B.r J. Pharmac. (1973), **49**, 258–267.

[^{3}H]-adenine nucleotide and [^{3}H]-noradrenaline release evoked by electrical field stimulation, perivascular nerve stimulation and nicotine from the taenia of the guinea-pig caecum

M. KUCHII, J. T. MIYAHARA AND S. SHIBATA

Department of Pharmacology, School of Medicine, University of Hawaii, Honolulu, Hawaii 96822, U.S.A.

Summary

1. The release of [^{3}H]-noradrenaline and adenine nucleotide evoked by electrical field stimulation (20–60 V, 30 Hz), perivascular nerve stimulation (20–80 V, 60 Hz) and nicotine (10, 100 μM) was studied in the taenia of the guinea-pig caecum under various conditions.

2. Electrical stimulation at high intensity (60 V) caused the release of [^{3}H]-adenine nucleotide; however, the inhibitory action of electrical stimulation was proportional to [^{3}H]-noradrenaline release.

3. The intensity of the inhibitory effect of stimulation of the perivascular nerves was directly related to [^{3}H]-noradrenaline release and not associated with the release of [^{3}H]-nucleotide.

4. Cold storage for more than 8 days, cooling (19° C) or tetrodotoxin treatment (1 μg/ml) abolished the inhibitory responses to electrical stimulation and to nicotine. After these treatments, nicotine and electrical stimulation elicited only contractions; the release of [^{3}H]-noradrenaline, but not that of [^{3}H]-adenine nucleotide, was inhibited.

5. The dissociation of the inhibitory effects of electrical stimulation and nicotine from [^{3}H]-nucleotide release does not support the hypothesis that ATP or a related nucleotide is the humoral transmitter of the non-adrenergic inhibition in the taenia of the guinea-pig caecum.

Introduction

The classical view maintains that the neurogenic inhibition of the intestinal smooth muscle results solely from the activation of postganglionic sympathetic neurones. However, recently Burnstock and his colleagues (see Burnstock, 1972) have postulated that the inhibitory control of the mammalian intestinal tract, although there are adrenergic inhibitory fibres present, consists mainly of neurones which are neither adrenergic nor cholinergic. Thus, in the taenia of the guinea-pig caecum, the perivascular pathway mediates the adrenergic inhibitory effects, whereas the responses to transmural stimulation and to application of ganglion-stimulating agents such as nicotine and dimethylphenylpiperazinium (DMPP) are mediated predominantly by intramural non-adrenergic neurones. Since the intestinal smooth muscle preferentially accumulates adenine nucleotide and the

relaxation in response to electrical transmural stimulation is associated with the release of adenine nucleotides, it was proposed that the non-adrenergic neurohumoral transmitter may be ATP or a closely related nucleotide (Burnstock, Campbell, Satchell & Smythe, 1970). Furthermore, Su, Bevan & Burnstock (1971) have shown that the isolated taenia of the caecum incorporates ^{3}H-adenosine from the bathing medium. However, Hattori, Kurahashi, Mori & Shibata (1972) recently demonstrated that in the cold-stored taenia of the guinea-pig caecum, the disappearance of the inhibitory action of nicotine, DMPP and transmural stimulation is most probably attributable to a reduction of releasable endogenous catecholamines caused by the degeneration of adrenergic nerves. Moreover, several investigators still attribute the inhibitory activity in the intestinal tract, at least that evoked by ganglionic stimulants, to the stimulation of adrenergic nerves (Gillespie & MacKenna, 1960; Weiss, 1962; Weisenthal, Hug, Weisbrodt & Bass, 1971).

The present study was undertaken to compare the inhibition evoked by transmural stimulation and by nicotine, paying particular attention to the release of [^{3}H]-noradrenaline and [^{3}H]-adenine nucleotide under various conditions. An essential aspect of the investigation concerned the effects of cold storage, cooling, tetrodotoxin and adrenergic blocking agents on the modification of the mechanical responses and of the release of the putative transmitters. A preliminary report of some of the results has been presented elsewhere (Miyahara, Kuchii & Shibata, 1972).

Methods

Adult guinea-pigs of either sex, weighing 400 to 600 g, were used. The animals were stunned by a blow on the head, exsanguinated, and the taenia dissected from the caecum. Strips of 0·3–0·5 cm resting length were used since such short preparations show little variation in mechanical activity. To examine the effects of drugs on the mechanical response, the strip was suspended vertically in a 50 ml organ bath by tying the lower end to a holder at the bottom of the chamber and connecting the upper end to a Grass strain-gauge force transducer (FT .03); the responses were recorded on a Grass model 7 polygraph. The bathing medium was Krebs bicarbonate buffer solution of the following composition (mM): NaCl, 120·3; KCl, 4·8; $CaCl_2$, 1·2; $MgSO_4$, 1·3; KH_2PO_4, 1·2; $NaHCO_3$, 24·2; glucose, 5·5, bubbled with a gas mixture of 95% O_2 and 5% CO_2 and maintained at 37° C; the pH was 7·4. In the drug tests, the taenia strips were exposed to nicotine for about 1–1·5 min with an exposure cycle of 30 minutes. After each application, the tissue was washed three times by changing the bathing medium. Antagonists were added to the bath fluid 20 min before testing the responses to nicotine or electrical transmural stimulation.

For electrical transmural stimulation, the taenia strip was suspended between two parallel platinum wire electrodes; the tissue was superfused with Krebs solution at a rate of 2·2 ml/min by a Harvard infusion pump. Electrical stimuli were rectangular pulses of 0·15 ms duration at 30 Hz for 30 s at variable voltage, from a Grass model 5 stimulator. The perivascular nerve was stimulated supramaximally for 10 s with pulses of 1·5 ms duration at 60 Hz. Intervals between stimulation were at least 5 min to ensure complete recovery of the tissue.

The experiments began after the taenia strips had been equilibrated in the tissue bath, 1 h for fresh preparations and at least 2 h for preparations which had been stored in the cold. For cold storage, the taenia strips were placed in 250 ml of Krebs solution at $2 \pm 0{\cdot}5°$ C for 5–10 days (Fukuda & Shibata, 1972).

For radio-isotopic studies a 2–3 cm taenia strip was equilibrated in the Krebs solution for 30 minutes. The strip was then transferred into the incubation medium containing (−)-[7-^{3}H]-noradrenaline (specific activity 6·6 Ci/mmol, Amersham/Searle Corp.) or [^{3}H]-adenosine (G) (specific activity 12·0 Ci/mmol, Amersham/Searle Corp.) at 0·1 μM for 1 hour. The incubation medium with [^{3}H]-noradrenaline also contained 0·1 mg/ml of ascorbic acid and 1·5 μg/ml of disodium edetate to prevent autooxidation of noradrenaline. The taenia was then rinsed three times with non-radioactive Krebs solution, cut into strips of about 1–1·5 cm and mounted in the organ bath for transmural stimulation. To determine the release of labelled compounds, the superfusion fluid flowing over the taenia strip was collected in vials containing 10 ml of Bray's scintillation solution (2,5-diphenyl-oxazole; PPO, 5 g; 1,4-bis [2-(4 methyl-5-phenyloxazolyl)] benzene; dimethyl POPOP, 0·15 g; naphthalene, 50 g; dioxane, 1 litre). The radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer and expressed as μCi g wet weight^{-1} min^{-1}. Three control samples were collected before and five samples during and immediately after transmural stimulation. For stimulation with nicotine, 0·2 ml of 1 mM solution was delivered in drop-form over a period of 30 s directly on the superfused taenia strip.

Results

Responses to electrical stimulation

Although the stimulus for threshold and maximum response varied in different taenia strips, transmural stimulation at 20 V and 30 Hz caused a response in the two preparations shown in Figure 1. This slight relaxation was accompanied by an outflow of [^{3}H]-noradrenaline from the strip loaded with labelled noradrenaline. On the other hand, the level of [^{3}H]-adenine nucleotide output from the strip loaded with labelled nucleotide was not greater than that during the period when the strip was not stimulated. Similar results were observed in four taenia strips. When the intensity of the stimulus was increased to 40 V, the relaxation and release of [^{3}H]-noradrenaline were quite marked; the release of [^{3}H]-noradrenaline continued throughout the period of relaxation. Stimulation of the strip loaded with [^{3}H]-adenine nucleotide produced a similar relaxation; however, the release of labelled nucleotide was still only barely greater than the background level. Only a maximal stimulation of the taenia strip at 60 V caused a significant release of [^{3}H]-adenine nucleotide; [^{3}H]-noradrenaline release was not greater than at 40 V although the relaxation was increased at 60 V.

When the perivascular nerves were stimulated, the relaxation of the taenia strip was associated with a proportionate increase in the release of [^{3}H]-noradrenaline (Figure 2). On the other hand, the release of [^{3}H]-adenine nucleotide was not consistent and not easily detectable; however, a trace of [^{3}H]-adenine nucleotide appeared in the superfusion fluid when the perivascular nerves were stimulated supramaximally. Even with a maximal relaxation, the release of [^{3}H]-adenine nucleotide after perivascular nerve stimulation was never as great as after transmural stimulation.

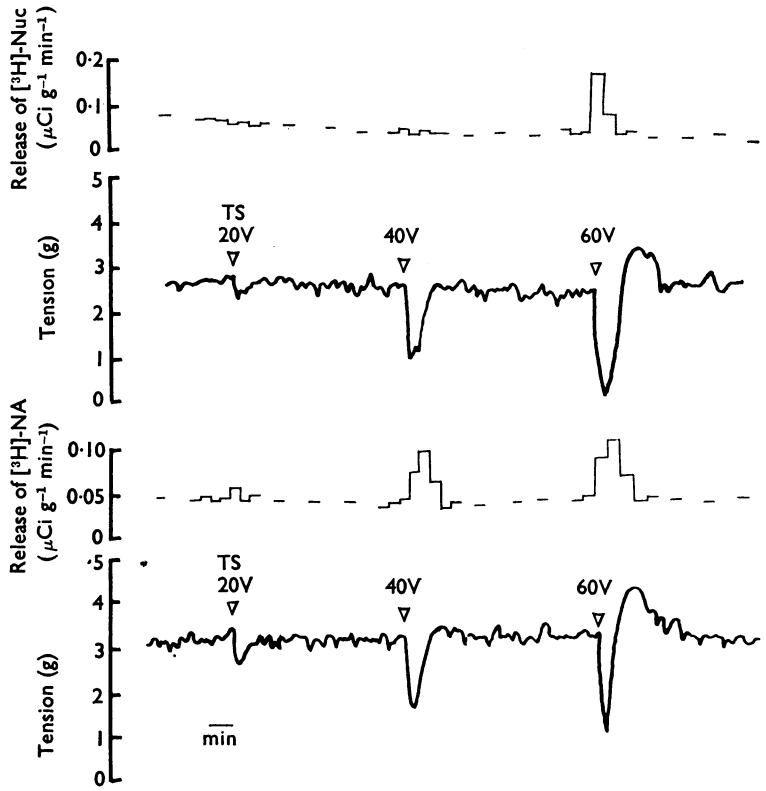

FIG. 1. Release of ^{3}[H]-adenine nucleotide ([^{3}H]-Nuc) and [^{3}H]-noradrenaline ([^{3}H]-NA) from the taenia of the guinea-pig caecum during the inhibitory response to electrical field stimulation (TS 0·15 ms, 30 Hz, 30 s) at 20–60 Volts. The upper record shows tritium release in superfused fluid; the lower shows mechanical activity. Tissues were first incubated with [^{3}H]-adenosine or [^{3}H]-noradrenaline for 1 hour.

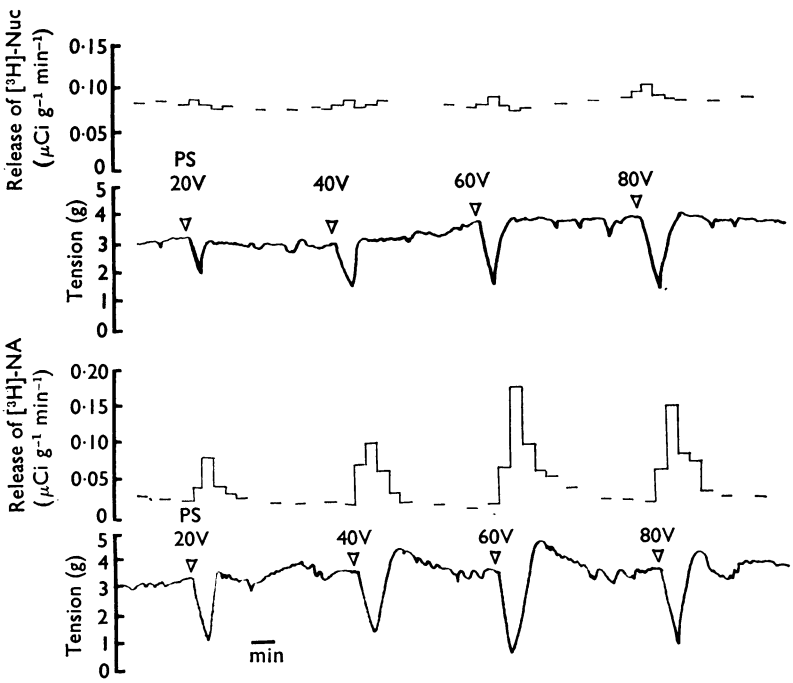

FIG. 2. Release of [^{3}H]-adenine nucleotide ([^{3}H]-Nuc) and [^{3}H]-noradrenaline ([^{3}H]-NA) from taenia of guinea-pig caecum during the inhibitory response to perivascular nerve stimulation (PS, 1·5 ms, 60 Hz, 10 s) at 20–80 Volts. The upper record shows tritium release in superfused fluid; the lower shows mechanical activity. Tissues were incubated first with [^{3}H]-adenosine or [^{3}H]-noradrenaline for 1 hour.

Effect of cold storage

In two fresh preparations, maximal electrical stimulation (60 V, 30 Hz for 30 s) produced a comparable relaxation of the taenia strips, associated with a release of [^{3}H]-adenine nucleotides and [^{3}H]-noradrenaline (Figure 3). When these two taeniae preparations were tested after cold storage at 2° C for 8 days, electrical stimulation resulted in a small relaxation, followed by contraction. There was no longer a release of [^{3}H]-noradrenaline, whereas the efflux of the tritiated nucleotide was not diminished. At this intensity and frequency of stimulation, the rebound excitatory effect curtailed part of the inhibitory response, as application of atropine unmasked a greater relaxation.

Nicotine solution (1 mM, 0·2 ml) applied as drops to give a final concentration of 0·1 mM enhanced the release of the tritiated nucleotide and of [^{3}H]-noradrenaline in the fresh taenia (Figure 4). The release of [^{3}H]-noradrenaline was similar to that found after the electrical stimulation; nicotine caused a greater release

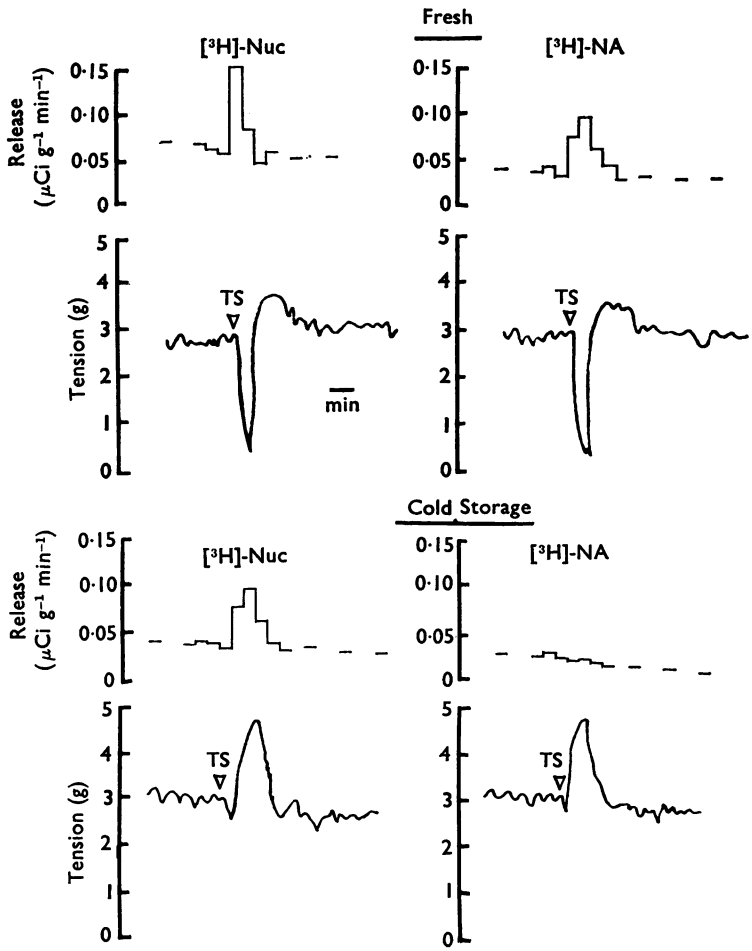

FIG. 3. Effect of cold storage for 8 days on the release of [^{3}H]-adenine nucleotide ([^{3}H]-Nuc) and [^{3}H]-noradrenaline ([^{3}H]-NA) and tension changes in guinea-pig taenia caecum after electrical field stimulation (TS 0·15 ms, 30 Hz, 30 s, 60 Volts).

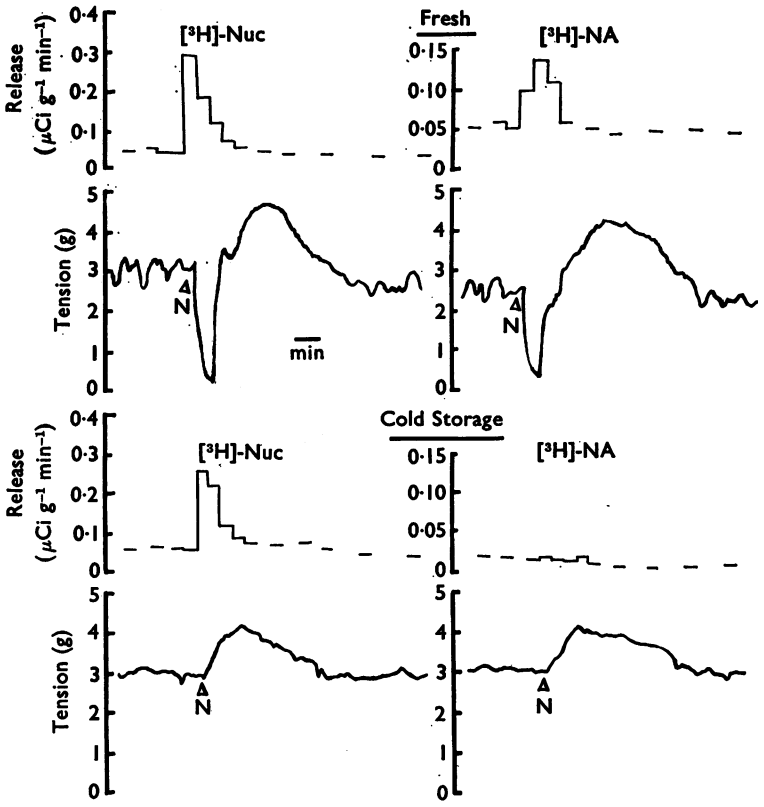

FIG. 4. Effect of cold storage for 8 days on the release of [³H]-adenine nucleotide ([³H]-Nuc) and [³H]-noradrenaline ([³H])-NA) and tension changes in guinea-pig taenia caecum after application of nicotine (N, 0·1 mM). Nicotine solution (1 mM, 0·2 ml) was applied as drops to give a final concentration of 0·1 mM.

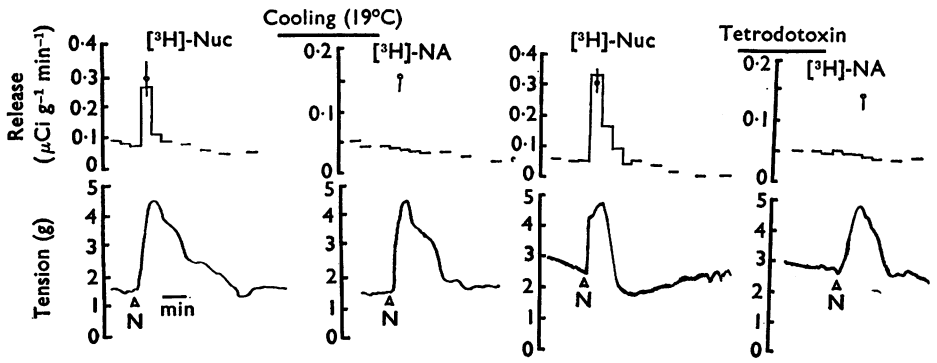

FIG. 5. Effect of cooling (19° C) and tetrodotoxin (1 μg/ml) on the release of [³H]-adenine nucleotide ([³H]-Nuc) and [³H]-noradrenaline ([³H]-NA) and tension changes in guinea-pig taenia caecum after application of nicotine (N, 0·1 mM). Open circles with vertical lines indicate the mean values ± S.E.M. (n=5) of the release of [³H]-nucleotide and [³H]-noradrenaline by nicotine from tissues before cooling or treatment with tetrodotoxin.

of the [^{3}H]-adenine nucleotide. After cold storage, nucleotide release was scarcely diminished but [^{3}H]-noradrenaline was barely detectable in the superfusate. In stored taenia the response to nicotine was purely excitatory or biphasic with a small initial relaxation.

Effect of tetrodotoxin and cooling

After 20 min treatment with tetrodotoxin (1 μg/ml) or after lowering the bath temperature to 19° C, nicotine (0·1 and 1 mM) did not relax but contracted the taenia. The contraction was associated with a release of [^{3}H]-adenine nucleotide but no [^{3}H]-noradrenaline was detectable in the perfusate (Figure 5).

Similarly, the inhibitory effect of transmural stimulation in taenia was blocked by tetrodotoxin or by cooling. In these strips there was a release of [^{3}H]-adenine nucleotide but no [^{3}H]-noradrenaline was detected (Figure 6). The relaxation produced by nicotine (0·1 mM) and transmural stimulation before treatments were $2{\cdot}8 \pm 0{\cdot}6$ g ($n=5$) and $2{\cdot}4 \pm 0{\cdot}4$ g ($n=5$), respectively.

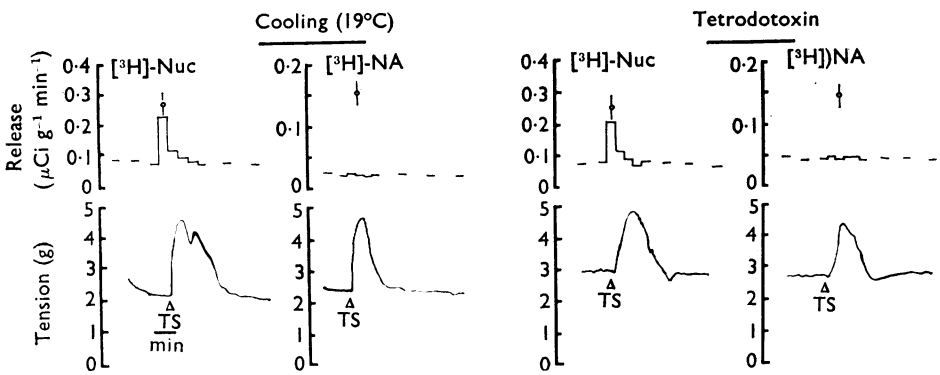

FIG. 6. Effect of cooling (19° C) and tetrodotoxin (1 μg/ml) on the release of [^{3}H]-adenine nucleotide ([^{3}H]-Nuc) and [^{3}H]-noradrenaline ([^{3}H]-NA) and tension changes in the guinea-pig taenia caecum after application of electrical field stimulation (TS, 0·15 ms, 30 Hz, 30 s, 60 Volts). Open circles with vertical lines indicate the mean values ±S.E.M. ($n=5$) of the release of [^{3}H]-nucleotide and [^{3}H]-noradrenaline by transmural stimulation from tissues before the cooling or treatment with tetrodotoxin.

Discussion

Freshly dissected taenia from the caecum of the guinea-pig responds with relaxation followed by rebound contraction to nicotine or electrical field stimulation. The responses elicited by these stimuli are similar in time course and configuration and are distinct from those produced by perivascular nerve stimulation which causes long lasting relaxation with less after-contraction.

Histochemical and pharmacological investigations (Shibata, Hattori & Timmerman, 1970; Hattori *et al.*, 1972) showed that prolonged cold storage, which causes physical degeneration of the adrenergic nerves, inhibits the relaxation induced by nicotine and electrical field stimulation. Therefore, the inhibitory effects of both types of stimulation may be due mainly to the response of the adrenergic neurones. Fukuda & Shibata (1972) showed that, after cold storage of the taenia for more than 7 days, nicotine fails to cause membrane hyperpolariza-

tion, whereas activation of the adrenoceptors by catecholamines can still hyperpolarize the muscle membrane.

Weisenthal *et al.* (1971) considered the possibility that the inhibitory effect of nicotine and of electrical field stimulation on the taenia of the caecum may be due to catecholamines released by stimulation of the intrinsic nerves. On the other hand, Burnstock, Campbell & Rand (1966) proposed that nicotine and transmural stimulation activate non-adrenergic intramural neurones which release a specific transmitter substance other than noradrenaline. Since muscle relaxation in the taenia (Su *et al.*, 1971) and other intestinal tissue (Burnstock *et al.*, 1970) is associated with efflux of adenine nucleotide, it is thought that the transmitter of the non-adrenergic neurones may be ATP or a related nucleotide.

The results from previous experiments indicate that treatment with adrenoceptor blocking agents fails to abolish the inhibitory effect of nicotine and electrical field stimulation in the taenia of the caecum (Hattori *et al.*, 1972). In the present experiments, nicotine and electrical stimulation still cause relaxation of the taenia in the presence of atropine even after prolonged cold storage, but the inhibitory responses to both stimuli are much smaller than in the untreated preparation. These results lead us to speculate that the remaining inhibition may be evoked by a non-adrenergic mechanism but the results obtained with release of [^{3}H]-nucleotide do not support the view that the neurotransmitter is an adenine nucleotide. The relaxation elicited by electrical stimulation at high voltage is associated with the release of [^{3}H]-adenine nucleotide, whereas the inhibitory response to electrical stimulation at low voltage is not accompanied by an increase of [^{3}H]-adenine nucleotide above the resting level. On the other hand, the release of [^{3}H]-noradrenaline by transmural stimulation is proportional to the degree of relaxation. A similar relationship between [^{3}H]-noradrenaline release and relaxation was observed in the inhibitory response to perivascular nerve stimulation, which was accompanied by only a negligible [^{3}H]-nucleotide release.

The effects of cold storage, cooling by lowering of the bath temperature and tetrodotoxin on relaxation and on the release of adenine nucleotide and noradrenaline do not support the hypothesis that an adenine nucleotide is the non-adrenergic inhibitory transmitter. The association of nucleotide release with a contractile response is also not in accord with this hypothesis. The good correlation between the inhibitory effect of electrical stimulation and the release of [^{3}H]-noradrenaline is more in favour of noradrenaline being the sole inhibitory transmitter. Even after 15 days of cold storage, uptake and release of [^{3}H]-adenosine by the taenia of the guinea-pig caecum were not blocked, whereas uptake and release of [^{3}H]-noradrenaline were inhibited (Kuchii, Miyahara & Shibata, unpublished results). Since such prolonged cold storage probably causes the degeneration of all neuronal tissues, the site of uptake and release of the [^{3}H]-nucleotide must be extraneuronal. The fact that tetrodotoxin, lowering of the bath temperature, and prolonged cold storage had no effect on the release of adenine nucleotide also supports the hypothesis that the released [^{3}H]-nucleotide may be of extraneuronal origin. Although attempts were made to separate and identify the nucleotides released into the perfusate after stimulation, the results were inconclusive, because of the low radioactivity of the different fractions and, possibly, the chemical instability of the released nucleotide. However, since the predominant product of biotransformation of [^{3}H]-adenosine in the taenia is ATP

(Su *et al.*, 1971; Kuchii, Miyahara & Shibata, unpublished data), it is assumed that ATP makes up the major part of the radioactive material released from [^{3}H]-adenosine treated tissues.

Reprint requests to Dr. S. Shibata.

REFERENCES

BURNSTOCK, G. (1972). Purinergic nerve. *Pharmac. Rev.*, **24**, 509–581.

BURNSTOCK, G., CAMPBELL, G. & RAND, M. J. (1966). The inhibitory innervation of the taenia of the guinea-pig caecum. *J. Physiol., Lond.*, **182**, 504–526.

BURNSTOCK, G., CAMPBELL, G., SATCHELL, D. G. & SMYTHE, A. (1970). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerve in the gut. *Br. J. Pharmac.*, **40**, 668–688.

FUKUDA, H. & SHIBATA, S. (1972). The effect of cold storage on the inhibitory action of isoprenaline, phenylephrine and nicotine on the mechanical and membranal activities of guinea-pig taenia caecum. *Br. J. Pharmac.*, **46**, 438–448.

GILLESPIE, J. S. & MACKENNA, B. R. (1960). The inhibitory action of nicotine on the rabbit colon. *J. Physiol. Lond.*, **152**, 191–205.

HATTORI, K., KURAHASHI, K., MORI, J. & SHIBATA, S. (1972). The effect of cold storage on the adrenergic mechanisms of intestinal smooth muscle. *Br. J. Pharmac.*, **46**, 423–437.

MIYAHARA, J. T., KUCHII, M. & SHIBATA, S. (1972). ^{3}H-ATP-^{3}H-NE release inhibition in guinea-pig taenia caecum. Absts. Fifth Int. Congr. Pharmac., p. 160.

SHIBATA, S., HATTORI, K. & TIMMERMAN, D. (1970). The effect of cold storage on the response of guinea-pig taenia coli to certain catecholamines and other agents. *Eur. J. Pharmac.*, **11**, 321–331.

SU, C., BEVAN, J. A. & BURNSTOCK, G. (1971). ^{3}H-adenosine triphosphate: release during stimulation of enteric nerves. *Science, N.Y.*, **173**, 337–339.

WEISS, J. (1962). Biphasic responses of guinea-pig taenia coli induced by 1-1-dimethyl-4-phenyl-piperazinium (DMPP). *Acta pharmac. tox.*, **19**, 121–128.

WEISENTHAL, L. M., HUG, JR., C. C., WEISBRODT, N. W. & BASS, P. I. (1971). Adrenergic mechanisms in the relaxation of guinea-pig taenia coli *in vitro*. *J. Pharmac. exp. Ther.*, **178**, 497–508.

(*Received January* 23, 1973)