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Studies of the interaction of 5-hydroxytryptamine and the perivascular innervation of the guinea-pig caecum

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Summary

1. The action and interaction of noradrenaline (NA), 5-hydroxytryptamine (5-HT) and the sympathetic innervation was studied in the isolated taenia of the guinea-pig caecum.

2. Addition of 5-HT led to a contraction of the taenia while addition of NA or perivascular nerve stimulation resulted in relaxation. Responses to 5-HT or perivascular nerve stimulation were abolished by tetrodotoxin. Tetrodotoxin did not affect responses to applied NA. Hexamethonium and hyoscine converted the 5-HT response to a relaxation and augmented the relaxation which followed low frequency perivascular nerve stimulation. Hexamethonium and hyoscine did not affect the dose-response relationship for NA.

3. Fatigue of mechanical responses of the taenia to perivascular nerve stimulation was accelerated when nerves were stimulated in the presence of 5-HT or α-methyl-*p*-tyrosine (α-MPT). These two agents were additive in this action.

4. Reserpine, 6-hydroxydopamine and α-MPT all reduced the NA content of the taenia. However, only after 6-hydroxydopamine could adrenergic activity be related to NA content.

5. Segments of taenia were incubated with either tritiated NA or 5-HT. An increased rate of release of radioactivity followed perivascular nerve stimulation after incubation with either substance. This release did not occur when tissue was taken from animals given reserpine or 6-hydroxydopamine.

6. It is concluded that 5-HT activates neural elements exclusively while NA has a direct effect on smooth muscle. 5-HT can apparently be taken up by adrenergic axons, and appears to enter the releasable neurotransmitter pool. Since none of the actions characteristic of 5-HT are seen when it is released by adrenergic axons as a false neurotransmitter, the released amine probably fails to reach neuronal receptors for 5-HT.

Introduction

Both 5-hydroxytryptamine (5-HT) (Tafuri & Raick, 1964; Robinson & Gershon, 1971) and noradrenaline (NA) (Norberg, 1964; Jacobowitz, 1965; Åberg & Eränko, 1967) have been identified as endogenous constituents of the myenteric plexus. Both compounds have been found in elements of the plexus distinct from, but in close proximity to, ganglion cells. The elements which contain 5-HT can be distinguished from axons storing NA (Robinson & Gershon, 1971; Gershon &

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Altman, 1971) but the distances between storage sites of the two amines are quite small. Moreover, adrenergic axons have been found to be able to take up 5-HT under some circumstances (Owman, 1964 ; Lichtensteiger, Mutzner & Langemann, 1967 ; Snipes, Thoenen & Tranzer, 1968 ; Thoa, Eccleston & Axelrod, 1969 ; Jaim-Etcheverry & Zieher, 1969, 1971 ; Shaskan & Snyder, 1970 ; Robinson & Gershon, 1971 ; Zieher & Jaim-Etcheverry, 1971). Therefore, there seems to be ample opportunity for an interaction between 5-HT and the adrenergic sympathetic innervation of the gut.

Previous studies have implicated both 5-HT and NA as transmitters in neural inhibitory pathways to the smooth muscle of the gut. In the stomach of the guinea-pig and the duodenum of the mouse the action of 5-HT has been localized to non-adrenergic inhibitory ganglion cells (Bülbring & Gershon, 1967 ; Drakontides & Gershon, 1968). Two mechanisms have been advanced to explain the relaxation of the gut induced by NA. NA acts on smooth muscle directly (Gershon, 1967a) and it has also been shown to reduce the intestinal output of acetylcholine (Schaumann, 1958 ; Paton & Vizi, 1969 ; Kosterlitz, Lydon & Watt, 1970). However, the possible interaction of 5-HT and the adrenergic innervation has received little attention.

In the present study the interaction of 5-HT and the perivascular adrenergic innervation of the taenia of the guinea-pig caecum were analysed. The possibility that adrenergic axons might take up 5-HT and release it as a 'false transmitter' was investigated. If this can occur, then it is of interest to determine the physiological effects of such release. Uptake and release of 5-HT by adrenergic axons could be a means of modulation of sympathetic neural activity in the taenia. Moreover, since the action of 5-HT on the taenia is recognizably different from that of NA, 5-HT released as a 'false transmitter' might be useful as a marker substance to aid in the localization of sites accessible to transmitter released from adrenergic axons. In the course of these studies the sites of action of 5-HT on the taenia and the components of the perivascular innervation were studied initially. Parameters necessary for maintenance of the adrenergic responses were then examined, and the effect of 5-HT on the maintenance of adrenergic responses was determined. Finally, labelled 5-HT and NA were used in order to investigate release of substances by the perivascular nerves to the taenia in response to stimulation.

Methods

Male English short hair guinea-pigs, ranging in weight from 250 to 350 g were used. The animals were stunned by a blow on the head and exsanguinated from the carotid arteries. The caecum was quickly removed and placed in Krebs solution of the following composition (mM): NaCl 133, KCl 4·7, NaH_2PO_4 1·3 ; $NaHCO_3$ 16, $CaCl_2$ 2·7, $MgCl_2$ 0·17, dextrose 7·07. The pH of the Krebs solution ranged between 7·38 and 7·42. Segments of taenia coli 3 to 4 cm in length and the accompanying perivascular vessels were excised as described by Burnstock, Campbell & Rand (1966).

The perivascular vessels were passed through two loops of platinum wire embedded in epoxy resin which served as a bipolar electrode for perivascular nerve stimulation. This electrode was not in contact with the muscle. The preparation was placed in a 10 or 25 ml organ bath containing Krebs solution at 37° C,

bubbled with 95% O_2 and 5% CO_2. Mechanical activity was recorded isotonically by a Phipps and Bird Model ST-2 or a Clevite Brush transducer connected to a potentiometric recorder. A stainless steel wire mesh and a stainless steel needle were placed on either side of the taenia, and served as the electrodes for electrical field stimulation. Electrical stimulation was applied with a Grass model S8 stimulator. Perivascular nerves were stimulated supramaximally with rectangular pulses, 0·5 ms in duration, at a frequency of 5–30 Hz for 10 seconds. At the end of each experiment the tissue between the electrodes and the taenia was cut. The stimulus was then reapplied to check that the effects were not a result of current spread to the taenia. Electrical field stimulation was a supramaximal current applied at 5 Hz with a pulse duration of 0·5 ms and was continued for 10 seconds.

In the series of experiments in which isotopes were employed, segments of taenia were placed in 5 ml organ baths. Following the attainment of stable mechanical responses to perivascular nerve and electrical field stimulation the segments were incubated for 40 min in Krebs solution containing either tritiated 5-HT (3–6·2 Ci/mmol; Amersham Searle) or (±)-noradrenaline (16·7 Ci/mmol; New England Nuclear Corp.), each at 3×10^{-6} M. The contents of the bath were removed at the end of the 40 min incubation period, and replaced with fresh Krebs solution. A subsequent perfusion of Krebs solution through the chamber was maintained and 2 ml samples were collected every 3 minutes. Electrical stimulation was begun 45 min after the start of the perfusion and trains of stimuli, 3 min in duration, were applied at intervals of 30 minutes.

The 2 ml samples collected from the bath were mixed with 15 ml of liquid scintillator which contained 8 g 'Omnifluor' (New England Nuclear), 60 g naphthalene, 200 ml ethylene glycol monoethyl ether and 1,000 ml *p*-dioxane. The radioactivity of the samples was counted with a Picker Nuclear liquid scintillation counter. Samples were corrected for quenching by external standardization.

The concentration of NA in tissues was determined spectrophotofluorometrically after ion exchange chromatography and oxidation by the method of Häggendal (1963). Amberlite CG-120 (Na^+ form) was used in a resin bed 20 mm × 6 mm. Internal standards were run during each assay procedure. Results were corrected for recovery, which was 78·0 ± 4·9% S.E.

Segments of taenia prepared for studies of fluorescence were treated in the same manner as those discussed above. Incubation was in NA or 5-HT in concentrations of 5×10^{-6} M for 40 minutes. Following incubation the tissues were rapidly frozen in a mixture of liquid propane and ethane cooled by liquid nitrogen and dried at −40° C for 24 to 48 h in a Speedivac-Pearse Tissue Dryer (Model 1). The tissues were then treated with formaldehyde gas of 60% relative humidity at 80° C for 1 hour. Following formaldehyde treatment the tissues were embedded in paraffin, sectioned at 10 μ and mounted in Entellan (Merck) for fluorescence microscopy. A Leitz microscope equipped with a dark field condensor was used. Two Schott BG 12 2 mm filters were placed in the beam of a mercury vapour light source. The barrier filter had a maximum absorption below 510 nm.

The drugs used were: DL-α-methyl-*p*-tyrosine methyl ester-hydrochloride (α-MPT), (+)-amphetamine, cocaine hydrochloride, dihydroergotamine tartrate, eserine sulphate, L-β-3,4-dihydroxy-phenylalanine (DOPA), guanethidine monosulphate, hexamethonium chloride, 6-hydroxydopamine hydrobromide, 5-hydroxytryptamine creatinine sulphate (5-HT), hyoscine hydrobromide, (−)-noradrenaline

bitartrate (NA), pargyline, pheniprazine, (+)-propranolol hydrochloride, reserpine (Serpasil), and tetrodotoxin citrate (Sankyo).

Results

The actions of 5-hydroxytryptamine, noradrenaline and perivascular nerve stimulation on the taenia

The mechanical effects brought about by the addition of 5-HT or NA to the organ bath and by stimulation of perivascular nerves were analysed pharmacologically. This analysis is summarized in Figure 1. In preparations which had not previously been treated with another drug, the addition of 5-HT (tested in the dose range of $2{\cdot}5 \times 10^{-7}$–$2{\cdot}5 \times 10^{-5}$ M) led to a contraction of the taenia, whereas the addition of NA ($3{\cdot}1 \times 10^{-9}$ M–$3{\cdot}1 \times 10^{-6}$ M) or perivascular nerve stimulation (tested at frequencies of 5–30 Hz) were invariably followed by a relaxation. After these control responses were obtained, hexamethonium ($3{\cdot}6 \times 10^{-5}$M) and hyoscine ($2{\cdot}6 \times 10^{-7}$M) were added. These drugs were used to block nicotinic and muscarinic responses of the taenia and to remove any pre-existent cholinergic tone. In the presence of hexamethonium and hyoscine the response to 5-HT was one of relaxation. Occasionally a rebound contraction followed the relaxation induced by 5-HT. Therefore, cholinergic blockade unmasked an inhibitory effect of 5-HT.

Neither hexamethonium nor hyoscine, alone or in combination, had an effect on the responses of the taenia to NA. Thus the relaxant response to exogenous NA did not result from removal of cholinergic tone. Although hexamethonium and hyoscine had no effect on the response to high frequencies of perivascular

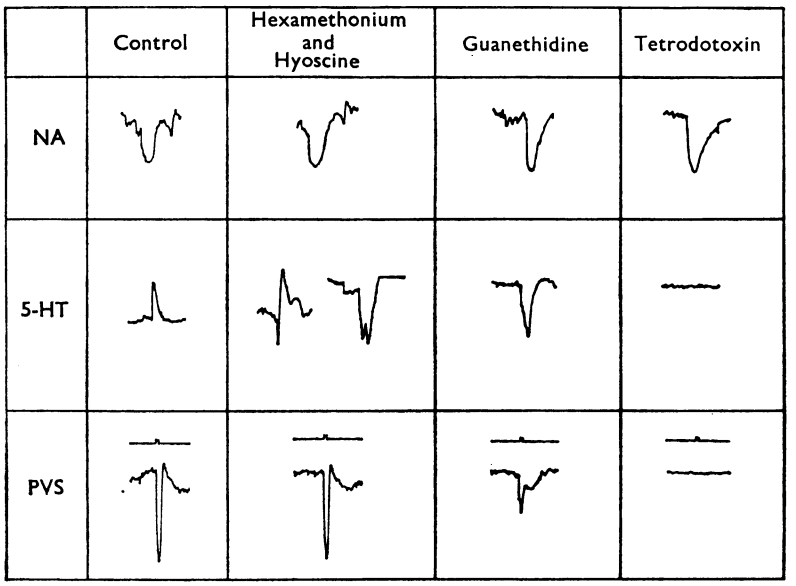

FIG. 1. Pharmacological analysis of the mechanical effects resulting from the application of NA ($3{\cdot}1 \times 10^{-7}$M) or 5-HT ($2{\cdot}5 \times 10^{-5}$M) and from stimulation (30 Hz, 10 s) of the perivascular nerves (PVS). Typical responses are illustrated under the conditions listed. Control: no prior treatment with drugs. Hexamethonium ($3{\cdot}6 \times 10^{-5}$M) and hyoscine ($2{\cdot}6 \times 10^{-7}$M) were present for 30 min prior to trial of NA, 5-HT, PVS. Guanethidine ($6{\cdot}7 \times 10^{-6}$M) was present for 20 min prior to test stimuli. Tetrodotoxin was used in a concentration of $3{\cdot}1 \times 10^{-7}$M.

nerve stimulation (greater than 20 Hz), these drugs did potentiate the relaxation of the taenia in response to lower frequencies (5–10 Hz) of perivascular nerve stimulation. This observation supports the view that there is a cholinergic component in the perivascular nerve supply to the taenia (Burnstock, Campbell & Rand, 1966).

Guanethidine ($6{\cdot}7 \times 10^{-6}$ M) did not affect the relaxant response induced by 5-HT after muscarinic and nicotinic blockade. Hence this response is probably not mediated by adrenergic axons. Similarly guanethidine did not antagonize the action of exogenous NA. However, guanethidine did antagonize responses to perivascular nerve stimulation. In 43 out of 50 (86%) preparations the antagonism of responses to perivascular nerve stimulation was incomplete and a residual relaxation remained. These small residual relaxations were not further altered by higher concentrations of guanethidine ($3{\cdot}3 \times 10^{-5}$ M), by the addition of dihydroergotamine ($7{\cdot}6 \times 10^{-8}$ M) and propranolol ($3{\cdot}4 \times 10^{-7}$ M), alone or in combination. These concentrations of dihydroergotamine and propranolol did antagonize the action of exogenous NA ($3{\cdot}1 \times 10^{-6}$ M). (+)-Amphetamine ($7{\cdot}4 \times 10^{-6}$ M) reversed the action of guanethidine and restored relaxant responses of perivascular nerve stimulation to control levels. The action of (+)-amphetamine was transient. These observations suggest that the perivascular nerve supply to taenia contains non-adrenergic, guanethidine-resistant inhibitory fibres in addition to adrenergic axons.

Tetrodotoxin was used to differentiate direct muscle responses from those elicited by nerve stimulation (Narashashi, Moore & Scott, 1964; Kuriyama, Osa & Toida, 1966; Kao, 1966; Gershon, 1967b). Both the contractile and the relaxant response to 5-HT were abolished by tetrodotoxin $3{\cdot}1 \times 10^{-7}$ M. Therefore, the action of 5-HT appears to be entirely neuronally mediated. In contrast, tetrodotoxin had no effect on responses to exogenous NA. Responses to perivascular nerve stimulation were abolished by tetrodotoxin. These observations again indicate that the relaxation in response to exogenous NA does not result from inhibition of neuronal activity.

Fatigue of responses of the taenia subjected to repeated intermittent perivascular nerve stimulation

Segments of taenia were incubated in Krebs solution containing hexamethonium and hyoscine. Perivascular nerves were stimulated with trains of stimuli delivered at 30 Hz for 10 s every 4 minutes. The response to perivascular nerve stimulation was well maintained and fatigued very slowly (Fig. 2). Fatigue was more rapid if trains of stimuli were delivered at shorter intervals. The rate of development of fatigue was enhanced by the tyrosine hydroxylase inhibitor, α-MPT (8×10^{-5}M) (Fig. 2). However, even in the presence of α-MPT complete loss of relaxant responses to perivascular nerve stimulation was never observed and the amplitude of responses tended to remain stable at 20–30% of maximum. After long periods (Fig. 2) of stimulation the magnitude of perivascular responses in control preparations gradually fell and approached that seen with α-MPT. However, the residual relaxant responses seen after 6–8 h of stimulation in the presence of α-MPT, in contrast to controls, were unaffected by the addition of guanethidine ($6{\cdot}7 \times 10^{-6}$ M) (Fig. 3). Therefore, since they were resistant to adrenergic neurone blockade with guanethidine, the responses to perivascular nerve stimulation in the taenia treated

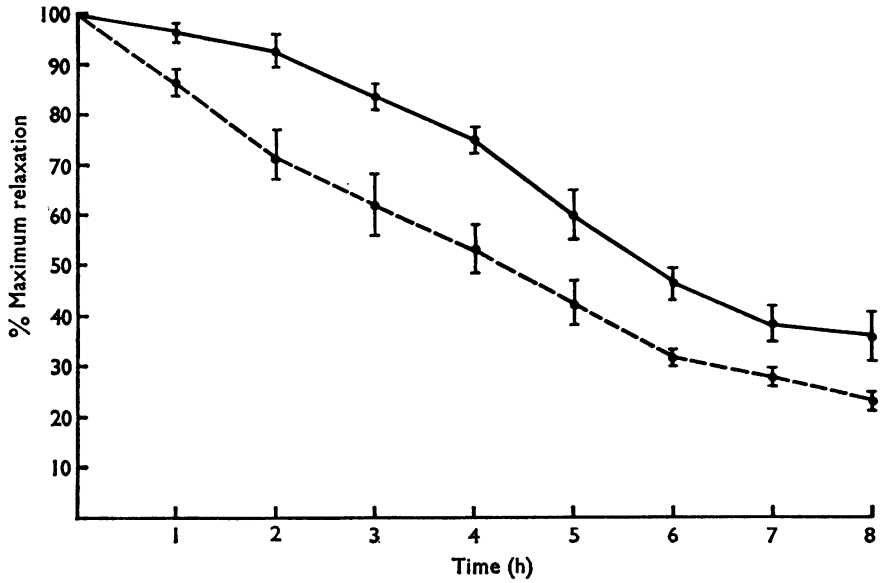

FIG. 2. The solid line represents the responses to stimulation of the perivascular nerves to the taenia coli at 4 min intervals. Tissues were incubated in Krebs solution in the presence of hexamethonium ($3{\cdot}6\times10^{-5}$M) and hyoscine ($2{\cdot}6\times10^{-7}$M). The dotted line represents the responses recorded under the same conditions in the presence of α-MPT ($8{\cdot}0\times10^{-5}$M). Baseline tone was not altered by α-MPT. Each point represents the mean of 5 experiments. The vertical lines are $\pm$S.E.

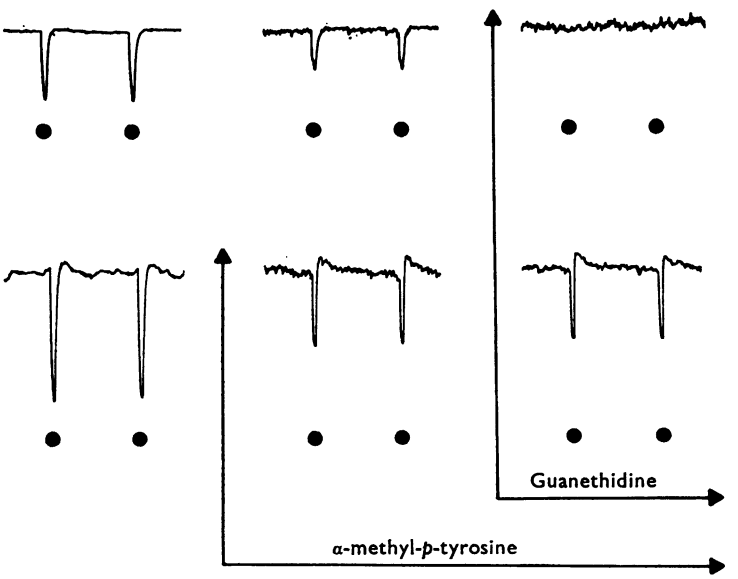

FIG. 3. Responses from two segments of taenia coli to perivascular nerve stimulation (indicated by dots) which were recorded simultaneously in the presence of hexamethonium ($3{\cdot}6\times10^{-5}$M) and hyoscine ($2{\cdot}6\times10^{-7}$M). The upper tracings show responses from an untreated segment of taenia. The first responses (upper left) were obtained at the beginning of the recording period. After 6 h (centre), the magnitude of relaxation was reduced. Following guanethidine ($6{\cdot}7\times10^{-6}$M) there was no response to perivascular nerve stimulation (upper right). The lower tracing represents responses recorded from a segment of taenia incubated with α-MPT ($8{\cdot}0\times10^{-5}$M). As in the control preparation, a reduction in the magnitude of responses occurred following 6 h of treatment (lower centre). However, guanethidine had no effect on subsequent responses to perivascular nerve stimulation (Lower right).

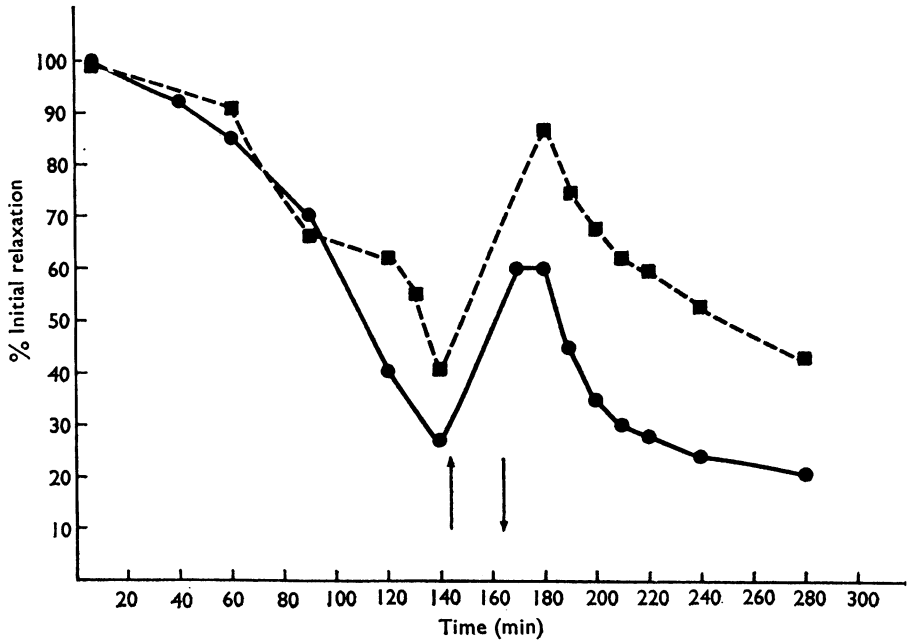

FIG. 4. Both curves represent responses to perivascular nerve stimulation from 2 segments of taenia coli in the presence of α-MPT ($8{\cdot}0\times10^{-5}$M). Between the points indicated by the vertical arrows NA ($3{\cdot}1\times10^{-6}$M) (solid line) and L-DOPA ($5{\cdot}1\times10^{-4}$M) (dotted line) were added to the organ baths and remained for 20 minutes. During the 20 min incubation period with NA and L-DOPA the perivascular nerves to the taenia were not stimulated.

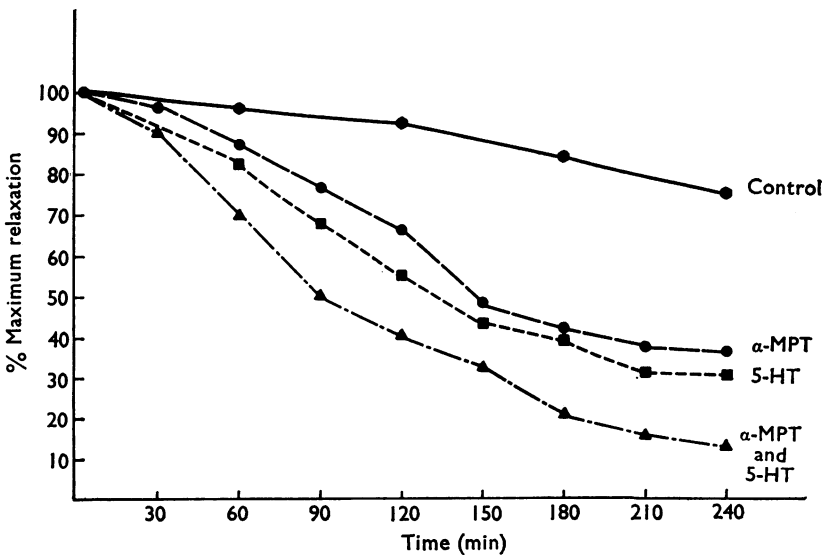

FIG. 5. The responses to perivascular nerve stimulation of taeniae in control, the presence of α-MPT ($8{\cdot}0\times10^{-5}$M), 5-HT ($5{\cdot}0\times10^{-5}$M) and the combination of α-MPT plus 5-HT. Each point represents the mean of 5 separate experiments. Although 5-HT itself caused a relaxation, it did not initially affect the magnitude of responses to perivascular stimulation.

with α-MPT were probably not adrenergic. It thus appeared that α-MPT had abolished the adrenergic component of the perivascular response. The complete abolition of responses to perivascular stimulation shown in the control preparation in Fig. 3 is unusual. The more usual effect of guanethidine was a reduction of responses, as noted previously and shown in Figure 1.

The dose-response relationship of the taenia to applied NA was not altered by treatment with α-MPT. Therefore, α-MPT does not affect adrenoceptors. However, after fatigue had been induced in the α-MPT treated preparations, the responses to perivascular nerve stimulation could be potentiated by the addition of either L-DOPA ($5{\cdot}1 \times 10^{-4}$ M) or NA ($3{\cdot}1 \times 10^{-6}$ M) (Fig. 4). This potentiation by L-DOPA or NA was transient and was not seen in controls of (α-MPT absent). These observations are consistent with the view that the increased rate of fatigue of perivascular responses in the presence of α-MPT was due to a decrease in releasable transmitter.

5-HT (5×10^{-5} M) like α-MPT (8×10^{-5} M), increased the rate of fatigue of perivascular responses when included in the incubation medium with segments of taenia (Fig. 5). 5-HT was about as effective as α-MPT in this regard, when both drugs were used at the concentrations indicated above. The addition of 5-HT in combination with α-MPT depressed responses to perivascular nerve

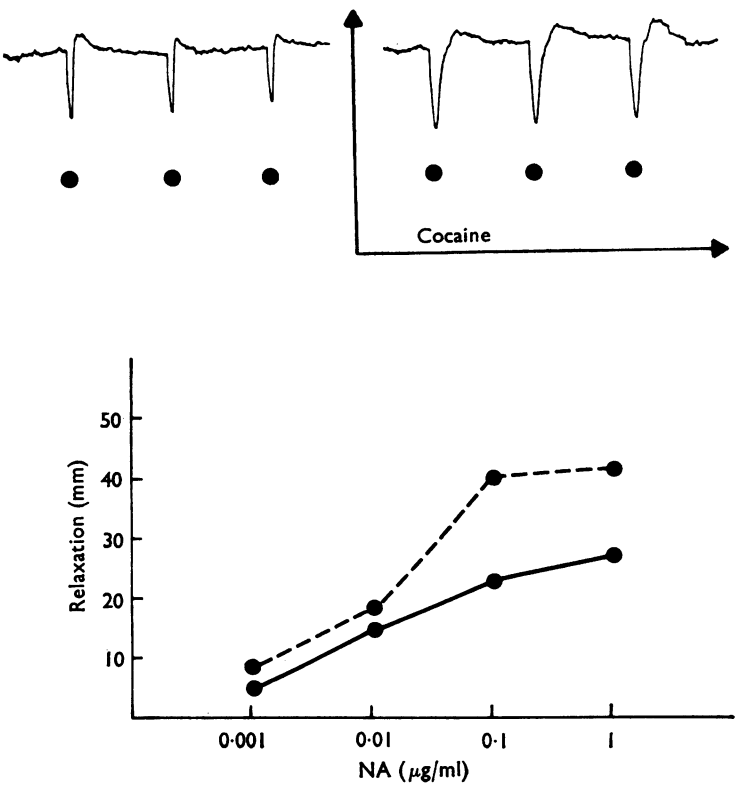

FIG. 6. In the upper half of the figure responses to perivascular nerve stimulation are shown before and 15 min after the application of cocaine ($2{\cdot}9 \times 10^{-6}$M). The lower portion of the figure represents the dose-response curve of the same taenia preparation to exogenous NA. The solid line represents responses prior to the administration of cocaine and the dotted line the responses observed in the presence of cocaine. Hexamethonium ($3{\cdot}6 \times 10^{-5}$M) and hyoscine ($2{\cdot}6 \times 10^{-7}$M) were present throughout.

stimulation more rapidly than either 5-HT or α-MPT alone (Fig. 5). Moreover, the rate of depression of responses to perivascular nerve stimulation was also increased when 5-HT was added to a preparation that had already been exposed to α-MPT. Again, like α-MPT, 5-HT did not affect the dose-response relationship of the taenia to applied NA. Therefore, 5-HT did not appear to affect adrenoceptors.

Since the effect of 5-HT might have resulted from inhibition of axonal re-uptake of NA, the actions on the taenia of cocaine, a drug known to inhibit uptake of NA, were compared to 5-HT. Inhibition of re-uptake of NA would be expected to potentiate adrenergic responses (Iversen, 1971). Unlike 5-HT, cocaine ($2{\cdot}9 \times 10^{-6}$ M) potentiated responses of the taenia to perivascular nerve stimulation and to applied NA (Fig. 6). Responses of preparations treated with cocaine fatigued slowly and did not return to control levels within 2 hours after addition of the drug. Thus cocaine did not resemble 5-HT in its actions.

The development of fatigue of responses to perivascular nerve stimulation was also studied in relation to the NA concentration in the taenia. Segments of taenia treated with α-MPT were assayed for their content of NA at the end of the experiments described above. These concentrations were compared with those of paired control segments of taenia. Control segments taken from the same animal were treated in the same way as experimental tissues except that α-MPT was omitted from the incubating solution. Although the content of NA in the taenia treated with α-MPT was significantly reduced when compared to the paired controls ($P<0{\cdot}001$), a considerable amount of NA remained (Table 1). Nevertheless, despite this residual NA, α-MPT had abolished guanethidine-sensitive adrenergic responses. The testing of adrenergic function with guanethidine itself affected the tissue content of NA. Tissues treated with guanethidine always had a higher NA content than equivalent tissues not exposed to the drug (Table 1).

TABLE 1. *Noradrenaline content and the effect of guanethidine on the responses of the taenia to perivascular nerve stimulation*

Treatment	NA content μg/g±S.E.	Guanethidine tested response	*P*
1. Control-non-incubated ($n=30$)	0·562±0·053		
2. Control (incubated) ($n=8$)	0·556±0·029		1 vs 2 $P>0{\cdot}9$
3. α-MPT ($n=8$)	0·392±0·024		2 vs 3 $P<0{\cdot}05$
4. Control-guanethidine-treated ($n=8$)	0·832±0·022	+	2 vs 4 $P<0{\cdot}01$
5. α-MPT-guanethidine-treated ($n=8$)	0·507±0·018	−	4 vs 5 $P<0{\cdot}001$
6. Reserpine-guanethidine-treated ($n=12$)	0·078±0·018	+	4 vs 6 $P<0{\cdot}001$
7. 6-Hydroxydopamine-guanethidine-treated ($n=12$)	0·013±0·001	−	4 vs 7 $P<0{\cdot}001$

n=Number of experiments. + = Response of the taenia to perivascular stimulation was reduced or abolished by guanethidine ($6{\cdot}7 \times 10^{-6}$ M). − =Response of the taenia to perivascular stimulation was not affected by addition of guanethidine ($6{\cdot}7 \times 10^{-6}$ M). All experiments lasted 6–8 h. α-MPT was added at the start and guanethidine 2 h before the end of the incubation period. Reserpine and 6-hydroxydopamine were given *in vivo* prior to beginning these experiments (see text).

The action of reserpine and 6-hydroxydopamine on responses of taenia coli to perivascular nerve stimulation and tissue content of noradrenaline

Treatment of guinea-pigs with reserpine (10 mg/kg) intraperitoneally, 17 to 19 h prior to the removal of the taenia, did not appear to alter responses of the taenia to perivascular nerve stimulation. Responses could not be distinguished from those of control animals. Neither L-DOPA ($5{\cdot}1 \times 10^{-4}$M) nor NA ($3{\cdot}1 \times 10^{-6}$M) potentiated responses to perivascular nerve stimulation in these tissues. Furthermore guanethidine antagonized responses to perivascular nerve stimulation in preparations from animals given reserpine. Reserpine also did not affect responses or the dose-response relationship of the taenia to applied NA or 5-HT. Therefore, reserpine in these experiments did not appear to interfere with adrenergic transmission from perivascular nerves to the taenia. However, reserpine did reduce, by about 90%, tissue stores of NA (Table 1). This reduction was greater than that produced by incubation with α-MPT, although adrenergic transmission appeared to fail after α-MPT but not after reserpine.

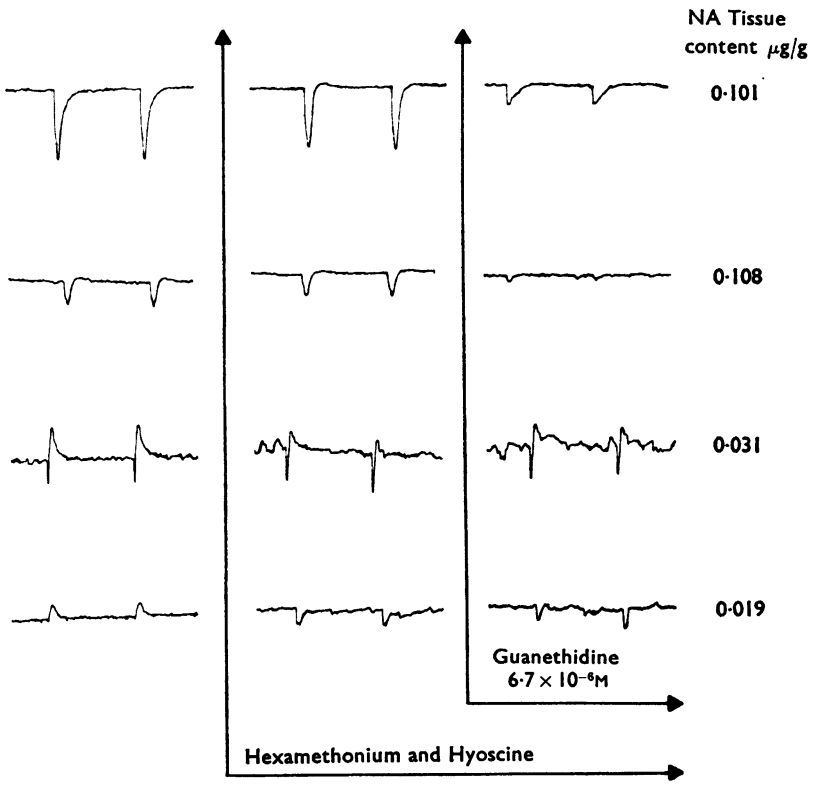

FIG. 7. The responses of 4 representative taenia, from animals treated with 6-hydroxydopamine to perivascular nerve stimulation are shown. In those preparations in which a contractile response was dominant, lower 2 records, hexamethonium ($3{\cdot}6 \times 10^{-5}$M) and hyoscine ($2{\cdot}6 \times 10^{-7}$M) converted the response to a relaxation and guanethidine was without effect. In the upper 2 records, in which only relaxant responses to perivascular nerve stimulation were present, guanethidine caused a substantial reduction in the magnitude of responses. The last column lists the tissue content of NA determined at the end of the experimental procedures. Although all the segments had reduced quantities of NA as compared to normal taenia, those segments in which guanethidine was without effect contained the least NA.

6-hydroxydopamine (4 doses 100 mg/kg) intraperitoneally was used in an attempt to destroy adrenergic axon terminals (Tranzer & Thoenen, 1968; Haeusler, Haefely & Thoenen, 1969). Three types of response to perivascular nerve stimulation of the taenia were seen in animals treated with 6-hydroxydopamine (Fig. 7). One type was purely relaxant. Another was an initial relaxation followed by a contraction. The third was purely contractile. The purely relaxant responses were unaffected by the addition of hexamethonium and hyoscine and were guanethidine-sensitive. When a prominent contractile component of the response was noted, this could be reduced or abolished by hexamethonium and hyoscine. A concomitant relaxant component was either potentiated (if initially present) or revealed by addition of these drugs. This latter relaxation was not affected by guanethidine and therefore was probably non-adrenergic.

There appeared to be a correlation between the content of NA in the taenia and the type of response to perivascular nerve stimulation seen after treatment with 6-hydroxydopamine. If the concentration of NA was less than 0·05 μg/g, responses to perivascular nerve stimulation showed a prominent contractile component. Relaxations, revealed by muscarinic blockade, were insensitive to guanethidine. These relaxant responses were, however, abolished by tetrodotoxin. Thus they were probably neural in origin.

Preparations from animals given 6-hydroxydopamine in which the response to perivascular nerve stimulation was primarily contractile were studied further. These responses were potentiated by eserine ($1{\cdot}5 \times 10^{-7}$ M). The potentiated contractile responses were blocked by hexamethonium and hyoscine revealing an underlying relaxation. The response of these preparations to exogenously applied 5-HT ($2{\cdot}5 \times 10^{-5}$ M) very much resembled responses to perivascular nerve stimulation. Again, the response showed a prominent contractile component following an initial relaxation. The contractile response was potentiated by eserine and the addition of hexamethonium and hyoscine again revealed an underlying relaxation. In no case did treatment with 6-hydroxydopamine alter the dose-response relationship of the taenia to applied NA. These experiments support the view that the perivascular innervation of the taenia has cholinergic excitatory and non-adrenergic inhibitory components, which are unmasked by 6-hydroxydopamine.

Effect of perivascular nerve stimulation and electrical field stimulation on the rate of release of radioactivity from taenia coli incubated with ^{3}H-5-hydroxytryptamine or ^{3}H-noradrenaline

Segments of taenia were incubated with tritiated NA or 5-HT (3×10^{-6}M) for 40 minutes. During the subsequent washout of radioactive material, the strips were subjected to perivascular nerve or electrical field stimulation. The rate of release of total tritium was measured. The monoamine oxidase inhibitor, pheniprazine ($6{\cdot}6 \times 10^{-5}$M) was added to the solution perfusing the taenia following incubation, during the washout phase of the experiments. In preliminary studies this concentration of pheniprazine prevented uptake of ^{3}H-NA. The drug was added to prevent deamination and re-uptake of labelled transmitter during nerve stimulation.

Both perivascular nerve stimulation and electrical field stimulation increased the rate of tritium release from taenia incubated with either ^{3}H-NA or ^{3}H-5-HT (Fig. 8). These increases were transient and were temporally related to the applica-

tion of the stimulus. Similar increases in the efflux of radioactive material were not seen in unstimulated control tissues, taken from the same animals and run simultaneously. Moreover, the increased rate of release of radioactive material in response to perivascular nerve stimulation was not prevented by perfusing segments of taenia with a solution containing hyoscine ($2{\cdot}6 \times 10^{-7}$M), which abolished the contraction produced by stimulation. The increased rate of release of radioactivity following perivascular nerve stimulation, from segments incubated either with ^{3}H-5-HT or ^{3}H-NA, but not that which followed electrical field stimulation, was blocked by tetrodotoxin ($3{\cdot}1 \times 10^{-7}$M). It seems likely, therefore, that the tritium release which followed perivascular nerve stimulation depended upon axonal conduction and was from a neuronal source. This cannot be said of the increased rate of tritium efflux which followed electrical field stimulation.

Reserpine and 6-hydroxydopamine

Preparations taken from animals previously treated with reserpine or 6-hydroxydopamine were incubated, as described above, with either ^{3}H-NA or ^{3}H-5-HT. No increase in the rate of release of radioactivity could be detected following perivascular nerve stimulation, in any of these tissues. When tissues were incubated

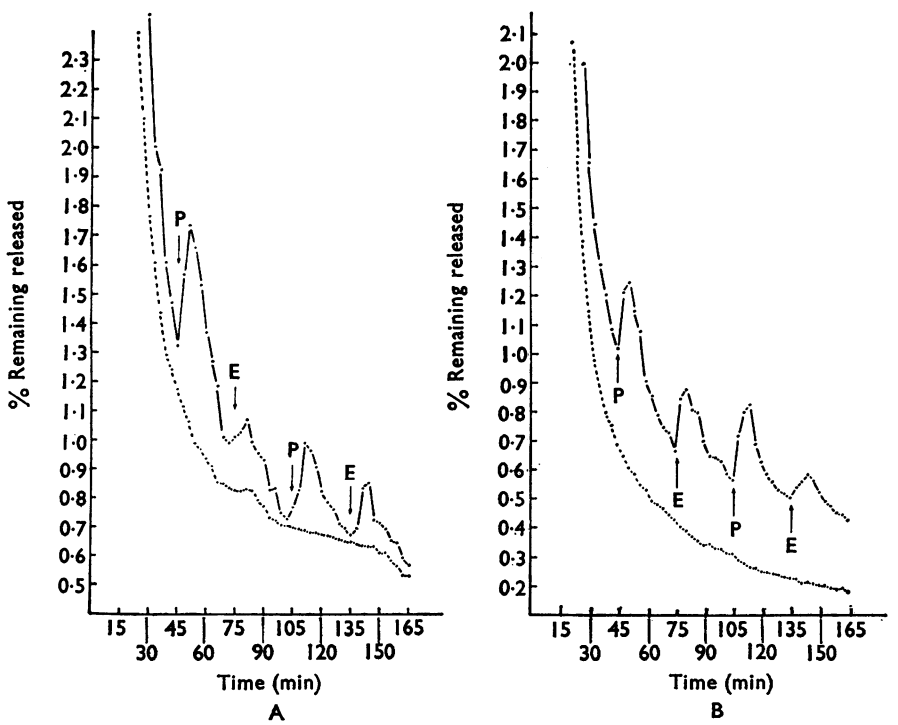

FIG. 8. Each curve in A and B shows the rate of release of radioactivity from segments of taenia plotted as a function of time. In A two strips from the same animal were preincubated for 40 min in ^{3}H-5-HT (3×10^{-6}M), and in B in ^{3}H-NA (3×10^{-6}M). The preparations were perfused with Krebs solution containing pheniprazine ($6{\cdot}6 \times 10^{-5}$M). The 2 strips in A and B were studied at the same time. At the intervals indicated by the arrows in A and B one preparation (upper line) was electrically stimulated for 3 min via the perivascular nerves (P) or electrical field stimulation (E) was applied. At the points of stimulation an increase in the rate of release of radioactivity from the stimulated segments occurred.

with ^{3}H-5-HT, no increase in the rate of tritium efflux was seen after electrical field stimulation. However, in preparations which had been incubated with ^{3}H-NA an increase in the rate of release of radioactivity was seen after electrical field stimulation. It is probable that some ^{3}H-NA but not ^{3}H-5-HT is taken up by smooth muscle. Histofluorescence studies, using the Falck-Hillarp technique (Falck & Owman, 1965) revealed a slight green fluorescence of the muscle following incubation with NA, but no corresponding yellow fluorescence was seen after incubation with 5-HT. It is possible that electrical field stimulation led to release of labelled NA or its metabolites from smooth muscle. Burnstock, McLean & Wright (1971) have recently demonstrated NA uptake by non-innervated smooth muscle.

In several experiments involving reserpine, pargyline (10^{-5}M) was added to prevent catabolism of labelled amine by monoamine oxidase (MAO) during incubation. Pargyline does not prevent uptake of ^{3}H-NA (Iversen, 1967). Results with pargyline were not different from those of experiments in which the drug was omitted. No increase in the rate of tritium release was seen following perivascular nerve stimulation in preparations from animals given reserpine.

Discussion

Responses of the taenia to applied 5-HT appeared to be entirely mediated by nervous structures. Thus, no response to 5-HT was seen in the presence of tetrodotoxin. Similar results have also been obtained by others (Rikimaru & Suzuki, 1971). Moreover, 5-HT appeared capable of activating both excitatory and inhibitory neurones. The excitatory neurones were probably cholinergic since contractile responses to 5-HT were blocked by hyoscine and potentiated by eserine. The inhibitory neurones were probably not adrenergic, since relaxant responses to 5-HT were unaffected by adrenergic neurone blockade with guanethidine and could be seen after adrenergic neurones had been destroyed by 6-hydroxydopamine. Treatment with 6-hydroxydopamine was considered to have successfully destroyed adrenergic axon terminals if the NA content of the taenia was lowered to a level below 0·05 μg/g (>90% depletion) and the response to perivascular nerve stimulation was primarily contractile. In these preparations responses to 5-HT mimicked those to perivascular nerve stimulation, and muscarinic blockade revealed a relaxant response. The action of 5-HT on the taenia of the guinea-pig caecum is similar to its action on the stomach of the guinea-pig (Bülbring & Gershon, 1967). In the small intestine, however, 5-HT appears to activate receptors on smooth muscle as well as those on neural elements (Gershon, 1967b ; Drakontides & Gershon, 1968).

Responses of the isolated taenia to NA on the other hand, appeared to result from a direct action of the amine on smooth muscle. No evidence could be obtained that inhibition of release of acetylcholine (Paton & Vizi, 1969), if it occurred in the taenia, contributed to the relaxation which followed addition of NA. For instance, prior treatment with hyoscine, hexamethonium, and tetrodotoxin all failed to affect the dose-response relationship to applied NA. In these experiments hyoscine should have antagonized muscarinic responses to acetylcholine before application of NA. Tetrodotoxin should have eliminated neural activity in the preparations and both tetrodotoxin (Ogura, Mori & Watanabe, 1966 ; Paton, Vizi & Zar, 1971) and hexamethonium (Paton, Vizi & Zar, 1971) themselves inhibit the release of acetylcholine.

The response of the taenia to perivascular nerve stimulation was complex and the perivascular nerve supply did not appear to represent a pure example of an adrenergic innervation. Evidence was obtained, in agreement with others (Burnstock, Campbell & Rand, 1966), that there was a cholinergic component to the innervation. Thus, at low frequencies of stimulation, hexamethonium and hyoscine potentiated the response to perivascular nerve stimulation. After destruction of adrenergic terminals with 6-hydroxydopamine the response was a contraction which was potentiated by eserine and blocked by hexamethonium and hyoscine. The perivascular innervation also seemed to contain a non-adrenergic inhibitory component, since guanethidine usually failed to abolish fully the relaxant responses of the taenia to perivascular nerve stimulation. In addition, a relaxant component of the response to perivascular nerve stimulation persisted after treatment with 6-hydroxydopamine and could then be revealed by muscarinic blockade. This component was also seen after the adrenergic response had fatigued following prolonged periods of stimulation in the presence of α-MPT.

Responses to stimulation of perivascular nerves fatigued slowly when the nerves were stimulated with 10 s trains of stimuli at 3 min intervals for several hours. During this period of activity *in vitro* there was no detectable fall in the NA content of the taenia. This ability of the taenia to maintain its transmitter store is probably due to the increased formation of NA from tyrosine which accompanies nerve stimulation (Alousi & Weiner, 1966; Gordon, Reid, Sjoerdsma & Udenfriend, 1966; Roth, Stjärne & Euler, 1967; Sedvall & Kopin, 1967; Weiner & Rabadjija, 1968). Re-uptake of NA by axon terminals may also contribute (Iversen, 1967; Langer & Vogt, 1971). In the guinea-pig vas deferens, re-uptake of the released transmitter may be more important to maintenance of NA stores during stimulation than is new synthesis (Thoa, Johnson & Kopin, 1971). The tyrosine hydroxylase inhibitor α-MPT was added to the bath in order to prevent new synthesis of NA. Fatigue of perivascular responses was accelerated and the NA content of the taenia fell. However, adrenergic (guanethidine-sensitive) responses appeared to be lost while substantial stores of NA remained in the taenia. Greater depletion of NA could be achieved by pretreatment of animals with reserpine, without, however, abolishing adrenergic responses. Once adrenergic transmission had failed, it could, in the presence of α-MPT be transiently restored by adding NA or L-DOPA to the bath. Thus, *in vitro*, continued synthesis of NA appears to be necessary for the maintenance of adrenergic responses. Since transmission failed with ample stores of NA remaining, a substantial portion of the transmitter store appears to be unavailable for release in response to nerve stimulation. Yet, since reserpine did not abolish adrenergic responses, only a small portion of the NA store seems to be necessary for transmission. This portion, selectively depleted by stimulation in the presence of α-MPT is probably the newly synthesized transmitter. Since exogenous NA can transiently restore transmission in the presence of α-MPT, newly taken up transmitter must also enter this releasable transmitter pool. Similar conclusions have been drawn by other authors from studies of different systems (Kopin, Breese, Krauss & Weise, 1968; Gewirtz & Kopin, 1970; Stjärne & Wennmalm, 1970; Thoa, Johnson & Kopin, 1971).

5-HT had a similar effect to that of α-MPT on adrenergic responses. When included in the incubation medium 5-HT was about as effective in diminishing responses to perivascular nerve stimulation as was α-MPT. Moreover, 5-HT and

α-MPT appeared to be additive in their action. Thus, if α-MPT had fully inhibited tyrosine hydroxylase, 5-HT must act against adrenergic transmission by a different mechanism. 5-HT did not affect adrenoceptors, nor did its action resemble that of cocaine, a drug known to inhibit uptake of NA. It is possible that the action of 5-HT was due to the uptake of the amine by adrenergic axon terminals as a 'false neurotransmitter'. Intraneuronal displacement and liberation of NA into the cytoplasm by a false transmitter can result in diminished synthesis of NA from tyrosine (Kopin, Weise & Sedvall, 1969). This mechanism would also decrease the availability of NA for release. If the 5-HT taken up by adrenergic axons could either not itself be released, or if it were released from axons but had no action post-synaptically, the accumulation of 5-HT by adrenergic terminals would reduce their effectiveness.

Preparations were incubated for 40 min with ^{3}H-NA or ^{3}H-5-HT in order to load adrenergic axons with radioactive amine. Release of all labelled substances was then followed. Langer (1968) has shown that for the calculation of the actual output of transmitter after stimulation of adrenergic nerves it is essential to include the sum of the metabolites and not to rely on the determination of ^{3}H-NA alone. Both labelled amines were taken up by the taenia and the rate of tritium release from the tissue was increased by perivascular nerve or electrical field stimulation. The release following perivascular nerve stimulation was abolished by tetrodotoxin and so was probably dependent on activation of neuronal elements. Thus 5-HT does seem to be taken up and can be released by perivascular axons. Since treatment with 6-hydroxydopamine prevents release of both labelled amines (and their metabolites) in response to perivascular nerve stimulation it seems likely that the release is primarily from adrenergic axons. It is interesting that reserpine, even after inhibition of MAO, also prevents tritium release in response to perivascular nerve stimulation from tissues incubated with either amine. Reserpine did not decrease amine uptake. The action of reserpine is primarily on amine storage vesicles (see Andén, 1968). In the presence of reserpine and an MAO inhibitor, therefore, most of the intraneuronal amine, ^{3}H-NA or ^{3}H-5-HT, was probably extravesicular (Van Orden, Schaefer, Burke & Lodoen, 1970). These observations would suggest that only amine stored in vesicles is available for release. The data also suggest that 5-HT must not only enter adrenergic terminals but also the pool of intravesicular material that can be readily released from the adrenergic terminal. This is not surprising since isolated NA storage granules have a strong affinity for 5-HT (Bertler, Rosengren & Rosengren, 1960; Euler & Lishajko, 1965). Ultrastructural studies have also suggested that exogenous 5-HT not only enters adrenergic axons, but the storage vesicles as well (Jaim-Etcheverry & Zieher, 1969, 1971; Zieher & Jaim-Etcheverry, 1971).

These studies indicate that 5-HT can influence motility of the taenia in several ways. The compound can activate excitatory and inhibitory neurones. It can also interfere with the adrenergic innervation. 5-HT appears to accomplish this by uptake into adrenergic axons as a 'false transmitter'. However, once taken up by adrenergic axons it appears to enter a releasable pool and is released on perivascular nerve stimulation. Since perivascular responses are diminished by the presence of 5-HT, the released 5-HT does not appear to reach a site which can respond to it. No responses characteristic of addition of exogenous 5-HT were seen when 5-HT had substituted for NA, as in those preparations incubated with 5-HT and α-MPT. It seems likely that 5-HT released from adrenergic axons

therefore does not reach 5-HT receptors on ganglia. Smooth muscle in the taenia does not seem to respond directly to 5-HT. Therefore it is possible that transmitters released from adrenergic axons in the taenia reach smooth muscle but not ganglia. If the true transmitter NA reaches only the same sites as the 'false transmitter', these observations would support the evidence presented previously (Gershon, 1967b) that endogenous NA acts directly on intestinal smooth muscle rather than on ganglionic transmission.

This direct action on smooth muscle has been associated with β-adrenoceptors (Kosterlitz *et al.*, 1970) or with both α- and β-adrenoceptors (Bailey, 1971).

The suggestion has also been made that NA might bring about relaxation of the smooth muscle indirectly by removing an excitatory cholinergic tone (Paton & Vizi, 1969; Kosterlitz *et al.*, 1970). This latter action has been associated with α-adrenoceptors. If so, the present experiments would indicate that such an action by endogenous NA is not effected by interference with ganglionic transmission. The ability of NA to block the release of acetylcholine by electrical field stimulation (Paton & Vizi, 1969; Kosterlitz *et al.*, 1970) also indicates that its action must be distal to the ganglion. These experiments also provide no support for the suggestion made by Weisenthal, Hug, Weisbrodt & Bass (1971) that field stimulation excites adrenergic neurones which terminate on smooth muscle while perivascular stimulation releases catecholamines which act on the excitatory ganglion cells of the myenteric plexus. Neither these experiments nor those of Weisenthal *et al.* (1971) provide evidence which bears on the further possibility of an axo-axonic relationship whereby adrenergic nerves may terminate on excitatory postganglionic endings.

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