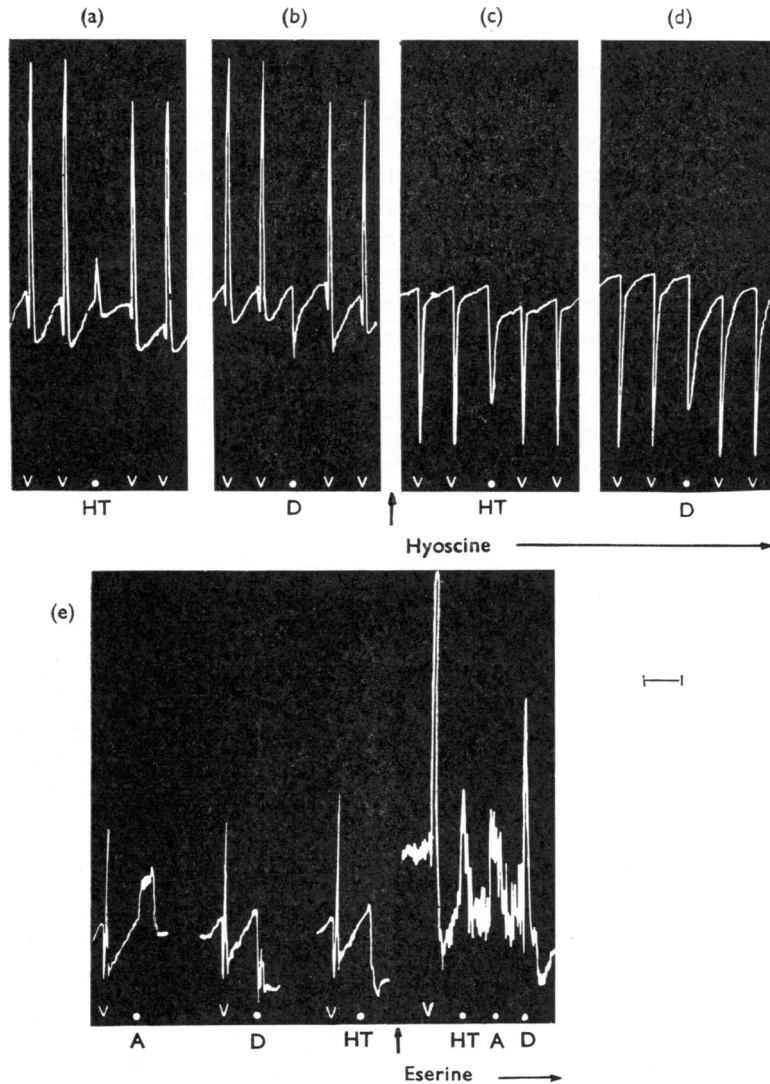
#### *Ganglion stimulants and acetylcholine*

The ganglion stimulants, nicotine, DMPP, and choline phenyl ether, varied in how they affected the different preparations, but a constant response was always noted for a given kind of preparation, and the responses to each of the three drugs were qualitatively similar. Thus all three compounds relaxed the guinea-pig stomach (Figs. 6 and 7), produced either no response or a feeble contraction of the mouse stomach, either contracted or relaxed the taenia coli (Burnstock *et al.*, 1966), and produced contractions in the four remaining preparations (rat duodenum, fundus of the rat stomach, rabbit jejunum and guinea-pig ileum). The relaxations produced in guinea-pig stomach or taenia coli by moderate doses of the three compounds ( $10^{-7}$  to  $5 \times 10^{-6}$  g/ml.) were potentiated by hyoscine ( $10^{-7}$  g/ml.), reversed to a contraction by eserine ( $10^{-7}$  g/ml.) (Fig. 6), and abolished by tetrodotoxin ( $10^{-7}$  g/ml.) (Fig. 7). The contractions produced in rabbit jejunum by these drugs in the same concentrations as above were potentiated by eserine, reversed to a relaxation by hyoscine, and again were abolished by tetrodotoxin. Contractions of guinea-pig ileum induced by these compounds were also potentiated by eserine, and were blocked by hyoscine as well as tetrodotoxin (Fig. 8). The fundus of the rat stomach and the rat duodenum responded only to high doses of ganglion stimulants, and the contractions were sensitive to tetrodotoxin.

If massive doses of the ganglion stimulants were given ( $>10^{-5}$  g/ml.) then effects were noted in the presence of tetrodotoxin (Fig. 7). In all preparations, in the presence of tetrodotoxin, DMPP and nicotine relaxed and choline phenyl ether contracted the smooth muscle. Where the tone of the muscle was very low, as in guinea-pig ileum, the relevant effects were seen only after the tone had been raised by histamine ( $10^{-8}$ – $10^{-7}$  g/ml.) (Fig. 8).

Both excitatory and inhibitory ganglion cells are present in the wall of the gut (Ambache, 1951; Paton & Vane, 1963; Martinson, 1965; Burnstock *et al.*, 1966). In moderate doses the ganglion stimulants stimulate both types of ganglia. The net effect then is a balance between cholinergic excitation and inhibition, the final mediator of which has not been definitely established. This balance may be shifted toward excitation by cholinesterase inhibition and toward relaxation by blocking the muscarinic receptor with hyoscine. These nerve-mediated effects are both abolished by tetrodotoxin. The non-specific effects of high doses of ganglion stimulants are also seen in nerve-free preparations (Evans & Schild, 1953; Zar, personal communication) and are probably direct effects on the smooth muscle itself.

All preparations were normally contracted by acetylcholine. The contractions were antagonized by hyoscine and potentiated by eserine. In all, except the isolated stomach preparations, tetrodotoxin  $10^{-7}$  g/ml. had no effect on the log-dose response relationship for acetylcholine. This is illustrated for guinea-pig ileum in Fig. 9. In the isolated stomach preparations, however, acetylcholine could be shown to produce nicotinic effects in high doses ( $10^{-6}$  to  $10^{-5}$  g/ml.) as shown in Fig. 10. Thus, in the presence of hyoscine  $10^{-6}$  g/ml., these high doses of acetylcholine relaxed the stomach (Fig. 10c). The effect



**Fig. 6.** Guinea-pig stomach. Intraluminal pressure. (a) Stimulation of the vagus nerves (V), 100 pulses, 1.0 msec at 10/sec, produces a triphasic response. 5-HT,  $10^{-5}$  g/ml. (HT) produces a contraction, followed by relaxation, and reduces the excitatory component of the vagal response. (b) DMPP,  $5 \times 10^{-6}$ , (D) causes relaxation and also reduces the excitatory component of the vagal response. (c) and (d) In the presence of hyoscine ( $10^{-7}$  g/ml.) the responses to vagal stimulation, to 5-HT and to DMPP are all purely inhibitory and the relaxation to each is augmented. (e) In another stomach preparation the response to vagal stimulation is again triphasic, that to ACh,  $10^{-7}$  g/ml. (A) is contraction, to DMPP and 5-HT relaxation. In the presence of eserine ( $10^{-8}$  g/ml.) the vagal response is enhanced; 5-HT and DMPP produce contractions.

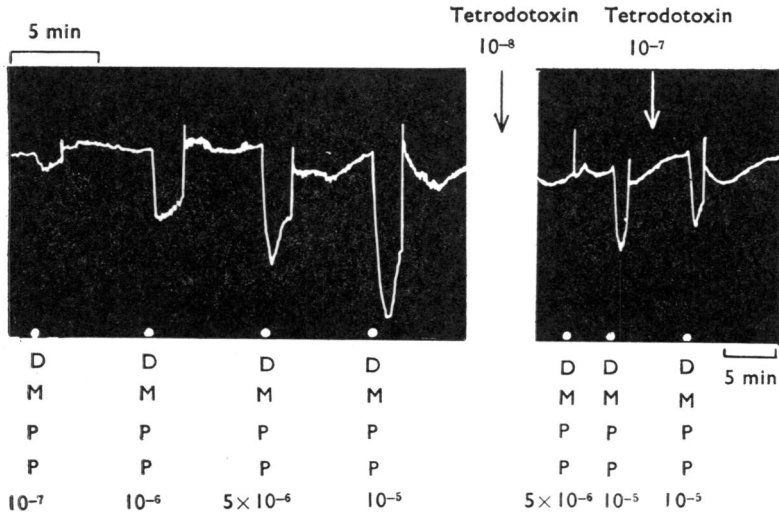


Fig. 7. Guinea-pig stomach. Intraluminal pressure. Tetrodotoxin ( $10^{-8}$  g/ml.) abolishes the responses to all but the highest concentrations of DMPP ( $10^{-5}$  g/ml.), which persist even when the concentration of tetrodotoxin is raised tenfold.

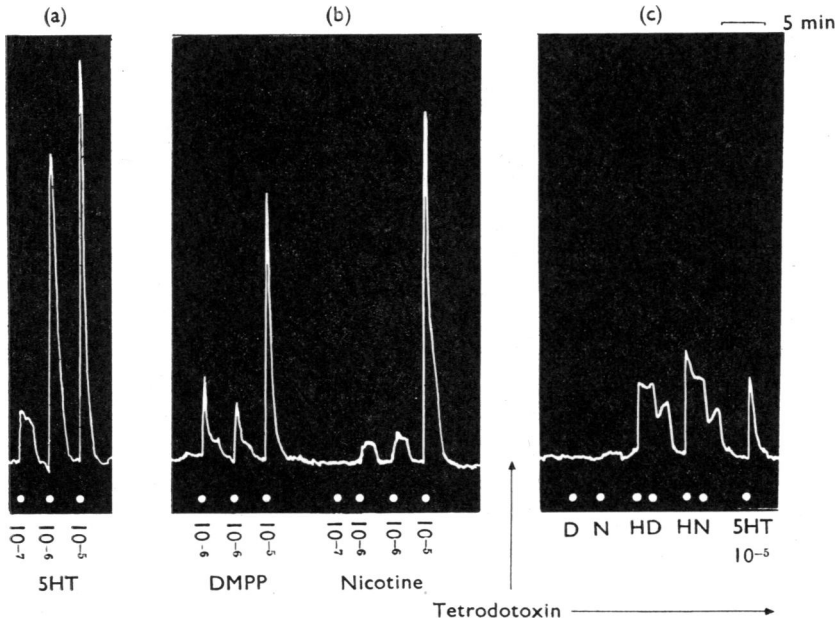


Fig. 8. Guinea-pig ileum. (a), (b) Contractions in response to increasing doses of 5-HT, DMPP and nicotine. (c) Tetrodotoxin ( $10^{-7}$  g/ml.) abolishes the contractions produced by DMPP and nicotine, but leaves a small relaxant action of both compounds which is seen when the muscle tone is raised by histamine. Tetrodotoxin reduces the 5-HT response to a small remnant. D=DMPP  $10^{-5}$  g/ml.; N=nicotine  $10^{-5}$  g/ml.; H=histamine  $2 \times 10^{-9}$  g/ml. The bath fluid was changed after each dose of 5-HT, DMPP or nicotine.

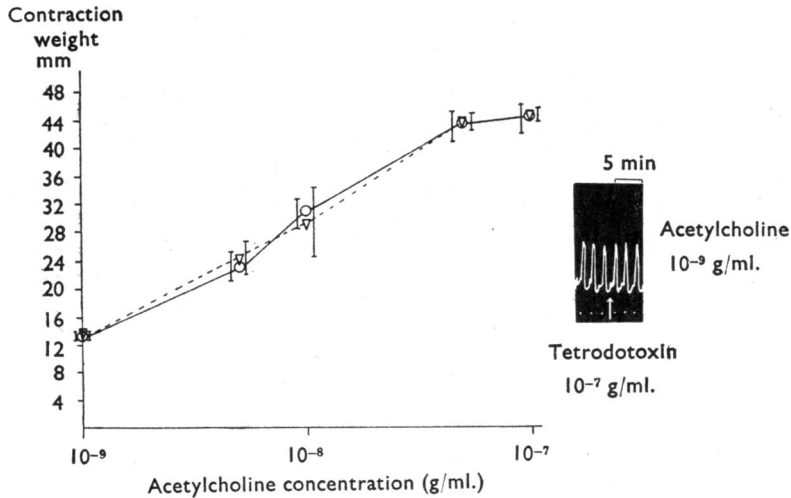


Fig. 9. Guinea-pig ileum. Tetrodotoxin ( $10^{-7}$  g/ml.) has no effect on the contractions in response to acetylcholine. Left. Dose-response curve to acetylcholine.  $\circ$ — $\circ$  = control;  $\nabla$ — $\nabla$  = in the presence of tetrodotoxin. Right. Constant doses of ACh ( $10^{-9}$  g/ml.). Vertical bars indicate standard errors of the mean of five determinations.

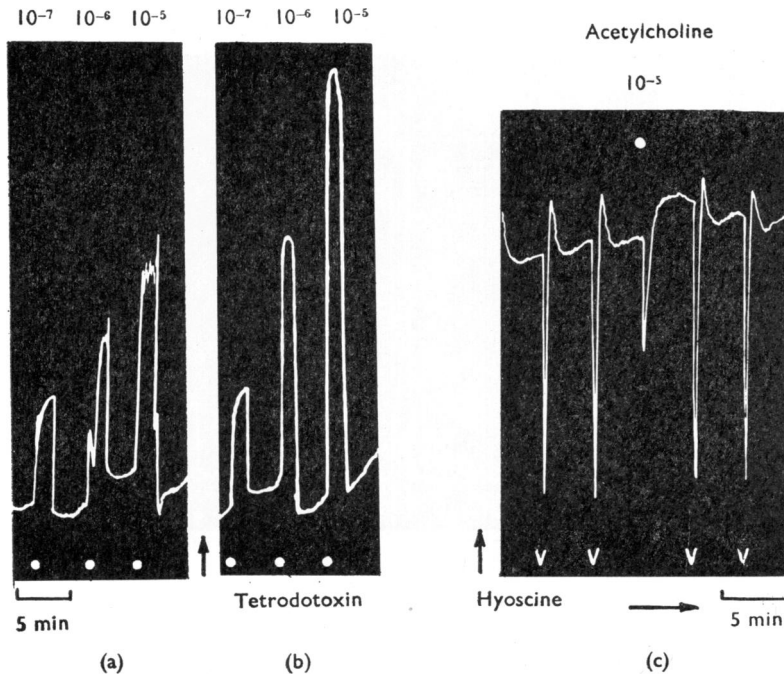


Fig. 10. Guinea-pig stomach. Tetrodotoxin potentiates the response to high concentrations of acetylcholine ( $>10^{-6}$  g/ml.) (a and b). This is interpreted as a removal by tetrodotoxin of an inhibitory action of high doses of acetylcholine. This action is revealed in the presence of hyoscine (c) and compared with that of vagal stimulation. The inhibitory action of acetylcholine is blocked by pentolinium ( $10^{-5}$  g/ml.) or tetrodotoxin (not shown).

of eserine ( $10^{-7}$  g/ml.) was then to increase the size of the relaxation produced by acetylcholine. This relaxation, similar to that produced by nicotine (and also produced by tetramethylammonium ion and carbachol), was abolished by tetrodotoxin  $10^{-7}$  g/ml. The effect of tetrodotoxin on the contractions produced by high doses of acetylcholine was to remove the attenuation due to the concurrent action on inhibitory neurones and thus to enhance the contractions (Fig. 10a and b). (The nicotinic action of acetylcholine on inhibitory neurones could not be seen in the absence of hyoscine, unless the distension of the stomach was increased by injecting 10 ml. saline. Occasionally then, the stomach was relaxed by acetylcholine.)

#### *5-Hydroxytryptamine*

The actions of 5-HT were similar, in some preparations, to those of the ganglion stimulants described above. Thus in the isolated guinea-pig stomach 5-HT ( $10^{-7}$ – $10^{-6}$  g/ml.) was also able to relax the preparation (Fig. 6). Frequently 5-HT caused a diphasic response, a contraction followed by a relaxation (Fig. 11). In the presence of hyoscine

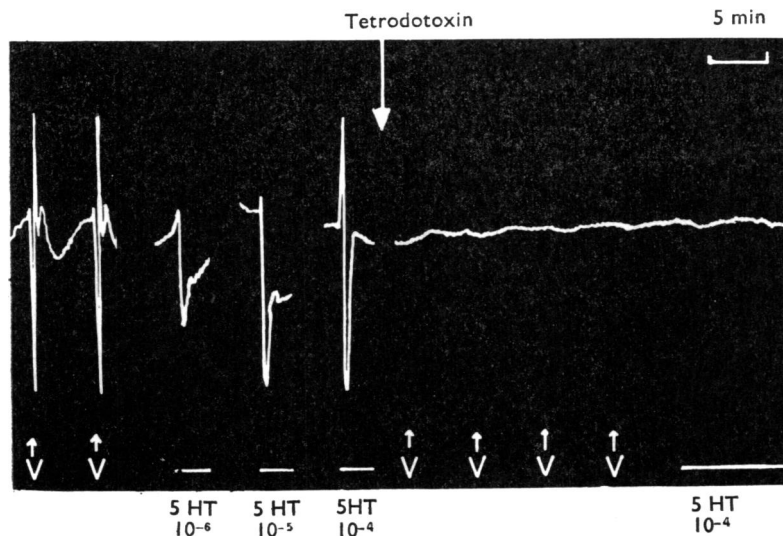


Fig. 11. Guinea-pig stomach. Tetrodotoxin ( $10^{-7}$  g/ml.) abolishes both the contractile and the relaxant component of the response to vagal stimulation as well as the response to 5-HT. V=vagal stimulation, 100 pulses, 1 msec at 10/sec. HT=5-HT and duration of exposure. Concentrations are in g/ml.

( $10^{-7}$  g/ml.) only the relaxation, which was much increased, remained (Fig. 6c). In the presence of eserine ( $10^{-7}$  g/ml.) only a contraction could be elicited by 5-HT (Fig. 6e). Tetrodotoxin ( $10^{-7}$  g/ml.) abolished all response to 5-HT (Fig. 11). Neither eserine nor hyoscine was able to prevent the blocking action of tetrodotoxin. When 5-HT was given in massive amounts ( $10^{-4}$ – $10^{-3}$  g/ml.) in the presence of tetrodotoxin, it produced a small relaxation in about 50% of the preparations.

In contrast to these results on guinea-pig stomach, the contractions of the fundus of the rat stomach and of the rat duodenum in response to 5-HT were not influenced by

hyoscine ( $10^{-7}$  g/ml.), eserine ( $10^{-7}$  g/ml.) or tetrodotoxin ( $10^{-7}$  g/ml.) (Fig. 12), nor by tetrodotoxin ( $10^{-6}$  g/ml.). The smooth muscle of these preparations was remarkably sensitive to 5-HT; the fundus of the rat stomach contracting in response to  $10^{-11}$ – $10^{-10}$  g/ml. and the rat duodenum to  $10^{-10}$ – $10^{-9}$  g/ml. Neither of these preparations could be relaxed by 5-HT.

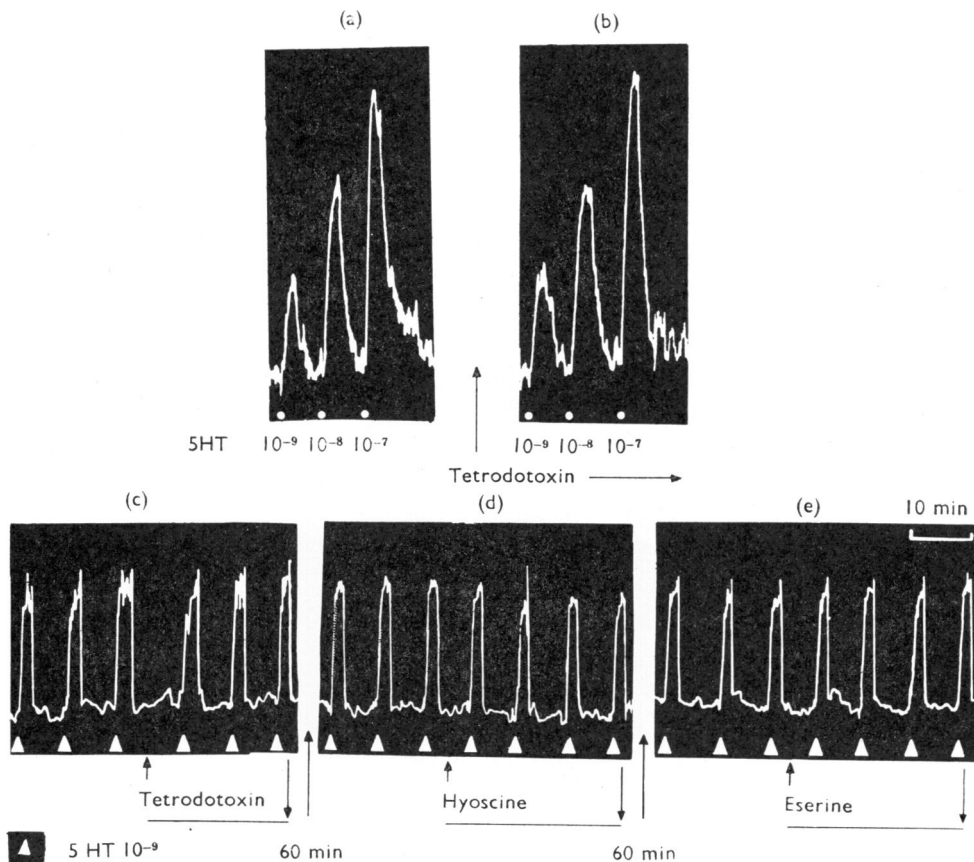


Fig. 12. Fundus of the rat stomach. (a) Responses to increasing doses of 5-HT, (b) in the presence of tetrodotoxin. (c), (d), (e) Responses to a constant dose of 5-HT are not affected by tetrodotoxin ( $10^{-7}$  g/ml.), hyoscine ( $10^{-7}$  g/ml.), or eserine ( $2 \times 10^{-7}$  g/ml.).

The guinea-pig ileum and taenia coli, and the rabbit jejunum were also contracted by 5-HT. The guinea-pig organs were approximately equally sensitive to 5-HT, responding to about  $2 \times 10^{-9}$  g/ml. The rabbit jejunum was somewhat less sensitive;  $10^{-8}$  g/ml. was usually the minimal concentration required to produce a response. Eserine ( $10^{-7}$  g/ml.) increased the size of the contractions to 5-HT in all three preparations and hyoscine ( $10^{-7}$  g/ml.) reduced them. Hyoscine was most effective at blocking the response of guinea-pig ileum to 5-HT (Robertson, 1953; Gaddum & Picarelli, 1957). In the rabbit jejunum hyoscine ( $10^{-7}$  g/ml.) blocked the response to concentrations of 5-HT up to  $10^{-7}$  g/ml., but 5-HT,  $10^{-6}$ – $10^{-5}$  g/ml. produced a contraction which was

often preceded by a relaxation of the smooth muscle, as shown in Fig. 2b and e. The taenia coli still responded to 5-HT in the presence of hyoscine ( $10^{-7}$  g/ml.); in most preparations the dose-ratio was about 10. In about one preparation in four the 5-HT response was refractory to hyoscine.

In many taenia preparations there was a long latent period (up to 1 min) after the 5-HT had been added to the bath before the taenia contracted (Bülbring & Burnstock, 1960). This latent period appeared to be longer in the presence of hyoscine and consequently 5-HT was left in contact with the tissue for 2 min. In order to avoid tachyphylaxis, 5-HT was given at intervals of not less than 30 min. Burnstock *et al.* (1966) have reported that atropine completely blocks the 5-HT contraction of the taenia coli, while only a partial block was observed with hyoscine in the present experiments. These authors did not indicate the time of exposure or the time between doses of 5-HT in their experiments and it is therefore possible that the discrepancy between the results may have been due to the long latent period or to tachyphylaxis. Very high doses of 5-HT ( $10^{-4}$  g/ml.) in the presence of hyoscine occasionally relaxed the taenia (Burnstock *et al.*, 1966).

Tetrodotoxin ( $10^{-7}$  g/ml.) incompletely antagonized the contractions in response to 5-HT of all three preparations, reducing them to about the same extent as did hyoscine. The relaxant effects of 5-HT were, however, blocked completely. The residual small contractions produced by 5-HT  $10^{-5}$  g/ml. in the presence of hyoscine or tetrodotoxin in guinea-pig ileum and rabbit jejunum were blocked by methysergide ( $10^{-7}$  g/ml.) or dibenamine ( $10^{-8}$  g/ml.). When the responses of the taenia coli to 5-HT were resistant to hyoscine ( $10^{-7}$  g/ml.) they were similarly resistant to tetrodotoxin ( $10^{-7}$  g/ml.). Conversely, if the responses were reduced by hyoscine they were also reduced by tetrodotoxin. These results are illustrated in Figs. 2 and 8.

#### *Barium and potassium ions*

Paton & Zar (1965) have reported that both  $\text{BaCl}_2$  and KCl produce contractions of denervated longitudinal muscle strips of the guinea-pig ileum. Since these contractions were reduced compared with those of fully innervated strips, part of the response to these salts must have been nerve mediated and part a direct effect on the smooth muscle. The effect of tetrodotoxin on the contractions induced by  $\text{BaCl}_2$  and KCl was studied on innervated longitudinal muscle strips from guinea-pig ileum in order to compare tetrodotoxin treatment with mechanical denervation. The responses to  $\text{BaCl}_2$  and KCl and the effect of tetrodotoxin ( $2 \times 10^{-7}$  g/ml.) are illustrated in Fig. 13, showing that a reduced response survived treatment with tetrodotoxin. These contractions could not be potentiated by eserine ( $2 \times 10^{-7}$  g/ml.) or further antagonized by hyoscine ( $2 \times 10^{-7}$  g/ml.). They probably result, therefore, not from liberation of acetylcholine from nerve endings, but from the direct action of  $\text{BaCl}_2$  and KCl on smooth muscle. Thus tetrodotoxin treatment has the same effect as mechanical denervation with respect to responses to these agonists.

#### *Polypeptides*

Zar (1966) has found that denervated and innervated muscle strips from the guinea-pig ileum respond equally well to bradykinin and concluded that bradykinin produced its effects solely by a direct action on the smooth muscle. This was confirmed in the present experiments. Neither tetrodotoxin ( $10^{-7}$  g/ml.), nor eserine ( $2 \times 10^{-7}$  g/ml.), nor hyoscine ( $2 \times 10^{-7}$  g/ml.) had any effect on the maximal contractions of the innervated longitudinal

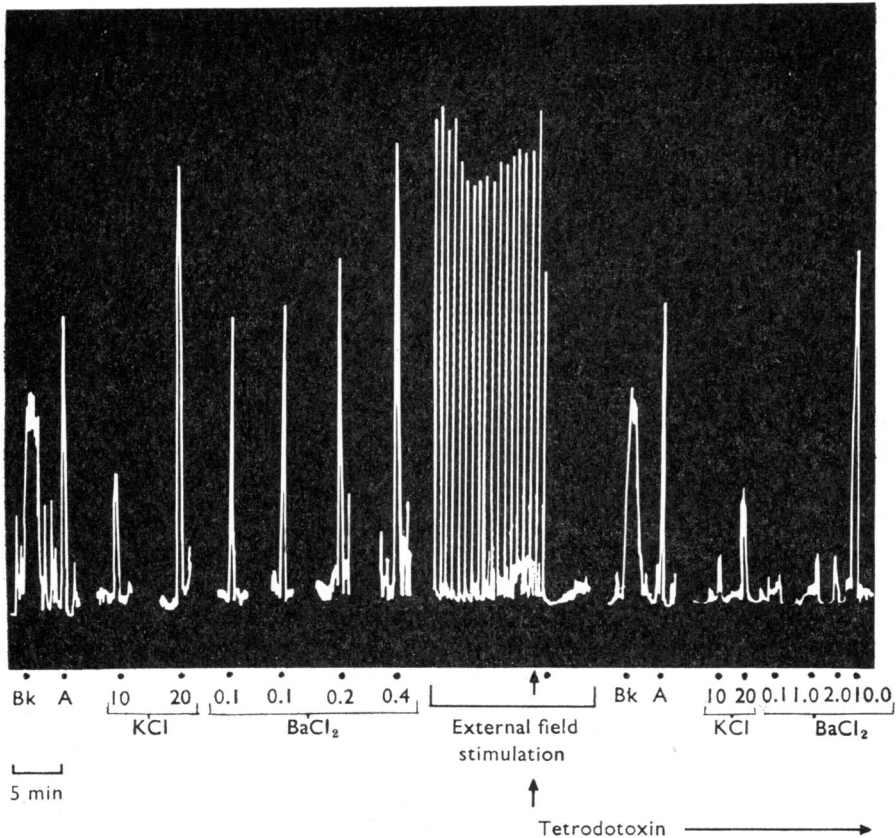


Fig. 13. Guinea-pig ileum longitudinal muscle strip. Tetrodotoxin ( $2 \times 10^{-7}$  g/ml.) abolishes contractions in response to electrical stimulation, does not affect responses to bradykinin or acetylcholine, and reduces, but does not abolish, responses to KCl and  $\text{BaCl}_2$ . BK = Bradykinin ( $4 \times 10^{-9}$  g/ml.); A = acetylcholine ( $10^{-8}$  g/ml.). Numbers indicate mg KCl or  $\text{BaCl}_2$  added to 5 ml. bath. External electrical field stimulation was given as single shocks of 0.1 msec duration.

muscle strip of the guinea-pig ileum produced by bradykinin,  $2.5\text{--}5.0 \times 10^{-8}$  g/ml. (Fig. 13). On the other hand, Zar (1966) has found that contractions in response to angiotensin are reduced, but not abolished, by mechanical denervation. Similarly, tetrodotoxin reduced, but did not abolish responses to angiotensin ( $10^{-8}$  g/ml.) (see Table 1).

#### *The action of tetrodotoxin on the vagus nerve*

Tetrodotoxin ( $5 \times 10^{-7}$  g/ml.) rapidly and reversibly abolished all components of the compound action potential of the vagus nerve. The resting potential, as far as could be judged from external recording with the sucrose gap method, was unaffected.

#### DISCUSSION

Tetrodotoxin appears to have a very specific effect on some excitable tissues. It prevents the rapid increase of sodium conductance and thus the conduction of action



potentials, but, in contrast to conventional local anaesthetics, it does not affect potassium conductance (Narahashi, Moore & Scott, 1964). Sucrose gap recording from nerves treated with tetrodotoxin (or tarichatoxin, which appears to be identical) reveals therefore no change in resting potential but abolition of conducted spikes (Kao & Fuhrman, 1963). In the present experiments on vagus nerve these observations were repeated and the effective concentration for complete abolition of the compound action potential was found to be  $5 \times 10^{-7}$  g/ml. A slightly lower concentration ( $10^{-7}$  g/ml.) was able to abolish the responses of the guinea-pig and mouse stomachs to vagal or perivascular nerve stimulation and the responses of the guinea-pig ileum and rabbit jejunum to transmural stimulation. Thus both preganglionic (vagal) and post-ganglionic nerves (perivascular nerves and the nerves excited by transmural stimulation (Paton, 1955)) are blocked equally. On the other hand, neither the action of the excitatory transmitter, acetylcholine, nor that of the inhibitory transmitter, noradrenaline, were blocked by tetrodotoxin. Similar observations of the effect of tetrodotoxin on vagal excitation of rat stomach (Ogura, 1963) and on the responses of the guinea-pig ileum to transmural stimulation, nicotine, 5-HT, acetylcholine and histamine, have been made by several other workers (Ogura, Hamada & Tsukada, 1960; Hamada, 1966—quoted by Kao, 1966; Ogura, Mori & Watanabe, 1966). Since, in addition, tetrodotoxin does not abolish the electrical activity of smooth muscle (Toida & Osa, 1965; Bülbring & Tomita, 1966; Kuriyama *et al.*, 1966) its antagonistic action on responses of these various smooth muscle containing organs evoked through their nerve supply may be ascribed to a block of conducted action potentials in the nerves. Thus tetrodotoxin, though inert to the smooth muscle, effectively paralyzes its innervation.

No pharmacological effects of tetrodotoxin were found which did not stem from its primary action on nerve conduction. Thus it had no antihistaminic properties in preparations where histamine affects the smooth muscle directly, such as the guinea-pig ileum (Paton & Zar, 1965), but it reduced the histamine response where it is, in part, nerve mediated, as in the guinea-pig stomach (Paton & Vane, 1963). Tetrodotoxin was in no instance adrenolytic or atropinic. It also had no effect against bradykinin nor on 5-HT, where 5-HT stimulates the smooth muscle directly, as in the fundus of the rat stomach (Vane, 1957) and the rat duodenum.

On the other hand, the effects produced by indirectly acting drugs, such as nicotine, DMPP, choline phenyl ether and, in the guinea-pig stomach, 5-HT, were abolished by tetrodotoxin. Where drugs have dual actions—that is, partly nerve-mediated and partly the nervous component. Thus, in guinea-pig ileum, contractions due to  $\text{BaCl}_2$ , KCl, angiotensin and 5-HT were reduced by tetrodotoxin and the residual contractions were not affected by hyoscine or eserine.

From the evidence so far presented, it would seem that tetrodotoxin effectively denervates smooth muscle preparations without otherwise affecting them. This is, however, not quite true. Since only the nerve action potential is abolished by tetrodotoxin and transmitter stores remain intact, it is certainly possible that part of the response to any given drug, in the presence of tetrodotoxin, might be due to transmitter release through an action of the drug not involving a conducted action potential in the nerves. Moreover, although tetrodotoxin reduced the acetylcholine output associated

with neural activity, it did not prevent the contraction of the muscle by a very large dose of eserine. This contraction, abolished by hyoscine, is due to accumulation of acetylcholine (Shelley, 1955) released from nerve endings (Paton & Zar, 1965; Zar, 1966). Therefore, in the intestine, as at the neuromuscular junction (Katz & Miledi, 1966), tetrodotoxin does not appear to interfere with spontaneous release of transmitter from nerve endings. Thus residual responses surviving treatment with tetrodotoxin could be either due to transmitter release or to a direct effect on the smooth muscle. In the case of cholinergic nerves it is possible to choose between these alternatives. Responses mediated by acetylcholine should be reduced by hyoscine and potentiated by eserine. These two drugs were therefore used in addition to tetrodotoxin in the analysis of drug responses. If a test drug produced a contraction in the presence of tetrodotoxin which was neither antagonized by hyoscine nor potentiated by eserine it was considered to be produced by a direct action on the smooth muscle. The possibility that non-cholinergic excitatory nerves could be selectively stimulated by drugs in the presence of tetrodotoxin was not ruled out, but was thought to be unlikely in the intestine. A classification of the sites of action of a number of drugs in the various preparations was made using tetrodotoxin, eserine and hyoscine, and is given in Table 1.

It is striking that none of the indirectly acting drugs did in fact appear to be able to release acetylcholine in the presence of tetrodotoxin. Even nicotine, KCl and BaCl<sub>2</sub>, which might have been expected to act on nerve endings, did not. Moreover, results obtained from experiments with tetrodotoxin on the guinea-pig ileum are the same as those reported by Paton & Zar (1965) with mechanical denervation. Thus, in this preparation, acetylcholine, histamine and bradykinin act exclusively on the smooth muscle, KCl, BaCl<sub>2</sub>, angiotensin, 5-HT and choline phenyl ether act partly on the nerves and partly on the smooth muscle, and nicotine and DMPP induce contractions exclusively through the nerves.

It is far more difficult to reach conclusions about the inhibitory than the excitatory effects of drugs if these inhibitory effects persist in the presence of tetrodotoxin. Not all inhibition is adrenergic (Martinson, 1965) and adrenergic blocking agents are less specific than hyoscine. Residual actions, such as the relaxations produced by high doses of nicotine or DMPP in the presence of tetrodotoxin, could therefore be due to the liberation of inhibitory transmitter, if the inhibitory nerve endings, unlike cholinergic endings, were susceptible to these drugs. Since nicotine relaxes the nerve-free smooth muscle of the chick amnion and the chronically denervated circular muscle of the cat intestine (Evans & Schild, 1953) and nicotine and DMPP also relax the denervated guinea-pig ileum (Paton & Zar, personal communication), it is probable that the direct action of those drugs in high doses is to relax smooth muscle. Day & Vane (1963), however, concluded the opposite—that is, that nicotine contracted smooth muscle by a direct action after denervation. Their conclusion, based on the effects of anoxia, was not substantiated by Paton & Zar (1965) on mechanically denervated muscle, and might have been due to incomplete cholinergic denervation by anoxia.

Tetrodotoxin can thus be used in pharmacological investigations to differentiate direct muscle responses from those elicited by nerve stimulation. In this respect it appears to be more specific than the conventional local anaesthetics, more complete than botulinum toxin in that non-cholinergic nerves are also blocked (Ambache & Lessin, 1955) and it

TABLE  
A CLASSIFICATION OF THE DIRECT AND INDIRECT ACTIONS OF DRUGS BY MEANS OF THE MODIFICATION OF THEIR RESPONSE BY ESERINE, HYOSCINE AND TETRODOTOXIN

C = Contraction; R = relaxation; B = blocked; 0 = unchanged; ↑ = increased; ↓ = decreased

Drugs	Responses of various preparations								Conclusion
	Guinea-pig stomach	Guinea-pig ileum	Rabbit jejunum	Guinea-pig taenia coli	Fundus of rat stomach	Rat duodenum	Mouse stomach	Guinea-pig stomach	
Acetylcholine	C	C	C	C	C	C	C	C	Normally affects smooth muscle only
Normal	B; R > 10 <sup>-6</sup>	↑C	B	B	B	B	BC	B	Nerve mediated R by high doses
Eserine	C	↑C	↑C	↑C	↑C	↑C	↑C	↑C	Stimulates both nerve and muscle
Tetrodotoxin	0; ↑C > 10 <sup>-6</sup>	0	0	0	0	0	0	0	
Angiotensin		Slow C							
Normal		↓C							
Hyoscine		↓C							
Eserine		↓C							
Bradykinin		Slow C							Stimulates only smooth muscle
Normal		0							
Hyoscine		0							
Eserine		0							
Tetrodotoxin		0							
Choline phenyl ether	R		C	C or R	Small C	C	C or R	C	Below 10 <sup>-5</sup> g/ml. stimulates nerves only. Inhibitory predominate in stomach
Normal	↑R		R	R	↓C	↓C	R	↓C	
Hyoscine	↑R		↑C	C or ↑C	↓C	↓C	C or ↑C	↑C	
Eserine	B <sup>+</sup>		B <sup>+</sup>	B <sup>+</sup>	↓C	↓C	B <sup>+</sup>	↑C	
Tetrodotoxin	R		C	C or R	Small C	Small C	C or R	C	Below 10 <sup>-5</sup> g/ml. stimulates nerves only. Inhibitory predominate in stomach
DMPP and nicotine	↑R		R	R	B	B	R	B or R	
Normal	↑C		↑C	C or ↑C	↑C	↑C	C or ↑C	↑C	
Hyoscine	B <sup>+</sup>		B <sup>+</sup>	B <sup>+</sup>	B <sup>+</sup>	B <sup>+</sup>	B <sup>+</sup>	B <sup>+</sup>	
Eserine	0		0	0	0	0	0	0	
Tetrodotoxin	0		0	0	0	0	0	0	
Histamine	0 or ↓C		Irregular response	C	C	C	C	C	Normally affects smooth muscle only
Normal	0 or ↓C		0	0	0	0	0	0	May stimulate cholinergic nerves in stomach
Hyoscine	0 or ↓C		0	0	0	0	0	0	Acts on nerves only in guinea-pig stomach; both nerves and muscle in guinea-pig
Eserine	0 or ↓C		0	0	0	0	0	0	and rabbit intestine; only on muscle in rat fundus and duodenum***
Tetrodotoxin	↑R		C	C	0	0	↑C	0	Direct relaxant effect on smooth muscle
Normal	↑C		↑C	↑C	0	0	↑C	0	
Hyoscine	↑C		↑C	↑C	0	0	↑C	0	
Eserine	B		↑C	↑C	0	0	↑C	0	Stimulates both nerve and muscle
5-HT									
Normal									
Hyoscine									
Eserine									
Tetrodotoxin									
1-Noradrenaline	R		R	R	R	R	R	R	
Normal	0		0	0	0	0	0	0	
Hyoscine	0		0	0	0	0	0	0	
Eserine	0		0	0	0	0	0	0	
Tetrodotoxin	0		0	0	0	0	0	0	
Normal									
Hyoscine									
Eserine									
Tetrodotoxin									

\* Doses of more than 10<sup>-5</sup> g/ml. produce a small contraction in the presence of tetrodotoxin.  
 \* Doses of more than 10<sup>-6</sup> g/ml. produce a small relaxation in the presence of tetrodotoxin.  
 \*\* A relaxation may be seen only if the tone of the smooth muscle is first raised (see text).  
 \*\*\* A dose which is subthreshold for nerve stimulation fully contracts the muscle directly.

is more convenient and generally applicable than the separation of intestinal longitudinal muscle from Auerbach's plexus (Paton & Zar, 1965). Results should be interpreted with some caution however. Responses resulting from stimulation of nerve will be abolished by tetrodotoxin but, although this has not yet been demonstrated, residual responses may still be due to transmitter release which does not require the participation of a conducted action potential.

#### SUMMARY

1. The action of tetrodotoxin was studied on the responses, *in vitro*, of various smooth muscle preparations from guinea-pig, mouse, rat and rabbit, to drugs and to stimulation *via* their nerve supply.

2. The effects produced by nerve stimulation were abolished by tetrodotoxin. They recovered after removal of tetrodotoxin.

3. Tetrodotoxin abolished action potentials in the vagus nerve.

4. No actions of tetrodotoxin were found which could not be explained by a block of nervous activity.

5. No response to any drug, except acetylcholine, tested in the presence of tetrodotoxin was modified by either eserine or hyoscine. Responses to drugs in the presence of tetrodotoxin must, therefore, have been direct effects on the smooth muscle and not due in part to the release of acetylcholine.

6. Responses to drugs which act only through stimulation of nervous structures are abolished by tetrodotoxin. Responses to drugs which act partly on excitatory nerves and partly by direct excitatory action on the muscle are reduced by tetrodotoxin. Responses to drugs whose neural actions are antagonistic to the direct excitatory muscle action, as with acetylcholine, are potentiated by tetrodotoxin. If, as with choline phenyl ether, the antagonistic neural response is dominant, the muscle response is unmasked. Responses to drugs whose sole action is on the smooth muscle are unaffected by tetrodotoxin.

7. The responses of the guinea-pig ileum to drugs in the presence of tetrodotoxin are the same as those on denervated longitudinal smooth muscle strips. The action of tetrodotoxin is thus limited to the nervous tissue in innervated smooth muscle preparations.

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#### REFERENCES

- AMBACHE, N. (1951). 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. *Br. J. Pharmac. Chemother.*, **6**, 51-67.
- AMBACHE, N. (1954). Separation of the longitudinal muscle of the rabbit's ileum as a broad sheet. *J. Physiol.*, **125**, 53-55P.
- AMBACHE, N. & LESSIN, A. W. (1955). Classification of intestinomotor drugs by means of type D botulinum toxin. *J. Physiol.*, **127**, 449-478.
- ARMITAGE, A. K. & VANE, J. R. (1964). A sensitive method for the assay of catechol amines. *Br. J. Pharmac. Chemother.*, **22**, 204-210.

- BÜLBRING, E. (1955). Correlation between membrane potential, spike discharge and tension in smooth muscle. *J. Physiol.*, **128**, 200–221.
- BÜLBRING, E. & BURNSTOCK, G. (1960). Membrane potential changes associated with tachyphylaxis and potentiation of the response to stimulating drugs in smooth muscle. *Br. J. Pharmac. Chemother.*, **15**, 611–624.
- BÜLBRING, E. & TOMITA, T. (1966). Evidence supporting the assumption that the “inhibitory potential” in the taenia coli of the guinea-pig is a post-synaptic potential due to nerve stimulation. *J. Physiol.*, **185**, 24–25P.
- BURNSTOCK, G., CAMPBELL, G. & RAND, M. J. (1966). The inhibitory innervation of the taenia of the guinea-pig caecum. *J. Physiol.*, **182**, 504–526.
- DAY, M. & VANE, J. R. (1963). An analysis of the direct and indirect actions of drugs on the isolated guinea-pig ileum. *Br. J. Pharmac. Chemother.*, **20**, 150–170.
- ELMQVIST, D. & FELDMAN, D. S. (1965). Spontaneous activity at a mammalian neuromuscular junction in tetrodotoxin. *Acta Physiol. scand.*, **64**, 475–476.
- EVANS, D. H. L. & SCHILD, H. O. (1953). Reactions of nerve-free and chronically denervated plain muscle to drugs. *J. Physiol.*, **122**, 63P.
- FINKLEMAN, B. (1930). On the nature of inhibition in the intestine. *J. Physiol.*, **70**, 145–157.
- GADDUM, J. H. & PICARELLI, Z. P. (1957). Two kinds of tryptamine receptor. *Br. J. Pharmac. Chemother.*, **12**, 323–328.
- GERSHON, M. D. (1966). Effects of tetrodotoxin on innervated smooth muscle preparations. *J. Physiol.*, **186**, 4–5P.
- KAO, C. Y. (1966). Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. *Pharmac. Rev.*, **18**, 997–1049.
- KAO, C. Y. & FUHRMAN, F. A. (1963). Pharmacological studies on tarichatoxin, a potent neurotoxin. *J. Pharmac. exp. Ther.*, **140**, 31–40.
- KATZ, B. & MILEDI, R. (1966). The production of end-plate potentials in muscles paralysed by tetrodotoxin. *J. Physiol.*, **185**, 5–6P.
- KURIYAMA, H., OSA, T. & TOIDA, N. (1966). Effect of tetrodotoxin on smooth muscle cells of the guinea-pig taenia coli. *Br. J. Pharmac. Chemother.*, **27**, 366–376.
- MARTINSON, J. (1965). Vagal relaxation of the stomach. Experimental re-investigation of the concept of the transmission mechanism. *Acta Physiol. scand.*, **64**, 453–462.
- NARAHASHI, T., MOORE, J. W. & SCOTT, W. R. (1964). Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. Gen. Physiol.*, **47**, 965–974.
- OGURA, Y. (1963). The biological estimation of crystalline tetrodotoxin. III. On the isolated stomach-vagal nerve preparation of rat. *A. Rep. Inst. Food Microbiol. Chiba Univ.*, **15**, 93–96.
- OGURA, Y., HAMADA, J. & TSUKADA, O. (1960). Action of crystalline tetrodotoxin on nicotine and serotonin. *Folia Pharmacol. Jap.*, **56**, 35–36.
- OGURA, Y., MORI, Y. & WATANABE, Y. (1966). Inhibition of the release of acetylcholine from isolated guinea-pig ileum by crystalline tetrodotoxin. *J. Pharmac. exp. Ther.*, **154**, 456–462.
- PATON, W. D. M. (1955). The response of the guinea-pig ileum to electrical stimulation by coaxial electrodes. *J. Physiol.*, **127**, 40–41P.
- PATON, W. D. M. & RANG, H. P. (1965). The uptake of atropine and related drugs by intestinal smooth muscle of the guinea-pig in relation to acetylcholine receptors. *Proc. Roy. Soc. B.*, **163**, 1–44.
- PATON, W. D. M. & VANE, J. R. (1963). An analysis of the responses of the isolated stomach to electrical stimulation and to drugs. *J. Physiol.*, **165**, 10–46.
- PATON, W. D. M. & ZAR, M. A. (1965). A denervated preparation of the longitudinal muscle of the guinea-pig ileum. *J. Physiol.*, **179**, 85–86P.
- PATON, W. D. M. & ZAR, M. A. (1966). Evidence for transmission of nerve effects by substance P in guinea-pig longitudinal muscle strip. III. International Pharmacological Congress, Sao Paulo—Brazil, Abstracts No. 23, p. 9.
- ROBERTSON, P. A. (1953). An antagonism of 5-hydroxytryptamine by atropine. *J. Physiol.*, **121**, 54–55P.
- SHELLEY, H. (1955). A correlation between cholinesterase inhibition and increase in muscle tone in rabbit duodenum. *Br. J. Pharmac. Chemother.*, **10**, 26–35.
- TOIDA, N. & OSA, T. (1965). Spike generating mechanism of smooth muscle cell membrane. XX111. *Int. Cong. Physiol. Sci.*, Tokyo. Abstracts, No. 171, p. 94.
- VANE, J. R. (1957). A sensitive method for the assay of 5-hydroxytryptamine. *Br. J. Pharmac. Chemother.*, **12**, 344–349.
- ZAR, M. A. (1966). Factors influencing the output of acetylcholine. D.Phil. Thesis. University of Oxford.