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# The effect of cold storage on the adrenergic mechanisms of intestinal smooth muscle

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**Summary**

1. In the guinea-pig taenia caecum, fluorescent adrenergic fibres terminate in both muscle layers. The density of these fibres is greater in the taenia than in the underlying circular muscle layer. The myenteric plexus and individual ganglion cells are also densely innervated by intensely fluorescent adrenergic nerve fibres.

2. After three days of cold storage, the specific fluorescence disappeared from all tissue layers of the taenia caecum and smooth muscle fibres. In contrast, cholinesterase active substances were still demonstrable in all tissue layers even after seven days of cold storage but the density of these substances was decreased.

3. Cold storage (3–7 days) decreased the tissue noradrenaline content and did not modify the cholinesterase enzyme activity (4 days).

4. In cold stored strips, the inhibitory response to nicotine, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) or electrical transmural stimulation was abolished and enhancement of the contractile response occurred. Cold storage also inhibited the inhibitory action of tyramine. Similar results were observed after reserpine treatment.

5. In fresh taenia, the relaxation produced by nicotine, DMPP and electrical transmural stimulation was inhibited by adrenoceptor blocking agents and bretylium. In cold storage preparations, contraction produced by these stimuli was blocked by parasympathetic blocking agents and potentiated by anti-cholinesterase. These results indicate that the inhibitory response to these stimulants is mediated by stimulation of the adrenergic nerve system more than by non-adrenergic nerves ; the excitatory effect is probably due to stimulation of cholinergic nerves.

6. These results suggest that the adrenergic mechanisms of the taenia caecum are more labile in cold storage than the cholinergic mechanisms. Thus, the inhibitory action of cold storage on the relaxation produced by nicotine, DMPP, and transmural stimulation is probably explained by selective physical degeneration of the adrenergic nerve terminal. Also, enhancement of the contractile response to these stimulants in cold stored preparations is explained by the lack of adrenergic inhibitory mechanisms.

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## Introduction

Previously, Shibata (1969) reported that prolonged cold storage (2° C) potentiated the response of vascular smooth muscle (rabbit aortic strip) to noradrenaline and adrenaline but inhibited the response to tyramine and nicotine. Prolonged cold storage did not affect sensitivity of taenia from guinea-pig caecum to isoprenaline, adrenaline, noradrenaline and phenylephrine (Shibata, Hattori & Timmerman, 1970). Nicotine, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), and electrical transmural stimulation are believed to affect smooth muscle by an indirect action involving stimulation of nerves. While investigating the relationship between cold storage and the mechanical activity of the guinea-pig taenia caecum, it was noticed that the response of the strip to nicotine, DMPP, and electrical transmural stimulation was reversed after prolonged cold storage.

Because this phenomenon might help to elucidate the mechanisms and sites of action of such stimuli as well as the mechanism of the effect itself, modification of the responses to nicotine, DMPP, and tyramine after prolonged cold storage was studied.

## Methods

Male guinea-pigs, weighing between 500–700 g were used. Reserpine-treated strips were obtained from guinea-pigs that had been injected intramuscularly with reserpine, 4 mg/kg (Serpasil, CIBA), 24 h before killing. The animals were stunned and bled to death. The whole anterior and posterior taeniae were then dissected from the caeca.

### *Histochemical studies*

*Noradrenaline.* The histochemical technique of Falck (1962) was used to demonstrate the presence of noradrenaline. The guinea-pig taeniae caeca were frozen in isopentane, cooled in liquid nitrogen and freeze-dried at $-35°$ C at $1 \times 10^{-3}$ mmHg for one week. Preparations were treated with paraformaldehyde gas at 80° C for 1 h, then infiltrated *in vacuo* with paraffin at 60° C for 10 minutes. Sections of 4 $\mu$ were cut, placed on nonfluorescent microscope slides, deparaffinized by careful addition of xylene and mounted in Entellan (Merck).

The slides were examined in a Zeiss fluorescence microscope with an Osram high pressure mercury lamp (HB0200) as a light source. A Schott-BG-12 was used as an excitation filter and a Schott 0G4 was used as a barrier filter. Micrographs were taken on Kodak colour film with an exposure time of 5 minutes. The term 'noradrenaline fluorescence' as used here refers to a specific green fluorescence. To distinguish between noradrenaline fluorescence and nonspecific autofluorescence, preparations were treated with sodium borohydride (Corrodi, Hillarp & Jonsson, 1964).

*Acetylcholinesterase.* Acetylcholinesterase was stained by the thiocholine method of Jacobowitz (1965). Tissues were removed during anaesthesia and frozen with solid $CO_2$. Sections of 15 $\mu$ were cut with a cryostat at $-15°$ C and placed on slides. The slides were then placed in the appropriate preincubation solutions for 30 min at 37° C, following which they were incubated for 1 h at 37° C. Butyrylcholinesterase was selectively inhibited by preincubation with 15–20 nM di-isopropylfluorophosphate (DFP). Some slides were counter-stained with eosin.

### *Catecholamine chemical assay*

*Noradrenaline.* Quantitative determination of the noradrenaline content was carried out by the trihydroxyindole method. The taeniae caeca were blotted with filter paper, weighed, minced and homogenized in ice cold 0·4 N perchloric acid. The homogenate was centrifuged at 10,000 *g* for 10 minutes. The supernatant fluid was adjusted to pH 4·0 with NaOH. The noradrenaline in the extract was absorbed onto 200–400 mesh, Dowex 50 × 4 resin in a 6 mm × 6 mm column (Bertler, Carlsson & Rosengren, 1958) and then eluted with 8 ml of 1 N HCl. The trihydroxy reaction was performed with ferricyanide as described by von Euler & Floding (1958). An Aminco-Bowman spectrophotometer was used to measure fluorescence at the activation and fluorescence wavelength for noradrenaline (396–517 mμ). Recoveries in duplicate of 1 μg noradrenaline were carried through in each experiment and averaged 85–90% ; no correction was made for these. In each experiment, the taeniae caeca from two guinea-pigs were pooled and the tissue noradrenaline content was measured.

*Cholinesterase activity.* The cholinesterase activity of the tissue was measured with the electrometric method (Michel, 1949). The tissue was homogenized in five times its volume of 0·9% saline solution in a glass grinder, which had been placed in an ice bath. To lyse the cells, 0·04 ml of the homogenate was diluted with distilled, deionized water to a total volume of 2 ml. Acetylcholine substrate (0·4 ml acetylcholine chloride, 0·11 M) and 20 ml buffer (0·02 M sodium barbitone, 0·004 M $KH_2PO_4$, 0·6 KCl ; adjusted to pH 8·1 by 0·1 N HCl) were added to the homogenate and incubated for 20 min at 25° C. The enzyme reaction was allowed to proceed for 1 h, and then the final pH of the solution ($pH_2$) was determined.

### *Mechanical activity*

When placed in the Ringer solution at room temperature (about 24° C), the taeniae caeca of the guinea-pigs shortened to approximately one half of their original length *in situ*. Unstretched segments of a length of about 0·5 cm were cut. Ligatures were fastened to both ends of the muscle segments, one to be attached to a glass rod (holding device) and the other to a force displacement transducer. The preparation was suspended in an organ bath containing 15 ml of Ringer solution of the following composition (mM): NaCl, 154 ; KCl, 5·4 ; $CaCl_2$, 2·4 ; $NaHCO_3$, 6 ; dextrose, 11 ; in distilled, deionized water. The bath fluid was maintained at 37° C and equilibrated before and during the experiment with a gas mixture of 95% $O_2$ and 5% $CO_2$ (pH 7·4) in the tissue baths.

Whilst in the refrigerator, the preparations were kept in Ringer medium without an exogenous oxygen supply at 2° C ± 0·1° C for one to seven days. Upon removal from the refrigerator, the preparations were immediately supplied with oxygen and allowed to warm for 30 min to room temperature. The strips were then transferred to warmed Ringer solution (37° C) and equilibrated for 3 hours.

After this period, the tension of the preparation was adjusted to 1·5 g basic tension 30 min prior to beginning the experiment. This basic tension level was maintained throughout the experiment. Tension changes were recorded with a strain gauge (Grass, FT.03) transducer on a six channel Grass polygraph. With this equipment it was possible to examine simultaneously six preparations in separate tissue baths.

The test agents were added to the bath and allowed to act for 5 min before terminating the response by consecutively washing each preparation three times with Ringer solution (37° C). The volume of each single addition of an agent to the organ bath was 0·15 ml. The inhibitory and excitatory responses of the smooth muscle strips to test agents were measured in terms of the reduction or increase in the tone produced by the respective agents.

### *Transmural stimulation*

Transmural stimulation was applied to the tissue preparation as described by Su & Bevan (1970). The tension changes were recorded on a Grass polygraph.

The taenia caecum tissue (1 cm in length) was mounted vertically in a moisture bath between a stationary, supporting glass rod and the transducer. The strip was placed between a pair of parallel (2 mm apart) platinum wire electrodes (0·5 mm in diameter). Gaps between the electrodes and the tissue were wide enough to allow unimpeded smooth muscle contraction and yet sufficiently narrow to be filled consistently by part of the superfusate retained by capillary action, thus insuring continual electrical conductivity. The Ringer solution, previously warmed and equilibrated with the $O_2$-$CO_2$ gas mixture, was allowed to drip onto the strip at a constant rate of 0·382 ml/minute. At the appropriate times, the various agents were added to the drip solution. A total of 50 ml of each drug solution (10 $\mu$M) was allowed to drip onto the preparation.

The nerve terminals in the smooth muscle of taenia caecum were excited by a train of biphasic rectangular pulses from a Grass SD5 stimulator. The pulses were passed through the electrodes at 30 Hz for 30 s, each pulse being 0·2 ms in duration and 40–50 V in strength. The relatively short pulse duration ensured selective excitation of the neurones but not the muscular elements and this was confirmed by the observation that tetrodotoxin (500 ng/ml)) and hexamethonium (1 $\mu$M), or bretylium (1 $\mu$M), blocked such stimulation. The voltage was slightly in excess of that necessary for maximal response to nervous excitation.

The following drugs were used: atropine sulphate, nicotine (base), 1,1-dimethyl-4-phenylpiperizinium iodide (DMPP), tyramine monohydrochloride, hexamethonium bromide, bretylium tosylate, hemicholinium (HC-3), reserpine (Serpasil, CIBA), phentolamine mesylate, (−)-propranolol hydrochloride, phenoxybenzamine hydrochloride, physostigmine sulphate. All agents were prepared from concentrated stock solutions (refrigerated at 2° C) immediately before use with distilled, deionized water. The concentrations of these agents are expressed as the final concentration in the tissue bath.

## Results

### *Histochemical study*

Figure 1a, a cross section of the guinea-pig taenia caecum, shows specific catecholamine fluorescence. Numerous nerve structures with intense fluorescence are visible in the taenia (T), while fewer such nerve structures are present in the circular muscle layer (C). Auerbach's plexus (A) also exhibited an intensive specific catecholamine fluorescence. Specific catecholamine fluorescence containing fibres were the characteristic synaptic varicosities in the myenteric plexus.

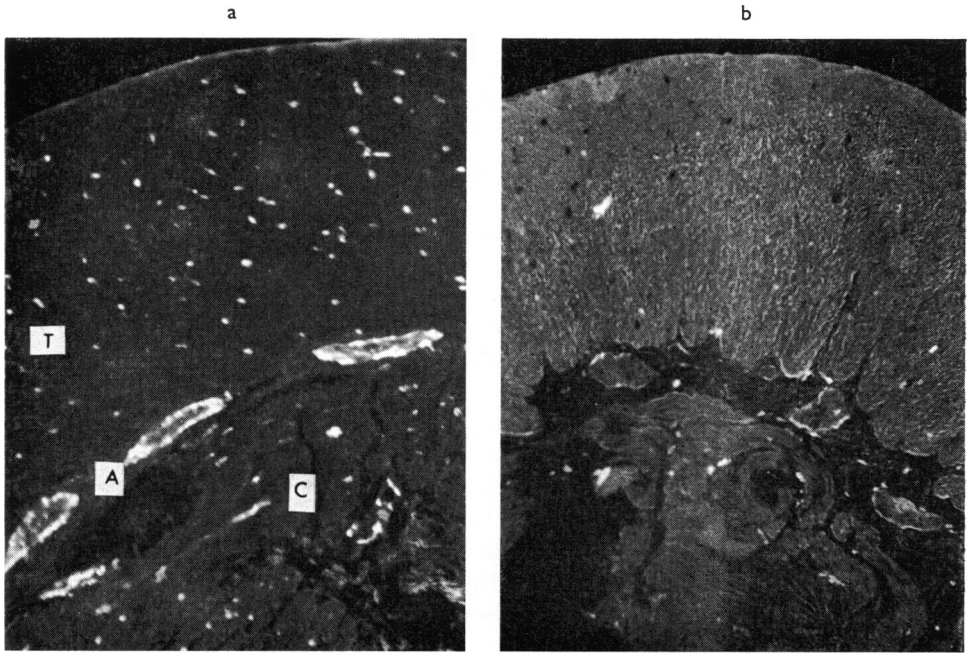


FIG. 1. (a) Fresh guinea-pig taenia caecum: catecholamine fluorescence in the taenia (T) and circular (C) muscle layers, and Auerbach's plexus (A). The dense distribution of catecholamine fluorescence is seen in the taenia and is also present around the Auerbach's plexus (cross section × 50). (b) Cold stored taenia caecum: after five days of cold storage, the catecholamine fluorescence is not seen in any of the tissue layers (cross section × 50).

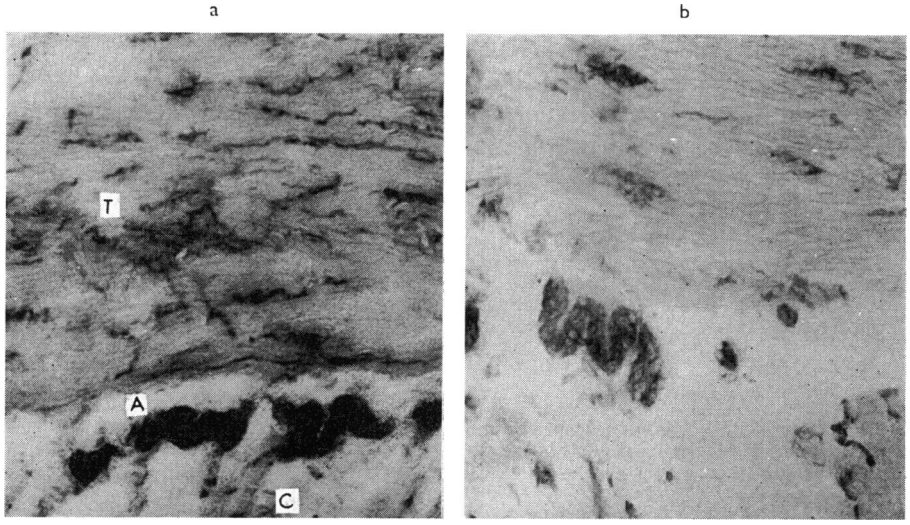


FIG. 2. (a) Fresh guinea-pig taenia caecum—acetylcholinesterase staining: heavy staining in Auerbach's plexus (A), moderate staining in the longitudinal muscle layer (T), and light staining in the circular muscle layer (C) (longitudinal section × 100). (b) Cold stored guinea-pig taenia caecum—acetylcholinesterase staining: after seven days of cold storage, low intensity staining is seen in all tissue layers (longitudinal section × 100).

After one day of cold storage, the fluorescent substances in taenia and in Auerbach's plexus were decreased but still demonstrable. After 5 days of cold storage, the specific fluorescence was difficult to demonstrate in any of the tissue layers (Fig. 1b) After reserpine treatment, the specific fluorescence had also disappeared from the taenia, circular muscle layer and Auerbach's plexus.

Figure 2 (a & b) indicates the effects of prolonged cold storage on the cholinesterase positive substances of taenia, the circular smooth muscle layer, and Auerbach's plexus. In the fresh preparation (Fig. 2a), the specific brown colour, which indicates a cholinesterase positive fibre, was observed in all tissue layers, but was especially dense in distribution in the taenia and in the Auerbach's plexus. The smooth muscle cells of the taenia also showed some enzyme activity. Even after seven days of cold storage, this cholinesterase reaction was still demonstrable in all tissue layers but the density of cholinesterase staining colour was much less than that of fresh preparations (Fig. 2b). Most of the ganglion cells around Auerbach's plexus were located between the taenia and circular muscle layers. The histopathological changes in Auerbach's plexus after cold storage were also studied. In each experiment, five taeniae from different guinea-pigs were used. The preparations were fixed in 10% neutral formalin, embedded in paraffin, and stained with haematoxylin and eosin. They were then cut into longitudinal and cross sections (4 $\mu$) with a rotary microtome and histopathological changes were examined under a light microscope. After 2–3 days of cold storage, the ganglion cells were characterized by marked enlargement and vacuolization and the nuclei were either crowded or had disappeared. Thus, the histopathological haematoxylin studies indicated that prolonged cold storage causes chromatolysis of ganglion cells.

### *Catecholamine content and cholinesterase activity*

Table 1 indicates the effects of prolonged cold storage or reserpine treatment on the noradrenaline content of the taenia caecum tissue. After three days of cold storage, the tissue noradrenaline content was decreased to 58% of the control. After five to seven days of cold storage, the tissue noradrenaline content was reduced to 38%. Reserpine treatment reduced the noradrenaline content to 18% of that of the fresh, untreated preparation. On the other hand, even after four days of cold storage, the cholinesterase activity of taenia caecum was not affected (Table 2).

TABLE 1. *Tissue noradrenaline content of fresh, cold stored and reserpine treated taenia*

| Noradrenaline (mean±S.E.M.) $\mu$g/g | | | |
|---|---|---|---|
| Fresh | Reserpine | 3 days cold storage | 5–7 days cold storage |
| 0·48±0·08 (7) | 0·09±0·01* (7) | 0·28±0·02* (7) | 0·18±0·02 (7) |

* $P$ value is significantly different from the mean value of the control (fresh) ($t$ test, $P<0{\cdot}001$). The number in parentheses indicates the number of experiments. Each experiment utilized three guinea-pig taeniae caeca.

TABLE 2. *Cholinesterase activity of fresh and cold stored taenia*

| Cholinesterase activity △pH (mean±S.E.M.) | |
|---|---|
| Fresh | 4 days cold storage |
| 0·18±0·008 (12) | 0·19±0·006 (12) |

The number in parentheses indicates the number of experiments. Each experiment utilized three guinea-pig taeniae caeca.

*Spontaneous mechanical activity*

After 60 min incubation in Ringer solution at 37° C, the intrinsic, rhythmic activity and increased tension of fresh and cold stored (2–7 days) preparations was restored and reached a maximum level in 15 to 30 minutes. At the end of the 3 h equilibration period, the intrinsic activity of both types of preparation became very small or ceased entirely. The intrinsic, rhythmic activity of the cold stored preparations was usually less than that of fresh ones. Previous workers have reported that the tone and spontaneous activity of the taenia caecum tissue varies considerably from one preparation to another (Weiss, 1962; Burnstock, Campbell & Rand, 1966). However, in the present experiment, no marked variations were observed in intrinsic tension, presumably because of the short preparations used.

The sensitivity of the taenia caecum tissue to noradrenaline did not change and consistent responses were obtained throughout the 8 h experiments.

*Response to nicotine.* The effect of ganglion stimulants such as nicotine and DMPP on the isolated taenia caecum of the guinea-pig varies with the tone of the preparations; accordingly, contraction, relaxation or a complex response consisting of both of these effects have been obtained with these substances (Akubue, 1966; Weiss, 1962; Burnstock *et al.*, 1966). Under the conditions of the present experiment, the fresh taeniae caeca showed two different types of response to nicotine (10 $\mu$M and 100 $\mu$M): relaxation followed by a small contraction and relaxation alone (Fig. 3A). Both types of response appeared in an equal ratio. Nicotine at 1 $\mu$M caused only relaxation in all preparations (10 experiments).

After two days of cold storage, nicotine (10 $\mu$M and 100 $\mu$M) caused a small relaxation followed by a large contraction of the taenia caecum (Fig. 3B). After

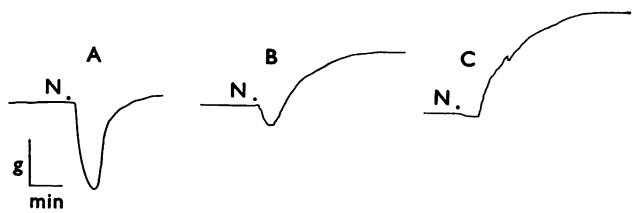


FIG. 3. The effect of nicotine on the guinea-pig taenia caecum. Fresh preparation (A), 2 days cold storage (B), and 4 days cold storage (C). The black dots indicate the point of administration of 100 $\mu$M nicotine (N).

four days of cold storage, nicotine produced only a large contraction (Fig. 3C). The amplitude of this contraction was one-third to two times higher than that of strips which had been stored for only two days. In fresh taenia caecum, the contractile phase of the response to nicotine was inhibited by pretreatment with atropine (100 nM) (Fig. 4 (1) and (2)). After two days of cold storage, in eight out of twelve strips, atropine pretreatment (100 nM) for 20 min caused a relaxation following nicotine (100 $\mu$M) administration. Application of this procedure to strips which had been stored for three days yielded similar results but of much lower magnitude (Fig. 4 (3) and (4)). After seven days of cold storage, atropine pretreatment did not cause any relaxation.

Relaxation of fresh taeniae caeca to nicotine (100 $\mu$M) was reduced by 75–87%

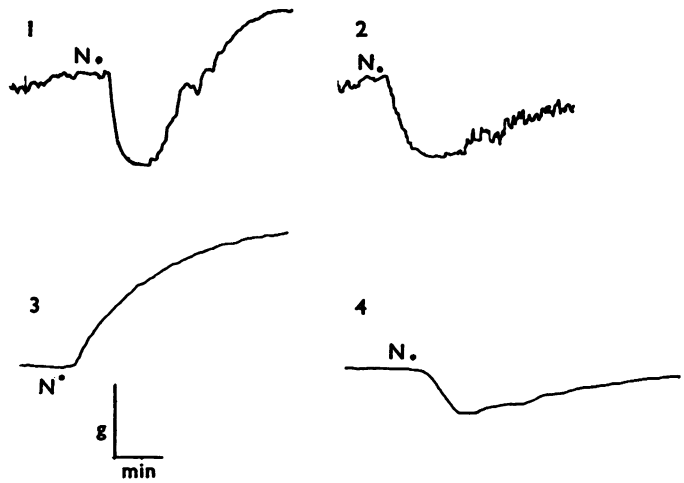


FIG. 4. The nicotine response of the guinea-pig taenia caecum in the presence of 100 nM atropine. 1 and 2 indicate the response of the fresh preparation before and after atropine treatment respectively. 3 and 4 indicate the response of cold stored strips for 3 days before and after treatment with atropine, respectively. Black dots indicate the administration of 100 $\mu$M nicotine (N).

following combined treatment with propranolol (10 $\mu$M, a $\beta$-adrenoceptor antagonist) and phentolamine (10 $\mu$M, $\alpha$-adrenoceptor antagonist) for 20 min; the contractile component of the response was unaffected by nicotine (100 $\mu$M) or slightly increased. Ten experiments were run for each drug. After pretreatment with bretylium (10 $\mu$M), which probably inhibits the release of noradrenaline from the nerve ending, the relaxation produced by nicotine (100 $\mu$M) was decreased by 40–50% (10 experiments) but the inhibitory action of bretylium was less effective than that of the adrenoceptor antagonists. Bretylium treatment did not affect the contractile response to nicotine.

In fresh preparations both the relaxant and contractile responses to nicotine (100 $\mu$M) were abolished after 20 min treatment with hemicholinium (10 $\mu$M). Similar treatment of preparations which had been cold stored for four days decreased the contractile response to nicotine (100 $\mu$M) by 80% (10 experiments).

*Response to DMPP.* In fresh taeniae, DMPP (10 and 100 $\mu$M), like nicotine, caused a relaxation followed by a small contraction, whereas after prolonged cold storage, the relaxation produced by DMPP (100 $\mu$M) was not evident and a contractile response only was observed (Fig. 5 (5) and (6)). In fresh preparations, 20 min pretreatment with phentolamine or propranolol (both 10 $\mu$M), significantly decreased the relaxation produced by DMPP (100 $\mu$M) but did not alter the contractile phase of the DMPP response (Fig. 5 (3) and (4)). The relaxation of fresh taeniae caeca strips produced by DMPP (100 $\mu$M) was decreased after 20 min pretreatment with bretylium (10 $\mu$M) (Fig. 5 (1) and (2)). The concentration of bretylium did not modify the relaxation of fresh taeniae caeca produced by noradrenaline (100 nM). The inhibitory action of the combination of propranolol and phentolamine on the relaxation produced by DMPP (100 $\mu$M) did not differ from that of single treatment with either phentolamine or propranolol.

After 20 min incubation in the medium containing atropine (10 $\mu$M) or hemi-

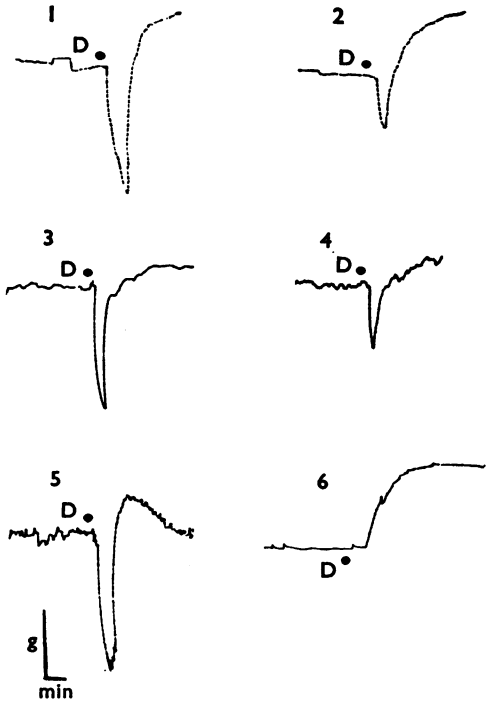


FIG. 5. The effect of bretylium (10 $\mu$M), the combined treatment with propranolol and phentolamine (both 10 $\mu$M), and cold storage (7 days) on the DMPP response of guinea-pig taenia caecum. The left column (1, 3 and 5) indicates the response before the treatment. The right column indicates the response after treatment with bretylium (2), combined treatment with adrenoceptor blocking agents (4), and cold storage (6). Black dots indicate the administration of 100 $\mu$M DMPP (D).

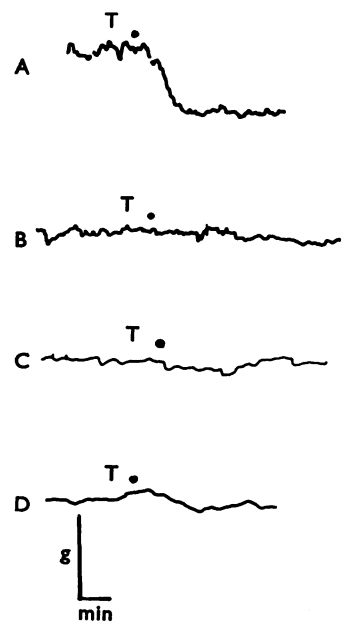


FIG. 6. The effect of tyramine on the guinea-pig taenia caecum. A, indicates the control experiments; B, C, and D indicate the response after treatment with 10 $\mu$M phentolamine, reserpine, and 4 days cold storage, respectively. Black dots indicate the administration of 100 $\mu$M tyramine (T).

cholinium (10 $\mu$M), the contractile response to DMPP (100 $\mu$M) was significantly diminished in those preparations which had been cold-stored for four days. After washing out these blocking agents, the responses partially recovered. In six out of ten strips stored for four days, atropine pretreatment followed by DMPP (100 $\mu$M) administration caused a small relaxation preceding the contraction.

*Response to tyramine.* Figure 6 indicates the response of fresh and cold storage taeniae caeca to tyramine. The relaxation produced by tyramine (100 $\mu$M) was observed in all of the fresh preparations (Fig. 6A). Tyramine (10 $\mu$M) produced a relaxation in four out of ten fresh preparations. Pretreatment with phentolamine (10 $\mu$M) or propranolol (10 $\mu$M) for 20 min inhibited the relaxation produced by tyramine (100 $\mu$M) in fresh preparations (Fig. 6B). After two and four days of cold storage, tyramine (100 $\mu$M) did not cause a relaxation of the tissue (Fig. 6D).

*Influence of reserpine treatment.* The effects of nicotine, DMPP and tyramine (all 100 $\mu$M) were examined on fresh tissue obtained from reserpinized guinea-pigs. Reserpine treatment reduced the relaxation produced by nicotine or DMPP and abolished the relaxation produced by tyramine (Fig. 6C). This treatment also increased the contractile response to nicotine and DMPP. Reserpine treatment modified neither the spontaneous, mechanical, rhythmic activity and tone of taeniae caeca nor the inhibitory response to noradrenaline (100 nM).

*Effect of physostigmine.* Twenty minutes pretreatment with physostigmine (10 nM) increased the contractile component of the response of fresh preparations to

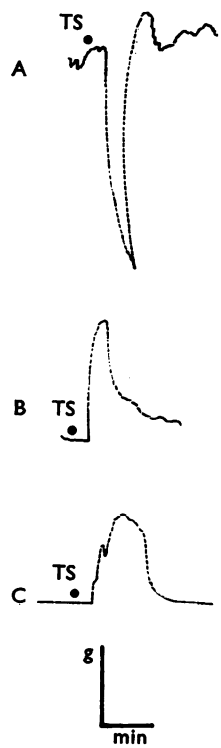


FIG. 7. Effect of transmural stimulation on the guinea-pig taenia caecum. A, B, and C indicate the response of fresh, 7 days cold stored, and reserpine treated preparations, respectively. Black dots indicate the point of transmural stimulation (TS).

nicotine or DMPP (both 100 $\mu$M) and slightly decreased the relaxation component. After seven days of cold storage, similar treatment augmented the contractile response to nicotine and DMPP (both 100 $\mu$M).

*Electrical transmural stimulation*

In fresh taeniae caeca, transmural stimulation caused relaxation followed, in six out of twelve different preparations, by a small contraction (Fig. 7A) similar to that induced by nicotine and DMPP. After prolonged cold storage (seven days), the relaxation produced by transmural stimulation was abolished and only the contractile response remained (Fig. 7B). This contractile response was blocked by treatment with hemicholinium, atropine, or hexamethonium (all 10 $\mu$M) for 30 minutes. Reserpine treatment also abolished (Fig. 7C) or reduced the inhibitory effect of transmural stimulation. In fresh preparations, after treatment with phentolamine or phenoxybenzamine (both 10 $\mu$M) for 1 h, the relaxation produced by the transmural stimuli was decreased to 25–30% (ten preparations) of that of the untreated preparations.

## Discussion

A schematic representation of the gastrointestinal innervation has previously been presented (Norberg, 1967), according to which 'the sympathetic inhibition of intestinal motility seems to be controlled by three neurones in a chain: one preganglionic cholinergic neurone in the spinal cord, one postganglionic adrenergic neurone in a prevertebral ganglion and finally a postganglionic parasympathetic neurone in the intestinal wall, terminating in the smooth muscle layers'. The histochemical study presented here, however, demonstrates a dense distribution of specific catecholamine fluorescence in the longitudinal and circular muscle layers and also in Auerbach's plexus of the guinea-pig taenia caecum. The evidence of the presence of extrinsic adrenergic fibres in very close proximity to smooth muscle fibres indicates the existence of an alternative pathway to the neurone chain postulated by Norberg (1967). The results provide evidence for the possible existence of a direct action of adrenergic fibres on the effectors in addition to an indirect action through the intramural nerve cells. Similar alternative pathways have also been suggested by Gabella & Costa (1969). The presence of cholinesterase active substances further indicates a rich innervation of the smooth muscle by cholinergic fibres originating from the ganglion cells of Auerbach's plexus, as described by Åberg & Eränkö (1967).

After prolonged cold storage, the specific fluorescence disappeared from all tissue layers of the guinea-pig taenia caecum but the cholinesterase positive fibres were still demonstrable. This observation was entirely consistent with our observation that while cold storage decreased the tissue noradrenaline content, it had no effect on cholinesterase activity.

Similar effects of cold storage were observed on the specific catecholamine fluorescence and noradrenaline content of vascular smooth muscle (Shibata, Hattori, Sakurai, Mori & Fujiwara, 1971). Disappearance of noradrenaline containing fibres with specific fluorescence from all tissue layers of taenia caecum was also observed after reserpine treatment. Mori & Shibata (unpublished results) have shown, with histochemical techniques, that the reduction in specific catecholamine

fluorescence which follows cold storage (5 days) could not be reversed by incubation in a medium containing noradrenaline. On the other hand, the reduction of specific fluorescence by reserpine treatment was readily restored by incubation in the same noradrenaline-rich medium. Evidence therefore favours the conclusion that cold storage results in a disintegration or degeneration of nerve structures rather than a simple reserpine-like catecholamine depletion without impairment of nerve function. Similar conclusions were previously drawn from cold storage experiments involving vascular smooth muscles (Shibata *et al.*, 1971).

The rather complex innervation indicated by histochemical studies of guinea-pig taenia would suggest that the pharmacological responses of this tissue might be equally complex. Although such studies have not yet provided direct evidence for complete selective inactivation of nervous elements under the conditions of the *in vitro* experiments, it seems probable that cold storage may simplify the innervation of the taenia caecum by selective destruction of adrenergic nerve elements.

In the present experiments with taenia caecum, cold storage treatment inhibited the relaxation normally produced by nicotine and DMPP. Moreover, after cold storage, the contractile responses to nicotine and DMPP were potentiated. These facts suggest that a special autonomic mechanism is present in this smooth muscle preparation. The relaxing effects of nicotine and DMPP were inhibited by reserpine, bretylium, or adrenoceptor antagonists, and the contractile response to both agents was blocked by parasympathetic blocking drugs. In addition, anticholinesterase (physostigmine) augmented contractile responses of both cold stored and fresh tissue to nicotine and DMPP. These results suggest that it is possible for one stimulating substance to elicit both adrenergic relaxation and a cholinergic contraction in the same preparation.

In fresh tissue, tyramine, which acts indirectly through a catecholamine release mechanism, caused relaxation. After cold storage, tyramine-induced relaxation could not be demonstrated. Other investigations provided evidence to support the adrenergic nature of the relaxation produced by nicotine and DMPP (Gillespie & Mackenna, 1960; Greef, Kasperat & Oswald, 1962; Jarrett, 1962; Burn & Gibbons, 1964; Weiss, 1962). These results strongly support the view that loss of inhibitory response to nicotine, DMPP, and tyramine is probably due to deterioration of the adrenergic nerve structures during cold storage. Since cold storage treatment preferentially affects ganglion cells, it seems more probable that the contractile response is directly mediated by stimulation between the ultimate synaptic junction and the muscle cell. This concept is supported by the finding of Gillespie & Wishart (1957) that cooling of the rabbit colon first inhibits the response due to the preganglionic stimulation of the pelvic nerves, the action of nicotine and lastly, that of acetylcholine. Similar concepts, based on results obtained from cold storage experiments on intestinal smooth muscle, have also been presented by Innes, Kosterlitz & Robinson (1957). Since adrenergic inhibitory mechanisms might occur primarily at the myenteric plexus (Jacobowitz, 1965), it might be assumed that enhancement by cold storage of the contractile response to DMPP and nicotine is due to impairment of such an inhibitory adrenergic system not only at the ultimate synaptic junction, but also at the myenteric plexus. It is possible to reject the hypothesis that the inhibitory action of cold storage is due to interference at postsynaptic effector sites, since prolonged cold storage did not

modify the sensitivity of the taenia to noradrenaline, phenylephrine and isoprenaline (Shibata *et al.*, 1970).

The possibility that the contraction elicited by nicotine and DMPP might be a 'myogenic rebound' secondary to the preceding relaxation has been considered but rejected since the contractile response was modified by anticholinesterase and persisted even when the relaxation phase had been blocked by cold storage or reserpine pretreatment. Previous experiments with the taenia caecum (Shibata *et al.*, 1970) have also demonstrated that cold stored preparations remain responsive to exogenous acetylcholine. It is therefore assumed that while adrenergic activity is lost early in cold storage, the responsiveness of cholinergic nerves to both electrical and pharmacological stimuli is resistant to prolonged cold storage.

Histochemical studies indicate that 4 days of cold storage did not alter the cholinesterase activity of taenia, whereas decreased density of cholinesterase was observed in strips stored for 7 days. In addition, the anticholinesterase agent was still effective in the strips after prolonged cold storage, indicating residual enzyme activity.

The inhibitory action of electrical transmural stimulation has been considered (Burnstock *et al.*, 1966; Burnstock, Campbell, Bennett & Holman, 1964; Day & Warren, 1968; Bianchi, Beani, Frigo & Crema, 1968) to result from stimulation of inhibitory nerves within the intestine. The generally accepted concept that the inhibitory nerves of the intestine are adrenergic sympathetic fibres receives further support from our observation that the inhibitory action of transmural stimulation was blocked by bretylium, reserpine, adrenoceptor blocking agents and also by ganglion blocking agents. In contrast, Holman & Hughes (1965) and Burnstock *et al.* (1966) found that the response of the taenia caecum to electrical transmural stimulation was retained even after prolonged cold storage, and it has been concluded that this response was mediated by non-adrenergic inhibitory neurones of parasympathetic or intrinsic origin (Burnstock, Campbell, Satchell & Smythe, 1970; Holman & Hughes, 1965; Burnstock *et al.*, 1966; Su, Bevan & Burnstock, 1971) which may release ATP or related nucleotides (Burnstock *et al.*, 1970). Furthermore, Holman & Hughes (1965) and also Burnstock *et al.* (1966) have postulated that the relaxation produced by DMPP and nicotine results from stimulation of non-adrenergic and non-cholinergic inhibitory nerve structures.

Our results are not consistent with this interpretation. The inhibitory responses to nicotine, DMPP and transmural stimulation were inhibited by cold storage. Although reasons for this discrepancy are not clear, they must be presumed to arise from differences in experimental conditions. Thus, it may be suggested that non-adrenergic inhibitory neurones exist but are labile in cold storage as are the adrenergic neurones; prolonged cold storage treatment decreased the inhibitory action of exogenous ATP in taenia (Shibata *et al.*, 1970). In addition, adrenoceptor blocking agents or reserpine block the inhibitory action of nicotine, DMPP or transmural stimulation on the taenia. Chemical sympathectomy of guinea-pig taenia caecum with 6-hydroxydopamine also markedly reduced the relaxation produced by nicotine, DMPP or tyramine (unpublished data). Thus in particular, it is suggested that the inhibitory action of nicotine, DMPP or tyramine in taenia is mostly attributable to the response to stimulation of the adrenergic nerves. However, the remaining inhibition may be due to non-adrenergic mechanisms. Further experiments are at present being carried out to resolve this question.

The present histochemical and pharmacological data suggest that prolonged cold storage causes selective and complete degeneration of the adrenergic mechanisms of the guinea-pig taenia caecum. This probably explains the loss of inhibitory action of nicotine, DMPP, tyramine and transmural stimulation by cold storage on the guinea-pig taenia. The persistence of peripheral cholinergic mechanisms may lead to the enhanced excitatory action of nicotine, DMPP and transmural stimulation in the cold stored taenia.

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