STUDIES ON

THE PERISTALTIC REFLEX

A thesis submitted for the degree of

Doctor of Philosophy

by

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TO MY MOTHER

DECLARATION

I declare that this thesis is a record of original work and contains no material which has been accepted for the award of any other degree or diploma in any University.

To the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Wolfgang Flachsenberger

November 1985

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CONTENTS

All figures and tables with roman numerals are displayed in the appendix.

CORRIGENDA:

Page 9, 2nd para, line 1: "neurones" should read "nerves".

Page 40, 3rd para, line 6: "... small animals of approximately 200g".

Page 46, last sentence should read "... very similar to whole chain responses."

Page 82, line 8: block should read reduce.

Page 112, 2nd para, line 3: should read "... relaxation of the circular muscle was ..."

Page 112, 2nd para, line 13 should read "The relaxation response ..."

Table 5, line 2: "contraction" should read "relaxation"

10 mV equals 1 mm tissue displacement in figures: 1-3, 6-8, 9-23, 39, 42 and 43.

CHAPTER I

GENERAL INTRODUCTION

Gastrointestinal function is one of the fundamental mechanisms of animal physiology, consisting of digestion, absorption and transport of nutrients, salts and water from the external to the internal environment, and finally the excretion of waste products. Intestinal motility is a vital part of the transport system, and some aspects of its neural complexity, especially control of the descending colonic inhibition, form the subject of this research work.

Historical Progression

Modern day scientific examinations of gastrointestinal functions, and of drugs that interact with it, started more than a hundred years ago. In 1880 B. von Anrep a scientist travelling across the Andes was greatly impressed by observations and reports of the miraculous effects that the chewing of coca leaves had on native porters: they could endure immense strain and hard work for days without much sleep or food intake. He decided to test this drug on various physiological systems in dogs. One of his tests involved observing the effect of cocaïne - the name given to the organic base extracted from coca leaves - on the gut *in situ*.

The abdominal cavity of the anaesthetized animal was opened, filled with warm 0.6% saline and then covered with a glass plate. This precaution avoided cooling and evaporation while allowing the viewer to observe intestinal activity. It could be seen that medium doses of cocaine (30mg/kg) led to "intensified movement", which decreased gradually, while a higher concentration (75mg/kg) initiated a short burst of peristaltic excitation followed by weakening and cessation of activity.

The above described method of either filling the abdominal cavity with saline, or submerging the whole animal in a temperature controlled salt solution, was widely used in intestinal research at the time, although Salvioli had already shown in 1880 that an excised rabbit or dog small intestine segment of 10cm to 15 cm long could survive for hours when perfused via its vascular system with a mixture of 30% calf blood and 70% of 0.75% saline. He used a "grasstalk", which was subsequently replaced by a straw as a writing lever, and a Kymograph to record changes in peristaltic movement when he added nicotine-, opium-, and atropine-containing blood to the perfusate.

In 1882 Nothnagel made a famous, subsequently often employed, discovery. Using cats and rabbits, celiotomized and submerged in 0.5% - 0.6% saline at 38^{o}C, he observed the effects of stimulating the intestinal muscles by touching the serosa with crystals of various salts. He found that sodium salts caused a longitudinal and circular muscle contraction, which always moved in the oral direction. Stimulation with potassium salts, however, resulted in a localized circular muscle contraction. The effects of stimulation with different Na or K salts varied only in their intensity.

Peristalsis research was further advanced by Lüderitz, who worked mainly on celiotomized rabbits submerged in 0.6% saline. In 1889 he concluded that fluid movement in the gut was due to distension of the intestinal wall. He tied the gut off orally, occluded it temporarily 6 cm - 10 cm below with forceps, and injected 5 ml - 8 ml saline in ileal sections, or 10ml - 20 ml in colonic sections. Thus he correlated distension pressures with the threshold of peristaltic activity. He also introduced small 5 mm rubber balloons through cuts into the dog gut and inflated them to 25 mm - 30 mm. Lüderitz reported that the balloon distension method was first used by Legros and Onimus in 1869 who inserted balloons into the small intestine via the stomach of anaesthetized animals. The balloon experiments of Lüderitz showed that a contractile wave of the circular muscle spreads orally as well as anally. He also tried a relatively new stimulation technique - the "galvanic method", which resulted in contractions of the circular muscle at the anode, and longitudinal muscle contraction at the cathode.

During another series of experiments in 1890 Lüderitz tied off 4 cm to 10 cm lengths of empty duodenal sections of rabbit intestine and cut all vascular and neural connections along the mesentric border. The resulting stimulatory effect was assumed to be due to cyanosis acting on intrinsic nerves. He observed anally propagated circular and longitudinal contractions, where the longitudinal contraction occurred before the circular one. Since all extrinsic influences had been severed he concluded that the peristaltic effects were due to an intrinsic "excitomotorischer Nervenapparat".

This intrinsic neural control theory was supported by Langley and Anderson (1895-1896), who found that, although resection of sacral nerves in dogs led to constipation and cessation of voluntary defaecation, artificial bowel-emptying was not necessary. They concluded that extrinsic neural control was not essential for colonic propulsion.

However, the greatest advances towards the elucidation of peristalsis were due to the research by Bayliss and Starling (1899) who worked on the small intestine of dogs. The whole animal, except the head, was submerged in warm saline after celiotomy. They then either inserted a rubber balloon filled with water, observing and recording the pressures exerted on it, or attached recording levers of an "enterograph" to a gut section, thereby allowing them to graphically record longitudinal and circular muscle changes.

They found that two types of movements existed in the gut: pendulum movements and the peristaltic wave. Salt introduced intraluminally produced strong peristaltic waves, whereas the pendulum movement was found to be entirely myogenic; waves did not move upwards - "there is no antiperistalsis". When the gut was fatigued the most striking difference observed was "the absence of inhibition below the bolus". Longitudinal and circular muscle contracted simultaneously upon stimulation. Although inhibition could ascend as well as descend, ascending inhibition was never observed as a result of stimulation by a bolus. They found that the descending inhibitory reflex travelled at 10 cm s^{-1}. They also tested the intestinal reaction to morphine, muscarine, atropine, cocaïne, and nicotine. Their most important achievement, however, was the formulation

of the law of the intestine based on their research results: "...if cerebro-spinal reflexes be excluded, excitation at any point of the gut excites contraction above and inhibition below".

In 1900-1901 Bayliss and Starling continued their work on the small intestine with research into the effects of pinch-, balloon- and bolus-stimulation of the colon. They found that the colon was also under "ascending excitatory and descending inhibitory control". Experiments in which cerebro-spinal control had not been severed showed that the whole colon would contract on stimulation of the visceral nerves, and relax when the splanchnic nerves were stimulated - demonstrating the opposing effects of parasympathetic and sympathetic control.

Magnus (1904) worked on the excised small intestine of dogs, cats and rabbits. He used Ringer-Locke-solution in which he submerged the gut section attached to two hooks permanently fixed 4 cm apart on a glass rod. Above the two fixation points he tied two fine cotton threads leading to his Kymographion levers in order to record circular muscle changes. For longitudinal muscle experiments he simply fixed one end of the gut to a glass rod and the other end again with a fine cotton, to a recording arm. The gut sections were open-ended and devoid of their mesentery. He used bolus-, pinch-, or salt crystal stimulation, usually in the middle between the two fixation points. His observations confirmed the two main movements - pendular and peristaltic waves. Magnus found that mechanical stimulation (by bolus or pinch) resulted in circular muscle relaxations preceding contractions mainly below, but sometimes above and below, the stimulation point. Salt crystal stimulation was

followed by oral relaxation and anal contraction in the cat gut, but by oral contraction and anal relaxation with subsequent contraction in dog intestinal sections. Cyanosis, fluid distension and temperature fluctuations were the main influences causing changes to the normally observed peristaltic response. Magnus tested these parameters exhaustively. Cyanosis stopped pendular movement whithin approximately 100 minutes. Excessive fluid distension (60 cm Hg) resulted in doubling the intestinal section's length and diameter but had no effect at all if the gut was already contracting at a 'lively' rate. Temperature changes from 14°C to 42°C had an effect only on the frequency not on the strength of contractions. Above and below these limits peristalsis ceased. In order to test whether "degenerative section of mesenteric nerves" had an effect on peristaltic movement Langley and Magnus (1905-1906) performed a number of experiments, subjecting the gut to pinch-, balloon- and pea-bolus stimulation using the influence of neuroactive drugs on these effects to show that there were no significant differences in the results before and after resection of the extrinsic nerve supply.

Cannon, in 1912, also observed a contraction of intestinal circular muscles above the point of stimulation but postulated that an active dilation occurred below. He named this "local reflex of the alimentary canal" the "myenteric reflex" because the nerve net between the longitudinal and circular muscle layers was called the "myenteric plexus". In contrast to Bayliss and Starling's observations he claimed that antiperistaltic waves occurred quite normally.

In 1917-1918 Trendelenburg devised a new stimulation method, which later became quite commonly used. His main objection to previous methods was that the stimulation 'dosage' could be neither measured nor controlled. His apparatus consisted essentially of a bottle, which could be raised or lowered by a micro-advance-mechanism, and which was connected to a submerged gut section, thus allowing a "...fortgesetzte Füllung des Darmrohres unter linear verlaufendem Durckanstieg.." (continual filling of the gut section with linearly increasing pressure). He recorded three types of intestinal movements: peristaltic waves, tonus fluctuations, and pendulum movements. Trendelenburg also observed that the longitudinal muscle contracted when the circular muscle relaxed, but that half a second after the longitudinal contraction started, the circular muscle contracted. Moreover, after its maximal contraction the longitudinal muscle relaxed, and this was followed by the circular muscle relaxation, which allowed the gut section to be filled again. Because the longitudinal contraction preceded the circular contraction by a quarter to half a wavelength, there appeared to be a phase difference.

A new point raised by Trendelenburg was that the "Dehnungslast ist vom Wandwiderstand abhängig" (distension pressure is dependent on the wall resistance). It thus followed that the critical point or threshold pressure for the onset of peristalsis was tonus dependent, which in turn was subject to a number of excitatory or inhibitory factors. He elucidated the effect of this dependence through the changes of pressure necessary to initiate peristalsis after the addition of various alkaloids to the bath medium. His statement that the human small intestine is pharmacologically more closely related to the guinea-pig small intestine

than to any other mammalian small intestine examined, has surely contributed greatly to the deaths of innumerable guinea-pigs.

Baur (1923), working with sections of guinea-pig small intestine submerged in Tyrode solution, improved Trendelenburg's method when measuring the peristaltic pressure and collecting the excreted fluid in a glass cylinder in order to measure the fluid volume expelled by every peristaltic wave, with the aid of an overflow system in a second cylinder. He kept his stimulation pressure constant at a 2.5 cm head of water. No changes occurred at temperatures between 28°C - 42°C. He observed interference phenomena when peristaltic and antiperistaltic waves met. At the above distension pressure the gut segments worked for hours stopping occasionally for "rest periods" possibly due to tonus decrease and subsequent peristaltic threshold increase.

That the bidirectional contractions become mainly caudally polarized was thought to be due to a metabolic gradient of the intestine (Alvarez, 1928). He compared the downward movement of peristaltic waves to the waves of a stone thrown into a fast flowing river. A graded rhythmic rate was observed in the small intestine - the duodenum contracted twice as fast as the ileum. An anatomical gradient was seen by Alvarez in the fact that ganglia became fewer and coarser aborally in the myenteric plexus of the small intestine. He even suggested that 'minute electric currents', produced by the graded metabolic rate, might cause an electrical potential gradient. Although he observed occasional, rather shallow reverse peristaltic waves travelling along a third to half the colon's length, he noticed that they never pushed any material orally.

Magee and Southgate (1929) described experiments, during which they observed that the duodenum had a significantly higher threshold pressure than the ileum and that the small intestine of carnivora required a four to twelve times greater pressure to initiate peristaltic movement than those of herbivora, a phenomenon previously overlooked when peristaltic records of these two major animal groups were discussed.

By electrically stimulating neurones in mesenteric flaps, which he had left attached to rabbit ileal and duodenal sections, Finkleman (1930) found that 'slow' stimulation 2-4 pulses s^{-1} resulted in atropine - sensitive parasympathetic longitudinal contractions, whilst 40 pulses s^{-1} caused sympathetic inhibition that could be mimicked by adrenaline. In an ingenious experiment, where Finkleman let the perfusate drip from the electrically stimulated (40 pulses s^{-1}) segment onto another non-stimulated segment causing a similar response, it became obvious that the latter electrical stimulation caused the neurally mediated release of an adrenaline-like substance.

Raiford and Mulinos (1934a) developed a completely new method. Female dogs were subjected to a colectomy. The resected 8 cm - 10 cm sections of colon (mesentery, vascular- and neural-supply intact) were cut open 'antimesenterically' to form a rectangular flap, attached serosally to the animals' abdominal subcutaneous layer. When the animals were ready for experimental use, 8 to 10 days later, enterograph levers were attached to this flap. Submucosal stroking was employed as the stimulation procedure. Longitudinal stroking resulted in longitudinal muscle contractions, while transverse stroking caused longitudinal and

circular muscle contractions. The longitudinal muscles contracted 0.2 s before the circular muscles. They concluded that these contractions must have been a local reflex response, because severance of neural and vascular connections did not alter the response.

Another series of experiments by the same team (1934b), again using 'exteriorized' gut sections, confirmed that "...mucosal irritation elicits contractions of the longitudinal muscles first and then of the circular". This was their first direct clash with postulates of Bayliss and Starling. A second contradictory viewpoint emerged when they studied the effects of stimulation more closely; Raiford and Mulinos never observed a relaxation, but always a longitudinal muscle contraction at the stimulated point and below, followed by a circular muscle contraction above. They concluded that the previously reported descending inhibition was an artifact, because, in a whole gut preparation, a longitudinal muscle contraction would appear to be circular muscle relaxation due to a bunching effect. They suggested that one of two paired sensory afferent nerves always projected from the mucosa directly to the longitudinal muscle fibres above and anally from any given point, whilst the second one went directly to the circular muscle layer and orally from the point of reference. Raiford and Mulinos were unable to offer an explanation for the circular muscle contraction delay. They also observed that after food intake by the animal the exteriorized colonic graft had a much lower irritation threshold. In conclusion they proclaimed the new law of the intestine: mucosal stimulation is followed by a longitudinal muscle contraction at and below the stimulation point, and by a contraction of

the circular muscle at and above it. The first precedes the latter by 3-5 seconds.

Raiford and Mulinos' views were supported by Hukuhara et al. (1936) who in their experiments used submerged dogs which used two inflated balloons inserted into the duodemum, from which pressure recordings were obtained. Pinch and distension stimulation resulted only in contractions of longitudinal and circular muscles above and below. This occurred both before as well as after neural connections to the central nervous system (CNS) were severed. They observed interference effects when pendular movements interacted with peristaltic waves, but no relaxation was recorded in front of a bolus. They claimed that Bayliss and Starling's law of the intestine was not tenable.

Bozler (1938) was one of the first reseachers to demonstrate, with intracellular electrophysiological recordings, that action potentials were propagated in visceral and ureter smooth muscle, thus showing that smooth muscle fibres is syncytically connected. He found that action potentials of smooth muscles differed from those of nerves or striated muscle only quantitatively. More importantly, however, he found differences in discharge patterns for pendular movements and peristaltic waves. Pendular movements were preceded only by bursts of impulses, whilst a continuous discharge pattern emerged during peristaltic wave activity.

On the basis of in vitro experiments Bozler (1949a) differentiated between simple and peristaltic waves caused by a 'myenteric reflex'. He

called the latter effect 'reflex peristalsis'. He observed that after electrical- and pinch-stimulation, or mucosal stroking, the contractile wave spread at first bidirectionally and that the orally directed contractions stopped after a few millimeters while only the aboral wave continued. These observations correlated well with Alvarez' gradient theory. Moreover, the descending inhibition described by other researchers was, according to Bozler, neither a reliable nor a polar phenomenon, possibly not even a part of the myenteric reflex.

Bozler (1949b) also carried out electrophysiological recordings on excised canine duodenal or colonic sections. He used non-metallic, non-polarizable recording electrodes and a varnished lead bolus, as the means of stimulation. He found that the bolus stimulus increased rhythm and strength of contractions mainly orad with little anal effect. During peristalsis the spike discharge also increased in rate and strength when compared to the action potentials during normal rhythmic contractions. At maximal peristalsis, spikes condensed into a prolonged, fused discharge.

In 1953 Schaumann reported that the longitudinal muscle relaxation was an active neurogenic process which he could mimic with cold stimulation (29°C) or application of 'Sympathin I', and which he could also antagonize with morphine. Schaumann theorized about a cholinergic-adrenergic interaction of longitudinal and circular muscle contraction-relaxation due to interneuronal presynaptic counter-effects. Although he found that a slow longitudinal muscle stretch caused a tonus elevation, he did not place any physiological importance on this

effect because there are no fixed points in the gut in situ for stretch to work against.

Further electrophysiological research, this time carried out by Bülbring (1955), revealed increased spike discharge during muscular activity or stretch stimulation in guinea-pig taenia coli. This effect persisted even after nerve and ganglionic blocking agents had been applied, suggesting that it was entirely myogenic in origin.

Kosterlitz et al. (1955) looked at the question of whether the apparently sequential longitudinal and circular contractions were interdependent. They discovered that longitudinal muscles would still contract even if circular muscles were deactivated by hexamethonium (a ganglionic blocker), or by storing the gut for 24 hours at 4°C. They also found that lumen distension stimulation must have been active, due to radial distension rather than to the lengthening of longitudinal muscles. Kosterlitz et al. (1956) subsequently confirmed their previous findings, showing, by selectively blocking either longitudinal or circular muscles, that the apparently fixed pattern of interaction of the two muscle layers was not interdependent. They hypothesized that a stimulus first triggers the longitudinal muscles, due to their greater sensitivity, and subsequently the circular muscle layers, their explanation for the delayed reaction being that a greater pressure threshold is needed by circular muscles in order to react. More recent findings suggest that out-of-phase coupling of electrical oscillators in longitudinal and circular muscles leads to control, in time and space, of the excitability of intestinal smooth muscles (Daniel, 1975), who also noted that the

circular muscle contraction could raise the intraluminal pressure to 20 cm H_2O and that longitudinal muscle contractions were not involved in the efficacy of intestinal emptying.

Innest et al. (1956) also reported that the seemingly fixed pattern of circular and longitudinal muscle interaction was not interdependent, and that blocking of longitudinal muscles by high acetylcholine doses did not affect fluid expulsion from the lumen.

Ontogenetic work by Takita (1957), on human and bovine foetuses, showed that peristalsis occurs in response to injected fluids in the eighth week, before intramural ganglia develope, the contractions spreading in either or both directions. Orally and anally directed peristaltic waves intensify after the ninth week, due to longitudinal muscle and intramural ganglia development. After the 19th week there is strong peristaltic movement, with only very weak antiperistaltic waves on rare occasions.

In order to find the actual site from whence a stimulus triggers the intrinsic reflex arc, Bülbring et al. (1958b) used intraluminal pressure stimulation on ileal segments from guinea-pigs, cats, and rabbits. They claimed that sensory nerves travelling from the mucosa to motor neurons in the muscular coats, the existence of which they had shown anatomically, are excited by pressure changes and that when pressure thresholds are exceeded, they trigger the peristaltic reflex. They concluded that sensory impulses from the mucous membrane are most likely responsible for peristaltic onset, because asphyxiation of the mucous

membrane, local intraluminal anaesthesia, using cocaine, or removal of the mucous membrane abolishes the peristaltic reflex. Hukuhara et al. (1958) partly confirmed this previous conclusion. Using mainly dog, but also cat and rabbit ileal segments, which were still connected to the animals' vascular systems but had had their neural connections severed, they found that mechanical (stroking) or chemical (0.1NHCl) mucosal stimulation resulted in both hexamethonium-sensitive contractions above and relaxation below the stimulation point. The same stimulations when applied to the outer muscle coat resulted in a hexamethonium-insensitive oral as well as anal contraction inhibition. In 1959 Hukuhara and Miyake repeated the above experiments, using colon sections, and obtained identical results.

In order to test whether internal pressure or radial distension was responsible for triggering the peristaltic reflex, Kosterlitz and Robinson (1959) slipped perspex or glass tubes over guinea-pig ileal segments, and stimulated by distension of an intralumenal balloon. They observed that hexamethonium-resistant contractions were diminished or absent. This result led to the conclusion that the distension stimulus is due to physical distortion of sensory receptors that were crosslinked with longitudinal muscle fibres and therefore forced apart during the usual balloon distension.

Sperelakis and Prosser (1959) elucidated the question of stimulus transmission by recording peristaltic and electrical activity. One of their experiments consisted of placing two circular ileal muscle rings next to each other on a glass rod and then stimulating one of them

electrically or chemically (isotonic KCl or $BaCl_2$). They always found that electrical activity, and often also contractile movement, was propagated to the second muscle ring. This effect led them to the conclusion that stretch was not the mode of stimulus transmission from one muscle fibre to the next. They also assumed that transmission could not be due to nervous conduction in visceral smooth muscles, that, however, chemical transmission could not be excluded, but that the response was most likely due to electrical conduction. Additional findings were that peristaltic wave propagation occurred at 0.5 cm - 7.5 cm s^{-1} and that contractile units consisted of circular muscle fibre fasciculi of 0.1 mm diameter and 1.2 mm length.

In contrast Burnstock and Prosser (1960) concluded, from experiments in which 2 g - 4 g pull was applied in 'quick stretch' experiments (10% of the length) on a number of smooth muscle strips, usually 2 mm - 5 mm wide and 3 cm - 6 cm long, that the quick stretch always elicited a contractile response while slow stretch was effective only in highly sensitive taenia coli and bladder strip preparations. They observed electrophysiologically a high frequency discharge pattern after every quick stretch event.

In 1964 Kosterlitz and Lee contradicted these findings and claimed that neither slow nor sudden stretch cause a longitudinal smooth muscle contraction. They declared that peristalsis is caused by cholinergic activation and that the longitudinal muscle relaxation is due to an active inhibition, the effect of an unknown neurotransmitter.

Hukuhara et al. (1969) using a modified Trendelenburg's technique on jejunal loops in conjunction with two floating microelectrodes, placed 1 cm apart, reported a direct relationship between intraluminal pressure and action potential propagation velocity (v), which they found to be $v(mm\ s^{-1}) = 1.5$ to 2.2 x mm H_2O, e.g. 4 cm H_2O resulted in a 60 mm to 80 mm s^{-1} conduction velocity. Acetylcholine and physostigmine accelerated propagation, while atropine slowed it down.

Crema et al. (1970) used stimulation by balloon inflation on cat and guinea-pig colon segments. According to their results, longitudinal and circular muscles act in association with each other but also respond independently; tetrodotoxin (TTX), a neuroblocker, completely stops contractions above and relaxation below the stimulus point. They also claimed that atropine and hyoscine blocks the descending inhibition of guinea-pig and cat colons selectively, but not the contractions.

Kosterlitz and Watt (1975a) reported that stretch at a constant rate might result in contractions or relaxations in visceral smooth muscles, which may be myogenic or contain a neurogenic component. The same researchers (1975b) postulated that peristalsis begins with longitudinal muscle contractions (preparatory phase) followed by circular muscle contractions (travelling anally), then the longitudinal muscles relax (emptying phase) and subsequently the circular muscles also relax (filling phase).

It was reported by Code (1968) that mucosal stimulation results in both oral contractions and aboral relaxation, which are 'essential features

for any movement' in the intestine. Frigo and Lecchini (1970) demonstrated that a balloon filled with varying amounts of warm water could be used as a solid bolus of changing size, which resulted in a 'graded localized intraluminal stimulus' initiating propulsive activity. The velocity of propulsion could then be used as a quantitative determinant of propulsive activity in the isolated guinea-pig or cat colonic section. At maximal distension they found the mean colonic velocity of propulsion of the cat to be 1.08 mm s^{-1} and 1.51 mm s^{-1} for the guinea-pig. Removal of mucosal and submucosal layers resulted in stopping circular muscle movement, and, although longitudinal activity continued, no propulsion occurred. They concluded that stimulus-sensitive receptors are situated in either the mucosa or submucosa.

Jacobson and Schanbour (1974) reported that sensory mucosal input into the myenteric plexus results in excitatory cholinergic and inhibitory non-adrenergic responses of the intestinal muscle layers. They also found that aganglionic muscle strips lack the inhibitory mechanism, and that there are no significant differences between ileal and colonic responses.

Davenport (1966) believed that the most important motion of the small intestine is segmentation, controlled by a basic electrical rhythm originating in the longitudinal muscle, and that peristalsis is an intrinsic reflex where the extrinsic innervation modifies only strength and occurrence of movements. Mucosal stroking or intraluminal pressure increase by 2 mm Hg resulted in a contraction 2 cm - 3 cm above and a relaxation a few centimetres below the stimulation point. Longitudinal muscle contractions were followed by circular muscle contractions with a

90° phase difference. Despite his general acceptance of these concurring reports he expressed doubt about the validity of Bayliss and Starling's "Law of the Intestine".

In 1975 Baldwin and Thomas expressed similar doubts in accepting the above mentioned "Law of the Intestine" because of the 'failure of most observers to elicit a relaxation below a point of stimulation'. In order to produce the 'mucosal reflex' - an inhibitory phase followed by a contracting phase - they recommended application of mucosal stroking, radial stretch, or chemical stimulation. They accepted peristalsis as being a myogenic rather than a neurogenic phenomenon, consisting of a mucosal and a muscular reflex.

Thus, despite experimental difficulties and contradictory results, a number of methods have been established to initiate reproducible oral contraction as well as descending inhibitory responses in isolated animal intestinal segments. Some results of the search for the neuro-transmitters responsible for the peristaltic reflex will be described in the following. Although a much greater variety of agents have been tested against peristalsis, this review will be limited to recounting some of the research into adrenergic, GABAergic, purinergic, peptidergic and prostanoid systems.

Pharmacological Background

The search for the neurally mediated contraction and inhibition was facilitated by the use of tetrodotoxin, the active principle of the puffer-fish poison, which was extensively investigated by Narahashi et al. (1964). Voltage clamp experiments using lobster giant axons showed that tetrodotoxin blocked Na^+-dependent action potentials at very low concentrations by selective inhibition of the sodium-gating mechanism in neurons, while the potassium gating mechanism remained intact.

Results of Kurriyama et al. (1966) confirmed Narashi's findings, and further showed that spike generation in longitudinal muscles of guinea-pig taenia coli and of heart pacemaker cells, is not blocked by tetrodotoxin, and hence argued that spike generation is Ca^{++}, and not Na^+, mediated in these two types of cells. In ventricular heart and skeletal diaphragm muscles, spike generation was blocked completely by tetrodotoxin, proving the sodium dependence of their excitable membranes. Gershon (1967) worked on seven different kinds of guinea-pig smooth muscle tissues, and proposed that effects produced by neural stimulation were abolished by tetrodotoxin. Drug responses, partly due to neural stimulation, and partly due to direct action on muscles, were reduced. In cases where antagonistic neural action had reduced a reponse, tetrodotoxin application resulted in a potentiation of that response. Drug effects were the same in denervated muscle strips as in tetrodotoxin treated strips. The above findings subsequently allowed researchers to differentiate between myogenic and neurogenic responses. Since most researchers had agreed on acetylcholine as being the major excitatory

transmitter in the intestine, more emphasis has been placed here on the inhibitory effects that persist despite adrenergic and cholinergic blockade.

Finkleman (1930) had shown that 'high' frequency (40 pps) electrical stimulation of innervated rabbit small intestine sections resulted in an inhibition which strongly resembled the inhibition caused by adrenaline. A similar inhibition was obtained if the perfusate of the innervated and stimulated section, or adrenaline containing perfusate, was run over a second, non-stimulated, gut section lacking its extrinsic neural supply. This similar although weaker response, permitted the speculation that the enteric inhibitory transmitter could be an adrenergic substance. However, histochemical studies by Jacobowitz (1965) showed only sparse adrenergic innervation of intestinal smooth musculature of cats and monkeys which led to the suggestion that adrenergic inhibition mechanism takes place primarily in the myenteric ganglia. North (1982) observed during electrophysiological experiments that noradrenaline inhibited firing of myenteric neurones, and he also reported comparable action of noradrenaline on neurones in the submucous plexus of the guinea-pig small bowel. In contrast Frigo et al. (1984) claimed that all adrenergic inhibitory effects on isolated guinea-pig distal colon segments were myogenic only, never neurogenic. Evidently the adrenergic inhibitory receptors present in the intestine are an integral part of only the extrinsic innervation, although exceptions could be those nerve-endings that release two neurotransmitters like ATP and noradrenaline (Burnstock, 1982b) or possibly noradrenaline and 5-hydroxytryptamine as reported by Hökfeldt et al. (1982). But whatever the purpose of adrenergic gut

innervation might be, it has to be ruled out as the main enteric inhibitory transmitter because Furness and Costa (1973) reported that the descending inhibition persists after the specific adrenergic blockers guanethidine phentolamine and propranolol had been applied. However, the inhibition of muscularis mucosae acitivity could be attributed to the adrenergic system (Hog and Baldauf, 1985).

The first researchers to describe the inhibitory action of purines on the small intestine were Drury and Szent-Györgyi (1929). When they found that extracts from minced bullock hearts injected into dogs or guinea-pigs produced a "sympathomimetic" response, they purified the active substance: adenylic acid. When they then compared the adenylic acid effect with that of adenosine they observed identical responses. An intravenous (i.v.) injection of 25 mg of adenosine in a cat abruptly blocked all peristaltic movement of the small intestine for about two minutes, which then returned equally spontaneously at a larger amplitude. A 0.002% adenosine bath concentration caused only a reduction of peristaltic wave amplitude in rabbit gut sections, not a total inhibition. Since their interest centred around the cardiovascular effect, the importance of intestinal responses, which they had noticed, was not then fully recognised.

An important phase in the search for the inhibitory intestinal transmitter began when Burnstock et al. (1970) found that the myenteric plexus of turkey gizzards released adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) upon stimulation. In guinea-pig taenia coli, as well as in twelve other

different types of gut segments among a number of drugs tested ATP and ADP produced the greatest inhibition. Tachyphylaxis to ATP depressed the non-adrenergic inhibition in rabbit ileum sections, while adrenergic responses remained untouched. These experiments led to their claim that ATP or a related nucleotide was the non-adrenergic inhibitory transmitter which was disputed by Crema et al. (1982). They found that colon segments - usually from mice - were not as responsive to ATP as were ileal preparations, and the former also lacked the biphasic response exhibited by the latter. They explained this difference with a longer diffusion pathway, allowing enzymatic breakdown.

Maguire and Satchell (1981) reported that conflicting results had been obtained in response to purine application. They believed that observed results were often interpreted wrongly because it was not realized that both muscle layers of the lamina muscularis may have responded and that the observed response was due to a composite effect. Putative antagonists: Quinicline, imidazole, methylxanthines, 2,2 pyridylisatogen, and apamine were all found to lack specificity. The rapid inactivation of ATP at receptors was explained by dephosphorylation to adenosine, and subsequent adenosine-uptake.

Cytochemical studies and ultrastructural identification techniques employed by Burnstock (1981, 1982a) appeared to demonstrate that ATP is the most likely candidate responsible for the descending inhibition during peristalsis, receptive relaxation of the stomach, and reflex opening of sphincters. Two types of purinoceptors P_1 and P_2 as well as subtypes appear to exist (Burnstock and Kennedy, 1985). P_1 receptors, acting through an adenylate cyclase system, are selectively activated by

adenosine and AMP, while the P_2 receptors are selective for ATP and ADP (Burnstock, 1983). However, the 'simplistic' single transmitter (Dale's principle) hypothesis has had to be revised and replaced by the 'co-transmission' theory. Thus coexistence of ATP and a prostaglandin activator was suggested by Burnstock (1981). Co-storage and release of ATP and noradrenaline or acetylcholine was put forward by Burnstock (1982b), and in 1984 Sneddon et al. proposed that ATP and noradrenaline acted as co-transmitters in some sympathetic nerves. Gustafsson et al. (1985) found that neurotransmission was inhibited in the guinea-pig ileum prejunctionally by adenosine. They concluded that it is "... a powerful modulator of autonomic neurotransmission by way of inhibition of transmitter release..."

5-hydroxytryptamine (5-HT), a 'sensory stimulant', was tested by Bülbring and Crema (1958) for its effects on the guinea-pig ileum. Using a new gut bath technique (Bülbring et al., 1958a), they found that 5-HT applied to the mucosa facilitated the peristaltic reflex by 'sensory receptor stimulation'. Applied to the serosa, 5-HT stopped peristalsis, an effect which in turn could be abolished by 'ganglion block'. Similarly, Bülbring and Lin (1958) reported that intraluminal application of low doses of 5-HT decreased the peristaltic threshold and increased the peristaltic wave frequency, as well as the volume transported. Hukuhara et al. (1960b) found, using two recording balloons in dog small intestine loops, that 5-HT applied to the mucosa or serosa elicited the intrinsic reflex, contraction above and inhibition below the site of stimulation.

Gershon et al. (1965), after observing that 5-HT stimulated peristalsis by triggering mucosal receptors, showed autoradiographically and with

results from radioactive release measurements, that the myenteric plexus is the only place to synthesize and bind 5-HT in the small intestine of mice. They suggested that 5-HT interneurones link sensory with motor neurones in the peristaltic reflex pathway.

Fishlock and Parks (1966) recognized different effects of 5-HT in the human intestines: the taenia coli and the circular muscle of the lower bowel relaxed in the presence of 5-HT, whilst the terminal ileum contracted. These responses could be completely abolished by lysergic acid diethylamide (LSD). Furness and Costa (1982b) found 5-HT neurones - 'polarized in the anal direction' - were possibly acting as interneurones connected with the descending inhibition.

Another avenue explored by Hobbiger (1958) was that gamma- aminobutyric acid (GABA), a normal constituent of the mammalian brain, appeared to have certain antagonistic properties in the enteric system. Besides antagonizing histamine and nicotine it also blocked contractions due to 5-HT in the guinea-pig ileum. GABA seemed to have many characteristics in common with the antagonism produced by morphine. Inouye et al. (1960), studying this antagonism, also recognized that GABA occasionally produced stimulation followed by relaxation, which could be abolished by atropine, LSD and picrotoxin. Peristalsis was blocked or depressed by GABA in the guinea-pig ileum. They suggested a close correlation between GABA and tryptamine receptors on neurones in the myenteric plexus. Curtis and Johnston (1974) recognized GABA as being an inhibitory neurotransmitter in the vertebrate central nervous system, which acts by raising the membrane chloride conductance causing hyperpolarization close to the

chloride equilibrium potential. This partially counteracts the effect of increased cation permeability, due to the excitatory transmitter activity, thereby reducing the chances of depolarization and thus consequently blocking or reducing excitation (Brown, 1979).

Krantis and Kerr (1981a) were able to block GABA elicited contractions of guinea-pig ileal sections with bicuculline (a reversible GABA antagonist) and also with picrotoxinin (a chloride ion transport blocker). Their conclusion that this GABA excitation-stimulating action was due to intrinsic cholinergic nerves, would suggest - according to the previously described mechanism - that an inhibitor acting presynaptically on cholinergic neurones was blocked by GABA. The same researchers found (1981b) that GABA-antagonism by bicuculline or tachyphylaxis reduced intestinal motility and the amplitude of the contractions involved in pellet propulsion. Thus the physiological effect of GABA appeared to be regulation of intestinal motility.

Bowery et al. (1981) distinguished between two GABA receptor sites: $GABA_A$ -receptors, for which GABA and muscimol are agonists, blocked by bicuculline and responsible for an increased chloride-ion conductance, and $GABA_B$ -receptors, for which GABA and baclofen are agonists, that lead to a bicuculline insensitive, chloride-ion independent, reduction in transmitter release. In the ileum, the former mediate a contractile response, whilst the latter bring about a depression of electrically induced twitch responses (Krantis et al., 1980; Giotti et al., 1983; Ong and Kerr, 1983a). Also Cherubini and North (1985) showed that benzodiazepines potentiate $GABA_A$ -receptor mediated actions on myenteric

neurones. Furthermore, Kerr and Krantis (1983) showed that electrical stimulation causes a calcium dependent release of tritiated GABA from isolated guinea-pig longitudinal-muscle myenteric plexus preparations which have been incubated with [^{3}H] - GABA, and that neural elements accumulating [^{3}H] - GABA could be revealed in dissected myenteric plexus preparations (Krantis and Kerr, 1981c).

More evidence that GABA is a neurotransmitter of the autonomic nervous system was provided by Jessen et al. (1983). They measured tritiated GABA release from myenteric plexus neurone cultures and they also showed, using autoradiographic methods, [^{3}H]-GABA labelling of ganglia and axons projecting into the gut musculature, results which led them to the conclusion that a percentage of the population of myenteric neurones was GABAergic. They postulated that these neurones were concerned with the 'presynaptic modulation of transmitter release'. Ong and Kerr (1984) found that naturally occurring rhythmic relaxations of guinea-pig ileal longitudinal muscles, a neurally mediated effect, were antagonized by bicuculline and pictrotoxinin as well as GABA desensitization. They concluded that this inhibitory response produced via $GABA_A$ - receptors is added proof of GABA's role of inhibitory neurotransmitter in the enteric nervous system, as is suggested by alterations in peristalsis resulting from GABA-antagonism (Krantis and Kerr, 1981b; Ong and Kerr, 1983a). According to Minano et al. (1985), GABA plays a major role in gastric cytoprotection. It was also shown by Tanaka and Taniyama (1985) that substance P (SP) will release GABA from the myenteric plexus of the guinea-pig small intestine.

It is believed that peptides may control muscle tone and contractillity of the isolated mouse colon by various interdependent mechanisms, including neurogenic cholinergic activity, neurogenic non-adrenergic, non-cholinergic activity and local prostaglandin synthesis (Fontaine and Lebrun, 1985). This important group of chemicals involved in the enteric system, is the neuroactive peptides, 16 of which were tested for their biological activity and location in the gut by Furness and Costa (1982a). The number of putative neurotransmitters suggested for the enteric nervous system, reflecting its tremendous complexity, is not surprising since they control or modulate at least 12 gastrointestinal reflexes (Rattan, 1981). The distribution of neuroactive peptides in the gastrointestinal tract, although mainly in the mucosa, was shown by Keast et al. (1985), the most prominent putative peptidergic neurotransmitters being glucagon, neurotensin, SP, vasoactive intestinal peptide (VIP), and enkephalins (dynorphin).

It is claimed that glucagon inhibits gastrointestinal, especially colonic, motility, but, intravenous doses of porcine glucagon (Evans et al., 1982) cause intense electrical activity resulting in circular muscle contractions and longitudinal muscle relaxations in dogs. Holmgren et al. (1982) using immunohistochemical methods found glucagon reactivity in intestinal and gastric endocrine cells, but none in the nerve fibres of rainbow trout intestines, while Morley and Levine (1985) found that glucagon was mainly involved in the peripheral satiety regulation.

Neurotensin was first purified from bovine hypothalami, and its amino acid sequence, established by Carraway and Leeman (1975a, b). In vivo experiments, by Hellström and Rosell (1981) on anaesthetized cats, revealed that, at high doses, neurotensin raised the muscle tone but

increased antiperistaltic motility of the proximal colon, whilst low neurotensin doses stimulated colonic peristalsis. Similarly, Fontaine and Lebrun (1985) saw that low neurotensin concentrations caused neurogenic non-cholinergic contractions, whereas high concentrations initiated a biphasic response, neurogenic relaxations and myogenic contractions, in the mouse colon. Eysselein (1984), however, maintains that the only gastrointestinal neurotensin effects known for certain are that it depresses peristaltic motility and that it causes rat fundus muscle contractions (Huidobro-Toro and Kullak, 1985), responses not blocked by atropine, hexamethonium, or methysergide.

Substance P (SP), a peptide first discovered by von Euler in 1931, is found in most parts of the enteric system but is most abundant in the colon. In anaesthetized cats it selectively and powerfully stimulates contractions in the distal colon at low doses, while a dosage increase initiates strong contractions of the proximal colon as well (Hellström and Rosell, 1981; Garzon et al., 1985). Couture et al. (1981) reported similar results for the human colon when they found SP receptor specificity mediating stimulatory action in the human colon. Immunohistochemical studies by Furness and Costa (1982a, 1982b), Costa and Furness (1982), Costa et al. (1981) and Dalsgaard et al. (1982), proved that in a number of mammalian species, SP neurones were present in specific populations of intrinsic and extrinsic enteric neurones (Bailey et al., 1982), having the possible role of a excitatory neurotransmitter. It was claimed by Bartho et al. (1982) and Dinoso and Murphy (1984) that SP was the atropine-resistant peristalsis initiator of the guinea-pig ileum. North (1982), using electro- physiological techniques, saw in guinea-pig myenteric nerve cells, that SP at very low concentrations caused depolarization resulting in slow excitatory

postsynaptic potentials (EPSP). These were 'slow in onset and time course' and could be abolished by SP desensitization. Donnerer et al. (1984a, b) concluded that SP is a likely neurotransmitter in the gut, coordinating the peristaltic reflex, but showed by the use of capsaicin that the stimulatory effect of capsaicin due to SP release from sensory nerve endings.

VIP, however, was soon to become very important in peristaltic research. It was believed by Rattan (1981) to be one of the inhibitory transmitters involved in regulating the 12 different reflexes of the enteric system. Said and Mutt (1970), the first researchers to isolate VIP from the small intestine of a dog, called it a 'polypeptide with broad biological activity'. They mainly tested its cardiovascular, respiratory and glycemic effects. Jaffer et al. (1974), working on guinea-pig ileal and duodenal longitudinal muscles with synthetic VIP, discovered that it had a contractile effect quite in contrast to its relaxant action on colonic muscles.

Furness and Costa (1979), however, testing and tracing VIP-immunoreactivity from the guinea-pig small intestine myenteric plexus along its anally directed projections in the circular muscle layer,concluded that these findings are consistent with VIP's being the transmitter of enteric inhibitory neurones. Isolated longitudinal and circular human colonic muscle strips relaxed after VIP application, according to Couture et al. (1981). However, their average dose of 10^{-7} appeares to be quite high for a peptide. Electrophysiological evidence by North (1982) also showed that VIP excites myenteric neurones at very low (pM) concentrations, but, on the basis of its distribution, VIP may be a candidate for the role of enteric inhibitory transmitter, possibly

also responsible for the slow EPSP. Fahrenkrug and Emson (1982) believe that VIP stimulates AMP formation and that VIP neurones represent the non-cholinergic, non-adrenergic descending inhibitory neurones causing circular muscle relaxation 'on the anal side' of the peristaltic wave. Angel et al. (1982), however, declared VIP to be the main neurotransmitter of the muscularis mucosae in the canine gastrointestinal tract, while Zafirov et al. (1985) also found that VIP excited myenteric neurones.

Bennett et al. (1984) proposed a counteractive system, based on their findings, that VIP functions as a non-cholinergic, non-adrenergic inhibitor on isolated guinea-pig colon circular muscle strips but not on longitudinal muscles, which they speculated could be purinergically activated, a theory, supported by Grider et al. (1985) as well as Dinoso and Murphy (1984). Combining the above with the fact that ATP relaxes circular but contracts longitudinal tracheal muscle fibres, they arrived at the conclusion that both the purinergic and peptidergic hypotheses may be true. Both systems could be linked in diametrically opposed actions.

Another large group of peptides detected in the gastrointestinal tract encompasses the opioid peptides, which are thought to have a modulatory role primarily inhibitory in nature (Bardon and Ruckebusch, 1985a; Hellström, 1985). It was believed that opioids inhibit gut motility by reducing the cholinergic stimulatory response (Rattan, 1981; Costa and Furness, 1982). However, Hirning et al. (1985) and Vaught et al. (1985) reported canine in vivo and in vitro small intestinal motility enhancement due to opioid ligand application. Hughes (1983) and Hughes and Kosterlitz (1983) again described reports from 1957 that morphine

inhibits ACH release from guinea-pig enteric neurones and this has been shown to apply for all three main opioid peptide families: the endorphins, enkephalins and dynorphins (Kromer and Schmidt, 1982; Kromer et al., 1981a and 1981b).

In 1982 North, using electrophysiological techniques, discovered the hyperpolarizing effect of morphine, as well as other opiates and opioid peptides which at low concentrations (nM) lead to the inhibition of firing of myenteric neurones. This inhibition takes place in cell processes rather than in the soma itself, thus preventing the propagation of action potentials. The same researcher reported in 1983 that 25% of nerve cell bodies in the myenteric plexus of most enteric regions of many species show immunoreactivity to enkephalins and other opioid peptides. Opioid-immunoreactive axons are also abound in myenteric ganglia, as well as secondary and tertiary plexus, and the circular muscle layer. He also detected that continuous stimulation causes gradual peristaltic fatigue, which can be counteracted by naloxone, an opioid antagonist (also Kromer and Woinoff, 1980). A central influence of enkephalins on colonic motility was noticed by Fioramonti et al. (1985).
Hughes (1983) recognized a clear association between opioid release and stress or pain events (also Akil, 1976). Thus it may be that the role of opioid peptides was a protective mechanism against excessive stress or traumatic damage, rather than a vital factor of normal propulsive motility.

The last group of chemicals, of which the historical background and possible physiological role is to be examined, represents the

prostaglandins (PG). Von Euler (1935) first purified a compound from human - later monkey - prostate glands, this newly discovered chemical lowered the blood pressure and contracted longitudinal muscles of isolated gut sections of rabbits, cats and dogs. He named it 'prostaglandin'. In 1937 he described a second type of prostaglandin, which he called 'vesiglandin'. It also had a hypotensive but very weak intestinal effect..

Vogt (1949) recognized that frog vagus nerve stimulation increased gastric production of an atropine-resistant substance effective in the gut, which he called "Darmstoff" and believed to be related to "Substance P". This chemical was subsequently demonstrated to be a prostaglandin (type F and E) mixture. In the following years a number of different prostaglandins were isolated and purified. According to Bennett and Fleshler (1970), there are 16 naturally occurring prostaglandins in the four classes E, F, A and B, but, according to Morrell (1976), only 14 - six primary (3 PGEs and 3 PGFs) plus eight secondary prostaglandins, most of which had different effects on different systems. For PGE and PGF diametrically opposed functions are often observed.

With regard to peristalsis or intestinal motility, PGE, especially E_2, has been considered to have the greatest effect. However, the role of PGE in peristalsis has remained obscure, ranging from claims that it is part of an enzymatic negative feedback system involving hormonal blockage resulting in cyclic AMP release inhibition, to its being a major neurotransmitter involved in intestinal motility (Bennett and Fleshler, 1970). On the other hand, Bennett and Posner (1971) have suggested that

PGE is not at all essential to peristalsis, with only a modulatory role, namely to promote full peristaltic activity. But Sanders (1978) and Sanders and Ross (1978) claimed that PGE antagonists enhance motility or the amplitude of ACH and K^+ induced, as well as spontaneous, cat ileal circular muscle contractions. Electrophysiological studies by Sanders (1984b) supported the above conclusions. The opposing action: contraction of longitudinal muscles and relaxation of circular muscles of the gastrointestinal tract by PGE_2 became known as the 'prostaglandin effect'. Bennett et al. (1975), Bennett et al. (1968) and Bennett (1975) and Horton (1979), however, believed that this 'prostaglandin effect' is a result of a direct action of PG on the intestinal muscle.

Sanders (1984a) postulated that the prostaglandins act as second messengers at the smooth muscle cell level, involving Ca^{++} release, but was not certain on which side of the plasma membrane the receptors would have to be located. Weeks (1972) determined that the prostaglandin effect could neither be blocked by anticholinergic, anti-adrenergic substances nor by TTX, morphine or histamine antagonists; then again Sanner (1982) reported that atropine and morphine decreased the PGE effect on ileal longitudinal muscles. It was also claimed by Burks and Northway (1982) that the PGs ability to contract intestinal smooth muscle was mainly due to a neural interaction involving cholinergic neurones, because it could be greatly reduced by atropine or TTX application. Furthermore, the PGE_2 mediated inhibition of noradrenaline release in the rat hypothalamus, as well as the presynaptic inhibition of splenic or cardiac noradrenaline release by PGE, was a neural effect (Dray and Heaulme, 1984). PG synthesis blockers inhibit neurotensin's colonic motility enhancing

effect (Bardon and Ruckebusch, 1985b), while PG inhibits the feeding drive by a central mechanism (Morley and Levine, 1985). This was supported by the findings that functions of PGE_2 include the centrally mediated anorectic and gastrointestinal motor effect observed in rats by Fargeas and Fioramonti (1984) and Bueno et al. (1985), as well as a cytoprotective element, which is not understood (Satoh et al., 1984; Lacy and Ito, 1982). Thor et al. (1985) found that prostaglandins inhibit intestinal motility, yet Wienbeck and Sperling (1984) reported contractile effects of PGE_2 and F_2 on feline colonic circular muscles at low concentrations. Cyclic AMP involvement in the PG response is unresolved (Speelman et al., 1985). Nevertheless, it is obvious that the PGs and especially PGE_2 are important, possibly involved in propulsive motility and other events in the gastrointestinal tract.

After nearly a hundred years of research into intestinal propulsive motility, leading to the development of a great number of experimental techniques allowing the testing of a large variety of drugs, vast areas of the neurophysiology of the gut were still not fully explored. It is commonly agreed that peristalsis is controlled by an intrinsic reflex arc and that the excitatory transmitter is acetylcholine (ACH), but some of the unanswered questions are: What is anti-peristalsis, does it exist, does activity in longitudinal muscles functionally interact with that in circular muscles, why does the descending inhibition occur, and what is the transmitter substance responsible for it?

CHAPTER II

SELECTION OF METHODS AND DESIGN

INTRODUCTION

In order to find a reliable and reproducible method for simulating the peristaltic reflex in the guinea-pig colon, five major techniques, some of which had been used during the past 100 years of intestinal research, were tested. Most of these techniques and their development were described in the general introduction. Trendelenburg's intraluminal fluid distension method was slightly modified and tried out first. Then various types of transmural electrical stimulation systems were employed to record effects of different drugs on intestinal relaxation and contractions. Peristaltic activity resulting from wall distension by stretch was examined next, followed by a method where single artificial pellets or pellet chains were inserted into segments of isolated colon; their propulsion as well as the drug interference with propulsive forces and velocities was then tested. Finally, the first ever reported intestinal reflex testing procedure - intestinal wall distension by balloon inflation - was used with a number of modifications. Of these, electrical transmural stimulation and balloon distension stimulation were found to be the most suitable experimental procedures for the intended investigations. They were improved and adapted to experimental

requirements and then employed to examine the roles of various putative neurotransmitter substances.

Materials and Methods

Modified Trendelenburg's method

Three colonic segments were removed from guinea-pigs killed by a blow on the head and subsequent bleeding for each experiment. Six to eight centimetre long segments were excised, the first five cm from the anus (distal segment), the next five cm from the caecum (proximal segment), and the middle six to eight cm were cut out of the remaining length of colon (medial segment). These were all kept in a carbogen (5% O_2, 95% CO_2) aerated modified Krebs solution (appendix A) at 36°C until used.

Segments of the colon were attached to a frame in a carbogen aerated gut bath at a temperature between 35°C and 37°C. The oral and anal ends were tied over inlet and outlet tubes respectively. At first, two and subsequently three isotonic Harvard smooth muscle torque transducers (H 386) were attached to the gut by threads and small hooks placed in the serosa (fig. I). Intraluminal pressure was applied by raising the Krebs reservoir, and opening a tap in line with the gut section and the bottle. Pressure increases could then be quantified by recording the fluid rise in a fixed pipette also connected to the bottle by flexible plastic tubing. Relaxations and contractions of the gut were relayed via the transducers to a four channel Grass Polygraph (79 D) and recorded on paper.

Electrical transmural stimulation

In order to record circular muscle activity resulting from electrical stimulation, a gut segment (approximately 6 cm long) was attached to a holding frame placed in a gut bath containing Krebs solution gassed with 95% O_2, 5% CO_2. Two torque transducers were connected to the intestinal segment serosally, one at the oral, the other at the anal end. In the centre, two semi-circular platinum electrodes 0.5 cm apart were in contact with the serosa of the intestinal loop (fig. IIa). Stimulation at various rates was effected by pulses from a Grass (S 48) stimulator, and the resulting activity was again recorded by the Grass Polygraph.

A flap (approximately 6 x 1.5 cm) produced by cutting the intestinal segment along its mesentery, was fixed in a frame and electrically stimulated on both sides. The resulting activity was recorded from longitudinal and circular muscle layers (fig. IIb).

Longitudinal muscle and circular muscle strip responses to electrical stimulation were obtained using four small individual gut baths, fitted in close proximity to each other. Pulses from stimulators (S 48) were delivered by platinum ring electrodes each set 0.5 cm apart. Resultant muscular activity was measured by Grass isometric force displacement transducers (FT 03 C) to which one end of the gut segment was tied, while the other end was fastened to the aeration tube at the bottom of the bath. Circular intestinal strips approximately 2 mm wide cut helically or in zig-zag fashion from colonic segments were tested in the same manner (fig. IIc). Responses were again recorded by a Grass Polygraph. Distal

ileal segments were also used in the above described apparatus during some of the later experiments. Furthermore, stimulation pulse parameters will be given in the respective results section, because these were changed to suit the neurotransmitter system under scrutiny.

Stretch distension

Stretch distension was employed in two different ways: firstly, by applying a pulling force with the use of weights (Furness and Costa, 1977). In this case, weight units were the quantifying criteria. Secondly, pull was exerted by stretch where the quantifying criterion was the linear-distance measurement (Kosterlitz and Watt, 1975a). In each method, the same arrangement was made, where each segment was attached to the frame by two hooks, above which the isotonic torque transducers were fixed to the serosa (Davison and Pearson, 1979). In the centre of the gut segment, the base of a triangular shaped wire holder (1 cm) was inserted, the lowest point of which was then fastened to the holding frame below, whilst an identical triangular holder was inserted into the colon section directly above the first one. To the apex of this holder was attached a thread to which either weights or a micro-advance stretching mechanism could be tied. In order to measure displacement due to stretch, another torque transducer, calibrated to record linear distance deflections, was also connected to the upper triangle (fig. IIIa).

However, to determine whether stretch distension was more effective when exerted on a localised area or large tissue section the following two alterations to the above method were employed. In the first experiment a gut section had its centre (2 cm) cut into a flap but this remained attached to approximately 2.5 cm of intact gut on either side. Both these tubular intestinal sections were secured to the frame by hooks and had the transducers attached as before. The bottom part of the flap was held to the frame by a wire triangle and the stretch was effected as explained before (fig. IIIb).

The second idea was to examine localized stimulation, which involved sewing the centre (2 cm) of the gut section into a wire cradle exposing approximately 3 mm of gut surface between the two cradle attachments; stretch with the previously described upper wire triangle was applied there (fig. IIIc). Results obtained by these stretch methods were recorded via torque transducers by the Grass Polygraph.

Bolus distension stimulation

To mimic peristalsis, boluses of three different sizes resembling natural guinea-pig pellets as closely as possible, were moulded from silastic rubber. The average (n25) adult male pellet size had been measured to be 6.2 mm x 16.9mm (artificial pellets 6 mm x 16.5mm) while average female pellet size was 5.3 mm x 13.2mm (artificial pellets 5 mm x 13mm). A third artificial pellet size (5.5 mm x 15mm) was used for small male animals. The oral end of the gut section was tied over a small plastic funnel.

Alternatively, oral and anal ends of the segments were again fixed to the frame with recording attachments directly above them. Single artificial pellets were entered into the gut through a funnel and at the oral end, their propulsion velocity was recorded. In order to overcome problems of jamming and stopping encountered with single pellets, chains of ten or twelve pellets were tied together and introduced into the intestinal segment. To get consecutive propulsion velocity readings, the first pellet of a chain was reinserted after the last pellet had entered the colon segment, thus maintaining peristaltic activity for up to some hours (fig. IVb). The effects of various chemicals on propulsion velocity could then be tested and noted.

Furthermore, the same gut arrangement was used with a single artificial pellet tied to an isometric force transducer (Ishizawa and Miyazaki, 1975), when this pellet was introduced into the test segment, this was found to be an efficient technique to elucidate the effect of drugs on the force exerted by the segment during propulsion of the bolus (fig. IVa).

Balloon distension stimulation

However, some of the techniques mentioned were either too removed from naturally occurring events during peristalsis, or else could not be initiated in a regular and repetitive manner. This problem was overcome by the use of inflatable balloons (Frigo and Lecchini, 1970), with Fogarty arterial embolectomy catheters connected to a saline filled

syringe inserted into gut segments which in turn were attached to a frame as explained before. The balloon at the tip of the catheter was then inflated by moving the plunger of the syringe forward, and subsequently deflated by returning the plunger to its original position, the plunger movement being automated with the use of a timer-controlled solenoid system (fig. V). Three frames with gut segments and catheters were activated synchronously, thus permitting simultaneous comparison of activity of the three gut sections removed from different parts of the colon. A Statham (P 23 AC) pressure transducer (0 to 75 cm Hg) was initially placed in line with the catheter in order to quantify the applied pressure, and to adjust the degree of distension necessary to obtain the desired effect. In guinea-pig colon experiments catheters AE3F (green) and AE4F (red) were used. Rabbit gut sections were distended with AE5F (white) and AE6F (blue) catheters. Table V gives the experimentally determined relationship between inflation volumes and the resultant degree of distension.

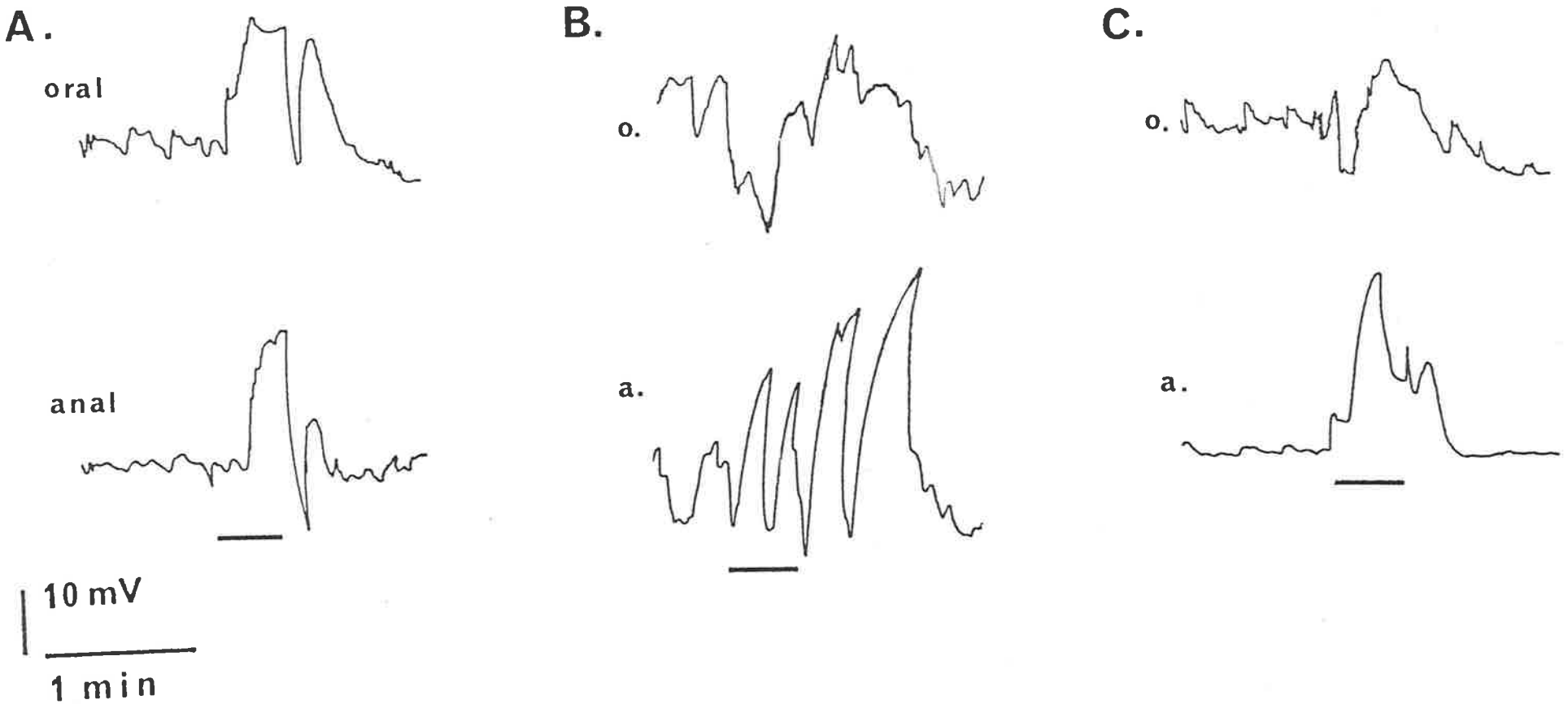
In order to determine whether the whole circular and longitudinal neural network of the myenteric plexus was involved in stimulus transmission in the oral or anal direction, approximately 90% of the centre 2 cm of the gut section was removed leaving only a thin strip (approx. 1 mm) of gut connecting the oral and anal intact gut sections. The distension balloon was inserted and inflated, first in the oral then in the anal section. Responses were each time recorded at the opposite section.

RESULTS

Trendelenburg's method

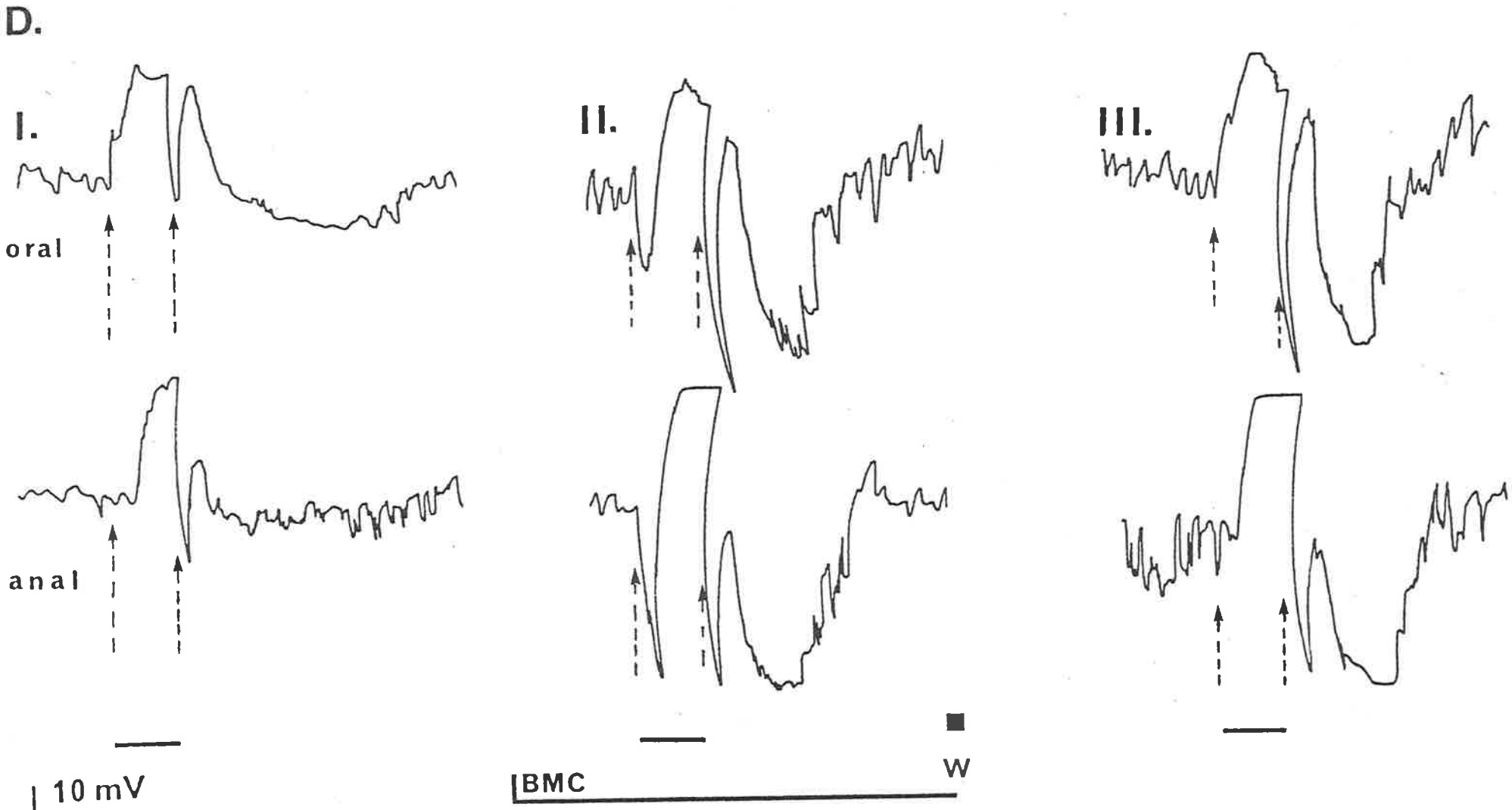
In experiments using the Trendelenburg method on the isolated colon the most commonly observed effects were an immediate contraction of the circular muscle at the oral (input) end following fluid influx, a sharply defined relaxation as soon as the intraluminal pressure was reduced, and a subsequent (rebound) quick contraction which preceded a slow shallow relaxation. The effect at the anal end differed basically only in the delayed onset (10 s) of the first contraction (fig. 1A). Quite often, during longer stimulation periods, an oral relaxation followed by a contraction was noted instead, while the anal end alternated between contractions and relaxations in an apparent pumping action (fig. 1B). On other occasions, the anal end remained contracted while a relaxation preceded a weak contraction at the oral end (fig. 1C).

Addition of bicuculline (5×10^{-6}M) synchronized both ends, so that they each relaxed abruptly at the onset of stimulation by fluid influx, then contracted strongly, and again relaxed sharply when stimulation was terminated. This was followed by another contraction - relaxation complex. Figure 1D shows a recording of this effect.



Effects of fluid distension stimulation (Trendelenburg's method) of 25 s on the oral (o.) and anal (a.) circular colonic muscle

Fig. 1 (A.-C.)



The effect of bicuculline (5×10^{-6} M) on the colonic response during a 25s (bars) Krebs infusions using the Trendelenburg method. (BMC = bicuculline w = wash)

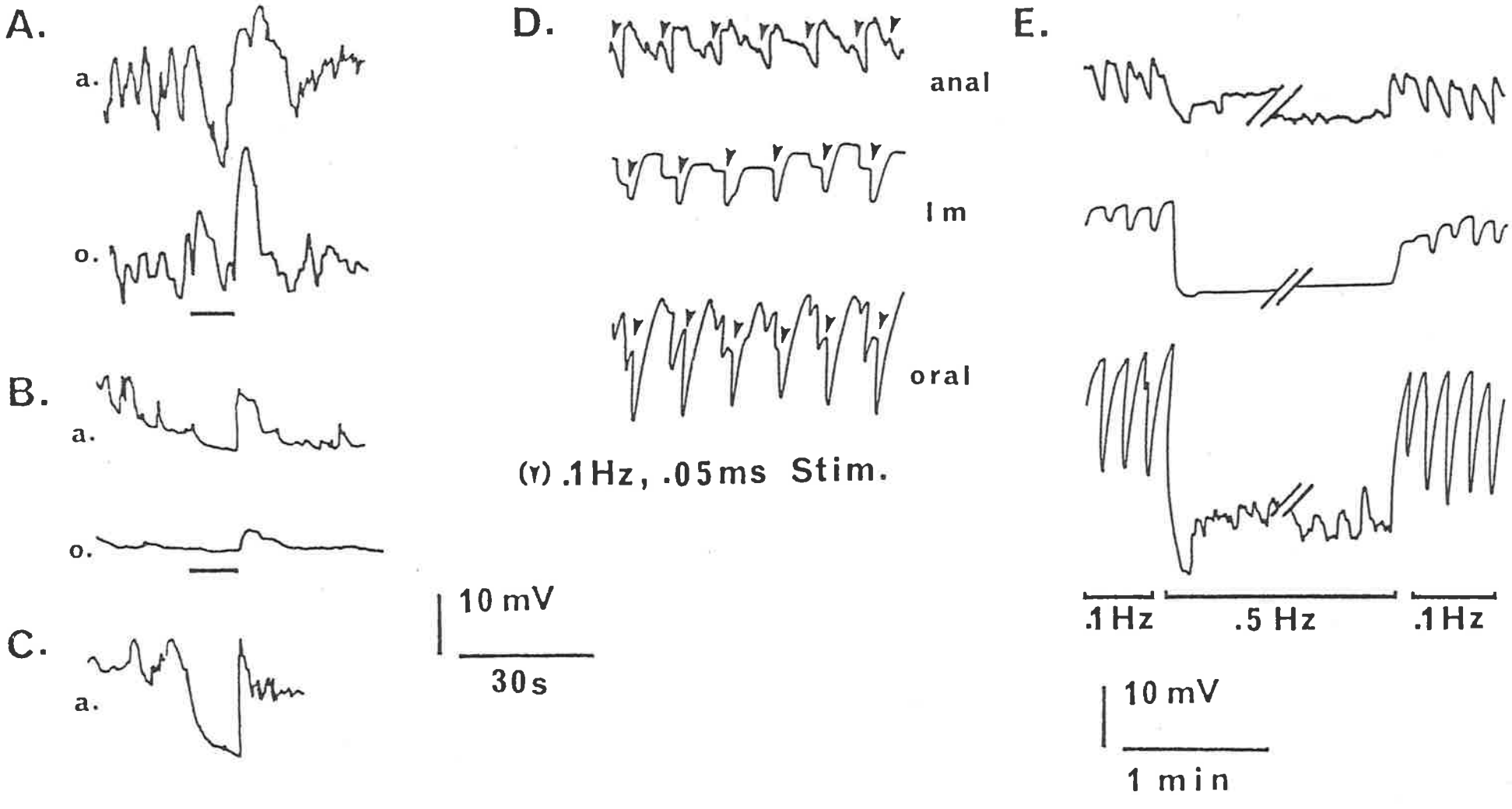
Fig. 1 D.

Transmural electrical stimulation

In whole gut segments, 1 ms pulses of 10 Hz at 50 V for 10 s produced oral contraction consistently, and an anal relaxation (fig. 2A), which at times changed to a contraction, but often returned as a relaxation. Atropine (10^{-6}M) abolished contractions completely but only reduced relaxations (fig. 2B). After a wash and subsequent histamine addition to the bath (10^{-7}M) resulting in an elevated tone, anal inhibition occurred at previous levels against the increased tone, while the oral contractions remained absent or were converted to small relaxations (fig. 2C).

When an intestinal flap was stimulated with electrodes on either side at 0.05 to o.1 Hz (1 ms, 50V), continuously repeatable and synchronized inhibitory responses were initiated (fig. 2D). Stimulation in excess of 0.2 Hz, i.e. 0.5 Hz (1 ms, 50 V) resulted in continuous inhibition of circular and longitudinal muscle (lm) for the duration of the stimulation period, which returned to the previous oscillating pattern when returned to the 0.1 Hz frequency (fig. 2E).

Figures D and E display anal circular (anal), central longitudinal (lm), and oral circular (oral) muscle responses. Tropicamide (3.6×10^{-6}M) reduced the amplitude of the inhibitory responses. Guanethidine (3×10^{-6}M) and propranolol (5×10^{-7}M) did not alter the inhibition due to electrical stimulation of circular or longitudinal intestinal muscles in either of the two above experiments.



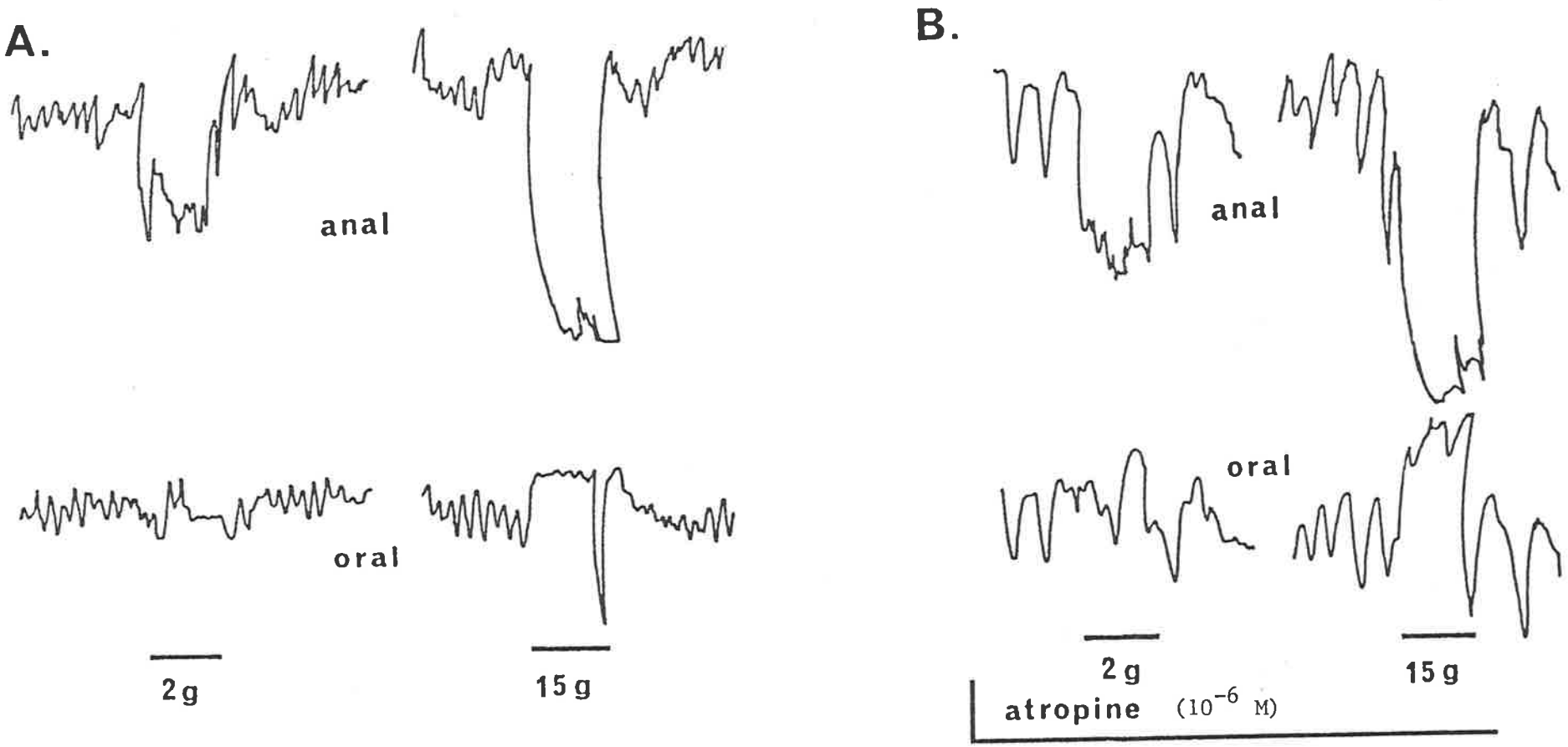
Transmural electrical stimulation of the colon at varying frequencies and durations

Fig. 2 (A.-E.)

Stretch stimulation

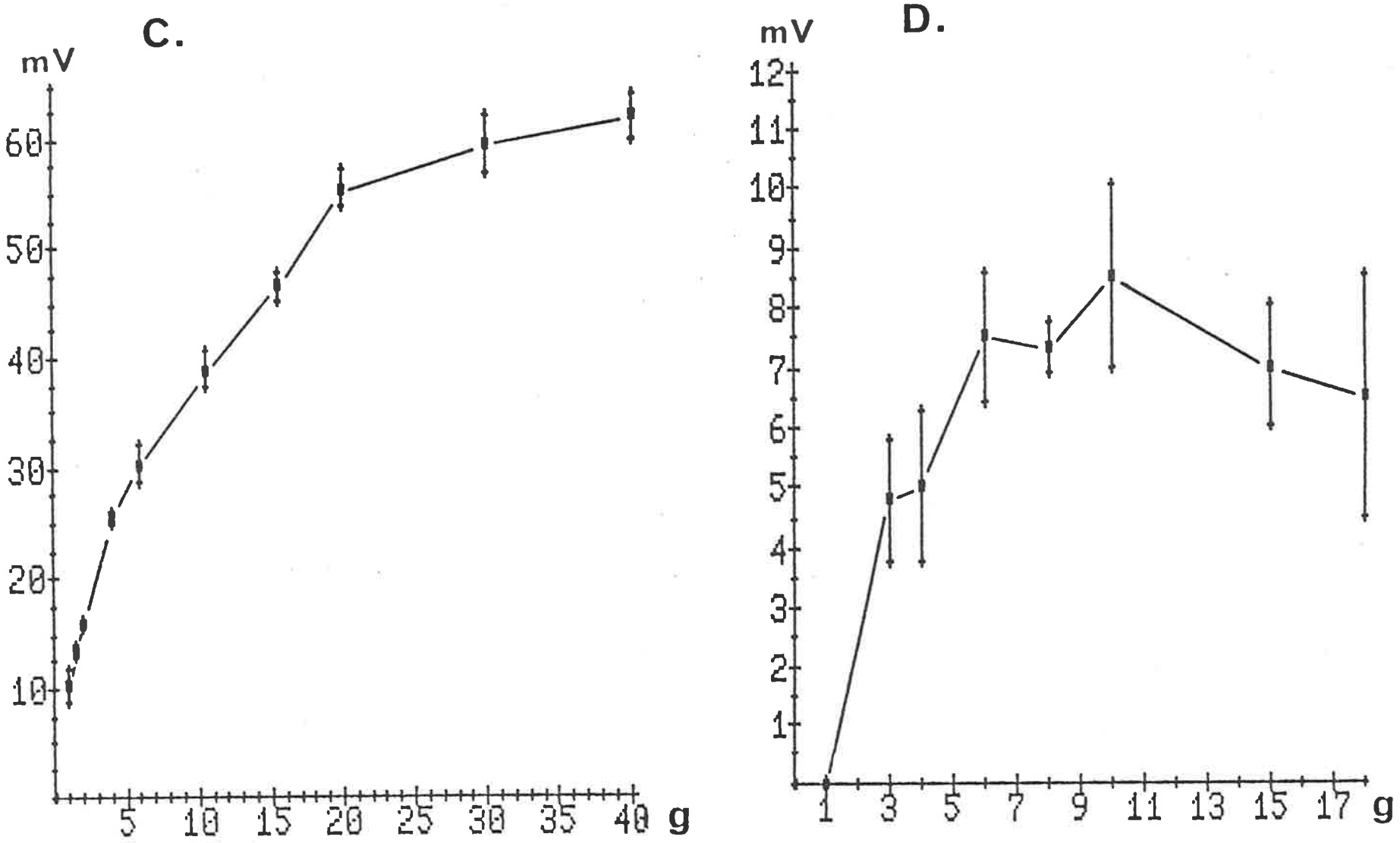
Distension caused by 2 to 15 g weights resulted in clearly defined relaxations of anal circular muscles. Although initially oral contractions were present, they gradually decreased in size. Atropine (10^{-6}M) did not influence the anal relaxation, but reinstated oral contractions (fig. 3A, 3B). In some experiments circular muscle relaxation was observed at both the anal and oral end. If a small weight (2 g) followed a heavy weight (15 g), the response to the small weight application was much larger than that followed a smaller weight, or when it had been applied repetitiously by itself. Subsequent use of the same sequence of weights resulted also in a proportionally greater response for each individual weight. If the response to increasing weight stimulation was plotted, the result was a curve which rose rapidly between 0 and 4 g and then started to plateau after 20 g (fig. 3C, table I).

Flap stretch stimulation produced only small anal and oral relaxations after approximately 3 hours of equilibration and intermittent stimulation. These responses then disappeared after some 30 min, and no further response could be elicited. Point stretch stimulation, at low weights, resulted in clearly defined relaxation responses anally. These responses appeared to plateau at 6 g, and the effect of 18 g was smaller than the one observed for 6 g (fig. 3D, table II). Oral contractions were unreliable at lower weight applications but appeared regularly when 15 or 18 g were used. The addition of atropine (10^{-6}M) or naloxone (2.5×10^{-7}M) to the bath did not significantly alter the anal relaxation,



Effects of weight distension stimulation on anal and oral colonic circular muscles

Fig. 3 (A., B.)



Stretch stimulation caused, weight dependent degree of descending inhibition

(C. whole colon segment, D. point stimulation)

Fig. 3 (C., D.)

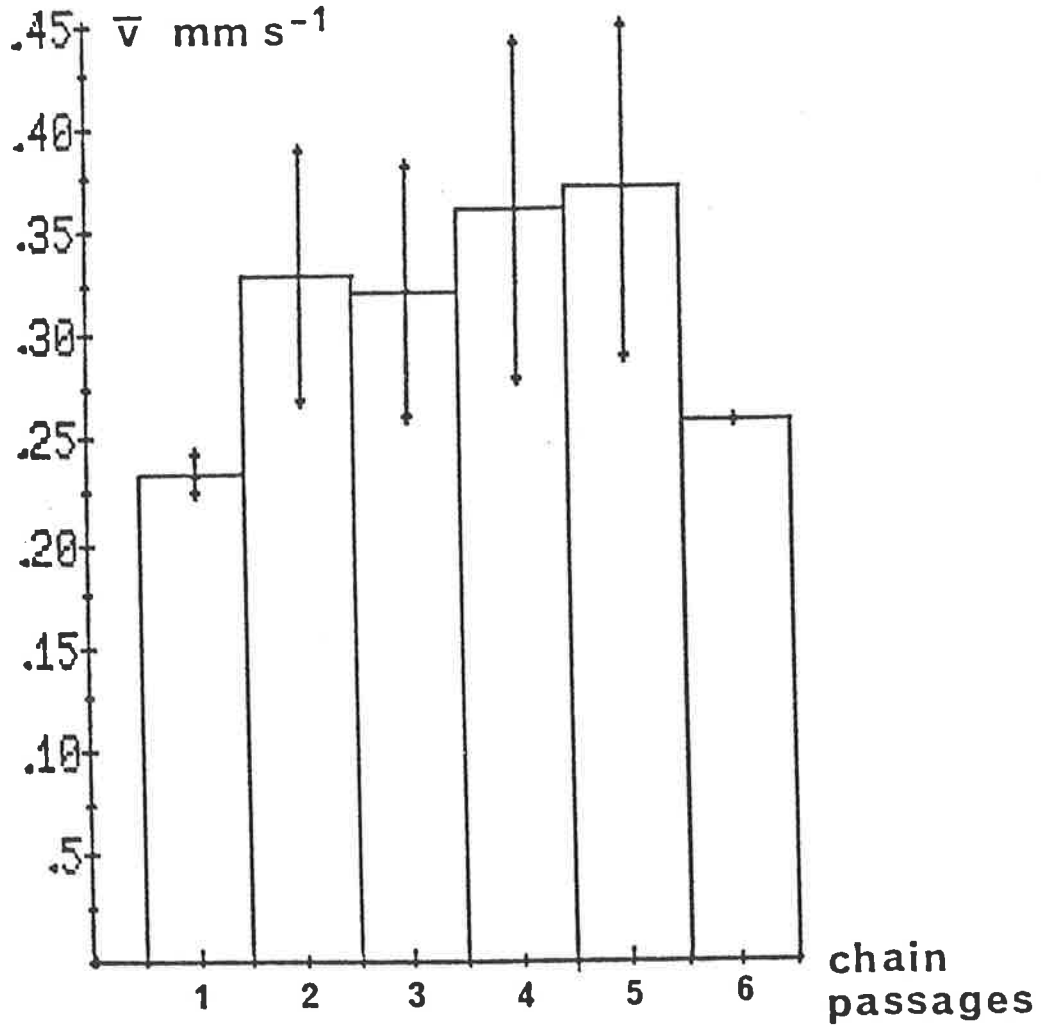
but a large contraction appeared following this relaxation in the presence of naloxone.

Stretch stimulation, by distending the intestinal lumen a certain distance, gave similar results to those with weight distension, the same stretch distance as that measured during weight distension stretch resulted in very similar responses. However, when this stretch distension stimulation was applied while relaxant or stimulant chemicals were added to the bath, responses became proportionally more pronounced.

Bolus distension stimulation:

The propulsive velocity of a single artificial pellet appeared to be remarkably constant. At the beginning of most bolus distension experiments, a single artificial pellet was passed through the gut segment. The mean velocity of a single pellet, averaging results from 22 experiments, was 0.65 (± 0.13) mm s^{-1}. Only pellets moving without hinderance were included in these results. The propulsion velocities of three passages of the pellet chain, consisting of 10 pellets each, through a colonic section were averaged and subseqently used as control values, the average velocity (n36) being 0.26 (± 0.05) mm s^{-1}. Mean velocities (n5) of six consecutive chain passages are depicted in figure 4A, table III. The three fastest consecutive pellets of a chain passage were also evaluated and it was found that responses to drug or other outside influences were the same or very similar. Various chemicals,

Fig. 4 A.



Averaged velocities (± S.E.M.) of a number of chain passages (n 17)

added to the bath, altered the propulsion velocities (table 1). An example of the effects of two doses of naloxone was shown in figure 4B, table IV. The correlation between oral and anal circular and centre longitudinal muscle events is shown in figure 4C (see discussion).

Experiments involving a single artificial pellet attached to an isometric force transducer were used to examine and evaluate the sequential functions of the two muscle coats, and the interference with these functions by various drugs. The pulling force - usually 15 to 25 g - exerted on the pellet and recorded via a force transducer appeared to be directly related to the longitudinal muscle contractions. The strongest pull registered was 39 g, 3 mm, 3 s respresenting, according to:

$$\text{Newton (N)} = \frac{\text{m x kg}}{\text{s}^2} \quad \text{a force of } 1.3 \times 10^{-5}\text{N}$$

although the average force, which the colon segment could exert continuously for more than 8 hours, was about 5×10^{-6} N. Means of six experiments showed that the peak output of 18 (± 3.2) g at 1 contraction per 4.5 (±1.3) minutes was reached between 5 and 7 hours after excision of the colon segment. An hour later its performance had decreased to 15.4 (± 2.9) g at 1 contraction per 18 (± 3.8) min, followed by 10.4 (± 3.1) g at 1 contraction per 27.3 (± 5.8) min 9 hours after excision. The temperature range for optimal results was quite narrow; below 34°C and above 37°C the pulling force diminished rapidly. At 31°C and 41°C no effective pull was exerted; although circular muscle contractions continued, longitudinal muscle movement had ceased.

Table 1

Drug interference with pellet propulsion velocity during chain passage experiments (n 6)

Drug	Dosage (M)	V *
Adrenaline	10^{-7}	82
	10^{-6}	14
Noradrenaline	10^{-7}	46
	5×10^{-6}	0
GABA	10^{-6}	104
	5×10^{-6}	18
	10^{-5}	0
3-Aminopropane-sulfonic Acid	5×10^{-6}	74
	10^{-5}	16
	10^{-4}	0
Baclofen	5×10^{-7}	0
	10^{-4}	0
Bicuculline	10^{-6}	0
ACH	5×10^{-6}	128
	10^{-5}	235
Carbachol	10^{-6}	148
	5×10^{-6}	256
Tropicamide	10^{-6}	0
Hexamethonium	5×10^{-5}	0
5-HT	10^{-6}	0
	10^{-5}	0
Morphine	10^{-7}	0
	10^{-6}	0
Naloxone	1.5×10^{-8}	106
	5×10^{-8}	92
	5×10^{-7}	14
SP	4×10^{-9}	87
	10^{-8}	0
TTX	10^{6}	0

* pellet propulsion velocity (mm/s) expressed as percent of control

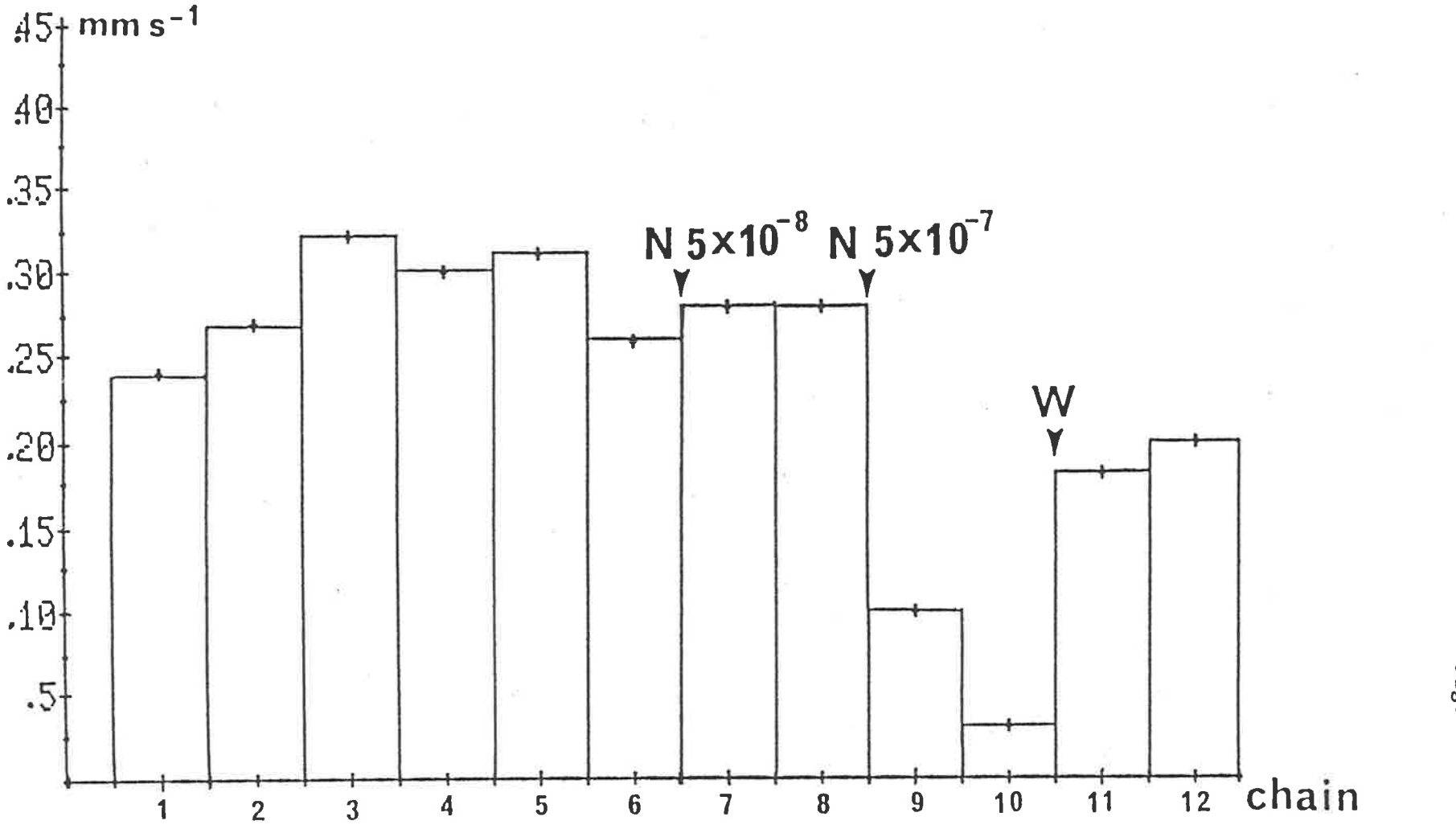
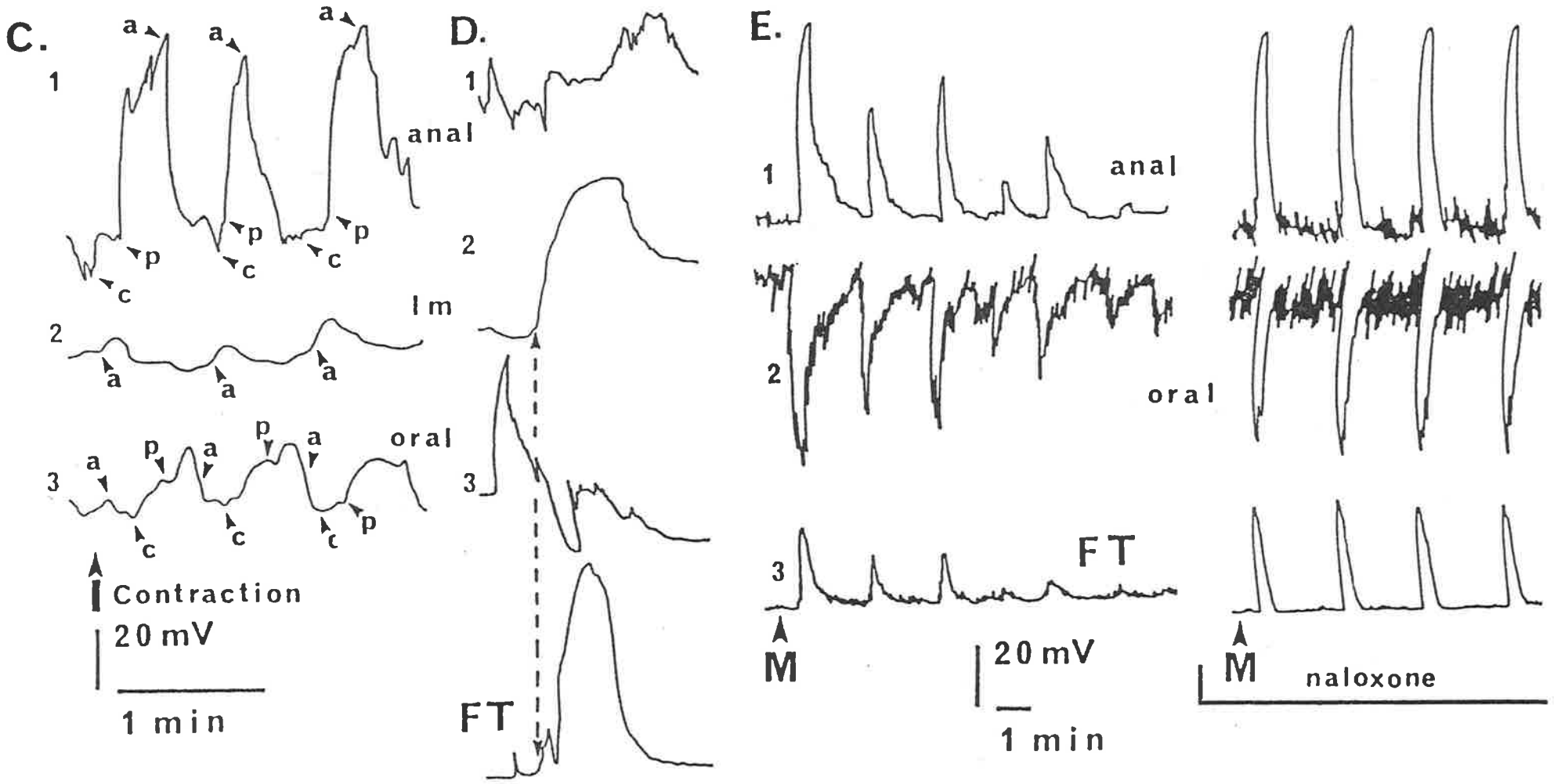


Fig. 4 B.

A typical result, testing drug interference with colonic motility
(N = naloxone, W = wash)

Gut segment activity recordings (fig. 4D) showed that the approaching pellet caused an immediate oral circular muscle contraction, an anal circular muscle relaxation and a longitudinal muscle contraction towards the oral end. When the pellet centre (C) had reached the centre recording hook, the oral circular muscle tension had dropped to baseline levels while the longitudinal muscle contraction had reached a peak, after which it relaxed and the gut wall returned anally to its starting position. When the pellet passed (p) the oral circular and longitudinal muscle recording point, a relaxation below the baseline ensued in the circular muscle, while the longitudinal muscle gradually returned to its baseline. The initial anal circular muscle relaxation was followed by a slow contraction. The development of the pulling force always coincided with the longitudinal muscle contraction. Events that selectively influenced the longitudinal muscle response also influenced the pulling force (table 2). If the longitudinal muscle remained in a contracted state, but the circular muscles oscillated between contractions and relaxations, the pulling force recording was in synchrony with the longitudinal contraction. When 5-HT decreased the longitudinal contraction amplitude the pulling force was also reduced. In general, when drug effects were being studied, the three recording hooks were spaced 2.5 cm apart; the responses therefore appeared to be out of phase. An example is given showing morphine- naloxone interaction (fig. 4E), and the results of 55 experiments each lasting approximately 8 hours were represented in table 2.



Effects of the passage of an artificial pellet chain (C.);a single artificial pellet connected to a force transducer (D.); a typical drug interference trace (E.); (M = morphine, FT = force transducer, a = approaching, c = centre, P = passing of a pellet, M - $5x10^{-7}$ M, Naloxone - $5x10^{-8}$ M)

Fig. 4 (C.-E.)

Table 2

The effect of drugs recorded during single pellet force transducer experiments (n 5).

DRUG	Dosage (M)	Prop.* force	Fre-** quency	Contraction LM	Contraction CM
ACH	10^{-6}	–	–	–	↑
Carbachol	10^{-6} to 5×10^{-6}	↑	↑	↑	↑
Bethanechol	1.8×10^{-5}	↑	↑	↑	↑
Methacholine	1.8×10^{-5}	↑	↑	↑	↑
Tropicamide	7×10^{-7} to 7×10^{-5}	0	0	0	0
Hexamethonium	10^{-4}	↓	–	↓	↓
GABA	5×10^{-6}	–	–	–	–
	10^{-5}	–	–	–	–
Glutamic acid	7×10^{-5} to 10^{-4}	–	–	–	–
Bicuculline	10^{-7} to 10^{-6}	–	–	–	–
	2×10^{-5}	BLOCKED ALL FOR 7 MIN THEN CONTD. WEAKER			
Picrotoxinin	3×10^{-6}	↓	↓	↓	–
5HT	1.5×10^{-6}	↑	–	↑	–
	3×10^{-6}	–	–	–	–
	5×10^{-6}	↓	–	↓	–
	5×10^{-5}	0	0	0	0
Methysergide	5×10^{-5}	0	0	0	0
5HT/GABA	5×10^{-5}	INHIBITS 5-HT (5×10^{-6}) BLOCKING EFFECT			
Morphine	10^{-8}	↓	–	↓	–
	10^{-6}	↓	↓	↓	↓
	3×10^{-6}	↓	↓	↓	↓
Naloxone	5×10^{-8}	BLOCKED MORPHINE (5×10^{-7}) EFFECT			
	5×10^{-6}	0	0	0	0
Noradrenaline	5×10^{-8} to 10^{-7}	0	0	0	0
Adrenaline	10^{-7}	0	0	0	0
ADP	10^{-8} to 10^{-6}	–	–	–	–
	10^{-4}	–	↓	–	–
ATP	10^{-8} to 10^{-5}	–	–	–	–
	10^{-4}	↓	↓	↓	–
Angiotension II	1.6×10^{-9}	0	0	0	0
	8×10^{-9} to 10^{-8}	–	–	–	–
SP	10^{-10} to 10^{-8}	–	–	–	–
	3×10^{-8}	↑	–	↑	–

– = No change; ↑ = Increase; ↓ = Decrease; 0 = Complete inhibition.

* Prop. force = propulsion force exerted on the force transducer.

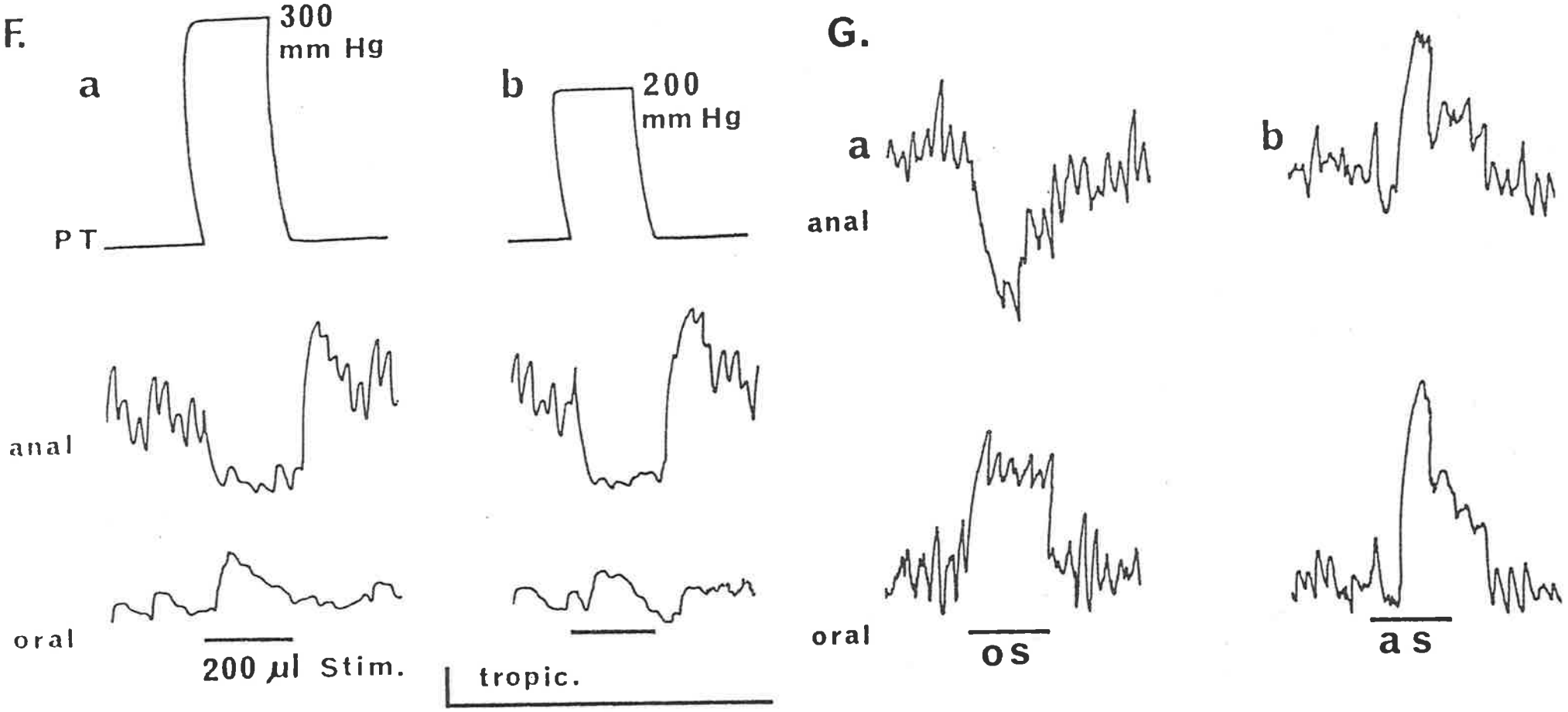
** Frequency = frequency of force exertion events.

Stimulation by balloon distension

The pressure transducer, in line with the syringe and catheter balloon, recorded pressure variations within the distended balloon due to change in muscle tonus of the gut segments (fig. 4F), especially in response to drugs, and in particular, those which did not alter the anal relaxation but abolished oral contractions. The pressure transducer was therefore useless as a means of quantifying stimulation. AE 3F (green) catheters distended with 200 ul to 5.8 x 6.5 mm and AE 4F (red) distended with 100 µl to 5.8 x 8 mm gave the most consistent stimulation results when using segments of the guinea-pig colon. All experimentally determined volume/ distension ratios for the Fogarty arterial embolectomy catheters may be seen in table V. Unfortunately, a 200 µl distension volume was the maximum recommended value for AE 3F catheters. Consequently they were very short lived and AE 4F (vol. max. 750 µl) were used since they had a much longer lifespan at 100 µl distension volume.

Colonic segments, in which only a narrow strip connected anal and oral segments, did not appear to have their neural transmission impeded due to surgical trauma. Anal relaxations followed distension-stimulation in the oral part (os), and oral contractions were present after balloon distension within the anal section (as) (fig. 4G).

Using three gut segments and three catheters at once, gave reliable and reproducible results, as described in relevant chapters where this method has been applied to test specific drug actions.



Effects of stimulation by balloon inflation
(red catheter 4F at 200 ul, tropic.= tropicamide (10^{-6}M)

Fig. 4 (F., G.)

DISCUSSION

The Trendelenburg technique did not produce consistently reliable results: orally, contractions alternated with relaxation responses while circular muscles at the anal end exhibited only contractions. Here, the infusate was pre-warmed to bath temperature levels (36° - 37°), whereas some earlier researchers might have used the infusate at ambient temperatures, thus perhaps causing stimulation due to temperature differences. The fact that anal relaxation only occurred after addition of bicuculline suggests that $GABA_A$ is possibly involved in blocking anal circular muscle relaxation during colonic fluid transport. Circular muscle contractions near the oral (influx) end were an often recurring cause of dysfunction, preventing distension and therefore stimulation at other points of the intestinal segment. In some experiments efflux of the distension fluid was blocked either by an anal circular muscle contraction or by mucous blocking the outflow tube, in which case stimulation could not be terminated until the pressure had gradually decreased by seepage. In addition to the above drawbacks, it must be mentioned that Trendelenburg's technique is an unnatural stimulant, probably unsuitable for colonic experiments, because fluid would not normally be encountered in the healthy distal colon.

However, electrical transmural stimulation of enteric neurones of a whole gut segment, a quite commonly used method, induced consistently reproducible effects, which could be used to test responses to various drugs and their antagonists, but with the disadvantage that intestinal muscles and neurones were exposed to a stimulant not naturally

encountered either in such magnitude or total pervasiveness. Nevertheless, it was used in subsequent experiments, mainly to test potencies of neuroactive agents and their antagonists.

When muscle flaps were stimulated electrically, good inhibitory responses were generated. Tropicamide and atropine appeared to reduce the inhibition due to electrical stimulation, a consequence most likely due to the lowering of muscle tone, because after the tone of the segment had been raised with histamine, the previous inhibitory amplitude was restored. Adrenergic blockers had no effect. It is therefore concluded that the observed inhibitory responses, as a result of transmural electrical stimulation at the described parameters, were non-cholinergic and non-adrenergic. Other researchers have recorded similar results (Brown et al., 1982; Sanger and Bennett, 1983; Szerb, 1982).

The next technique tested, namely stretch distension using weights, on whole gut segments, appeared to suffer from several problems and pitfalls. The major problem was encountered during repetitious stimulation sequences, where the responses seemed to decrease gradually when the same stimulus rate was applied repeatedly, leading to false conclusions if not accounted for.

Point stretch stimulation resulted in sharply defined responses, and appeared to be a good technique, except for the time and care needed to sew the gut section into the 'cradle'. If the gut had been pierced through to the lumen during fastening, experimental results became

erratic, possibly due to additional and irregular stimulation of mucosal receptors, consequently this method was not employed for drug tests.

The "flap stretch" procedure gave only poor results. It was first thought that this could have been due to the severance of 80% - 90% of longitudinal connections and subsequent reduction of longitudinal neural transmission, but this was shown later to be of little importance when using balloon distension experiments where 80% - 90% of intestinal tissue was excised. The trauma of the major cuts during the flap stretch technique preparations may have interfered with the stimulation/response complex at the stretch receptor level.

During experiments using stretch-distension as the stimulus, when distension distances were the quantifying criteria, altered response amplitudes at constant extents of distension were measured, here however, the major disadvantages were the occurrences of irregularities caused by fluctuations in response to fatigue, changing muscle tone, and spontaneous activity due to intrinsic events.

Furthermore, responses to distension stimulation technique, using a single artificial pellet as a bolus, were too erratic and unreliable to be useful. Pellets were often not transported past a certain point. Some pellets became lodged near the anal end because a tight muscular ring had been formed by inversion of the cut intestinal muscle layers, others became wedged in near the oral end because the longitudinal muscles had no attachment to pull against, if the oral end of the gut section was not tied to a funnel. Nevertheless, the average velocity, of 0.65 mm s-1 for

free moving pellets, was in agreement with reports by Frigo and Lecchini (1970) and comparable to migrating myoelectric complex velocities recorded for the guinea-pig ileum as 0.7 mm s^{-1} (Galligan et al., 1985).

The use of pellet chains, however, proved to be an efficient and reliable method of testing drug influences on propulsive activity of colon segments. The technique was, of course, closely related to naturally occurring events during peristalsis where a regularly spaced sequence of faecal pellets was often to be seen moving through the colon. Slitting the anal muscular inversion, and providing a flared funnel opening at the oral end reduced the chances of jamming. This technique was of sufficient sensitivity and reproducibility to allow drug actions on peristalsis to be examined, as outlined in table 1. Some of these results will be dealt with in more detail in the general discussion. Traces representing anal and oral circular, as well as longitudinal muscle responses (fig. 4C) showed that there was a circular muscle relaxation preceding every approaching pellet, which increased until the centre of the pellet had moved past the recording point. This relaxation was then followed by a massive contraction as soon as the pellet had traversed that particular point. Strong orally oriented longitudinal contractions, which started just before a pellet approached, pulled the intestinal wall over the pellet. The intestinal wall section subsequently travelled with the pellet back to previous starting positions.

Whilst the propulsion velocities of pellet chains provided the criteria for effects measured during the above chain experiments, the single pellet force transducer arrangement, however, allowed a quantification of changes of contraction frequency and amplitude, as well as force exerted

by the gut on the pellet; the level of intestinal propulsive activity, and the interference by various chemicals, could thus be registered. Since control responses differed in individual gut segments, relative changes only were recorded and averaged. Besides providing the results of drug effects (table 2), this method also led to the conclusion that longitudinal muscle contractions are of importance in the development of propulsive forces in peristalsis (vide infra).

The next technique tested - stimulation by distension with an intraluminal balloon - proved the most effective and reliable method, because the degree of stimulation could be accurately controlled and easily varied by changing the distension volume, and the stimulation/reponse complex could be reliably repeated over some hours. Precise repetition was achieved by timer controlled and solenoid-initiated syringe action for inflation, which was adjustable allowing stimulus duration as well as interval periods to be regulated. Consequently this method was adopted as the means of stimulation for most of the subsequent work, and the results so obtained will appear later in the appropriate sections.

Concluding this section, which has dealt with the advantages and disadvantages of various methods for eliciting peristaltic reflex activity, four techniques were found to yield satisfactory results.

Electrical stimulation allowed the testing of drug action on easily and reliably controlable inhibitory as well as excitatory responses; pellet chain experiments permitted examination of effects of drugs on pellet

propulsion velocities; single pellet/force transducer arrangements tested force, amplitude and frequency of propulsive waves; and finally, although test paramenters were limited during experiments using balloon distension this was found to be the most accurate and reliable method for testing the non-cholinergic, non-adrenergic anal relaxation phase in peristalsis.

Of these, electrical transmural stimulation, as well as balloon distension, was used in some of the subsequent experiments.

CHAPTER III

FREEZE SUBSTITUTION

INTRODUCTION

Events occurring during peristalsis have never been completely understood, and the relative contribution of circular and longitudinal muscles has always been a point of discussion, and often of disagreement (Lüderitz, 1890; Bayliss and Starling, 1899; Magnus, 1904; Trendelenburg, 1917-18; Raiford and Mulinos, 1934a and 1934b; Kosterlitz et al., 1956; Schneider, 1966; Frigo and Lecchini, 1970; Kosterlitz and Watt, 1975a). Prevailing thoughts are that the longitudinal muscles are not extensively, if at all, involved in intestinal emptying. This proposal stands in direct contrast to the present 'single-pellet-force-transducer' findings, where longitudinal activity seemed to exert the major propulsive force in the colon segment.

Another unexplained factor in intestinal motility has been 'reverse peristalsis' or 'anti-peristalsis', observed and mentioned by a number of researchers. 'There is no anti-peristalsis' (Bayliss and Starling, 1899). "Anti-peristaltic waves occurred quite normally.."(Cannon, 1912). Baur (1923) reported that sometimes every peristaltic event was preceded by an anti-peristaltic wave. Alvarez (1928) made an astute observation: he noticed that whilst rather shallow reverse peristaltic waves travelled along a part of the colon's length, yet these waves, never pushed

any material orally. Takita (1957) reported that young bovine fetuses exhibited contractile waves in both directions, but only very 'indistinct anti-peristaltic waves were observed on rare occasions' after the 19th week. The above quoted observations and comments of previous researchers provide only a small but representative sample of opinions regarding the relative importance of the two different muscle layers in peristalsis, and the existence of anti-peristalsis.

These two unresolved problems, together with an unexplained observation here, led to a search for a method making subsequent visualization of events during peristalsis possible. The observation, that could not be explained with the existing enteric model of muscle layer arrangements, was the formation of multiple bulges behind a pellet in excised colon segments during propulsive activity. Similar bulging of the colon was noticed during pellet chain experiments, and also when warm carbachol (10^{-6}M) containing Krebs solution was poured into the abdominal cavity of recently killed animals in order to promote emptying of the colon before excision.

To clarify the above phenomena, 'freeze-substitution', a technique designed to preserve tissue activity and constituents, was employed. Simpson (1941) had suggested a modification of existing freeze drying procedures by using dehydrating fluids that act also as a fixative, or which contain an additional fixative. He called this method "freezing-substitution", and recommended methyl cellusolve or absolute ethyl alcohol if temperatures between -40°C and -78°C were to be used. He had found that either -160°C and below, or approximately -60°C were most

suitable for intestinal tissue preparations, they "surpassed ordinary chemical fixation in excellence" (Simpson, 1941).

Freeze-substitution consists of five main steps (Pearse, 1980): a) quenching or vitrification, involving rapid freezing of the tissue samples; b) the substitution/fixation and c) dehydration steps, usually done simultaneously; followed by d) conventional sectioning and e) staining. Some researchers use the procedures in a different order, recommending sectioning of the tissue in a cryostat immediately after quenching, and then to take it through the substitution/fixation, dehydration and staining steps (Chang and Hori, 1961; Preece, 1972). Here, when trying to examine contractile events by 'freezing the action' of peristaltic motility, the latter sequence would not have been suitable, and the first described procedural design had to be employed. Most problems were envisaged arising during the first step, quenching. It was reported that rapid heat transfer during immersion in much colder than -37°C solvents (-160°C to -60°C) was prevented by a film of vapour forming around the specimen. The unobtainable cooling rate of 5000°C s^{-1}, initially to -100°C, would have allowed perfect vitrification. Nevertheless, it was found that extremely rapid cooling to between -80°C and -70°C would prevent ice crystal artifacts, distortion, or membrane fracture (Pearse, 1980). The main concern, that peristaltic events could be falsified, was relieved by the report that 'tissue constituents remained in their in vivo condition' (Feder and Sidman, 1958) and although 'contractile tissue often shortened during conventional fixation, it remained extended after freeze substitution'. Pearse (1980) also found that protein denaturation was minimal (5%-10%). The suggested

specimen size was optimal at a 3 mm diameter with a maximum of a 10 mm diameter (Feder and Sidman, 1958; Brown, 1978; Pearse, 1980), which was acceptable since the wall thickness of contracted intestinal sections would not ever have exceeded those values.

Another problem anticipated was that of the penetration rate of frozen specimens by substitution/fixation fluids, because of the enormous increase in viscosity of these fluids due to the low working temperatures. Acetone at -80°C and ethanol at -110°C were found to have a penetration rate of approximately 0.2mm per day while acetone at -43°C would progress 3 mm and ethanol at -70°C only 0.5 mm per day into a frozen section (Pearse, 1980).

It has been suggested that the most successful fixatives, during freeze substitution, are chromium trioxide (CrO_3) at 2%, osmium tetroxide (OsO_4) at a 1% concentration in acetone, or picric acid ($C_6H_2(NO_2)_3OH$) in ethanol (Feder and Sidman, 1958; Brown, 1978; Pearse, 1980). Penetration or maturation periods recommended by the above researchers for the acetone/osmium tetroxide, acetone/chromium trioxide, or ethanol/picric acid mixtures range from one to six days at -60°C to -75°C. The exact procedures used are described below.

MATERIALS AND METHODS

Into a polystyrene-foam vessel (6 litres), containing a dry ice/acetone mixture, was placed a glass jar (2 litres) also filled with the dry

ice/acetone coolant; the latter was connected to a vibrator and it accommodated a 300 ml wide-necked screw top bottle full of pure acetone. The temperature of the acetone was -77°C, 2% chromium trioxide (w/w) being added when the acetone had reached -7°C, because chromium trioxide reacts with acetone at room temperature. During some experiments, 2 bottles of 150 ml each were placed in the glass jar - one containing acetone/chromium trioxide, and the other ethanol/picric acid made by adding a 5% saturated picric acid water solution (v/v) to the ethanol (Pearse, 1980). The dry ice could be replenished as needed.

During initial experiments, weighted colonic segments, which had been tied off at both ends and which had been stimulated either by insertion of an artificial pellet or distended by air inflation, were thrown into the super-cool substitution/fixation mixture for quenching as soon as peristaltic activity or bulging had commenced.

During another series of experiments, a bath, containing precooled acetone or ethanol, was placed next to the freshly killed animal. As soon as the colon had been freed from its omentum and other connective tissue, a segment (appr. 20 cm) containing pellets and displaying peristaltic activity, but still connected to the animal, was rapidly quenched in the bath. The frozen region was then excised and divided into 2 cm pieces, using mainly those that showed peristaltic contraction rings, or bulging, behind a pellet. These selected pieces were then transferred to the substitution/fixation bottle. Since test samples, removed after one, two and three days, showed an equal degree of penetration, possibly due to a temperature rise from -77oC during the day to -55oC at night when dry

ice was not replenished, the substitution/fixation procedure was usually terminated after 24 hours.

After the temperature was raised from -77°C to -4°C, within three hours the tissue segments substituted in acetone were washed three times in pure acetone to remove excess fixative. Acetone-treated as well as ethanol substituted specimens were then put into pure ethanol, which was changed three times within 24 hours. The colonic segments were washed in chloroform to extract remaining traces of water, gradually brought from -4°C to room temperature and transferred to xylene. After three changes of xylene the specimens were then put into liquified paraffin wax (64°C) and embedded under reduced pressure, to remove trapped air bubbles. The tissue set in paraffin blocks was cut on a microtome longitudinally, sagitally, and crossectionally. The sections, 12 um - 20 um thick, were floated off in a warm water bath (56°C) containing 0.13% of a wetting agent (Kodak flow 200) and 1.7% of bacterial agar (0.3% concentration) and lifted onto albumin covered glass slides, which were left to dry overnight (Summer and Summer, 1969). Next day the slides were warmed and subsequently submerged in xylene to remove all wax, and brought down to water.

The tissue sections were then stained according to Delafield's Hematoxylin-Eosin staining procedure (Humason, 1962), except that the recommended 2 min - 5 min hematoxylin soaking time was found to be too long, the tissue sections having turned black so that regressive staining had to be applied. Subsequently, a 20 s - 30 s immersion was used, and resulted in the desired stain intensity. The connective tissue specific

Mallory-Heidenhaim stain was used on some sections, whilst in order to show up nerve cells and their processes, Golgi and Nissl (Gallocyanin) stains were also applied to others. Finished sections dehydrated, were covered with 'pix' gum solution and cover-slipped. After a 48 hour drying period the slides were ready for light microscope examination and photography.

During attempts to find a simpler method, the following technique was devised and found to be acceptable. The deep frozen gut segments (-77°C) were placed in a formol-saline (10%) mixture at -4°C for 48 hours, and the solution containing the tissue pieces was then gradually brought to 0°C, during which time fixation had occurred. The embedding, cutting and staining procedures were the same as described above.

RESULTS

Pictures 1 and 4, on plates 1 and 2, depict micrographs of longitudinal and crossectional sections respectively, which were fixed with chromium trioxide and subsequently stained with a hematoxylin/eosin stain. Mucosal and muscle tissue, mainly the nuclei in the latter, were stained red, whilst mucus, mesenteric and connective cells displayed a bright yellow appearance. The specimens' consistency ranged from hard to brittle making it difficult to cut thin slices (15 um). Chromium trioxide stained glycogen red, and hardened tissue, as has been reported by Humason (1962).

Longitudinal and crossectional tissue sections, shown in pictures 2 and 5 were picric acid fixed and also stained with hematoxylin/eosin. As expected, nuclei were brown, muscle and elastic tissue bright yellow (Humason, 1962). In both the above cases, hematoxylin/eosin staining only enhanced the coloration due to the chromium trioxide and picric acid fixation.

Pictures 3 and 6 represent two samples fixed in the formalin/saline mixture, and stained with hematoxylin/eosin. Nuclei ranged in colouring from blue to lilac, and cytoplasmic structures were rose and pink (Humason, 1962). Most gut components were readily recognizable. Specimens were easily cut at 10 um, whereas the picric acid fixed specimens had a lowest attainable thickness limit of 15 um.

In addition to, and of more importance than the above results, which pertained mainly to the different outcomes of the three fixation methods, the freeze-substitution techniques permitted clarification of hitherto unresolved major issues concerning the mechanism of peristalsis. Bulging (B), as seen in picture 1, plate 1, seemed to be held in place by the muscularis mucosae of longitudinal mucosal folds (pictures J, L, M; plate 4). Some aspects of another remarkable phenomenon - mucosal folds - were elucidated. In the colon, mucosal folding or plicae formation was found to be extensive. Plicae transversales were always observed oral of a pellet (pictures B, C, D; plate 3) while plicae longitudinales were mainly formed on the anal side of a pellet (pictures E, F; plate 3). If pellets were stationary, a circular muscle contraction in front of the pellet was visible (pictures E,F; plate 3). If pellets were moving, a

ABBREVIATIONS FOR PLATES

C	=	contraction
CM	=	circular muscle layer
CT	=	connective tissue
F	=	faeces
G	=	ganglion
LM	=	longitudinal muscle layer
Lp	=	lamina propria
Me	=	mesentery
Mm	=	muscularis mucosae
Mu	=	mucosa
P	=	pellet space
Pl	=	plicae longitudinales
Pt	=	plicae transversales
Sm P	=	submucosal plexus
V	=	vein
◄	=	direction of pellet movement

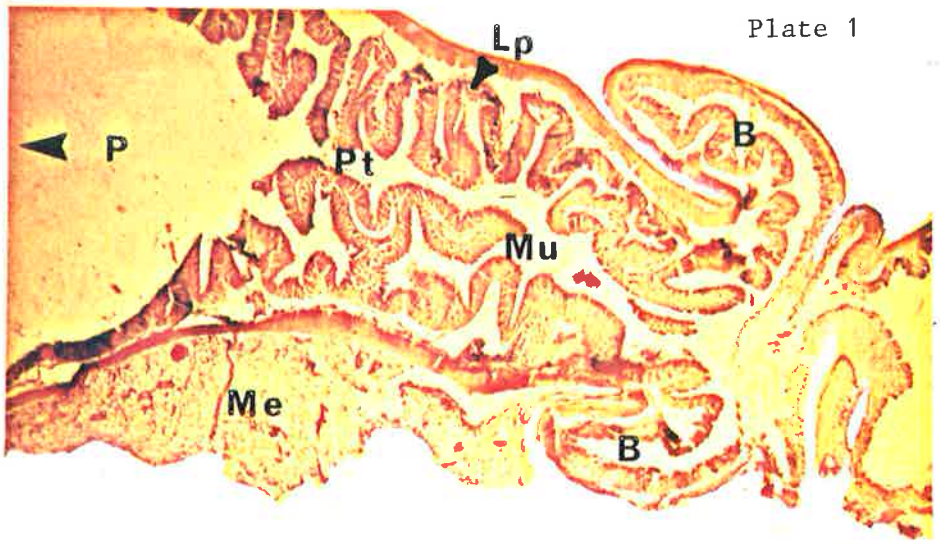
PLATE LEGEND

Plate 1

Picture 1: A chromium trioxide-fixed longitudinal section of the peristalting colon. Bulging and plicae transversales formation are obvious.

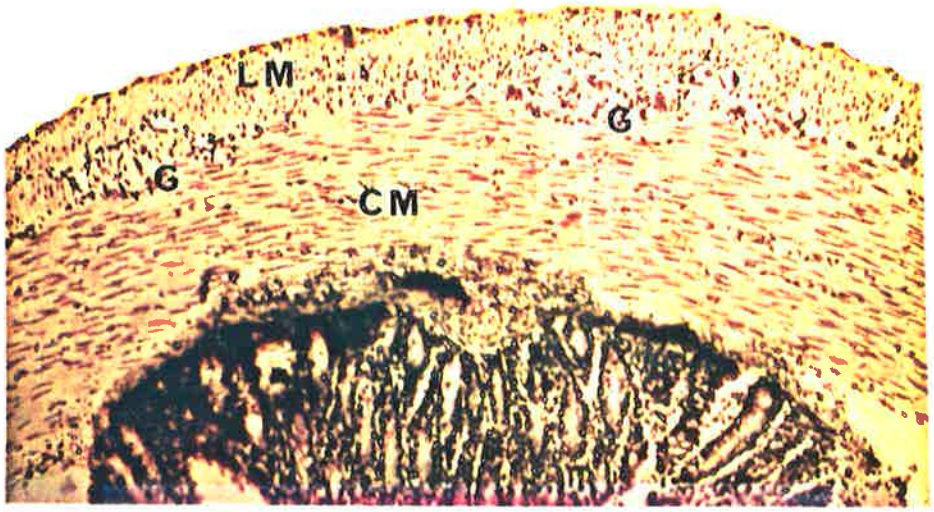
Picture 2: A colonic crossection of the relaxed picric-acid-fixed gut showing myenteric ganglia.

Picture 3: A formaldehyde-saline-fixed longitudinal section of a relaxed colon segment with myenteric ganglia.



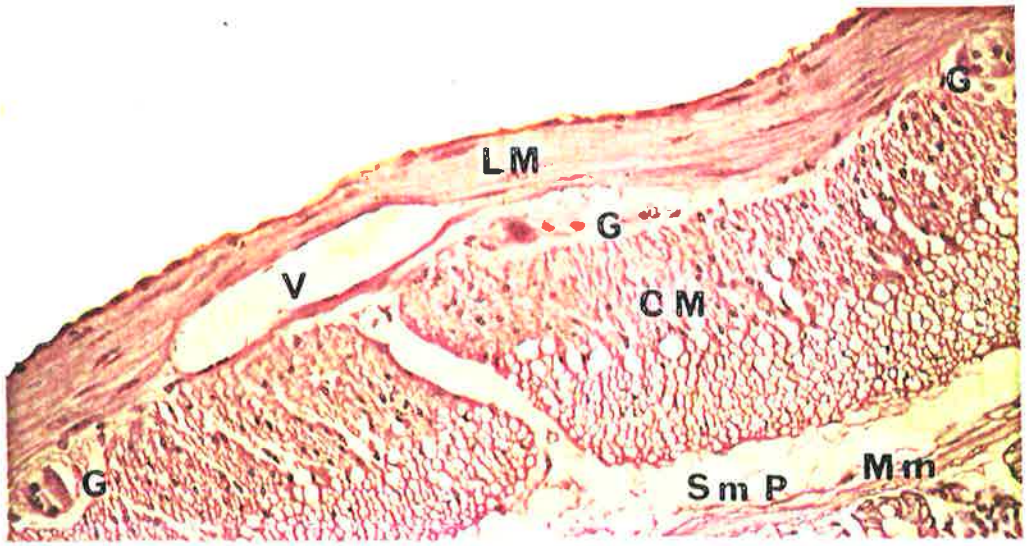
x 15

1



x 140

2



x 200

3

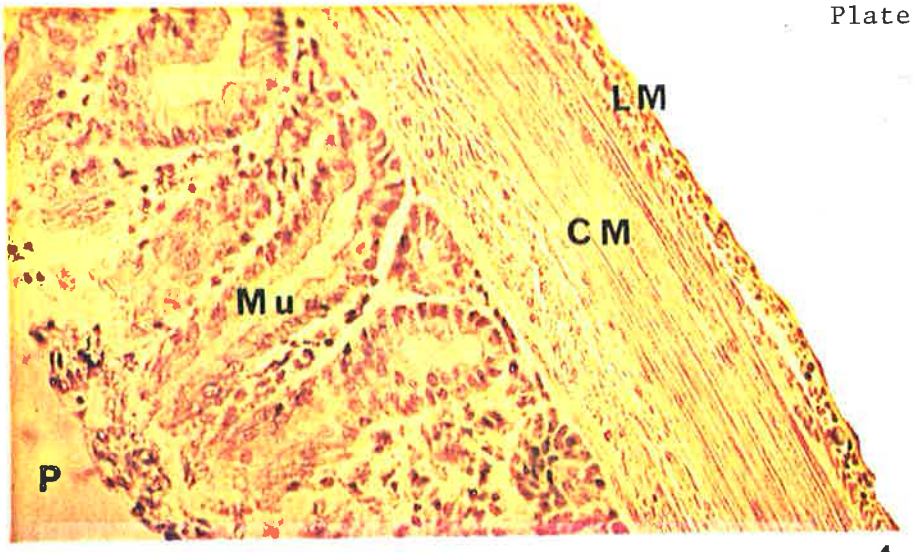
PLATE LEGEND

Plate 2

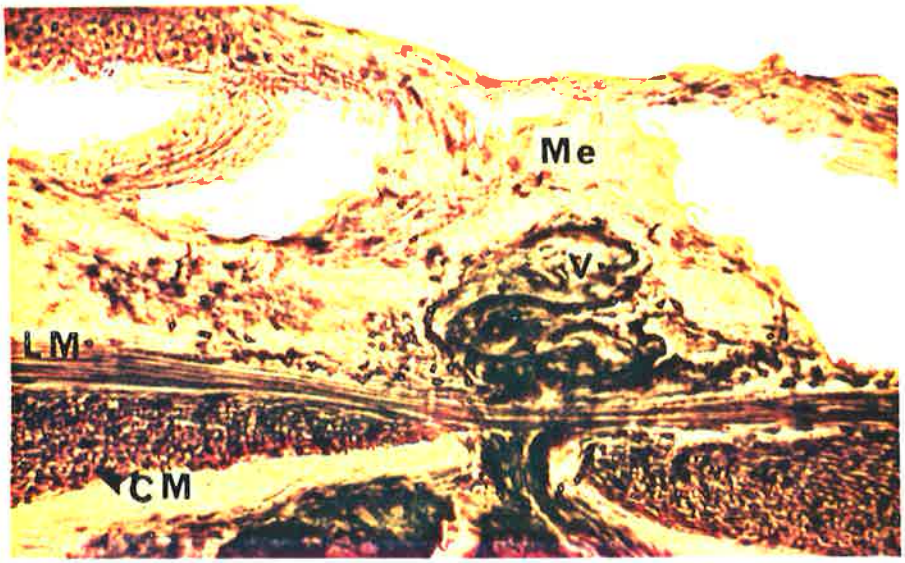
Picture 4: A chromium trioxide-fixed peristalting colon crossection.

Picture 5: A picric-acid-fixed longitudinal relaxed colon section displaying a folded neural or tubular unit.

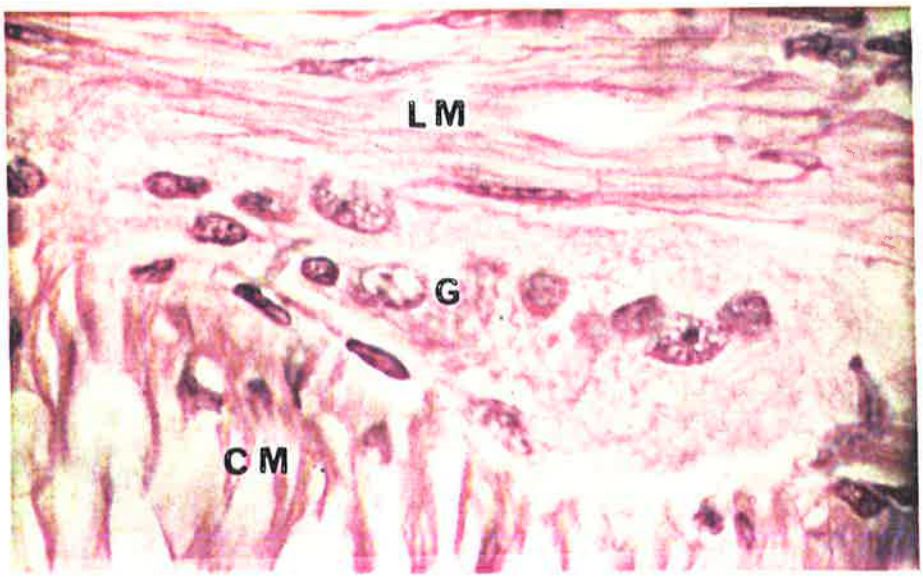
Picture 6: An enlarged myenteric ganglion in a longitudinal colon section which was formaldehyde-saline-fixed.



x 100 4



x 100 5



x 800 6

PLATE LEGEND

Plate 3

Picture A: A freeze-substituted, peristalting colon section.

Picture B: A longitudinal section of a freeze-substituted colon segment. The pellet had been removed.

Picture C: A longitudinal colon section with part of a pellet, and showing a plica transversalis oral of the pellet.

Picture D: Plica transversalis enlarged.

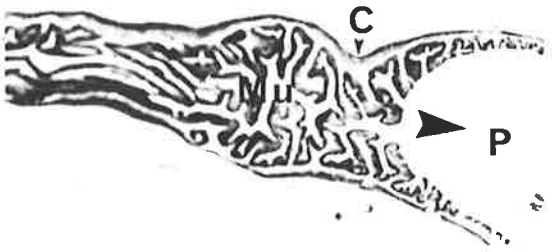
Picture E: Plicae longitudinales aboral of the approaching pellet.

Picture F: Plicae longitudinales with approaching pellet.

Plate 3

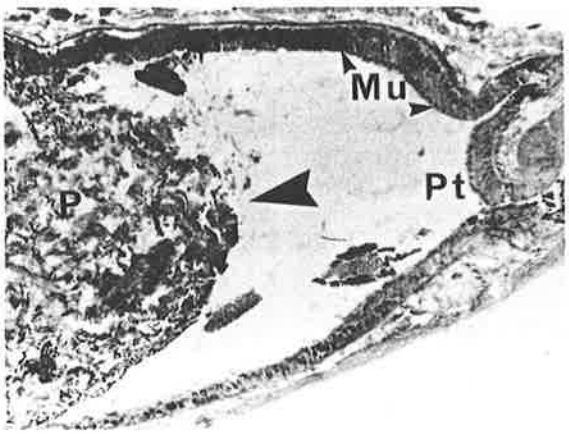
x 2

A



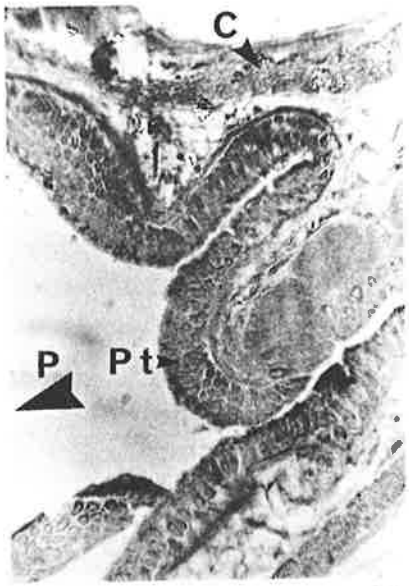
x 7.5

B



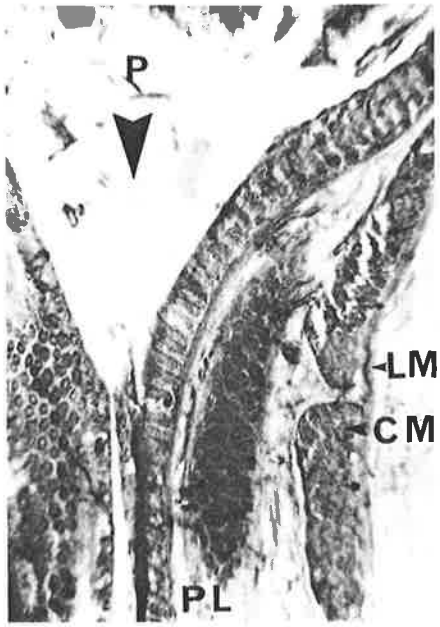
x 15

C



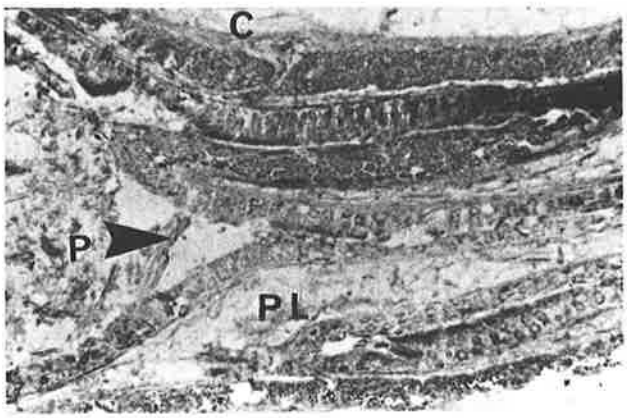
x 30

D



x 25

E



x 20

F

PLATE LEGEND

Plate 4

Picture G: A longitudinal colon section showing circular and longitudinal muscle contractions behind a pellet. Longitudinal muscles are of greatest thickness at the most contracted and therefore invaginated region.

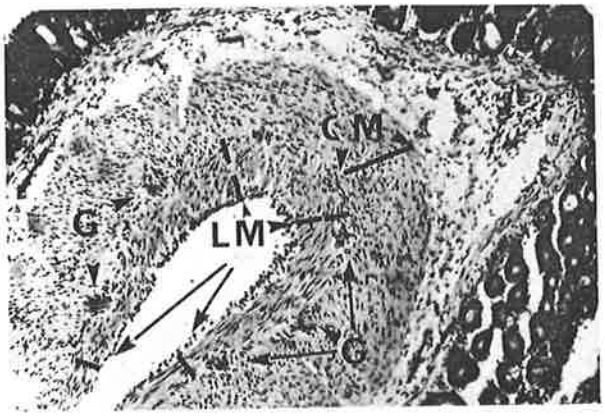
Picture H: A longitudinally cut colon piece, showing longitudinal muscles contracting to effect fold-over of the cut end.

Picture I: Shows the same effect as picture G on a different specimen.

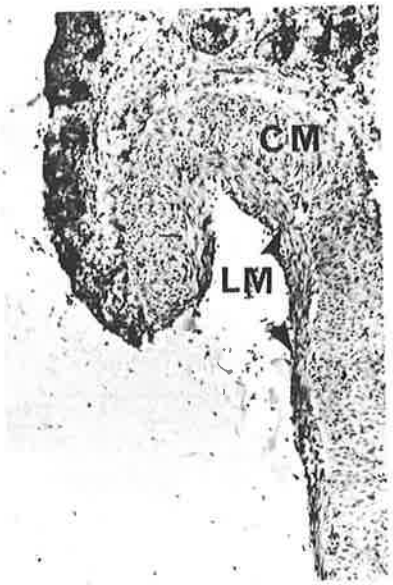
Pictures

J, L, M: Muscularis mucosae connecting oral and aboral walls of a colonic bulge.

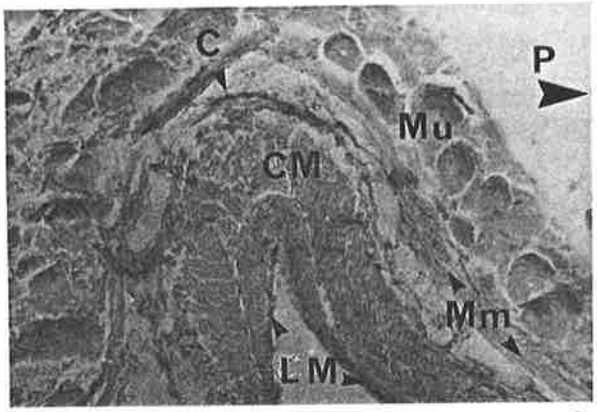
Picture K: Longitudinal section of an ileum section. No apparent plica formation.



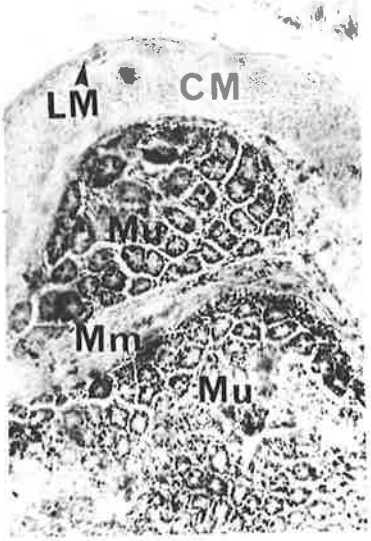
x 60 G



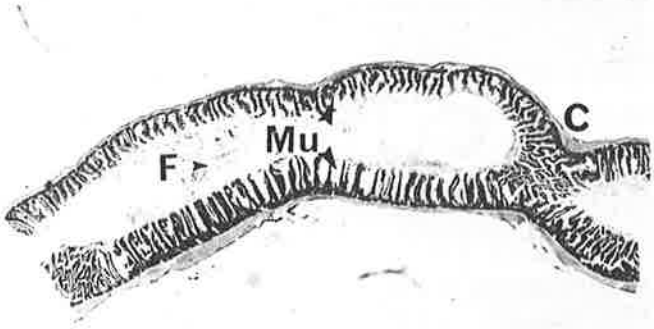
x 60 H



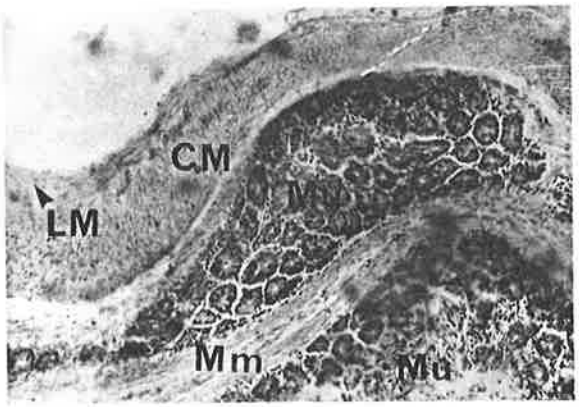
x 40 I



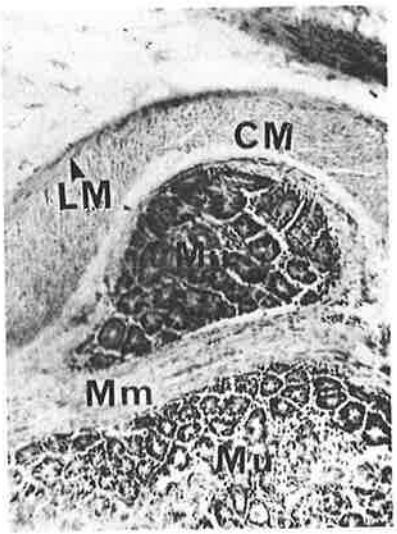
x 40 J



x 7.5 K



x 40 L



x 40 M

longitudinal muscle contraction could be observed in front of a pellet (pictures G, I; plate 4) combined with a circular muscle relaxation. The same effect could be noticed at the terminal section of the cut colon where the end section was folded back over itself (picture H; plate 4).

The lumen of sections of colonic segments that had been fully relaxed with tropicamide (10^{-5}M), was usually completely taken up by transverse mucosal folds (picture O; plate 5), and longitudinal mucosal folding was observed in only about 10% of the length of a given section (picture N; plate 5). Unoccupied, empty luminal space was rarely seen in relaxed colon sections. Ileal mucosa did not appear to be greatly folded, in comparison with colonic mucosa: this could be seen in a peristalting ileal segment, which was also freeze-substituted, picric acid fixed, sectioned and hematoxylin-eosin stained (picture K; plate 4).

Myenteric ganglia were quite obvious in sections that were formalin fixed and hematoxylin-eosin stained (picture 3; plate 1), or sections that were picric acid fixed and Nissl (Gallocyanin) stained (picture P; plate 5). This had coloured the Nissl substance in the cytoplasm of nerve cells bluish purple, as predicted by Humason (1962). The same author reported the appearance of black nerve cell processes on a light yellow background after application of the stain for Golgi bodies. Picture Q on plate 5 shows a Golgi stained crossection, with a multitude of black spots in the crypt area against a yellow background.

The connective tissue specific Mallory-Heidenhaim stain was used to reveal collagenous fibres blue on yellow-brown background between

PLATE LEGEND

Plate 5

Picture N: A crossection of a tropicamide-relaxed colon segment.

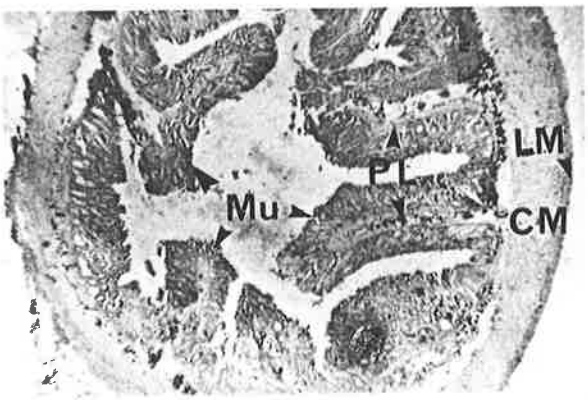
Picture O: A longitudinal section of a tropicamide-relaxed colon segment.

Picture P: A longitudinal colonic section during peristalsis, displaying clearly both muscle layers of the lamina muscularis, a large myenteric ganglion, a blood vessel, the submucosal plexus space, the muscularis mucosae, and the mucosa.

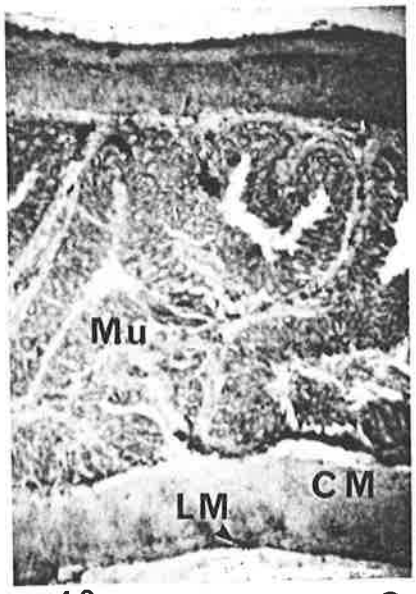
Picture Q: A colonic crossection featuring mainly the lamina muscularis and the submucosal space of a plica longitudinalis.

Picture

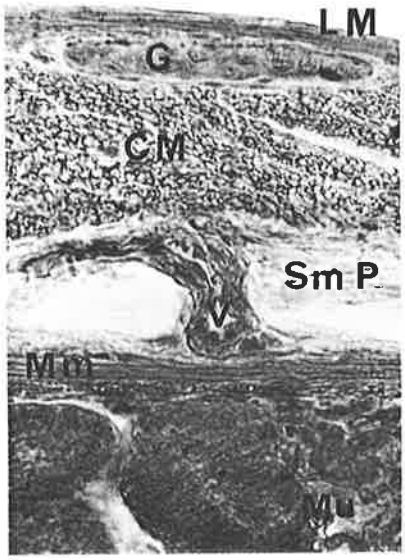
R, S: Concertina or web-like connective strands between the muscularis mucosae and the lamina muscularis.



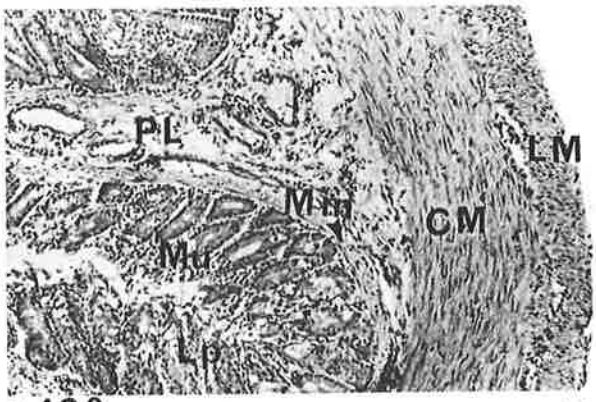
x 30 N



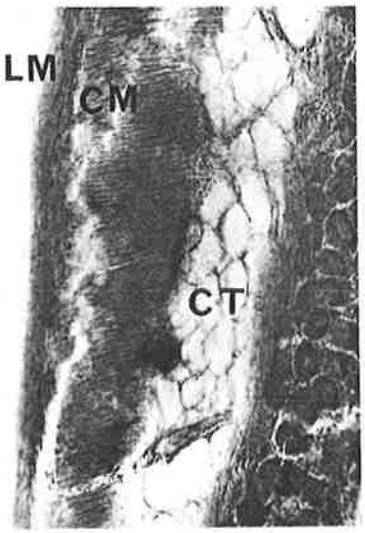
x 40 O



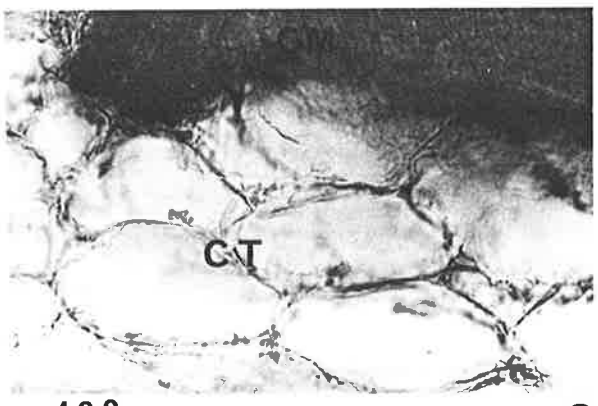
x 400 P



x 120 Q



x 100 R

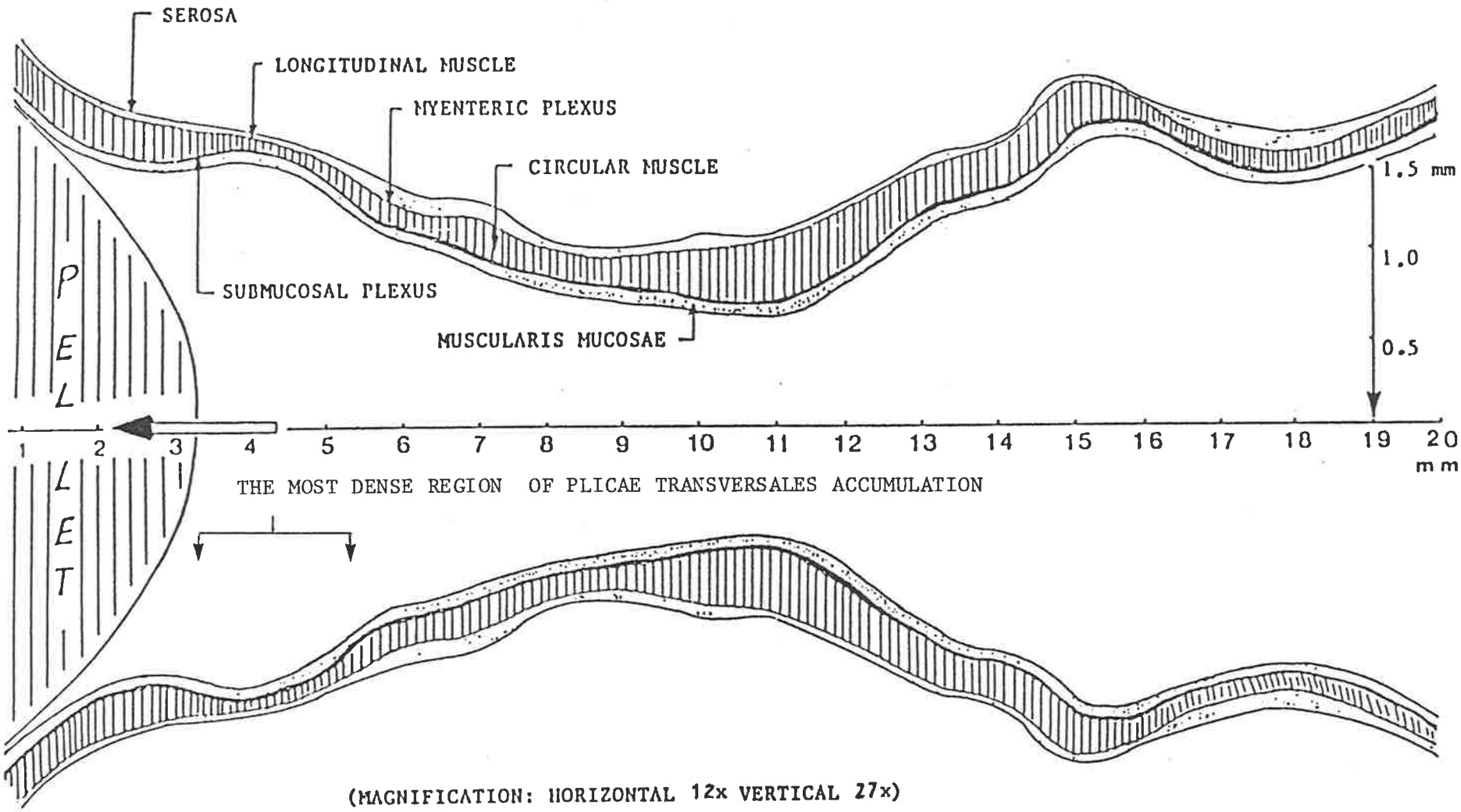


x 400 S

submucosa and the muscularis mucosae (Humason, 1962). Picture S is a sectional enlargement of picture R, plate 5 indicating the existence of a concertina like connective fibre system.

Measurements of longitudinal and circular muscle layers as well as of the muscularis mucosae were recorded from micrographs of eight peristalting colon segments prepared by freeze-substitution. They revealed several significant factors concerning propulsive movement. Values representing a typical colonic segment 'frozen' in action are graphically demonstrated in figure 5, where the second gut wall traces below the centre-line were drawn for easier visualization, with the same values used for the upper traces, and the direction of travel is indicated by the arrow. Along the 2 cm, spanning the distance from the last 3 mm of one pellet to just in front of the next pellet, muscle layer thicknesses were recorded at 1 mm intervals (table VI).

In order to simplify, the muscularis mucosae was drawn adjacent to the submucosa while in fact it had been measured in different folding positions within the lumen. Up to the 8 mm mark the mucosa was mainly folded in transverse folds, but from that point to the next pellet only longitudinal folds were noted and the thickness of the muscularis mucosae appeared to be reduced. The circular muscle was contracted near the end of the pellet (1 mm - 3 mm) and again in the middle of the section (10 mm - 15 mm). In contrast contractions of the longitudinal muscles were observed at 6 mm and 7 mm at 10 mm, 17 mm and 18 mm points.



Reproduction of colonic muscle layers according to measurements obtained by freeze-substitution (Table VI, appendix). Mucosal folding was omitted due to averaging difficulties

Fig. 5

DISCUSSION

Freeze-substitution 'arrested' peristaltic movement instantly, subsequent fixation and staining then allowed evaluation and study of propulsive activity. Chromium trioxide and picric acid fixation were more effective in retaining the original intestinal action unchanged, while a certain amount of relaxing of contracted tissue took place during formalin/saline fixation, possibly due to the higher temperatures at which fixation occurred. Staining, however, and therefore detail preservation was better in picric acid and also in the formalin treated sections than in the chromium trioxide fixed specimens. If the cutting difficulties of the chromium trioxide sections and the relaxation distortion of the formalin treated gut pieces were taken into account, it appeared that picric acid fixation was the best technique, wherever activity and structural detail was studied.

Bulging ('B', picture 1, plate 1) appeared to be a mechanism to accommodate slack in the colon between consecutive pellets, and in between phases of peristaltic activity. Bulges seemed to be held in place by the muscularis mucosae of a longitudinal fold (pictures J, L, M; plate 4): a difficult concept to explain, as will become more obvious in subsequent paragraphs. Bulging did not appear to possess any other obviously essential functions besides the above mentioned accommodation of slack.

Mucosal folding seemed, however, to play an extremely significant role in intestinal transport. It could possibly explain anti-peristalsis as well

as the sometimes observed ascending inhibition (Alvarez, 1928). Frigo and Lecchini (1970) found that, although upon removal of the mucosa and submucosa, muscle activity continued, yet no propulsion of material occurred, thereby demonstrating the necessity of these two layers for faecal transport. Möllendorf (1936) reported that the gut could be visualized as consisting of two tubes - the outer lamina muscularis and the inner mucosal tube - both of which could move independently of each other, connected only by a lose elastic submucosa. These elastic collagenous fibres are placed at approximately 45° or 135° to the gut axis (90° to each other), which allowed three-directioned movement so that one tube could glide within the other (Bloom and Fawcett, 1968; Ham, 1974; Möllendorf, 1936). Pictures S and R on plate 5 supported these claims. Nevertheless, the two questions which arose out of these concepts: a) how is the neural and vascular supply of the mucosal tube achieved and b) what is the purpose of mucosal sliding and folding - were not answered by any of these workers.

After inspecting more than 1200 sections from 20 freeze-substituted peristalting colon segments, by light microscope, it is concluded here that the neural and vascular supplies enter the submucosa at intervals - possibly up to 5 cm apart - as shown in picture 5, plate 2. The coiling, obvious in the picture, may allow freedom of movement of the mucosa to a certain degree, even at the connecting point. The suggested distance was estimated, however, because sections were generally about 4 cm long and only one such neural and vascular supply entry was to be seen in each.

Since, according to Scratcherd and Grundy (1984), absorption and secretion are the two main functions of the mucosal layer and also since, according to Möllendorf (1936), mucosal folding increases the surface area of the mucosa about threefold, the primary function of mucosal folds must be the increase to the efficiency of absorption and secretion. Möllendorf also suggested that folds are necessary for accommodation of mucosal slack.

However, observations in conjunction with pictorial evidence (picture 1, plate 1; picture B, C, D, plate 3) provided strong support for the theory that mucosal folding also plays an important role in colonic motility and transport. Circular folds appeared to be stacked orally of pellets so that the circular muscle contraction force, which acted with a 90° force vector in relation to the gut axis (i.e. propulsion direction) on a non-pliable solid object, would therefore be only marginally efficient, mainly due to the pellets' ovoid shape. This inefficient force could be converted to a straight pushing force in the direction of the gut axis, if the bunched-up circular mucosal folds acted as a piston behind the pellet, as was observed here. This formation of transverse plicae - by the use of mucosal slack - of course stretched the mucosa behind it. Therefore, longitudinal folds had to form in front of the following pellet (picture B, E, F; plate 3). If motility was stopped, circular muscle contractions in front of a pellet held it in place (picture E, F; plate 3). If mucus or fluid was transported - a rare occurrence in the colon - then this took over the role of mucosal bunching behind a pellet by providing the pliable 'cushion' necessary for converting the direction of force (picture C; plate 3). Because ileal

content is of a soft fluid consistency and efficient transport could therefore be achieved by squeezing activity of the circular muscle alone, extensive folding was never encountered in sections from freeze-substituted ileum (picture K; plate 4).

Anti-peristalsis had quite often been observed and reported (Cannon, 1912; Baur, 1923; Alvarez, 1928; Takita, 1957) but hardly ever explained. The theory that it might be a mechanism for transport of fluid from the colon back to the caecum (Scratcherd and Grundy, 1984) seems unlikely, since extracted fluid would usually have been absorbed and transported by the vascular system. Alvarez (1928) observed reverse peristaltic waves which never pushed any material orally. A more probable theory could be based on the fact that mucosal slack was observed travelling with pellets anally for the above expounded reasons. Pellet transport was also on occasion seen to stop after 3 to 5 pellets had been moved past a certain point, and that was the time when an ascending bulge (recorded as a relaxation), resembling an anti-peristaltic event, was usually seen travelling orally. It is proposed that this represents the "mucosal slack" being "taken-up", after it had been shifted anally during propulsive activity.

Measurements of events occurring during peristalsis, graphically recorded in figure 5, also support the above conclusions. Plicae circulares were seen from the 2 mm to 8 mm mark acting as the pellet- pushing-'piston', being squeezed against the pellet by the massive circular muscle contraction at 10 mm - 12 mm. The increased muscularis mucosae thickness at 7 mm - 8 mm was possibly due to the effort of pulling the mucosa

anally, which provided the slack for the circular folds (2 mm to 8 mm) and caused the longitudinally stretched mucosal folds from 8 mm to 20 mm points. Peristalsis also appears to be partly due to the longitudinal muscles pulling (6 mm and 7 mm) the muscle tube over the intestinal content while the circular muscle (2 mm and 3 mm) hold it in place (c.f. Möllendorf, 1936).

Another effect observed was that wherever the gut diameter was enlarged a longitudinal contraction was present (6 mm, 7 mm, 17 mm and 18 mm). By analogy two connected layers of pliable materials (e.g. bimetallic strip) would bend towards the direction of the one that contracted. This phenomenon was always observed in the intestinal muscle layer where the gut lumen widened in front of a pellet (picture G, I; plate 4) or when the cut end of a section folded over (picture H; plate 4). There appeared to be reciprocal events in action: the circular muscle coat seemed to be relaxed where the longitudinal layer was contracted and vice versa. This opposing colonic muscle activity pointed to a neurotransmitter with dual and opposing effects on the two main intestinal muscle layers.

It would be of interest to know how the myenteric and submucosal nerve nets change during the contraction and relaxation phases. Does the myenteric plexus change its conformation in accordance with the circular or longitudinal muscle layers, or with the whole of the lamina muscularis? Furthermore, does the submucosal plexus contract and expand with the muscularis mucosae? Freeze-substitution would have been an ideal technique to elucidate this question; although it was outside the stated

aims of this work, its clarification was attempted, but subsequently abandoned due to pressure of time.

CHAPTER IV

PUTATIVE TRANSMITTERS OF DESCENDING INHIBITION IN THE COLON

INTRODUCTION

The alternating activity of circular and longitudinal muscles, observed in freeze-substitution experiments, could be due to the actions of a single transmitter with opposing effects on these two muscle layers, which would provide a very efficient and conservative mechanism in nature. A transmitter system with counteractive effects, for which the two different innervation populations could be turned on or off selectively by presynaptic modulation, would represent an efficient control mechanism. Thus a search was undertaken for such an enteric neurotransmitter with opposing dual-effects in the two muscle layers, and a number of neuroactive drugs were screened.

a) Gamma Aminobutyric Acid

Gamma-aminobutyric acid (GABA) has been attributed with excitatory and inhibitory effects on the enteric nervous system, and therefore on intestinal motility (Hobbiger, 1958; Inouye et al., 1960; Curtis and Johnston, 1974; Brown, 1979; Krantis and Kerr, 1981a and 1981b). In 1981 Bowery et al. postulated a dual GABA receptor model, where $GABA_A$ - receptors mediate chloride-dependent GABA-actions, and $GABA_B$ - receptors

mediate a depression of transmitter output. Extensive studies of the effects of GABA on intestinal motility by Ong and Kerr (1983a and 1984) showed that GABA plays an important role in peristalsis and intestinal transport. They also noted that the main GABA action on the colon was the inhibitory $GABA_B$ - receptor mediated effect, in the absence of an excitatory $GABA_A$ response. Ong and Kerr (1983b) also found that $GABA_B$ - receptor mediated inhibitory actions on the longitudinal muscle of the ileum could be blocked by delta-aminovaleric acid (DAVA).

b) Prostaglandins

Prostaglandins (PGs), as von Euler (1935) showed, have the ability to stimulate or relax different intestinal muscle layers selectively. The previously quoted 'prostaglandin effect' (Bennett et al., 1968) - the PG's property to stimulate one muscle layer and relax the other at the same time - fits the requirements of the elusive inhibitory enteric transmitter. Since the circular-muscle relaxation, which is accompanied by a longitudinal-muscle contraction, was most effectively initiated by PGE_2 (Horton, 1979; Wilson, 1972; Bennett et al., 1981), causing a 'contradictory consequence' to the two muscle layers (Sanders, 1984a), it was therefore attempted to block the descending inhibition with a number of known prostaglandin synthesis inhibitors or antagonists.

c) Neuropeptides

Glucagon had been reported to have an inhibitory function on gastro-intestinal motility with opposing effects on circular and longitudinal muscles (Evans et al., 1982), while Mangel (1984) found glucagon to decrease colonic contractions.

Neurotensin, a drug with controversial effects on intestinal motility, blocks ileal longitudinal muscle (Goedert et al., 1984) but contracts longitudinal rat fundus muscles (Huidubro-Toro and Kullak, 1985). It relaxes colonic longitudinal muscles myogenically (Kitabgi and Vincent, 1981). It also stimulates acetylcholine (ACH) release in ileal longitudinal muscle- myenteric plexus strips (Yau et al., 1983; Garzon et al., 1985) and reputedly releases noradrenaline which reduces ACH release, causing inhibition of intestinal activity (Sakai et al., 1984). Taché (1984) classed neurotensin as a stimulator of colonic contraction, an effect in which prostaglandins are evidently involved since enhancement of colonic motility by neurotensin can be stopped by PG synthesis blockers (Bardon and Ruckebusch, 1985).

Gintzler (1980) considered that substance P (SP) is an atropine-resistant excitatory neurotransmitter in the gut, and it has been claimed to be the final non-cholinergic excitatory intestinal transmitter (Costa et al., 1985), having a neuronal as well as a direct effect on muscle (Chahl, 1985). SP has also been thought to be a peristalsis initiator (Hellström and Rosell, 1981), causing non-cholinergic excitation of intestinal longitudinal muscles (North, 1982; Bartho et al., 1982). In

contrast Fändriks and Delbro (1983) and Garzon et al. (1985) suggest that neurally-mediated colonic contractions due to SP are cholinergic in nature, but that some SP activity is myogenic. Differentiated effects on intestinal muscles were reported by Yokoyama and North (1983) when they found that the SP induced excitation of circular muscle is blocked by atropine but not the longitudinal muscle contraction.

The most likely neuroactive gut peptide, vasoactive intestinal polypeptide (VIP), has contractile effects on ileal muscles, but relaxant influence on colonic muscles (Jaffer et al., 1974). It has been concluded by most researchers working with VIP that it is the non-adrenergic, non-cholinergic inhibitory intestinal transmitter responsible for the aboral muscle inhibition (Furness and Costa, 1979; Fahrenkrug and Emson, 1982; Bennett et al., 1984). That VIP's neuronal pathway might act through ATP receptors was disproved by Satchell (1984).

d) Adenosine Triphosphate

Since it has also been claimed that ATP is the intestinal inhibitory transmitter (Drury and Szent-Györgyi, 1929; Burnstock et al., 1970; Burnstock, 1982a, 1983; Gillespie, 1982) theophylline (Brown et al., 1982; Hayashi et al., 1982) and Cibacron-Blue F3GA (Ciba-blue) (Apps and Gleed, 1976; Beissner and Rudolph, 1978; Weber et al., 1979; Kerr and Krantis, 1979) were used to examine the involvement of ATP in the colonic descending inhibition.

e) Miscellaneous

1) It was also necessary to show that the relaxations in response to balloon inflations were neurogenic. To this end TTX was employed, which, as reported before, acts as a potent neurotransmission blocker (Narahashi et al., 1964; Quandt et al., 1985).

2) Although it had been recorded and reported that descending inhibition (Mancinelli et al., 1983; Gillespie, 1982), as well as part of the ascending excitation (Costa and Furness, 1976), are neither adrenergically nor cholinergically mediated, the exclusion of these two most important neuro-active systems had to also be shown when using the present experimental techniques.

MATERIAL AND METHODS

Reliable, continually reproducible colonic circular muscle relaxation responses could be produced as described in chapter II by inflation of an intraluminal balloon. A colonic segment was attached to a frame by two hooks and placed in a bath containing heated (36°C) Krebs solution (fig. V). A Fogarty arterial embolectomy catheter (green) AE3F, which at 200 µl volume distended to 5.8 mm x 6.5 mm or at 180 µl to 5.4 mm x 6.5 mm, was used as the distending stimulus. This resulted in recognizable relaxations, which were recorded via tonic torque transducers by a Grass Polygraph. A 1 mm alteration in gut diameter caused a displacement of 0.38° of the torque transducer arm (r = 15 cm), which produced a 1 cm deflection at 10mV cm^{-1}. A 180 ul inflation volume was preferentially

used, because it allowed approximately 70 to 100 inflations before the balloon failed. Inflations were repeated at 3, 4 or 7 minute intervals.

An averaging technique was used to improve the signal-to-noise ratio of responses, against inherent activity; traces of the induced relaxations, together with trace sections 2 minutes before and 1 minute after the stimulation event,were fed into a PDP 11-40 computer as coordinate values, stored and averaged using the programme (DIGITX), designed for this work by Mr. Ron Read, Dept. of Physiology, University of Adelaide. The averaged composite traces were then drawn by a plotter (Hewlett Packard 7221 S).

A minimum of six animals was used for each drug. A proximal, medial and distal colon segment from each animal was examined each time. Drug sequences were applied more than three times to each segment, if the effect was reversible. Drug volume was always less than 1% of the bath volume.

It was tested whether the circular muscle relaxations, recorded, 15 mm to 25 mm anally from the inflated balloon, were either cholinergically or adrenergically mediated. Neither atropine (10^{-6}M), or tropicamide (10^{-6}M), both potent muscarinic blockers, nor guanethidine (10^{-5}M), propranolol (5×10^{-6}M) or phentolamine (5×10^{-5}M), specific adrenergic blockers, were able to antagonize the descending inhibition at concentrations of up to 5 mM. The neurogenicity of the descending inhibition was examined by application of tetrodotoxin (10^{-7}M - 10^{-6}M).

Tests for GABA involvement were carried out using the $GABA_B$ blocker, DAVA (Muyhaddin et al., 1982; Ong and Kerr, 1983a), at concentrations of 5×10^{-5}M to 5×10^{-4}M. During some experiments using possibly excessive stimulation, it was attempted to replenish GABA stores by adding GABA (10^{-7}M) to the bath 20 minutes before further stimulation.

Involvement of prostaglandins in the descending inhibition was tested by the addition of arachidonic acid (AA) at 10^{-8}M to 10^{-3}M concentrations (Bennett and Fleshler, 1970; Tulenko, 1981) to increase PG content, and application of the following known and putative prostaglandin-, or prostaglandin synthesis-blockers: Acetyl salicylic acid (Aspirin, ASA) at 6×10^{-4}M to 2×10^{-2}M (Vane, 1971; Bennett et al., 1975; Crocker and Willavoys, 1976; Sarosiek et al., 1984); Indomethacin (Ind) at 3×10^{-7}M to 5×10^{-4}M (Winder et al., 1967; Flower et al., 1972; Flower and Vane, 1974; Bennett et al., 1980b); racemic Trimethoquinol (Inolin) at 10^{-7}M to 10^{-5}M (MacIntyre and Willis, 1978; Bennett et al., 1980a) and its (+) entantiomer (gift: Dr. Kaiser, F. Hoffmann-La Roche & Co.); Phenyl phosphate (N-0164) (gift: Dr. V.J. Rajadhyaksha, Nelson Research Co.) at 10^{-7}M to 10^{-5}M (Eakins et al., 1976; Maas and Den Hertog, 1980); Disodium-Cromoglycate (Intal) at 10^{-5}M to 10^{-2}M (Cox et al., 1970); 6-Keto-Prostaglandin E_2 (gift: Dr. J.E. Pike, Upjohn Co) at 3×10^{-9}M to $3 \times 10^{-}$ M (Griffiths et al., 1982); Polyphloretin phosphate (PPP) (gift: Dr. B. Fredholm, Leo A.B.) at 10^{-7}M to 10^{-4}M (Bennett and Posner, 1971; Eakins et al., 1971; Weeks, 1972; Bennett et al., 1976); Suprofen (gift: Dr. Marlborough, Ethnor Pty. Ltd.) at 10^{-7}M to 10^{-5}M (Clerck et al., 1975; Janssen, 1975; Niemegeers, 1975; Van Nueten, 1976; Hadhazy, 1983); the dibenzo-oxazepine-hydrazide derivate SC 19220 (gift: Mr. P.A. Jamos,

G.D. Searle & Co.) at 10^{-5}M to 10^{-4}M (Sanner, 1969; Wilson, 1972; Weeks, 1972; Cranston et al., 1976; Sanner and Eakins, 1976; Laburn et al., 1977; Bennett et al., 1980a; Kennedy et al., 1982); HR 546 (same as S 732546) (gift: Dr. Schorr, Hoechst AG) at 10^{-7}M to 10^{-4}M (Cranston et al., 1976; Laburn et al., 1977).

Glucagon was applied at 10^{-10}M to 10^{-7}M in a single dose to induce an inhibitory effect, or a succession of applications was used in an attempt to block the descending inhibition by tachyphylaxis.

Neurotensin was employed at concentrations of 10^{-10}M to 10^{-7}M to observe its effect, or it was applied in succession to block the inhibitory response by 'desensitization'. In addition, [D-TRP^{11}] -neurotensin 2 x 10^{-7}M to 2 x 10^{-6}M was employed in an attempt to block the inhibition.

Substance P, the undecapeptide, was applied at concentrations of 10^{-12}M to 10^{-7}M, or desensitization to SP was induced by repeated high doses.

VIP was tried at 10^{-12}M to 10^{-8}M and also in a tachyphylactic design (5 doses of 10^{-9}M at one minute intervals).

Opioid involvement was tested with naloxone pretreatment at 10^{-9}M to 10^{-6}M. The effect of combinations of naloxone and DAVA on the descending inhibition was also investigated.

Theophylline, and a putative ATP blocker Ciba-blue (Reactive blue, Kerr and Krantis, 1979) as well as its purified components, were applied at

concentrations ranging from 10^{-5}M to 1.5 x 10^{-4}M, the latter in dose increments over several minutes to avoid loss of tone in the preparations.

The effects of methysergide (5 x 10^{-6}M - 5 x 10^{-5}M), mianserin (2 x 10^{-5}M) and DAVA (5 x 10^{-4}M) on the oral contractile responses to balloon inflation were also investigated.

To test neurogenicity of responses, possibly adrenergic and cholinergic involvements, the following drugs were used: TTX (10^{-6}M), phentolamine (5 x 10^{-5}M), propanolol (5 x 10^{-6}M), yohimbine (5 x 10^{-6}M), guanethidine (10^{-5}M), tropicamide (10^{-6}M) and atropine (10^{-6}M).

RESULTS

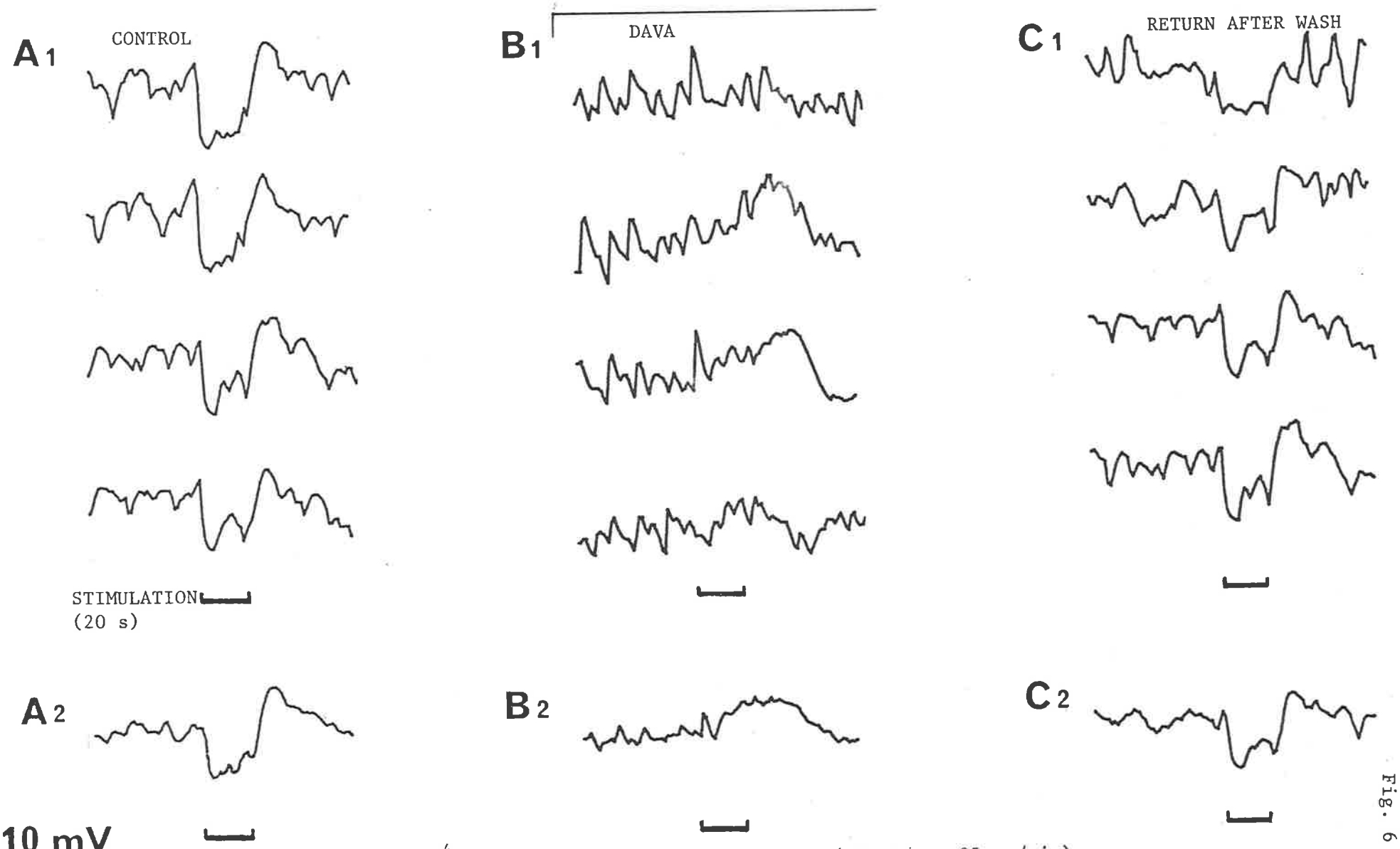
As detailed elsewhere, it was first, by the use of TTX, as well as cholinergic and adrenergic blockers, confirmed that the descending inhibition, elicited by intralumenal balloon inflation, was indeed neurogenic but neither cholinergic nor adrenergic in nature (vide infra, pp. 86-87).

Antagonism of the descending inhibition, in the isolated colon of the guinea-pig, elicited by 20 second inflations of the Fogarty balloon (bars) are shown in figure 6. The four traces (A_1), each an average of 3 responses to inflation and (A_2), averages of 18 such events, were taken as the control responses. A repeatedly reproducible series of circular

muscle relaxations in excess of one mm was demonstrated. The second row of 4 traces (B_1, n12), and the trace at the bottom (B_2, n18), show the alteration of the averaged responses when 5 x 10^{-4}M of DAVA had been added to the gut bath 7 minutes before the inflation stimulation, where the relaxations were abolished by DAVA. The gut segment was washed by flushing the bath twice, and, 20 minutes later, the relaxations in response to balloon inflations had returned to their previous level (C_1 and C_2). An "after-relaxation" that followed, was not affected. These results suggest a GABA involvement in the descending inhibition.

Additional evidence for a GABA involvement is presented in figure 7. Substantial relaxations, like the fourth trace (A_1), often deteriorated after excessive repetitions of the stimulation, and became less pronounced (traces 1-3, A_1), resulting in a small but recognizable averaged relaxation (A_2). GABA loading (20 minutes incubation at 10^{-7} M GABA) resulted in increased responses (B_1; n3 for each trace) and (B_2; n6). In these experiments the blocking effect of DAVA (5 x 10^{-4}M) reached a maximum after 10 minutes (C), and was reversed 20 minutes after washing the gut segment with Krebs solution.

In some preparations DAVA at 10^{-4}M did not completely block the relaxations induced by a 20 second inflation, but when a shorter (12s) inflation duration was employed, and results were recorded as in figure 8, strong control relaxations (A_1 n3 each and A_2 n18) were then seen to be blocked within 10 minutes by DAVA at 10^{-4}M (B_1 and B_2). This blocking effect was again abolished within 20 minutes after washing.



EFFECT OF DAVA (5×10^{-4}M) ON THE DESCENDING INHIBITION (10m V/cm, 25 mm/min)

Fig. 6

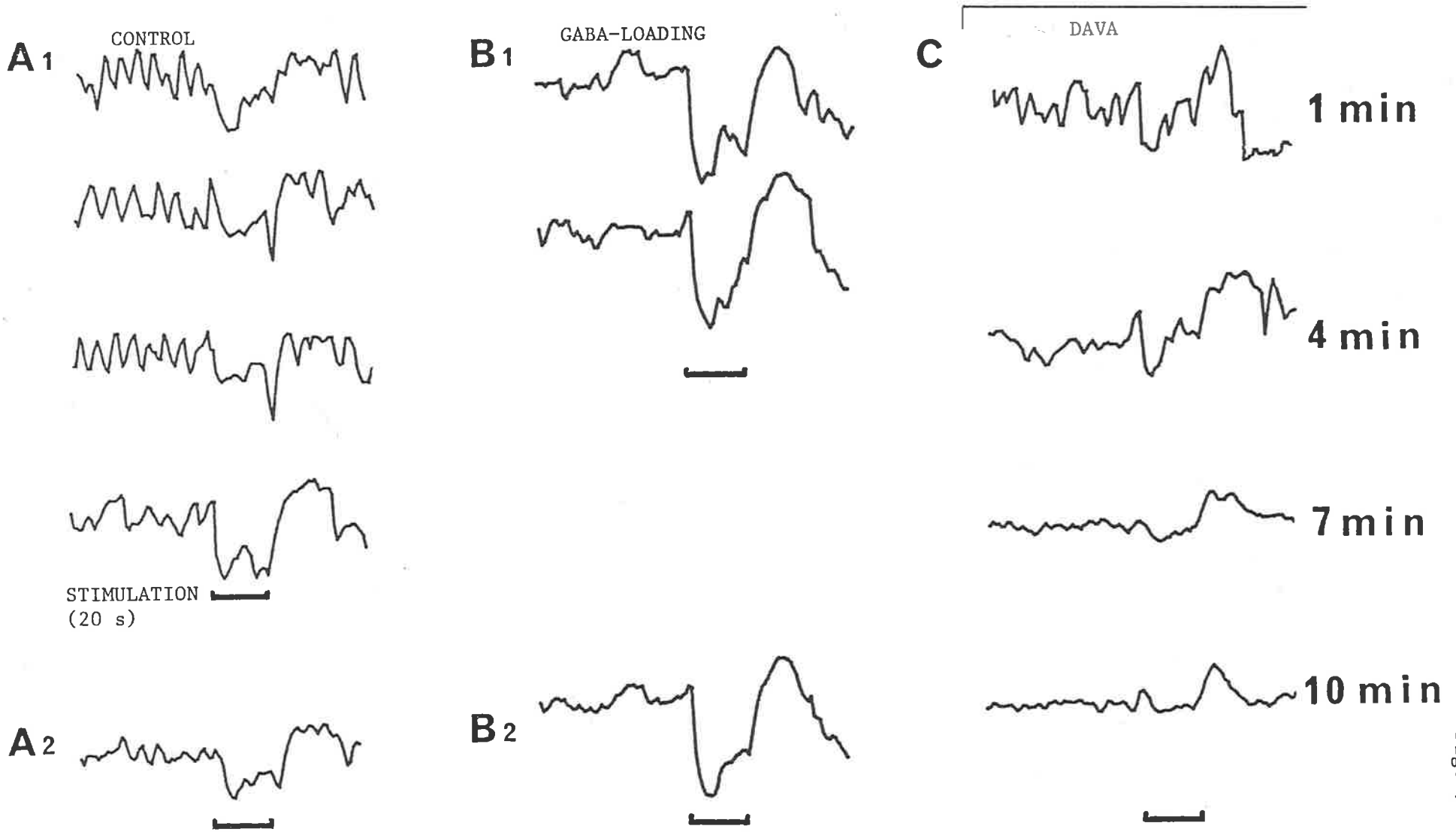


Fig. 7

GABA-LOADING AND TIME COURSE OF DAVA (5×10^{-4}M) EFFECT (10m V/cm, 25mm-min)

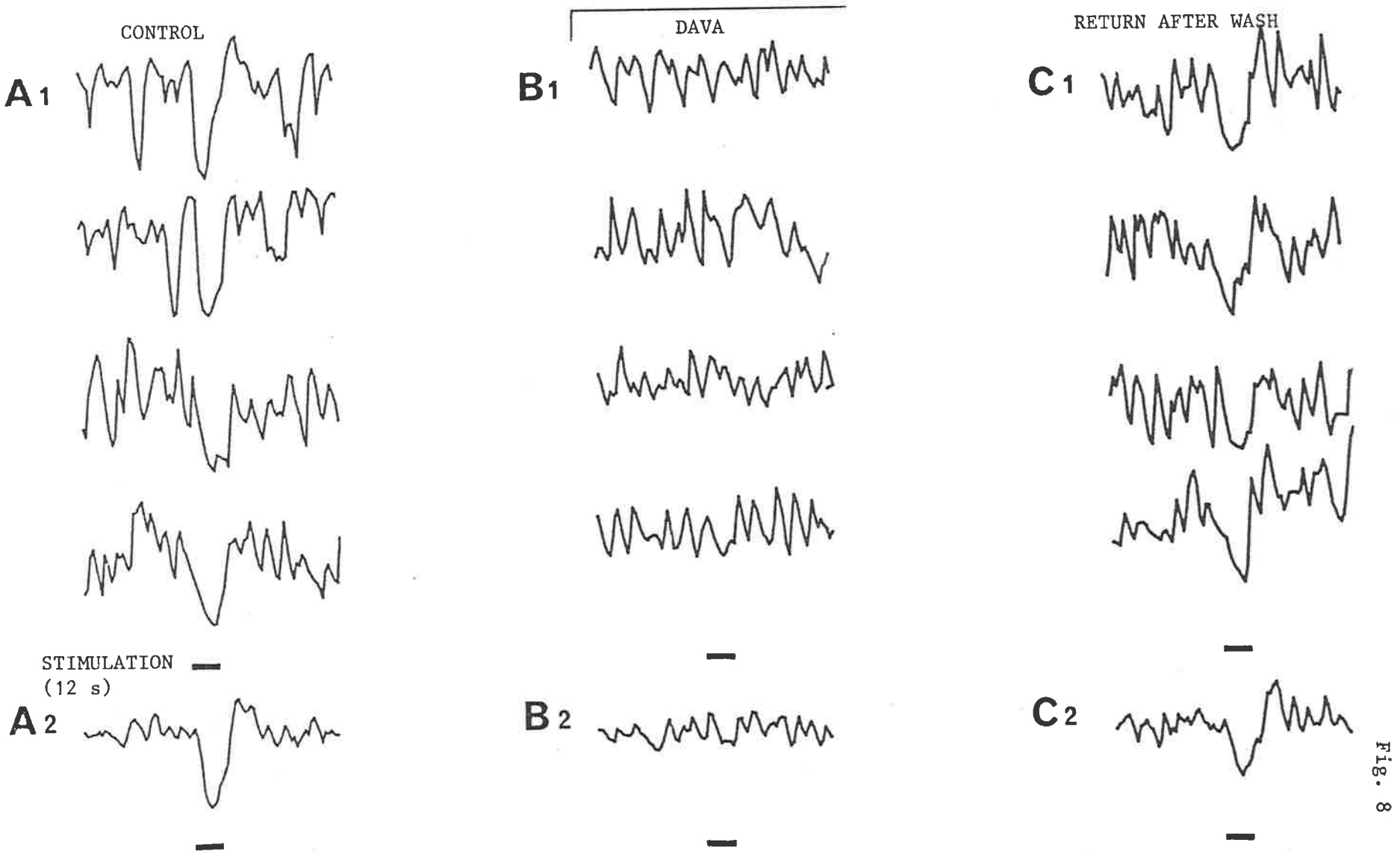


Fig. 8

DAVA (10^{-4}M) BLOCKING EFFECT (10m V/cm, 25 mm/min)

In yet other preparations, DAVA 5 x 10^{-5} M did not exhibit any blocking effect, even when using a 12 s stimulation duration, and on these, the efficacy of DAVA was tested when applied externally or intraluminally. In figure 9, strong control relaxations (A_1 and A_2) were registered (note that the sensitivity had been reduced to 20 mV cm^{-1}), and whilst DAVA (5 x 10^{-5}M) applied externally, by addition to the bath, did not have any significant effect (B_1 and B_2), yet the internal application of DAVA (5 x 10^{-5}M), injected within the lumen of the colon, did block the relaxation (C_1 and C_2). Even if DAVA did not completely block the descending relaxation, especially at lower concentrations, it did however usually reduce or abolish a later component of the relaxation induced by the balloon distension (fig. 14b, A_2).

However, a prostaglandin involvement is also likely: in gut sections fatigued by continual balloon inflations at one minute intervals for one hour, the responding relaxations often became minimal (fig. 10, A_1; averages A_2), but after addition of arachidonic acid (10^{-8}M), however, quite strong relaxations returned (B_1; B_2).

The effects of prostaglandins of the E and F series on the gut are well documented. Results, however, have not always been in agreement and for this reason it was decided to test them in the present experimental arrangement. It was found that prostaglandin E_2 gave the greatest relaxations at lowest concentrations, when guinea-pig colon sections were checked for their sensitivity in response to prostaglandin E_2, E_1 and F_2 The circular muscle relaxation appeared to be most pronounced at the medium dosage of 10^{-8}M (fig. 10, C_2). The longitudinal muscles' response

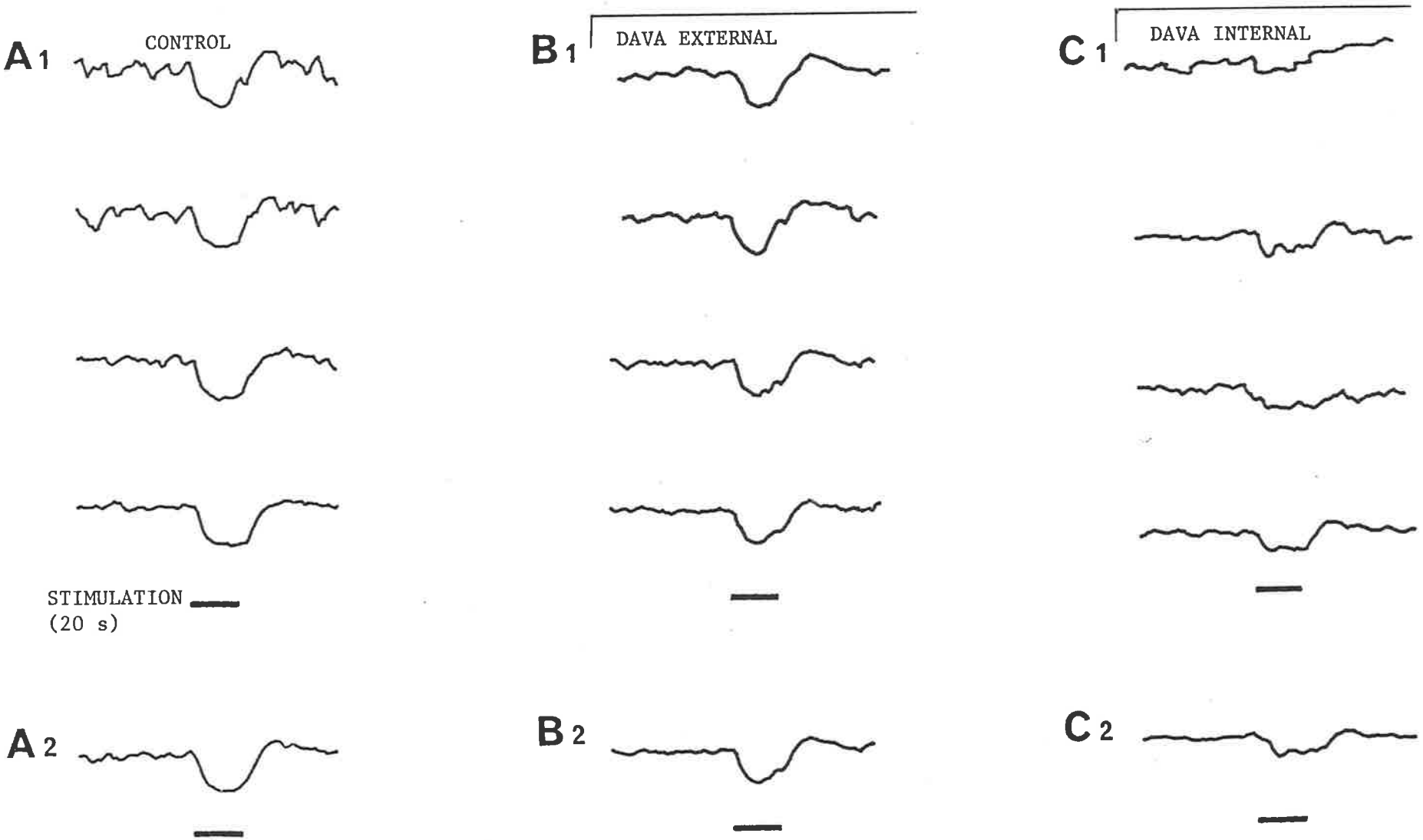


Fig. 9

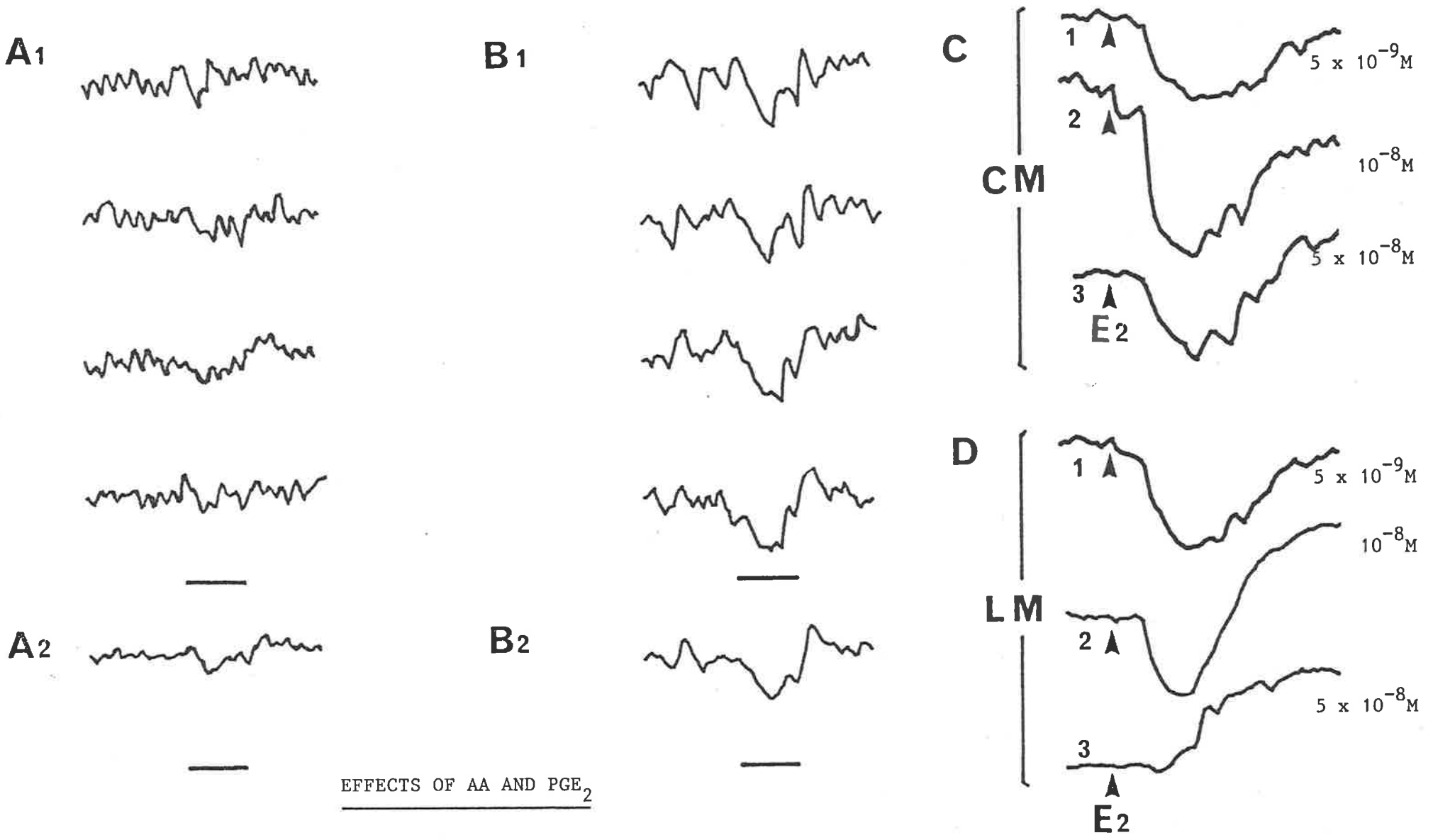
DAVA (5×10^{-5}M) EXTERNAL - INTERNAL APPLICATION (20mV/cm, 25mm/min)

changed from a relaxation at 5 x 10^{-9}M to a contraction at 5 x 10^{-8}M (fig. 10, D_1 and D_3), whilst at the medium dose of 10^{-8}M a relaxation followed by a large contraction could be observed (fig. 10, D_2).

The following traces showing results of tests carried out with various prostaglandin and prostaglandin synthesis blockers were computer averaged using results from 3 to 12 different animal records; although response variations existed between different animals, relative changes from control values demonstrated the effects of various drugs.

Aspirin (10^{-3}M) did not appear to have a significant effect on the relaxation initiated by balloon inflation, during the test periods from 3 min to 60 min (fig. 11, A_1 to A_4). Indomethacin also did not influence the relaxations at concentrations of 3 x 10^{-7}M to 10^{-5}M, even up to 40 min after addition to the gut bath (fig. 11, B_1 to B_4). Neither trimethoquinol nor its (+) entantiomer exhibited any relaxation blocking effect at 10^{-6}M for 40 min (fig. 11, C_1, C_3, C_4). However, Trimethoquinol markedly potentiated the inhibition after the first 5 min (fig. 11, C_2).

Neither N-0164 at 10^{-5}M nor Intal at 2 x 10^{-3}M caused any substantial changes in the responses for up to 40 min (fig. 12, A_1 to A_4; B_1 to B_4), whilst 6-Keto-PGE, potentiated the relaxation which returned to its control value after washing (fig. 12, C_1 to C_4). Suprofen (10^{-5}M), or SC 19220 (10^{-4}M), did not alter the relaxation at all within 40 min (fig. 13, A_1 and C_1), whilst HR 546 lowered the muscle tone and thus reduced the relaxation, but after the addition of 10^{-8}M carbachol the tonus as well as the inhibition returned to control levels (fig. 13, B_1 to B4).



EFFECTS OF AA AND PGE_2

Fig. 10

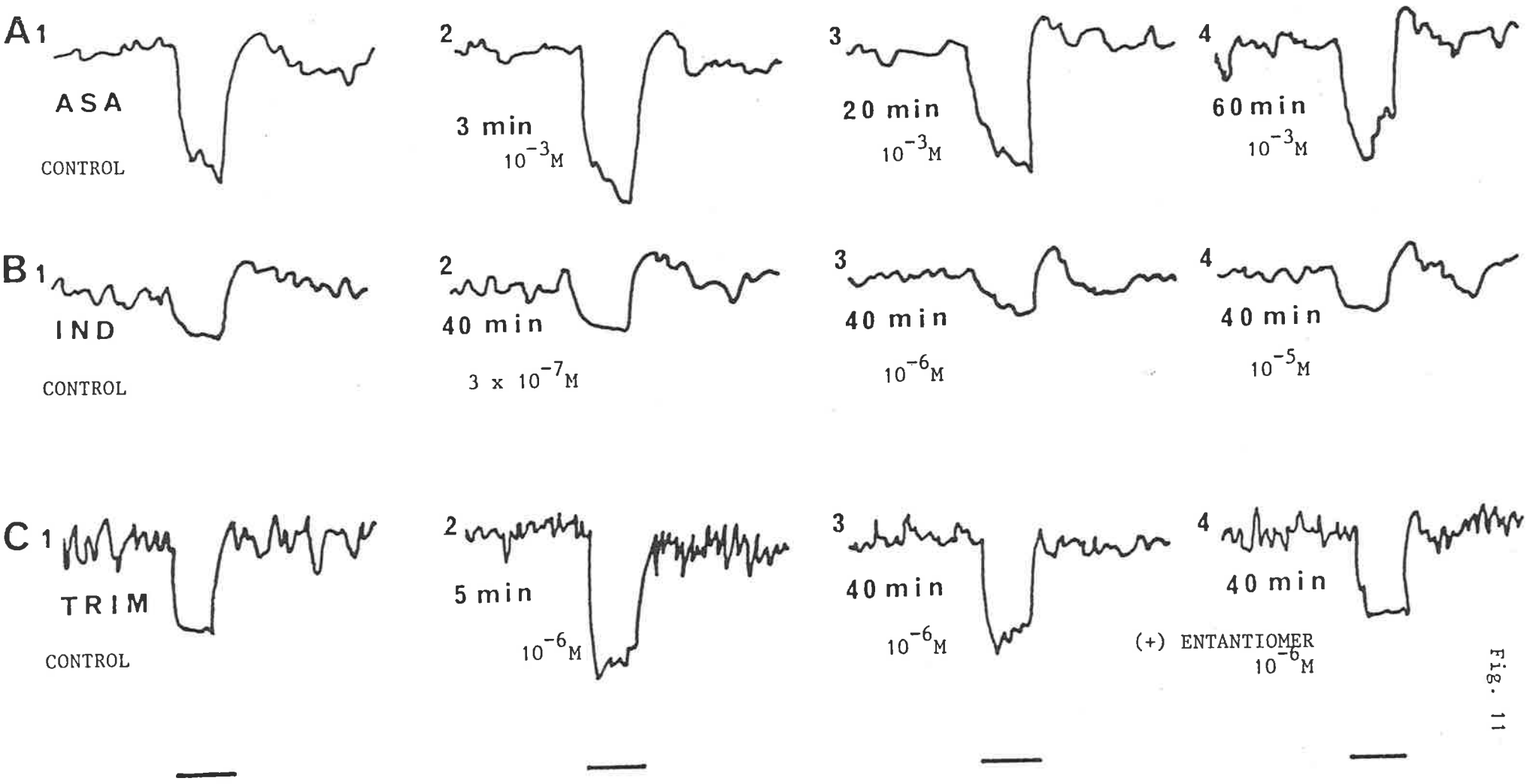


Fig. 11

PROSTAGLANDIN BLOCKERS: ASA, IND AND TRIM

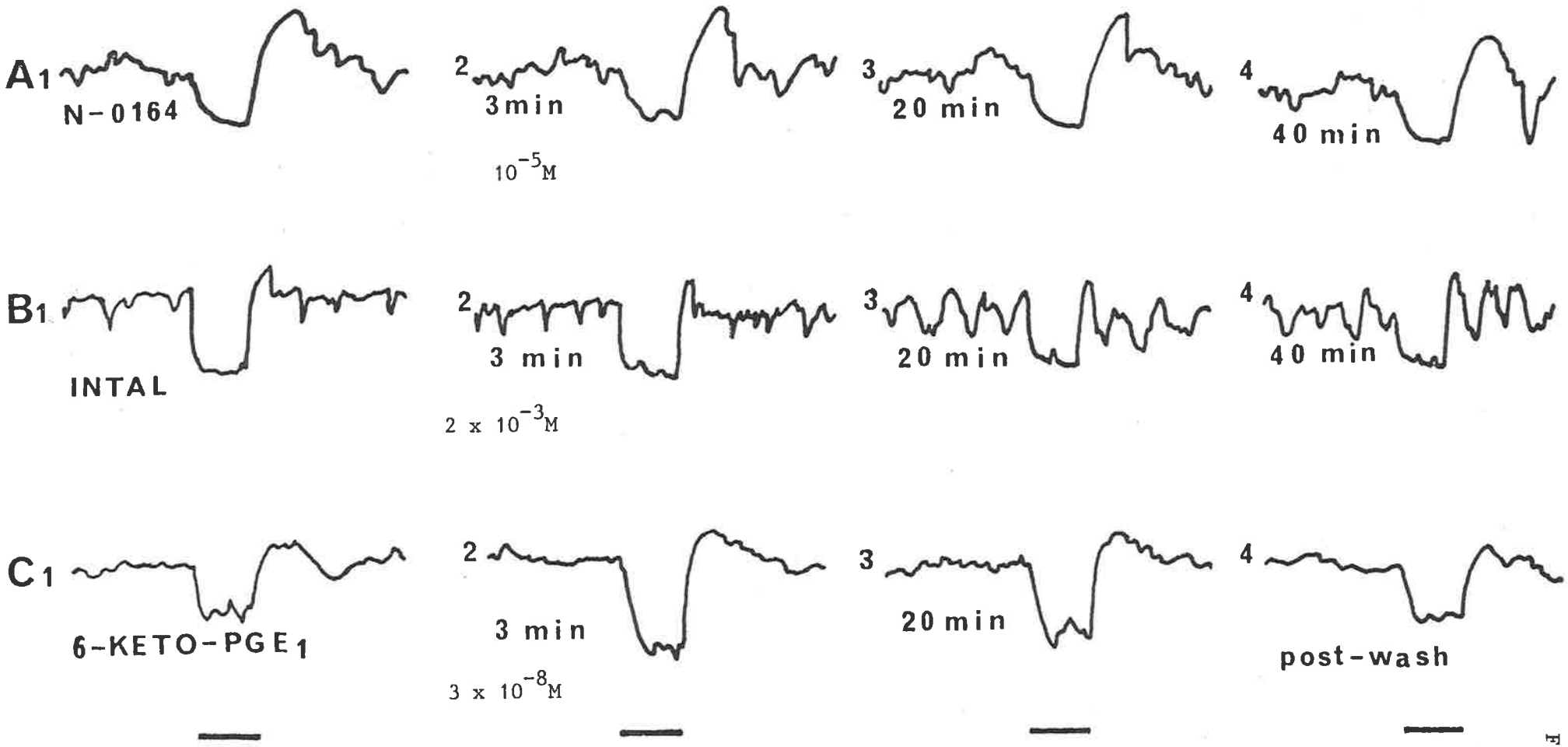
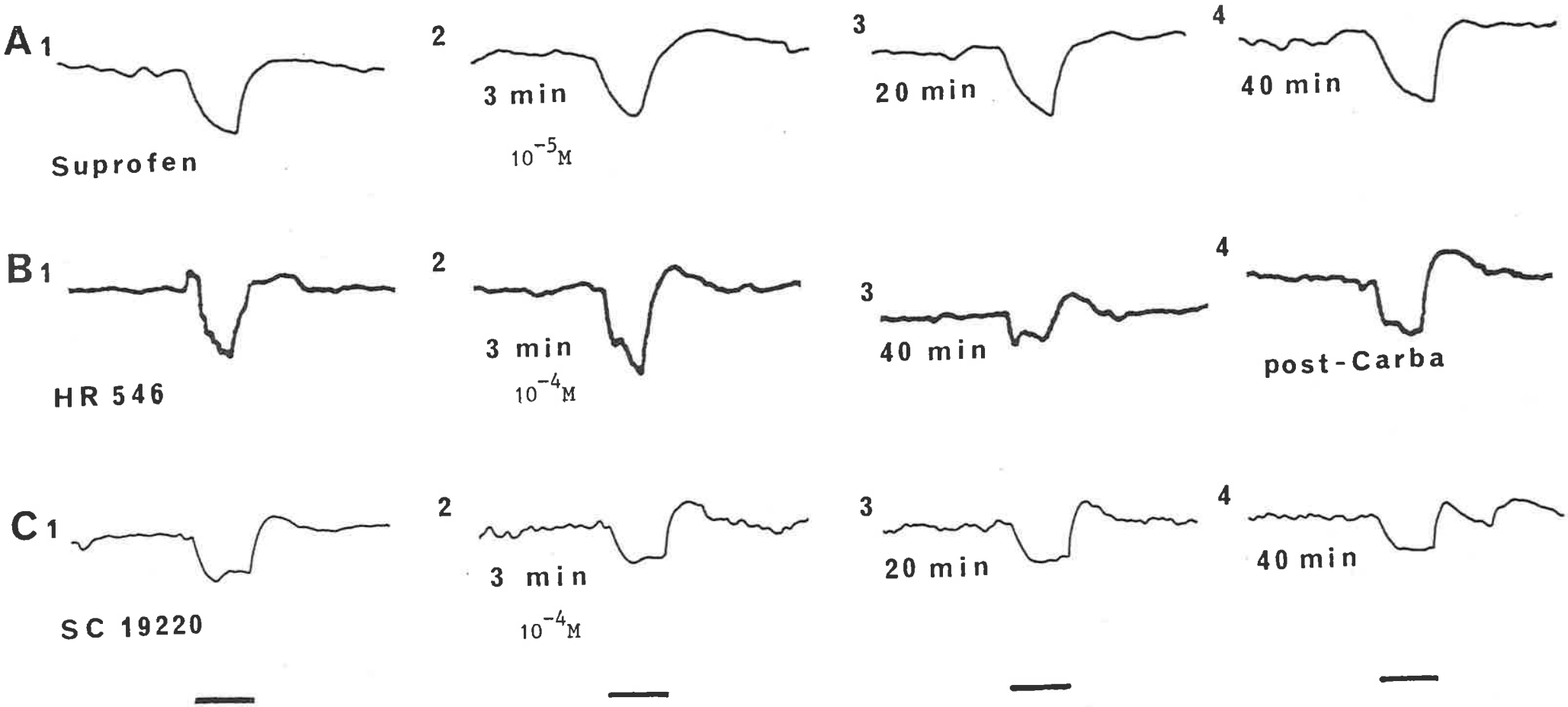


Fig. 12

PROSTAGLANDIN BLOCKERS: N-0164, INTAL AND 6-KETO-PGE

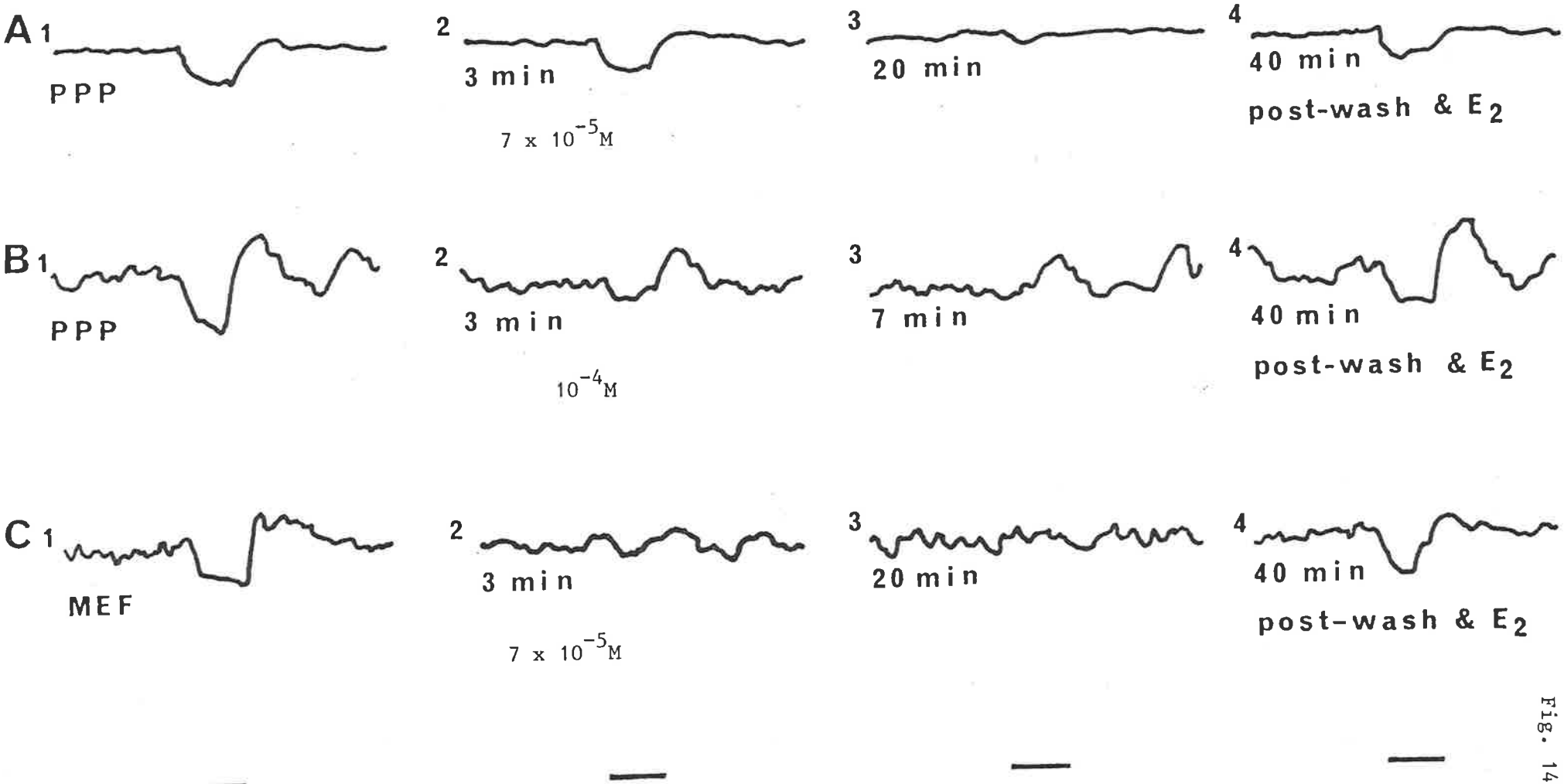


PROSTAGLANDIN BLOCKERS: SUPROFEN, HR 546 AND SC 19220

Fig. 13

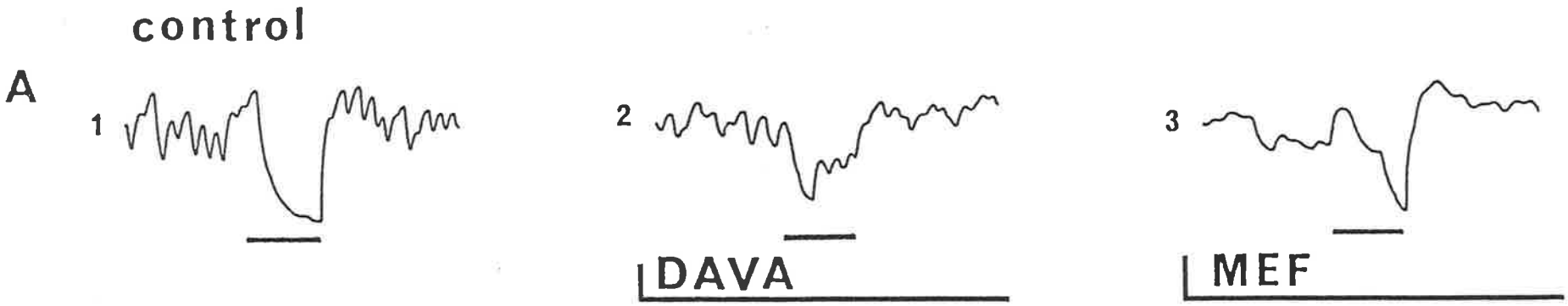
Two prostaglandin antagonists were found to block the balloon-inflation-induced descending relaxations, these were polyphloretin-phosphate (PPP) and sodium-meclofenamate (MEF). At 7×10^{-5}M, PPP took 20 min to block the relaxation which returned 40 min after PPP addition, several washes and PGE_2 supplement (fig. 14a, A_1 to A_4). PPP at 10^{-4}M had a full blocking effect after 7 min, which could again be reversed after 40 min by washing and PGE_2 addition (fig. 14a, B_1 to B_4). MEF at 1×10^{-5}M blocked the relaxation usually within 3 min to 7 min. An often associated decrease in muscle tonus was counteracted with carbachol, histamine or PGE_2 but the relaxation could never be elicited in the presence of MEF, and only thorough washing would permit the return of the inhibitory response; PGE_2 supplementation was also often found to be beneficial (fig. 14a, C_1 to C_4). It was also observed that whenever MEF blocked the relaxation, especially at low concentrations, it always affected the first part of the relaxation (fig. 14b, A_3). If MEF was left in the gut bath for times exceeding half an hour or more, the colon segment could not be induced to relax in response to further inflation stimuli.

The effects of varying concentrations of MEF 7 min after application are shown in figure 15, B_1 to B_4. A concentration of 2×10^{-5}M had a relaxation reducing effect and could be washed out quite readily (fig. 15, C_1), whilst the blocking effect of 4×10^{-5}M persisted somewhat even after 30 min washing (fig. 15, C_2); a similar effect was noted for MEF at 6×10^{-5}M (fig. 15, C_3), but blocking of the relaxation by MEF (10^{-4}M) largely persisted even after 30 min of washing (fig. 15, C_4). No prostaglandin supplement was added during the above dose/response



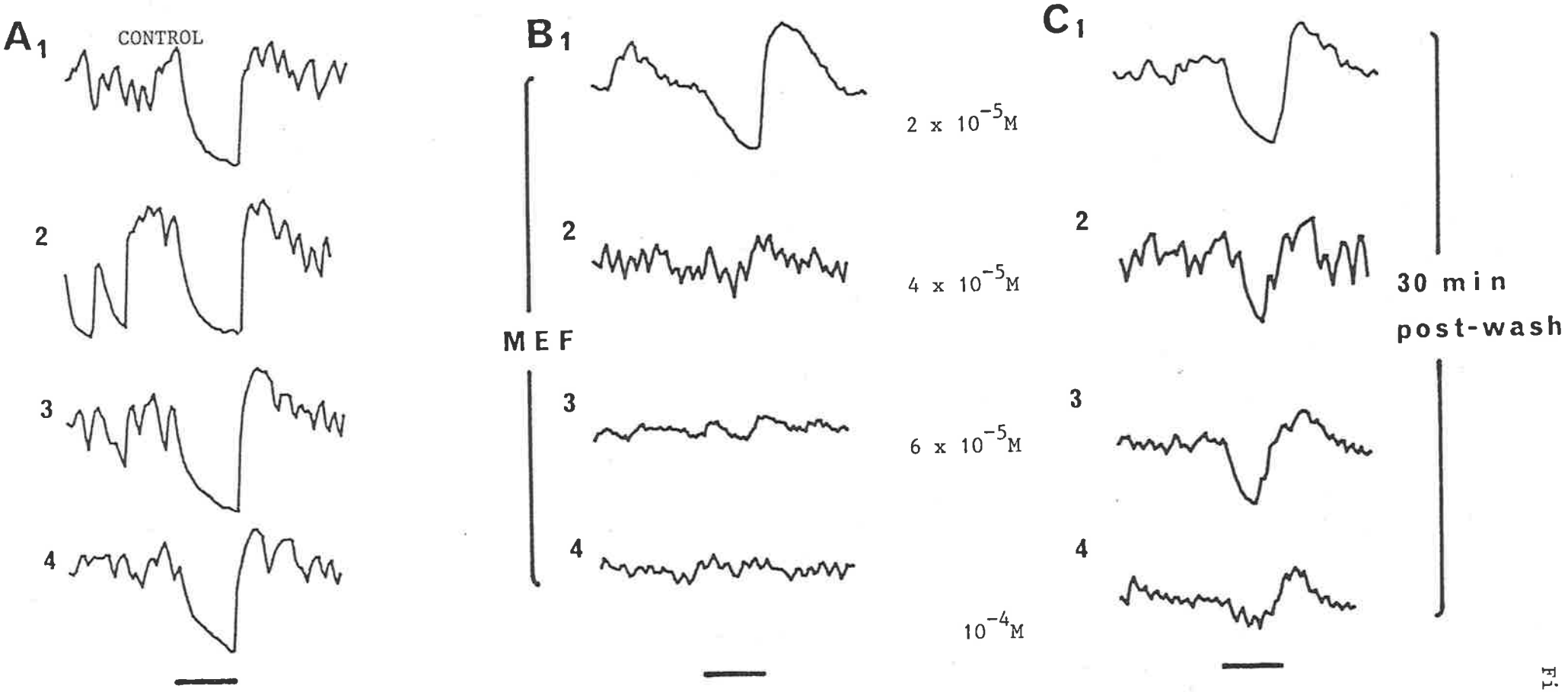
PROSTAGLANDIN BLOCKERS: PPP AND MEF

Fig. 14a



The partial blocking effect of the balloon distension evoked colonic relaxation by DAVA (5×10^{-5}M) and MEF (5×10^{-5}M)

Fig. 14b

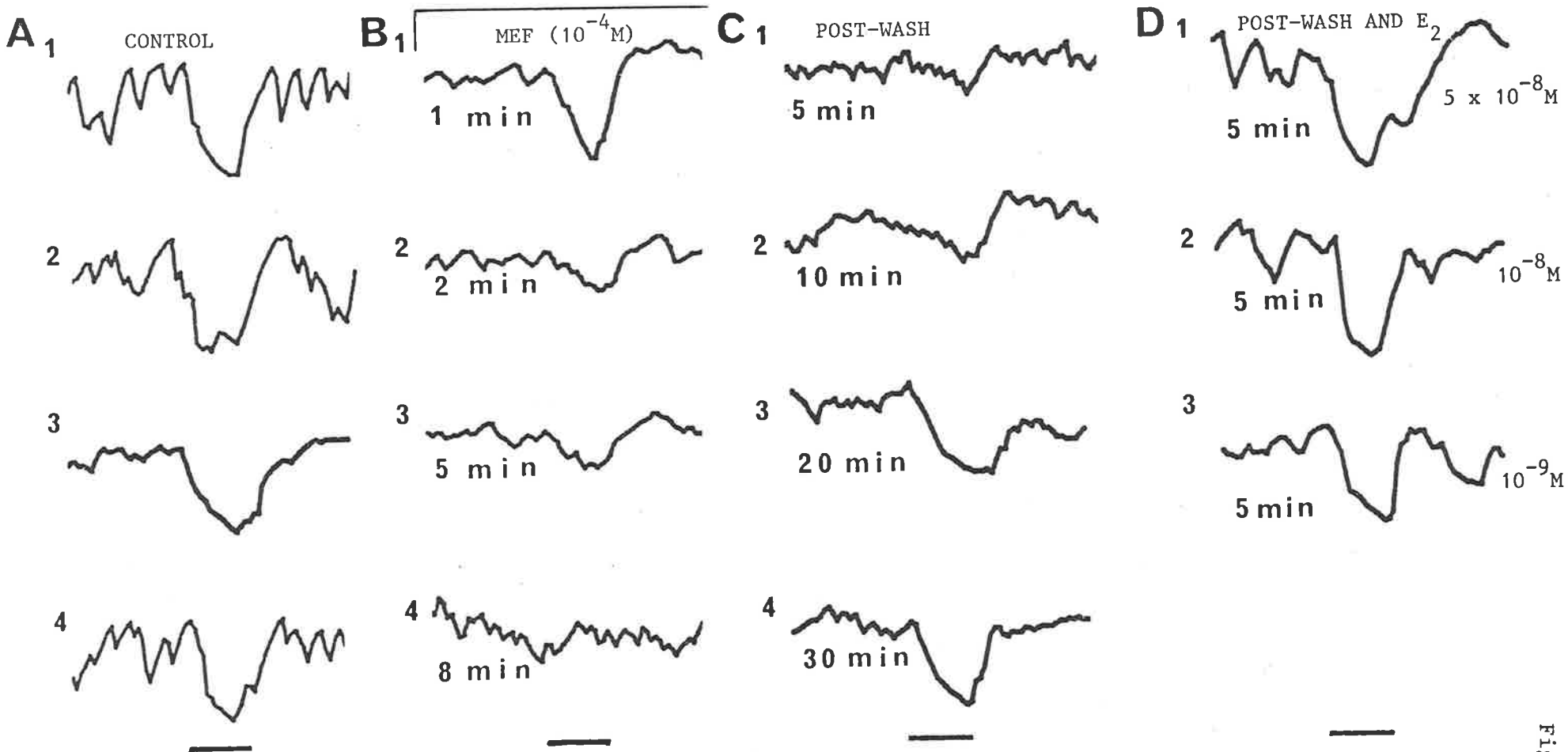


PROSTAGLANDIN BLOCKER MEF (AT VARYING CONCENTRATIONS)

Fig. 15

experiments. The MEF induced blockade of relaxation (fig. 16, B_1 to B_4) was time dependent. For each time trace (1 min, 2 min, 5 min and 8 min) six different responses were averaged, there being only a very slight impairment 1 min after antagonist application, whilst a massive and similar reduction of the response occurred after 2 min and 5 min. However, after 8 min the relaxations were converted to small contractions. Washing the colon segment resulted in a return of the relaxation to approximately control levels after 30 min (fig. 16, C_1 to C_4). If the gut segment was washed, and PGE_2 was also added, the relaxation returned to the pre-antagonist response level after 10 min. The size of the resultant relaxation effect was dependent on E_2 in a dose-dependent manner (fig. 16, D_1 to D_3).

The responses of neuroactive peptides are dealt with in the following paragraphs. Glucagon at concentrations of 10^{-10}M to 10^{-8}M had no effect, but desensitization with 5 aliquots of 2 x 10^{-8}M to a final bath concentration of 10^{-7}M caused slight potentiation of the inhibition (fig. 17, A_1 to A_4) in response to the balloon inflation. Neurotensin and [D-TRP^{11}]-neurotensin in themselves caused a significant but transient relaxation at 5 x 10^{-9}M and 10^{-9}M respectively (fig. 17, B_2 and C_2), although neither drug decreased the stimulus-initiated relaxation at concentrations up to 2 x 10^{-6}M and 10^{-6}M (fig. 17, B_3, B_4 and C_3, C_4). Substance P at concentrations of 10^{-12}M to 10^{-9}M (fig. 18, A_2, A_3) did not affect the inhibitory response, whilst desensitization with 5 aliquots of 1.5 x 10^{-9}M, to a final bath concentration of 7.5 x 10^{-9}M, resulted in an enhancement of the relaxation response (fig. 18, A_4). VIP had neither a marked effect on the inhibition at 10-12M to 10-9M, nor did



PROSTAGLANDIN BLOCKER: MEF (10^{-4}M)

Fig. 16

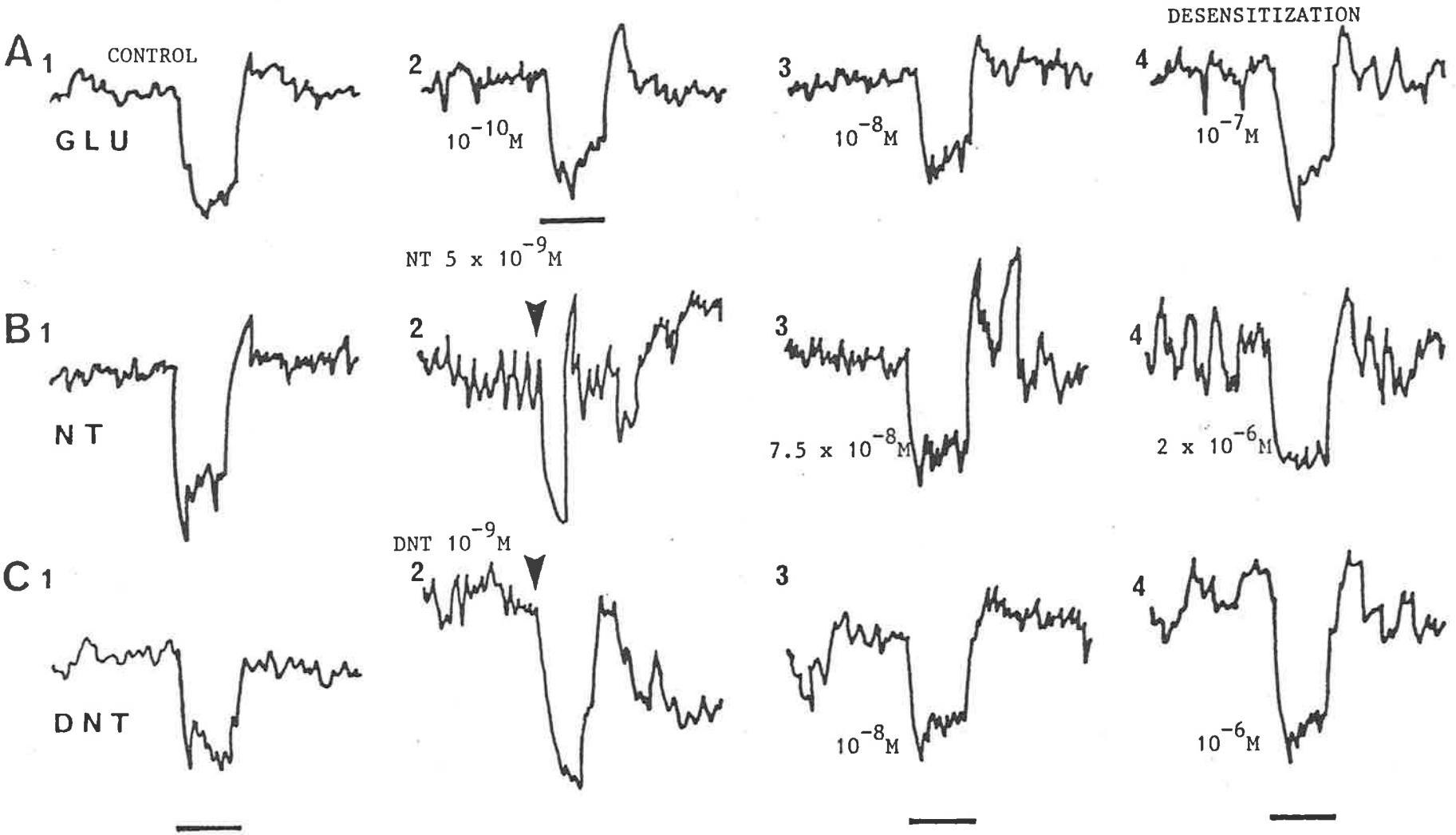


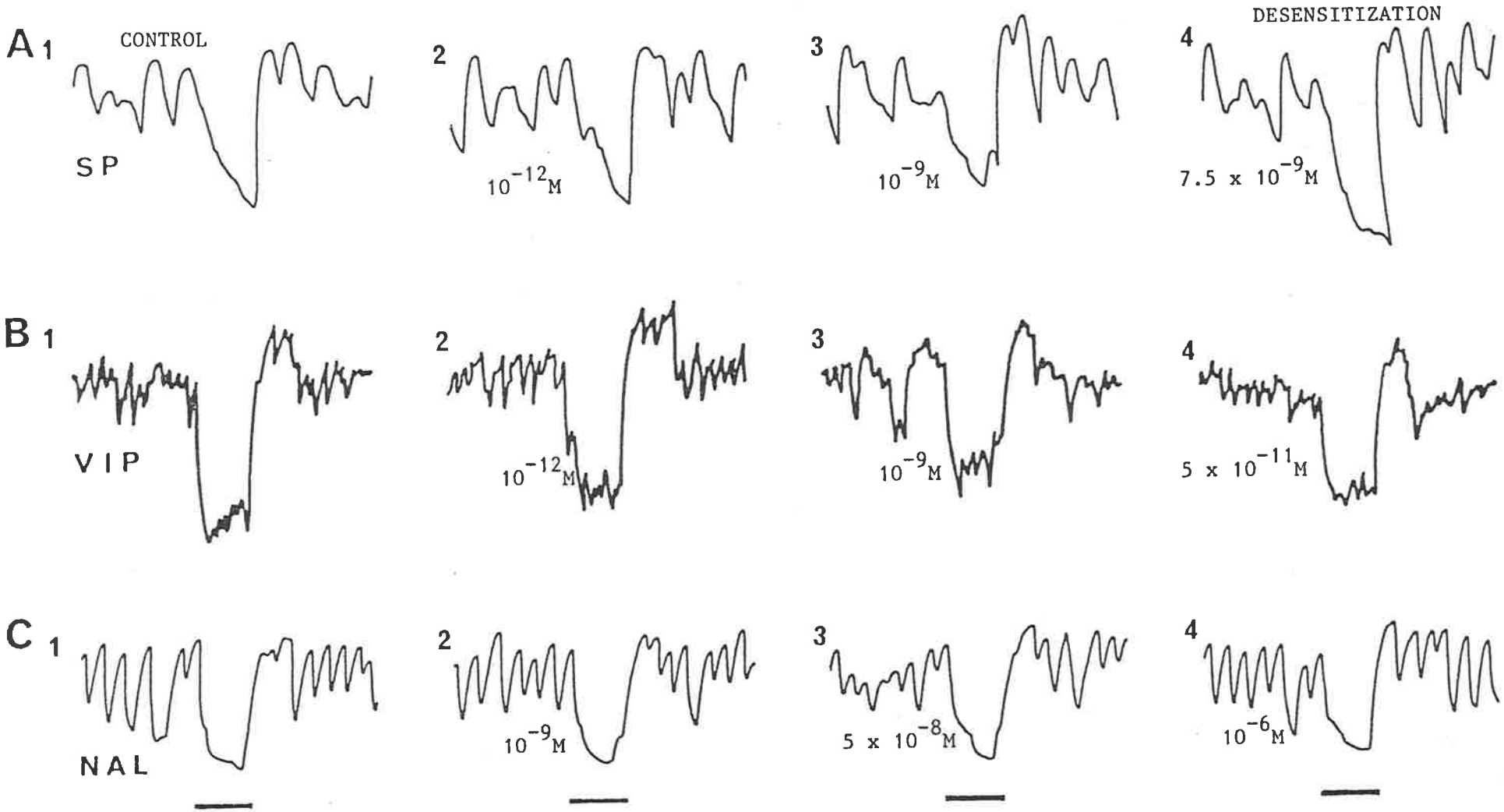
Fig. 17

NEUROACTIVE PEPTIDES: GLU, NT AND DNT

desensitization significantly alter the response to balloon inflation (fig. 18, B_1 to B_4), although the response was reduced. Naloxone, the opioid antagonist, caused no change in the response at 10^{-9}M to 5 x 10^{-8}M, whilst even a dose build-up to a final bath concentration of 10^{-6}M did not significantly reduce the relaxation (fig. 18, C_1 to C_4).

The cholinergic (muscarinic receptor) antagonists atropine and tropicamide were both applied at 10^{-6}M. Tropicamide had no blocking effect up to 25 min (fig. 19, A_1 to A_4). The trace in the presence of atropine (fig. 19, C_1 to C_4) is not an averaged one but a typical record showing on instant muscle tone decrease after atropine (10^{-6}M) application, which could be counteracted with histamine (5 x 10^{-7}M), but the relaxation response was not affected even after 60 min (fig. 19, C_1 to C_4). It should also be noted that the stimulus duration was reduced to 15 s.

Guanethidine, the 'false' adrenergic transmitter, was applied at 10^{-5}M and did not significantly alter the inhibitory response within one hour (fig. 19, B_1 to B_4). The α_1 and α_2 adrenergic receptor blocker phentolamine did not appear to affect the inhibition within 30 min at concentrations of up to 5 x 10^{-5}M (fig. 20, A_1 to A_4). Similarly the βadrenergic receptor blocker propranolol (5 x 10^{-6}M) caused only a passing reduction of the relaxation, which returned to control levels after 30 min (fig. 20, B_1 to B_4). Yohimbine an α_2 receptor antagonist did not appear to influence the inhibition within 30 min at concentrations of up to 5 x 10^{-6} M (fig. 20, C_1 to C_4). If a muscle tone reduction was caused



NEUROACTIVE PEPTIDES AND OPIOID INHIBITOR: SP, VIP AND NAL

Fig. 18

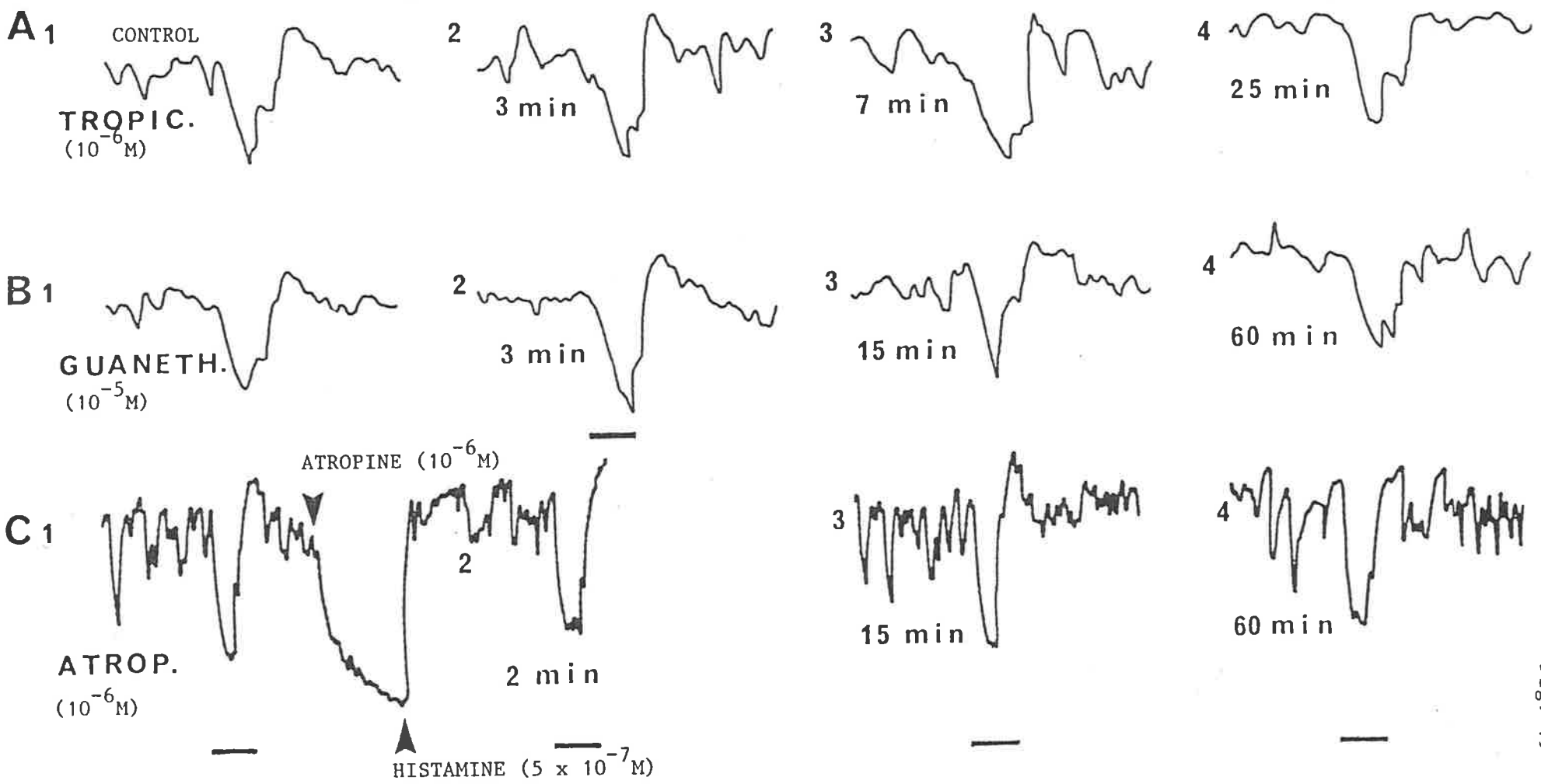


Fig. 19

CHOLINERGIC BLOCKERS AND 'FALSE' ADRENERGIC TRANSMITTER: TROPICAMIDE, ATROPINE AND GUANETHIDINE

by these adrenergic blockers, in some of the gut segments it was counteracted by 3 x 10^{-8}M carbachol application.

Tetrodotoxin, the neurotransmission blocker, totally blocked the colonic relaxation initiated by balloon inflation within 9 min at 10^{-6}M. The gradual reduction of neural transmission became obvious at the 3 min and 6 min inflations (fig. 20, D_1 to D_4). The tetrodotoxin-induced decrease of muscle tone was raised by barium chloride at concentrations of 5 x 10^{-5}M and 10^{-4}M, which acted directly on the smooth muscle.

Ciba-Blue, a suggested ATP antagonist, was added to the bath at 10^{-5}M and 10^{-4}M, which caused a dose-dependent reduction of the relaxation (fig. 21, A_2, A_3), whilst addition of 5 aliquots of 3 x 10^{-5}M, to a final bath concentration of 1.5 x 10^{-4}M, blocked the relaxation completely (fig. 21, A_4). Control relaxation responses, and the effects of the six major components of the dye, are shown in figure 21, B to G. The fraction Rf. .39 (fig. 21, D_1, D_2) and Rf. .45 (fig. 21, E_1, E_2) exhibited strong antagonism to the relaxation response. All these six chromatographically separated drugs, named according to their relative fronts (Rf.s), were applied in 5 aliquots of 3 x 10^{-5}M to reach a final bath concentration of 1.5 x 10^{-4}M.

Finally, it was noticed when 5-HT antagonists were used, that although they did not affect the descending inhibition, they nearly always counteracted the oral contractions. Methysergide at 5 x 10^{-6}M reduced the contraction (fig. 22, A_1, A_2) considerably, while 5 x 10^{-5}M abolished it (fig. 22, B1, B_2). Both responses returned to control levels after

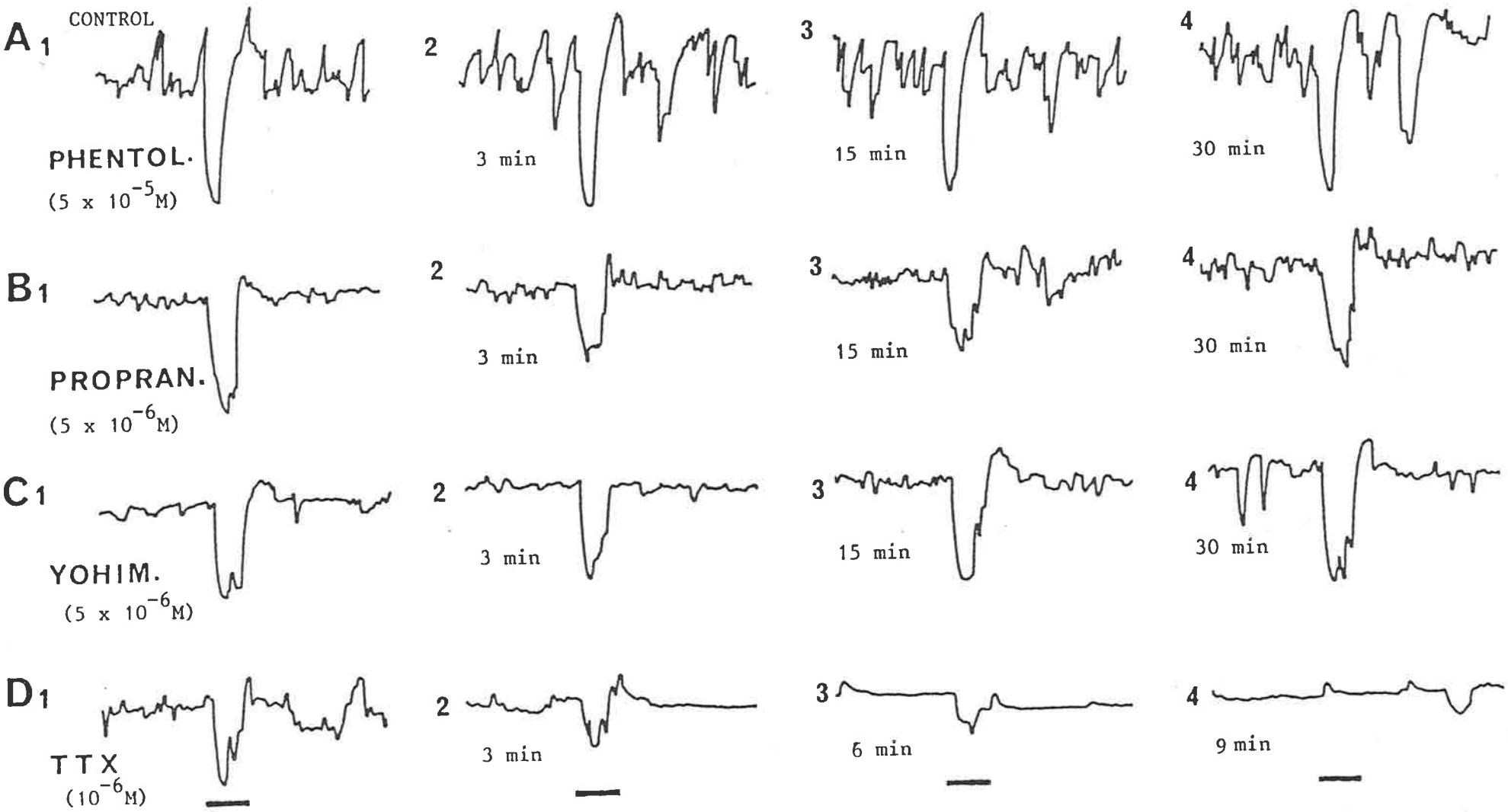
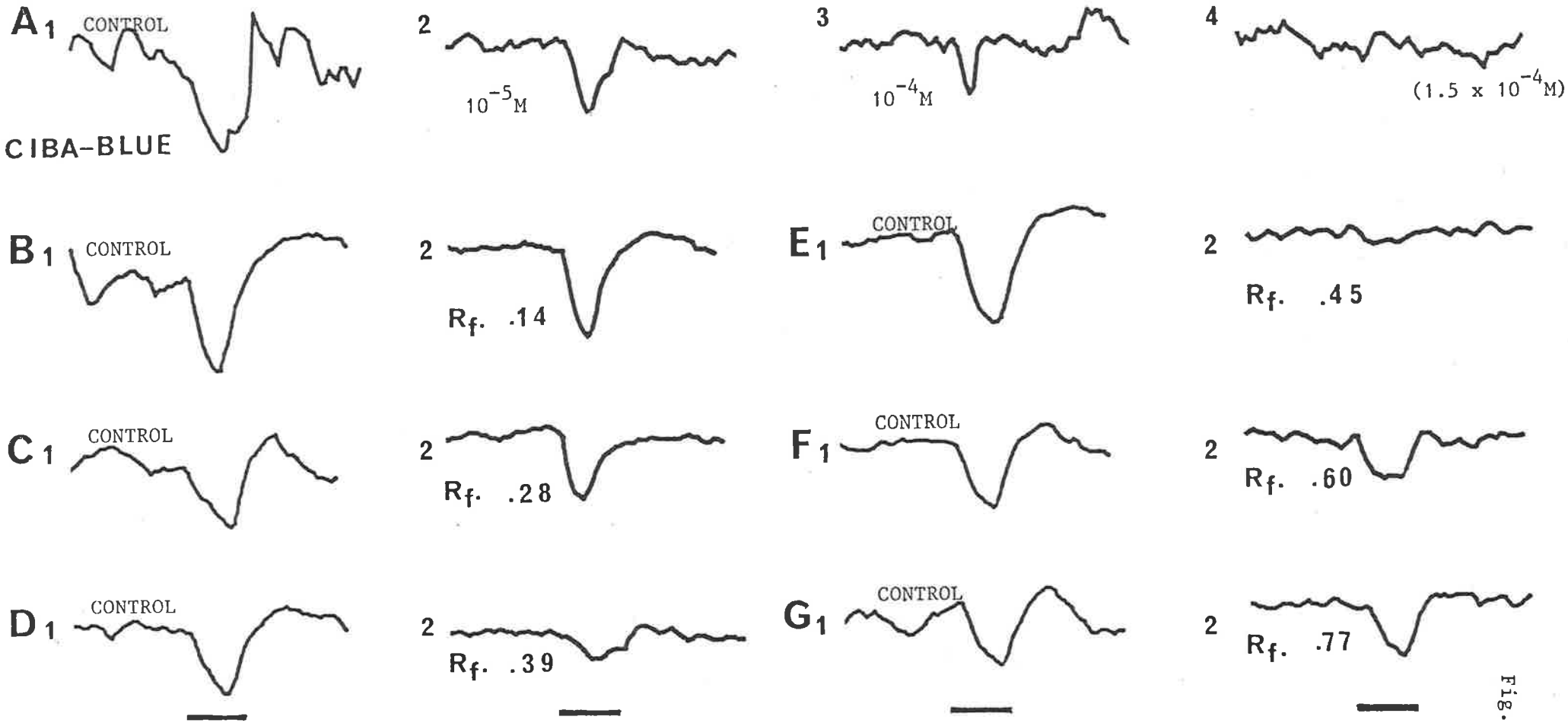


Fig. 20

ADRENERGIC AND NEUROTRANSMISSION BLOCKERS: PHENTOLAMINE, PROPRANOLOL, YOHIMBINE AND TETRODOTOXIN



EFFECTS OF CIBA-BLUE AND ITS MAJOR FRACTIONS (ALL FRACTIONS WERE APPLIED AT 1.5 x 10^{-4}M DESENSITIZATION)

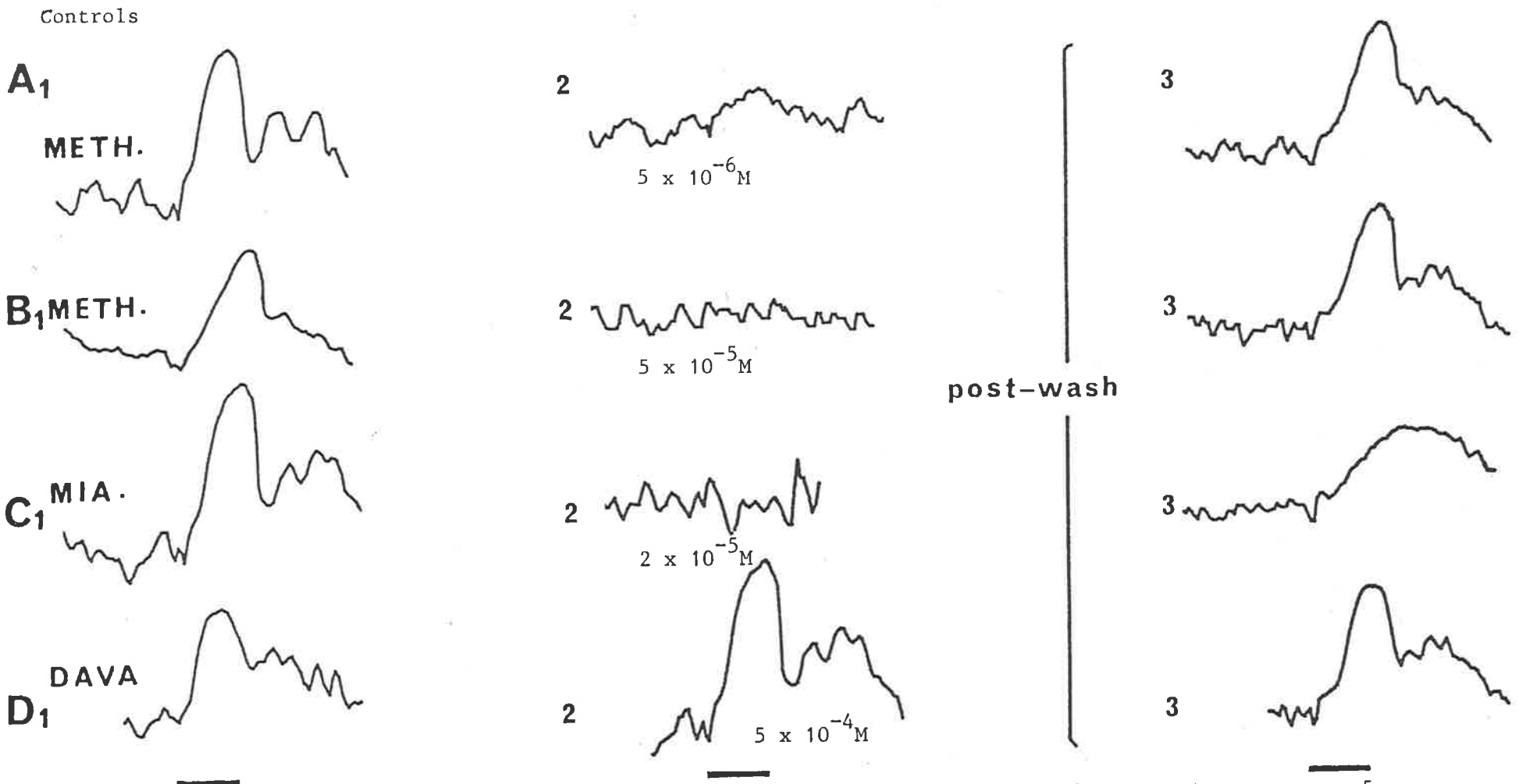
Fig. 21

washing (fig. 22, A_3, B_3). Mianserin at 2 x 10^{-5}M blocked the oral contractions (fig. 22, C_1, C_2), but could not be washed out (fig. 22, C_3). A potentiating effect (56%) on the oral contraction was observed for DAVA at 5 x 10^{-4}M (fig. 22, D_1, D_2), which returned to control levels after the wash (fig. 22, D_3).

DISCUSSION

Results presented in figures 6 to 9 point to the important role of GABA in the descending inhibition of the colon of guinea-pigs. The reasons for continuation of the search for the elusive inhibitory transmitter, after these findings that suggest a role for GABA in the reflex, were that a) comparatively high concentrations of antagonist (0.1 mM to 0.5 mM) had to be used to effectively block the relaxation and b) the blocking action of DAVA could not be achieved continuously with every experimental animal. Although the potentiation of the relaxation by GABA-loading, and the internal/external application results, were both suggestive, the lack of an effect of DAVA on the inhibitory response in some gut segments pointed to the possible involvement of another transmitter. DAVA's ability to block sometimes only the latter part of the descending inhibition (fig. 14b, A_2) also suggested a second, cooperating transmitter-effect.

Experiments with prostaglandins, of which PGE_2 was found to be the most effective, suggested some PG involvement in the descending inhibition. Relaxations in purposely fatigued gut sections returned to control value after arachidonic acid addition, thereby pointing to a



THE EFFECT OF METHYSERGIDE (5×10^{-6} TO 5×10^{-5}M), MIANSERIN (2×10^{-5}M) AND DAVA (5×10^{-4}M) ON THE ORAL CONTRACTION

Fig. 22

possible transmitter store replenishment due to precursor supplement. Differing circular and longitudinal muscle responses, due to varying concentrations of PGE_2, would allow the varying gradations and different activities executed by the gut wall, as observed and described in the previous chapter. It could be speculated that the two lower concentrations ($5 \times 10^{-9}M$ and $10^{-8}M$) could cause a complete relaxation of the lamina muscularis, while $5 \times 10^{-5}M$ could result in the observed active relaxation, or opening of the gut, ahead of a pellet, when the longitudinal muscle appeared to pull the relaxed circular muscle layer open. This speculation, and the dual as well as diametrically opposed effect of PGE_2 at $5 \times 10^{-8}M$ on the two muscle layers of the colon, led to the search for an effective and specific blocker of PGE_2 in the colon.

Surprisingly, most of the prostaglandin antagonists: ASA, Indomethacin, Suprofen, Sc 19220, N-0164, Intal, or 6-Keto-PGE_1 had no noticeable blocking effect on the descending inhibition at all. Trimethoquinol displayed an initial agonist action on the inhibitory response, but neither it nor its (+) entantiomer blocked the relaxation. HR 546 decreased the inhibition considerably but this was found to be due to its action in lowering muscle tone; after increasing the muscle tone with carbachol the inhibition could be initiated unimpeded in the presence of HR 546. MEF and PPP quite effectively blocked the relaxation induced by balloon inflation, but unfortunately only at quite high concentrations. Both appeared to have a tone decreasing side effect, which, however, could readily be counteracted by low doses ($10^{-9}M$ to $3 \times 10^{-8}M$) of carbachol. If PPP was left in the bath longer than 10 min, some effect - apparently secondary - made this drug irreversible even after PGE_2

addition. Since PPP was also less reliable than MEF in antagonising the relaxation, the latter was mainly used to test PGE involvement in the descending inhibition, as in detail in chapter VI. The relative rate of onset of blocking (2 min to 8 min) with MEF pointed to a receptor antagonism mechanism rather than to a synthesis blocking effect, which possibly was the irreversible secondary action of PPP. Since MEF could be washed out in a relatively short time (approximately 15 min to 20 min), a dissociation from receptors was indicated, rather than interference in the synthesis sequence, which usually involves direct, often destructive, action on an enzyme (i.e. cycloxygenase). The particularly fast (5 min) and dose-dependent return of relaxation after PGE_2 addition further seemed to support the idea that prostaglandins were involved in the descending inhibition pathway. Here again the partial blockage of the inhibition by MEF in some cases (fig. 14b, A_3) pointed to involvement of another transmitter.

Addition of neuroactive peptides GLU, NT, DNT, SP, VIP to the bathing solution, as well as desensitization attempts, had no significant effect on the balloon-inflation generated relaxation of the colon segments, although NT and DNT caused potentiation of the inhibition and also a very similar relaxation when compared to the distension stimulus. The neuroactive peptides tested did not appear to be involved in the descending inhibition of the colon. The same conclusion was drawn for the involvement of opioid substances, since NAL had had no blocking effect even at very high concentrations.

It could be shown, however, that the relaxation, or the transmission of the mechanical stimulus effect, were neurogenic and sensitive to TTX. If TTX was left in the bath for 10 min or less it could be washed out within approximately 60 min to 120 min, but if it was applied for 20 min or longer the stimulus induced relaxation could only be initiated again after 6 to 8 hours.

Cholinergic involvement in the relaxation was excluded by the addition of tropicamide and atropine to the bath solution. These two muscarinic antagonists did not block the inhibition due to balloon inflation.

Adrenergic involvement could also be excluded, as the relaxation persisted after the application of guanethidine, a 'false' adrenergic transmitter, phentolamine, an adrenergic α_1 and α_2 receptor blocker, propranolol, the specific adrenergic β-receptor antagonist, or yohimbine, also an α-receptor blocker.

In order to examine a possible role of ATP in the descending inhibition, theophylline was tested, but with negative results. It was suggested by Dr. D.I.B. Kerr to test Cibacron-Blue, because of its possible anti-ATP properties (Apps and Gleed, 1976; Weber et al., 1979; Kerr and Krantis, 1979). The crude Cibacron-Blue, and two of its components, blocked the relaxation when the dose slowly increased. This drug is dealt with in detail in the next chapter.

The possible blocking effect of a number of 5-HT antagonists on the inhibition was investigated. Only methysergide and mianserin had any

action and these did not block the relaxation, but methysergide (5 x 10^{-6}M) reduced the ascending oral contraction caused by the balloon inflation. Methysergide at 5 x 10^{-5}M and mianserin at 2 x 10^{-5}M blocked the oral contraction completely. This was in agreement with Costa et al. (1982) who described findings of the presence and storage of 5-HT in the guinea-pig myenteric plexus, which, they suggested, was possibly used for initiation or propagation of the ascending excitation (Costa and Furness, 1976). While the response to methysergide could be readily reversed by washing, mianserin produced a long lasting blocking effect on the ascending oral contraction. A marked increase (56%) of the oral contraction in response to DAVA application was also observed. This effect has not been reported before, and was possibly a result of the suppression of an inhibitory modulation of the ascending excitation by GABA, an apparent interaction of GABA and 5-HT in a major aspect of peristalsis of the colon. However, this phenomenon was not pursued any further, because, again, it was outside the aims of the project.

In conclusion, the most significant findings were: The descending inhibition or its signal transmission was found to be a neurogenic event and was not mediated via muscarinic or adrenergic receptors. The relaxation, 1.5 cm to 2.5 cm aboral from the distension stimulus point on gut wall, was blocked by DAVA, the $GABA_B$ - receptor antagonist, by PPP and MEF, both prostaglandin blockers, and by Cibacron-Blue, a putative ATP antagonist. GABA and arachidonic acid loading enhanced, whilst PGE_2, as well as NT and DNT, mimicked the inhibition response. Thus GABA, prostaglandin and ATP appeared to play major roles in the descending inhibition. The effects of blocking ATP, and prostaglandins, were

examined further as reported in subsequent chapters. Finally, the ascending oral contraction was apparently mediated by 5-HT and modulated by GABA.

CHAPTER V

PURIFICATION AND ANALYSIS OF AN ADENOSINE-TRIPHOSPHATE ANTAGONIST

INTRODUCTION

In 1976 D.K. Apps and CH.D. Gleed found that the dye, Cibacron Blue F3GA, interacted with adenine dinucleotide kinase. This was supported in 1978 by R.S. Beissner and F.B. Rudolph and in 1979 by B.H. Weber et al. Because of the affinity of this dye for the dinucleotide fold, it was thought by Kerr and Krantis (1979) that it, or one of its analogs, might antagonise ATP actions in the gut, particularly if these were mediated by some exo-enzyme. The crude dye was shown to be an ATP antagonist in the intestine (Kerr and Krantis, 1979). However, Weber et al. (1979) also found that different components, which they had separated by thin layer chromatography (TLC), had varying efficacies in reducing phosphoglycerate kinase activity. They also reported that Cibacron-Blue F3GA purchased from Pierce Chemical Co., Polysciences, Inc. and Ciba-Geigy appeared to consist of chromatographically different components.

Universal and specific ATP antagonists have not previously been found (as explained in the introductory chapter). Theophylline, which had been reported to be an effective enteric ATP blocker (Brown et al., 1982; Hayashi et al., 1982) but which had been found to be ineffective in the colon by Tonini et al. (1982), had had no effect here on relaxation

induced by balloon inflation. It was, therefore, decided to investigate three samples of Ciba-blue dye from different manufacturers, as well as their components, and to test their potential as ATP-antagonists, especially since preliminary tests with Cibacron-Blue and its major components (reported in the previous chapter) had been encouraging.

MATERIALS AND METHODS

Three different companies manufacture and distribute Cibacron-Blue F3GA - a trade name registered by Ciba-Geigy, here called Ciba-Blue for the sake of convenience. Quantities of the three blue dye products (10 g or more) were obtained from Ciba-Geigy Australian Ltd, Pierce Chemical Co. and Polyscience, Inc.

Descending one-dimension paper chromatography was used initially for separation and identification of the components of the three dyes. The solvent system consisted of 40% n-butanol, 32% H_2O and 28% ethanol (Beissner and Rudolph, 1978). 50 mg of dye was dissolved in 0.5 ml H_2O and streaked on a 60 x 22 cm sheet of 3MM Whatman chromatography paper. Since the laboratory temperature varied from 14°C to 22°C it took from 12 to 20 hours for the solvent front to traverse the paper. The type of chromatography tank (30 cm x 25 cm x 60 cm), allowed three sheets to be run simultaneously.

The major bands (plate 6) of each of the three dyes were cut out, eluted in water and subsequently freeze-dried. The three dyes and their

PLATE LEGEND

Plate 6

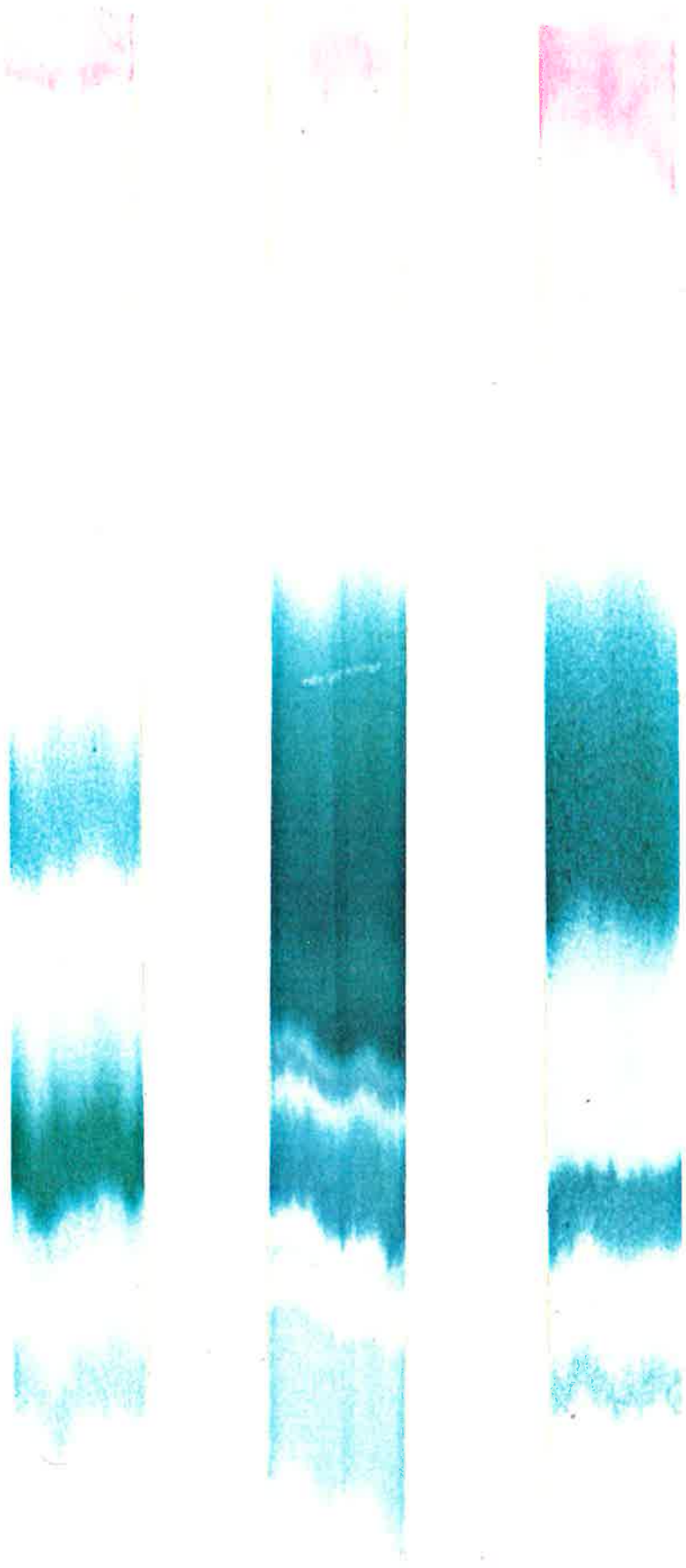
Relative fronts of the three crude Cibacron Blue dyes

Ci = Ciba-Geigy

Pi = Pierce Chemical Co.

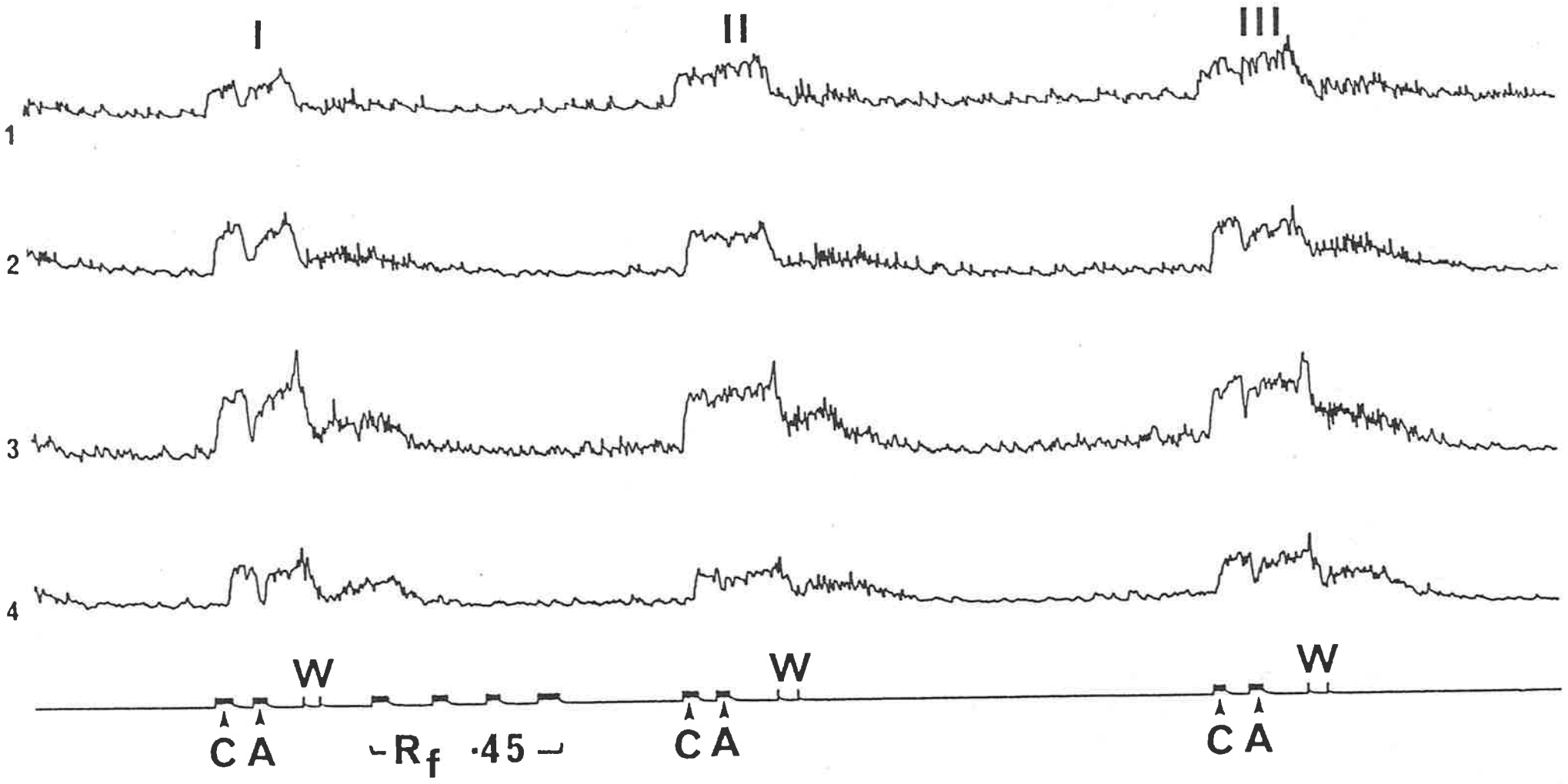
Ps = Polyscience Inc.

plate 6



constituents was tested in a four-bath design (without stimulating electrodes) as shown in figure IIc (appendix), the tone of the colon segments being raised with carbachol (10^{-9}M to 3 x 10^{-8}M). The fast relaxation caused by ATP (10^{-7}M to 2 x 10^{-6}M) was measured. The same procedure was repeated after the addition of Cibacron Blue or its fractions at various concentrations. Typical traces of one such procedure are shown in figure 23.

In order to obtain larger quantities of the most active fractions, column chromatography was employed. The method delineated by Weber et al. (1979) was tried, but found to be slow and with poor fraction differentiation. A modified version using a flush chromatography column reduced the column separation time from several hours to approximately 20 min but fraction differentiation was still not improved. A new method was therefore devised under the guidance of Professor R. Prager. In a typical purification and fractionization procedure, 2 g of Cibacron Blue was dissolved in an acetone: methanol (1:1; v:v) mixture and brought to boil while constantly being shaken. The mixture was cooled, filtered (Whatman, grade 4) and the solid as well as the fluid parts were retained. The fluid part was then dried by rotary evaporation. Thin layer chromatography (TLC) was used to monitor all fractions continuously. The resultant compound was adsorbed to 1 g of silica powder with water. The mixture was again dried by rotary evaporation. It was then placed in a flush chromatography column packed with silica gel. A mixture of ethylacetate : tetrahydrofuran : water (15:48:12; v:v:v) was the chromatographic eluting solvent. One litre was sufficient to flush the dye components through the column. The fastest running red and orange



$2\ mV\,cm^{-1}$

$1\ cm\ min^{-1}$

Traces of four colon segments showing the ATP/ciba blue interaction

Traces 1 = distal section, 2 and 3 = medial sections, 4 = proximal segment

C = carbachol (3×10^{-8}M), A = ATP (2×10^{-6}M), W = wash, R_f .45 = component of ciba blue (pierce)

Fig. 23

bands (100ml) were discarded. The following 700ml of blue and the next 200ml were each relatively homogenous, and their constituents showed quite different Rfs. when checked by TLC. Very similar differentiation could be obtained when the substance obtained from the first crystallization procedure was again crystallized, after having been dissolved and heated in the acetone/methanol mixture. Subsequent filtration tests showed that the TLC bands of the original fraction resembled those of the bands produced by the substance in the first 700ml collected and also those of Pierce Rf. .50. The fluid part was nearly the same as the contents of the last 200ml, which in turn corresponded to the bands of the Pierce Rf. .45.

The fronts of various Ciba-Blue components are shown on plate 7. In the top row, the relative fronts of the crude dye from Pierce can be seen (Pi) on TLC using DC Plastikfolien with silica gel 60 of 0.2mm thickness (Merck) and the previously mentioned ethylacetate-tetrahydrofuran-water solvent. The solvent front is not shown since it had advanced much further than the subsequent individual component fronts. The three Pi fronts Rf. .25, .45 and .60 (plate 7) had been re-touched, because the colour- photocopying technique did not show them, although they were present.

The next two runs, marked Ci .45 and Pi .45, record the Rfs. .45 obtained from Ciba-Geigy and Pierce Cibacron-Blue by paper chromatography. The last set in row one - PiF - is from the crude fluid component obtained by crystallization, and the first set in the middle row PiS is the crude solid component of the same procedure. The second run of the middle row

Pi .50, is from the Pierce .50 relative front extracted by paper chromatography, like the Pi .45 and Ci .45 it was also heterogenous. PiaS and PibS in the middle row represents the crystallization-separated, and the column-purified solid, component of Pierce Ciba-Blue, while PicS shows the only double crystallization separated and purified solid constituent. The first set in the bottom row Pi .45 is the constituent from the paper chromatography Rf. .45, separated and subsequently purified by column chromatography. PiaF and PibF are the crystallization separated and column chromatography purified fluid components, while PicF represents the only double crystallization purified and separated fluid constituent.

The test chromatographic results displayed on plate 7 were obtained by ascending chromatography. The Rfs. could not therefore be compared with those recorded for paper chromatography. Unless chemicals were run in the same gravitational direction on the same substrate using the same eluents, as was done with the components on plate 7, a totally different spread of fronts would result.

RESULTS

Paper chromatography resulted in a 29% to 37% recovery rate. The yield of different components varied greatly from one manufacturer to the other. Component ratio for Ciba-Geigy produced Cibacron Blue was Rf. .27 - 10%; Rf. .36 - 62%; Rf. .45 - 26%; Rf. .77 - 2%. Pierce Chemical Co. Rf. .24 - 2.5%; Rf. .33 - 3.9%; Rf. .39 - 18.5%; Rf. .45 - 18%; Rf. .50 - 52.5%;

PLATE LEGEND

Plate 7

TLC relative fronts of Cibacron Blue and its components

Pi - crude Cibacron Blue by Pierce Chemical Co.

Ci .45 - paper chromatography Rf .45 of Ciba-Geigy Blue

Pi .45 - paper chromatography Rf .45 of Pierce Ciba Blue

PiF - Pierce Ciba Blue fluid component
PiS - Pierce Ciba Blue solid component
} crystallization separated

Pi .50 - paper chromatography Rf .50 of Pierce Ciba Blue

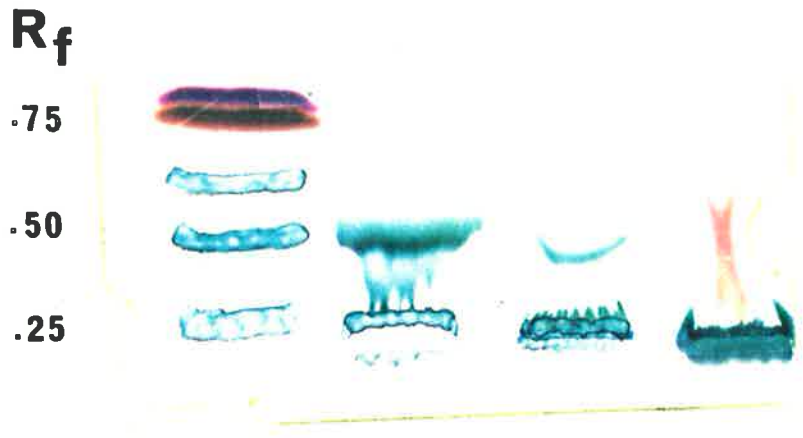
Pi .45 - column chromatography purified paper chromatography separated Rf .45

PiaS
PibS
} - Pierce Ciba Blue crystallization separated and column purified solid component

PicS - Pierce Ciba Blue double crystallization separated and purified solid component

PiaF
PibF
} - Pierce Ciba Blue crystallization separated and column purified fluid component

PicF - Pierce Ciba Blue double crystallization separated and purified fluid component



Pi Ci.45 Pi.45 Pi F

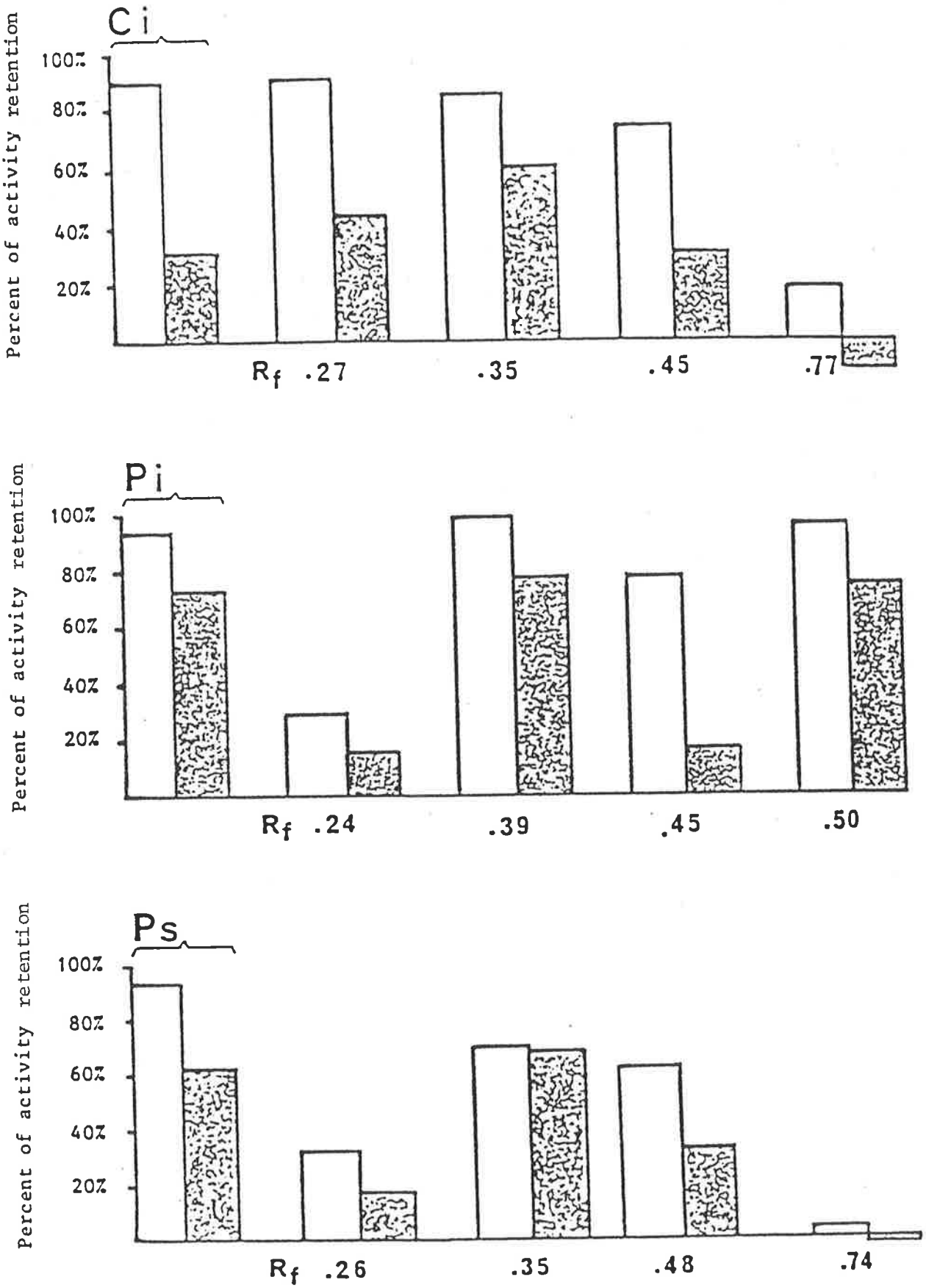
Pi S Pi.50 PiaS bS cS

Pi.45 PiaF bF cF

Rf. .73 - 4.6%. Polyscience Inc.: Rf. .26 - 8%; Rf. .33 - 17.5%; Rf. .48 - 71%; Rf. .74 - 3.5%. The averaged (n16) antagonising effects of the dyes and their components on the ATP inhibition, and also on the cholinergic tone-raising response on colonic segments, are graphically demonstrated in figure 24 and recorded in table VII (appendix).

Because of their advantageous ratio between purinergic blocking effect and the undesirable cholinergic antagonism, fractions Pi .50, Pi .45 and Ci .45 were selected for further purification and possible production in quantity. The end-products of the various separation techniques were continually checked for their physiological effect. Averaged results, usually, of eight to twelve experiments, are recorded in tables VIII to XX (appendix) and illustrated in figures 25 to 38. The ATP effect has been expressed as the percentage of inhibition of the carbachol control response. ATP antagonism by the Cibacron-Blue dyes and their components was seen in the degree of reduction of this inhibitory ATP effect on the cholinergically mediated contraction.

Figure 25 (tables VIII, IX; appendix) shows the effect of varying doses of ATP on contractile responses of colon segments to two different (10^{-8}M and 3×10^{-8}M) carbachol concentrations. ATP relaxed the contraction to carbachol 10^{-8}M with an approximate ATP ED_{50} of 5×10^{-8}M whilst the ATP ED_{50} with carbachol 3×10^{-8}M was approximately 10^{-6}M. Nevertheless, the higher carbachol concentration (3×10^{-8}M) was chosen, because responses were more uniform and could be measured with greater reliability. For similar reasons of dependability in the responses, an ATP dose (2×10^{-6}M), close to the ED_{50}, was selected to be used for testing the ATP-



ATP (shaded areas) and Carbachol (open columns) showing percentage of activity retained after the application of Cibacron Blue manufactured by Ciba-Geigy (Ci), Pierce chemicals (Pi) and Polyscience Inc. (Ps) and their major components.

antagonistic actions of the dye fractions obtained by paper chromatography. ATP/carbachol control curves were established each time for the intestinal segments of different animals during the experiments involving substances separated by column chromatography and crystallization. The points of control curves for ATP-antagonism are represented by filled squares (means of all experiments figs. 25 to 35b), while curves with open squares show the shift in the dose response curve with a given antagonist.

Figure 26 (table X; appendix) indicates the strong antagonism of the ATP-induced relaxation exerted by the Pierce Cibacron-Blue fluid-component of the crystallization process (PiF). The antagonistic depression by PiF (5 x 10^{-5}M) shifted the ATP response curve prominently to the right at concentrations exceeding 3 x 10^{-6}M, the parallel shift indicating competitive antagonism. Lower concentrations of the PiF fraction exhibited less dramatic but also parallel shifts to the right. Figure 27 (table XI; appendix) shows an antagonism of the ATP-induced relaxation, by PiF 2 x 10^{-5}M, whilst PiF (10^{-5}M) caused a lesser antagonism of ATP (fig. 28, table XII). But since PiF had also strong anticholinergic effects, which were believed to have been due to the pink component (Rf. .30 to Rf. .60) visible on plate 7 upper row, further purification was deemed necessary.

Effects of some active blue fractions separated by paper chromatography were tested, and their Rf. locations on TLC were established and compared with column or crystallization separated fractions. Figure 29 (table XIII) depicts the action of the most effective component with the fewest

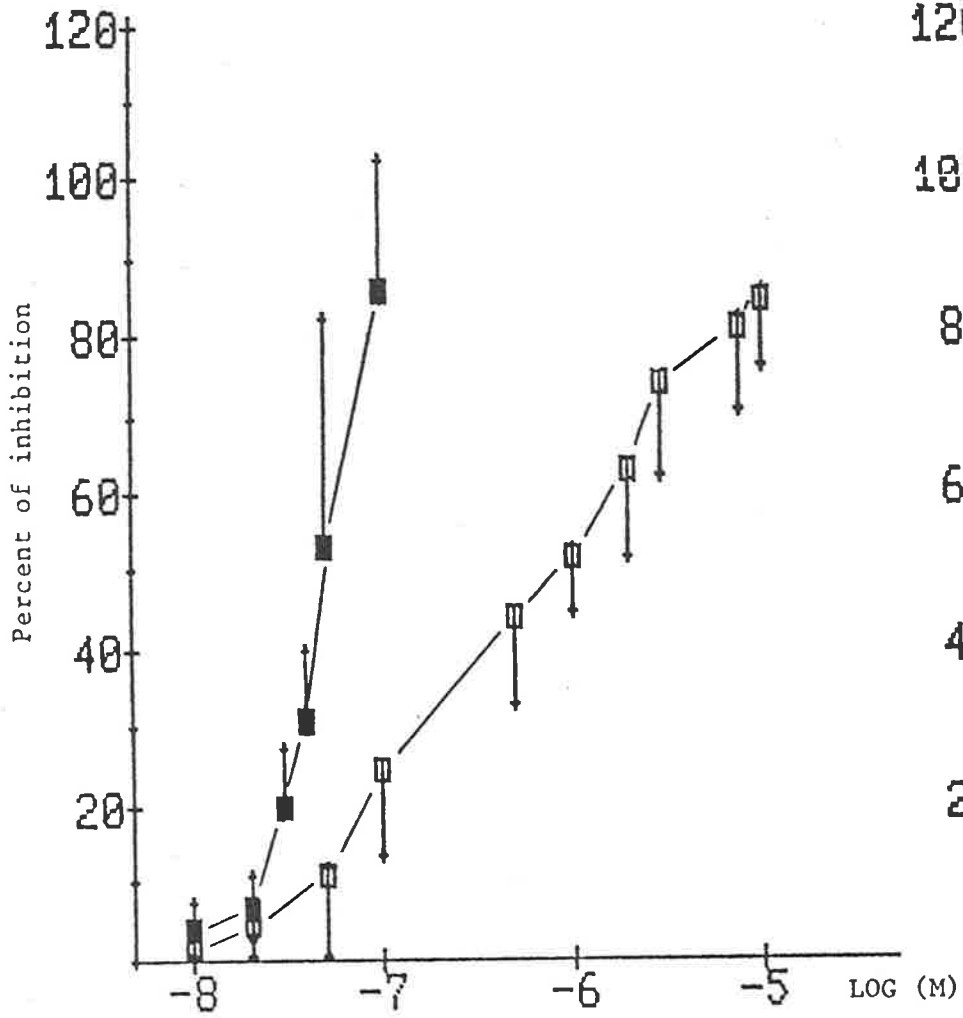


Fig. 25
Dose dependent inhibition of the carbachol response by ATP
(■——■ 10^{-8}M carbachol)
(⊞——⊞ 3 x 10^{-8}M carbachol)

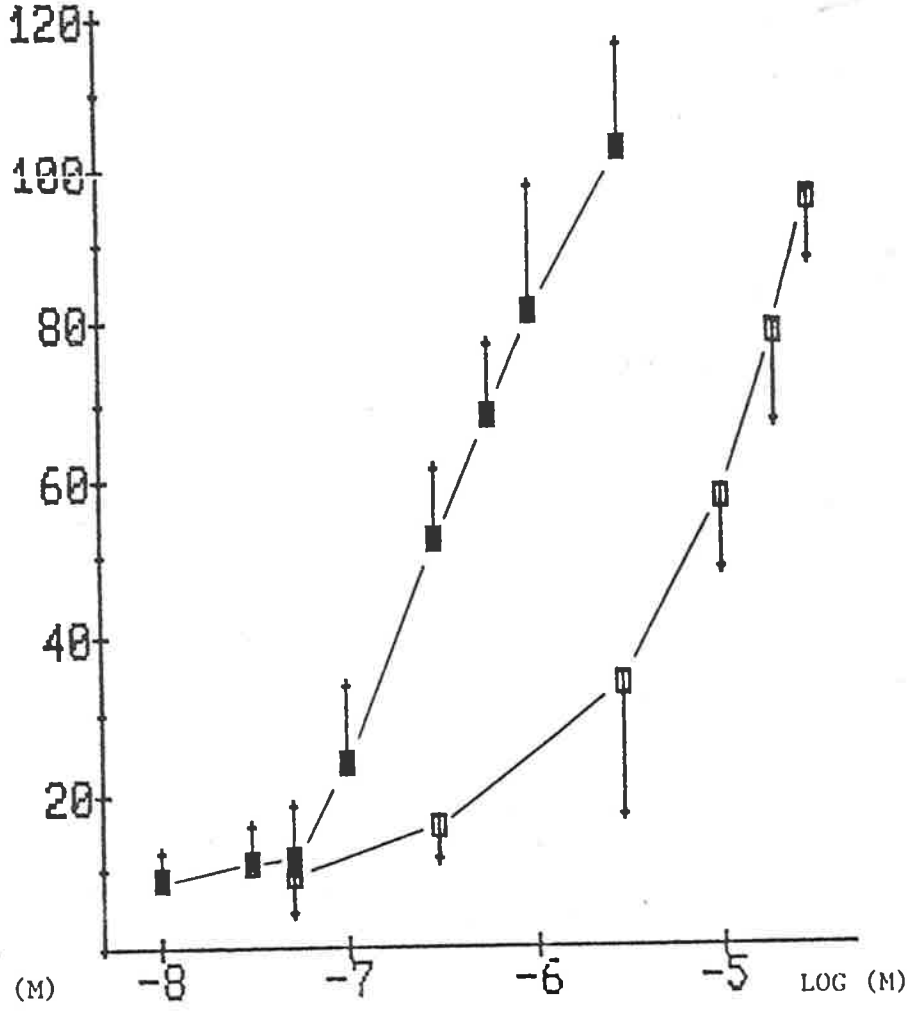


Fig. 26
Depression of ATP inhibition of the carbachol (3 x 10^{-8}M) response by PiF
(■——■ ATP)
(⊞——⊞ after PiF 5 x 10^{-5}M)

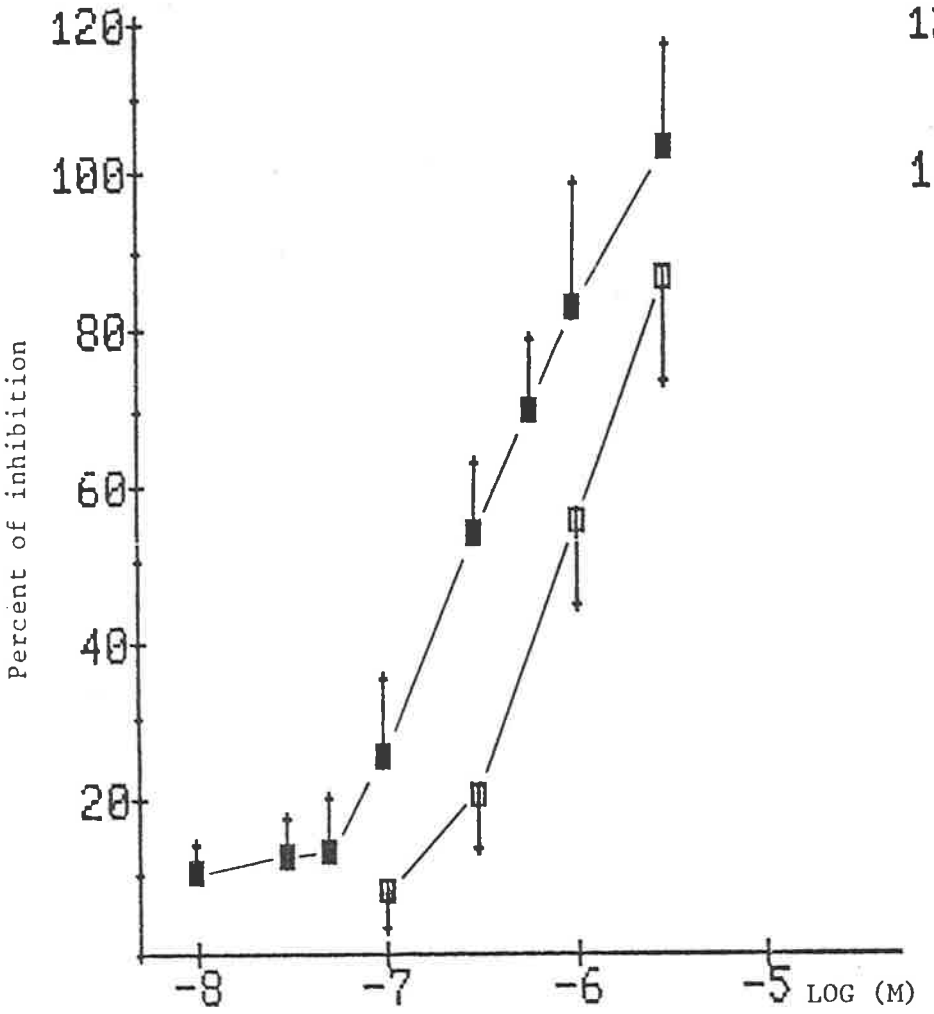


Fig. 27
Depression of ATP inhibition of the carbachol (3×10^{-8}M) response by PiF
(ATP)
(after PiF 2 x 10-5M)

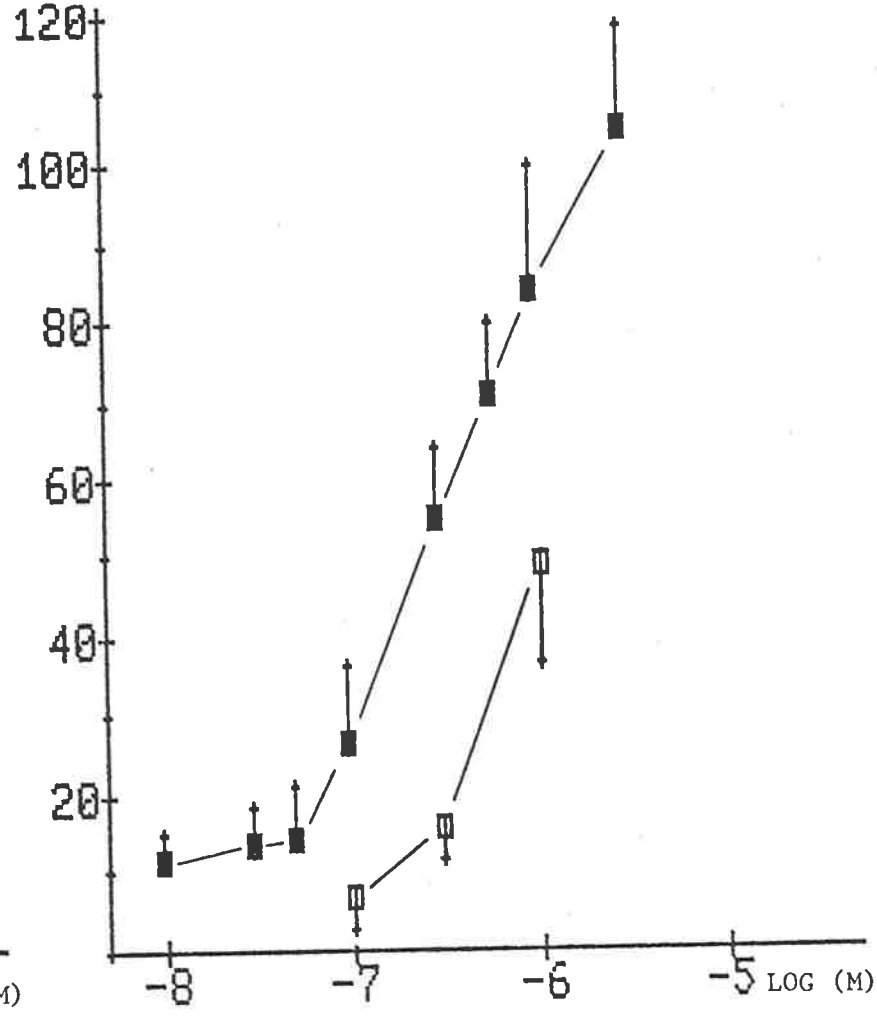


Fig. 28
Depression of ATP inhibition of the carbachol (3×10^{-8}M) response by PiF
(ATP)
(after PiF 10^{-5}M)

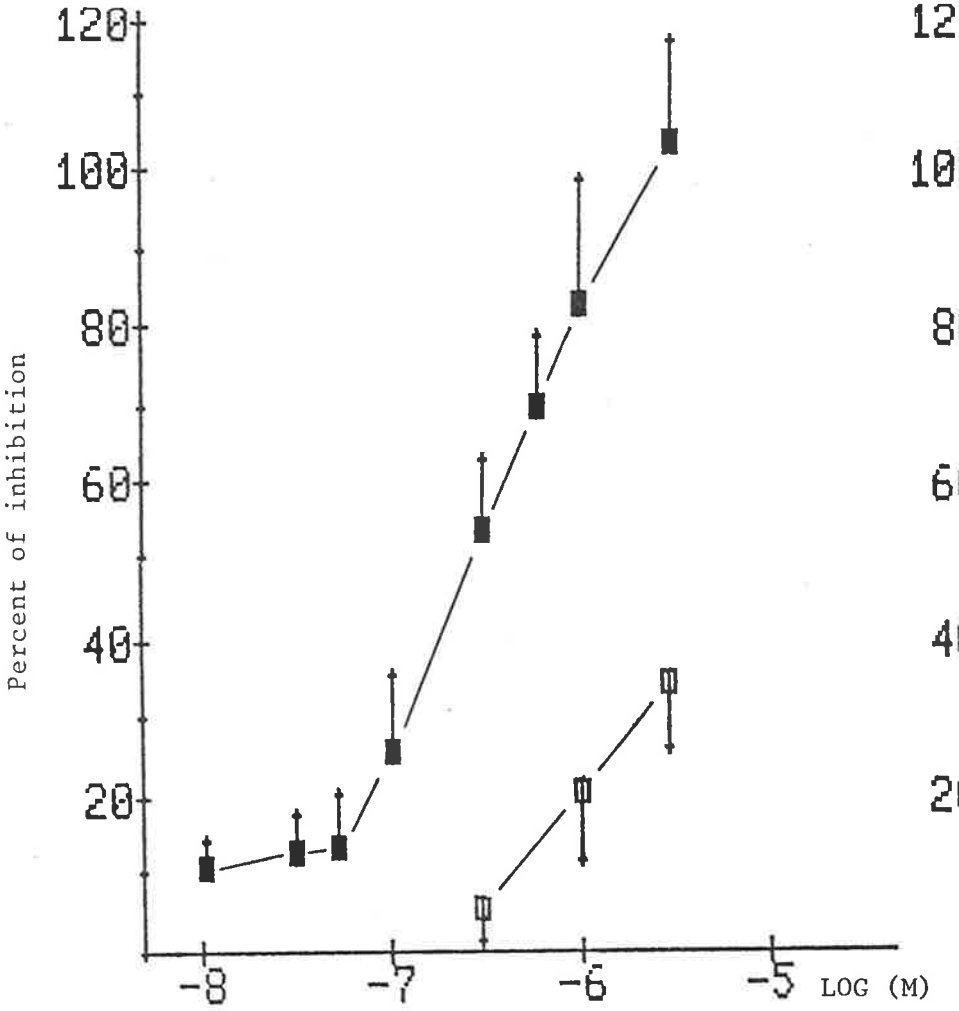


Fig. 29
Depression of ATP inhibition of the carbachol (3×10^{-8}M) response by Pi .45
(ATP)
(after Pi .45 (5×10^{-5}M)

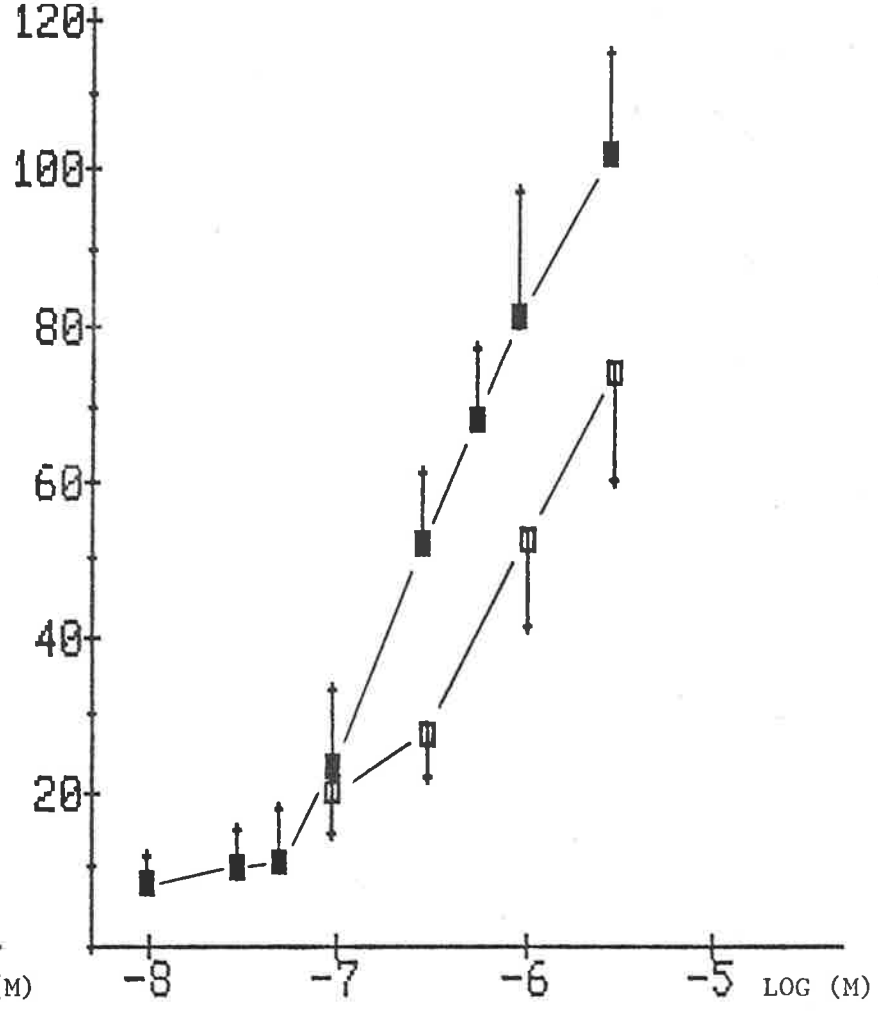


Fig. 30
Depression of ATP inhibition of the carbachol (3×10^{-8}M) response by Ci .45
(ATP)
(after Ci .45 (5×10^{-5}M)

side effects, Pi .45 (5 x 10^{-5}M) antagonising the ATP-induced relaxation, more potently than did Ci .45. (fig. 30, table XIV). Although the antagonistic action was significant, the shifted curve was not quite parallel. Also, Pi .50 produced a parallel shift (fig. 31, table XV). These three fractions had a second component in common, as can be seen in the upper and middle row of plate 7. This major component was very similar to that giving the relative front produced by the crystallization-separated solid parts PiS, PiaS, PibS and PicS (middle row, plate 7), and may have been responsible for the anti-cholinergic activity. All solid fractions mentioned above were tested and found to produce similar results to PiS (fig. 32, table XVI).

The fluid filtration products purified by different methods exhibited varying degrees of ATP-antagonism. Figures 33 and 34 (table XVI), the results of PiaF and PibF respectively, show strong antagonism, but did not shift the ATP dose-response curve in a parallel manner, possibly due to the presence of impurities of the Pi .50 or PiS type, which were obvious in their relative fronts (bottom row, plate 7). However, the most effective compound produced by double crystallization was PicF (10^{-5}M) (fig. 35, table XVII), which gave a parallel shift in the ATP-dose response curve, with a slight (12%) anti-cholinergic component. Figs. 35b, c give the data on an estimated pA_2 of 6.2 with PiF, which is essentially the same as PicF, for ATP-antagonism on the colon. The compound labelled Pi .45 (bottom row, plate 7) was actually the paper chromatography separated Pi .45, purified once by the crystallization process. It gave results identical to PicF.

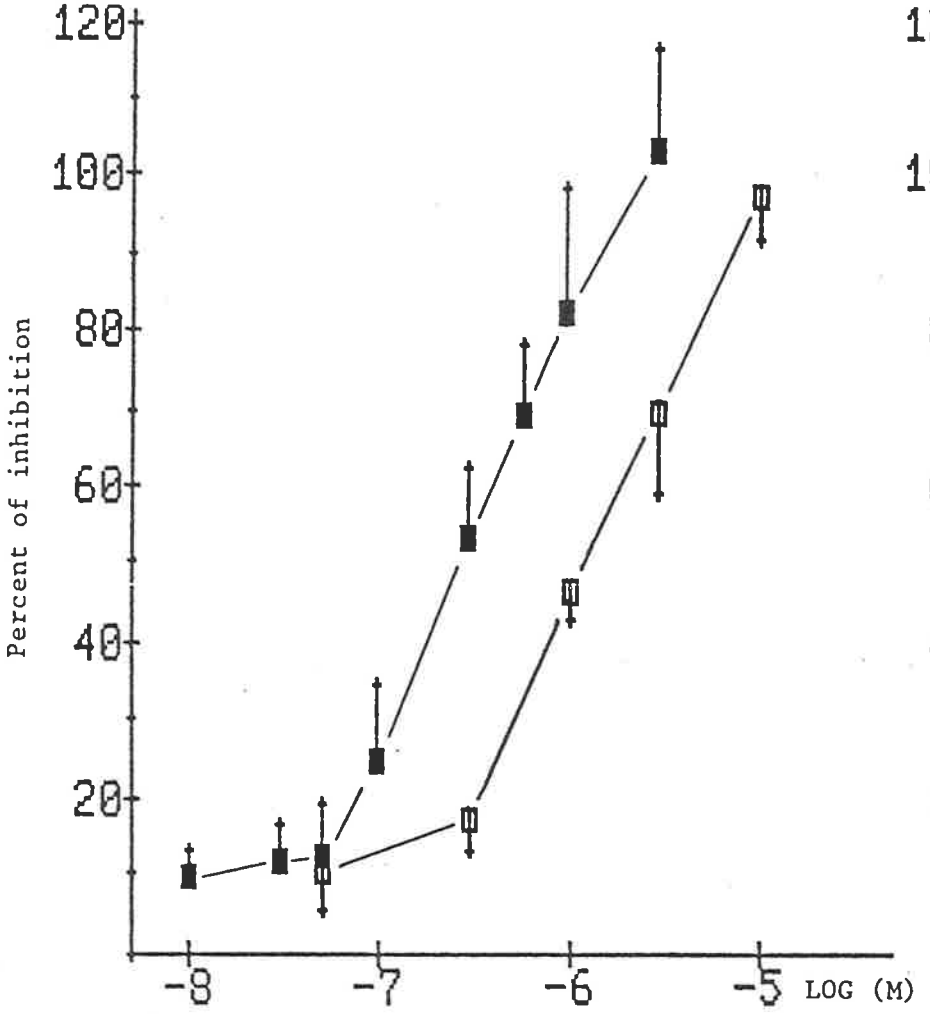


Fig. 31
Depression of ATP inhibition of the carbachol (3×10^{-8}M) response by Pi .50
(ATP)
(after Pi .50 (5×10^{-5}M)

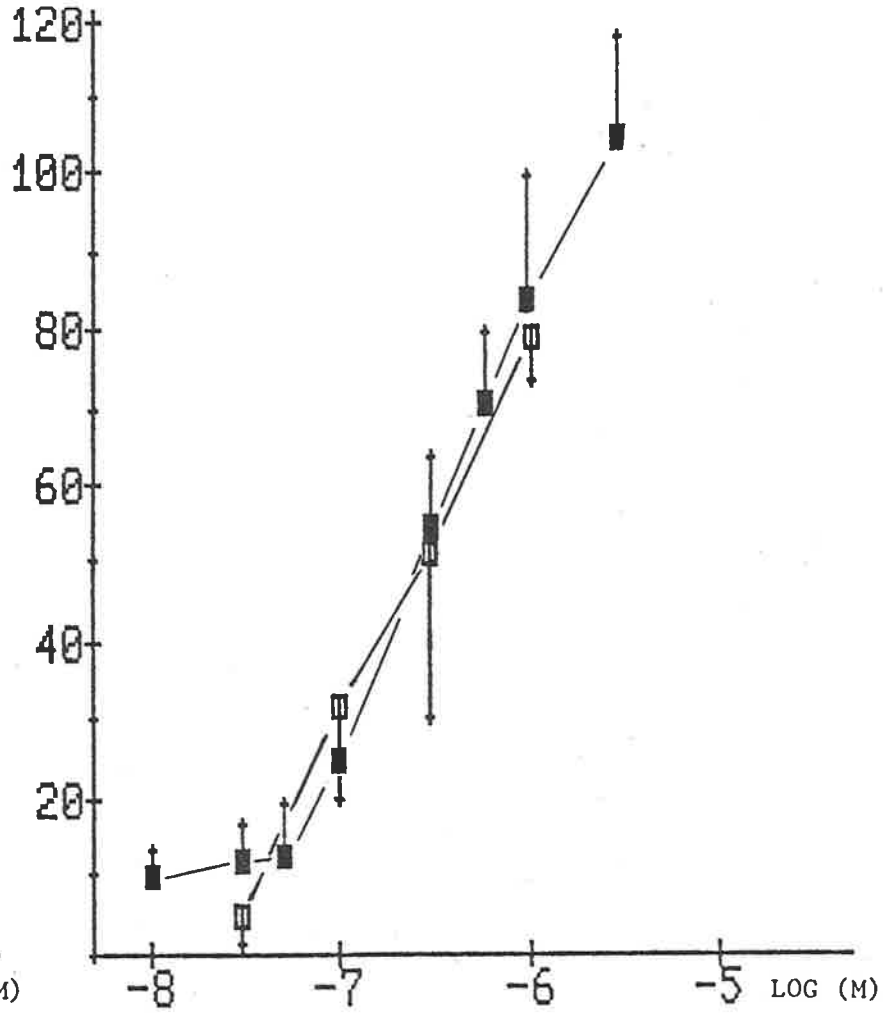


Fig. 32
Depression of ATP inhibition of the carbachol (3×10^{-8}M) response by PiS
(ATP)
(after PiS (5×10^{-5}M)

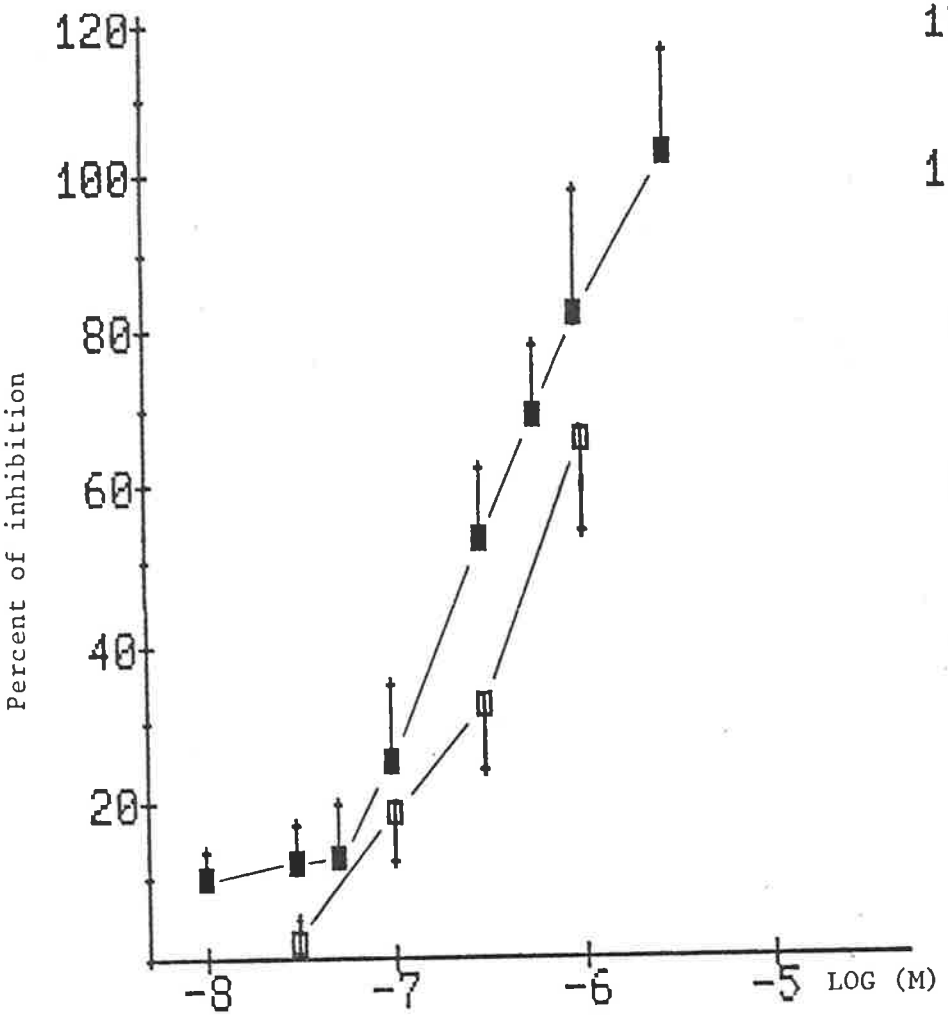


Fig. 33
Depression of ATP inhibition of the carbachol (3×10^{-8}M) response by PiaF
(ATP)
(after PiaF (10^{-5}M)

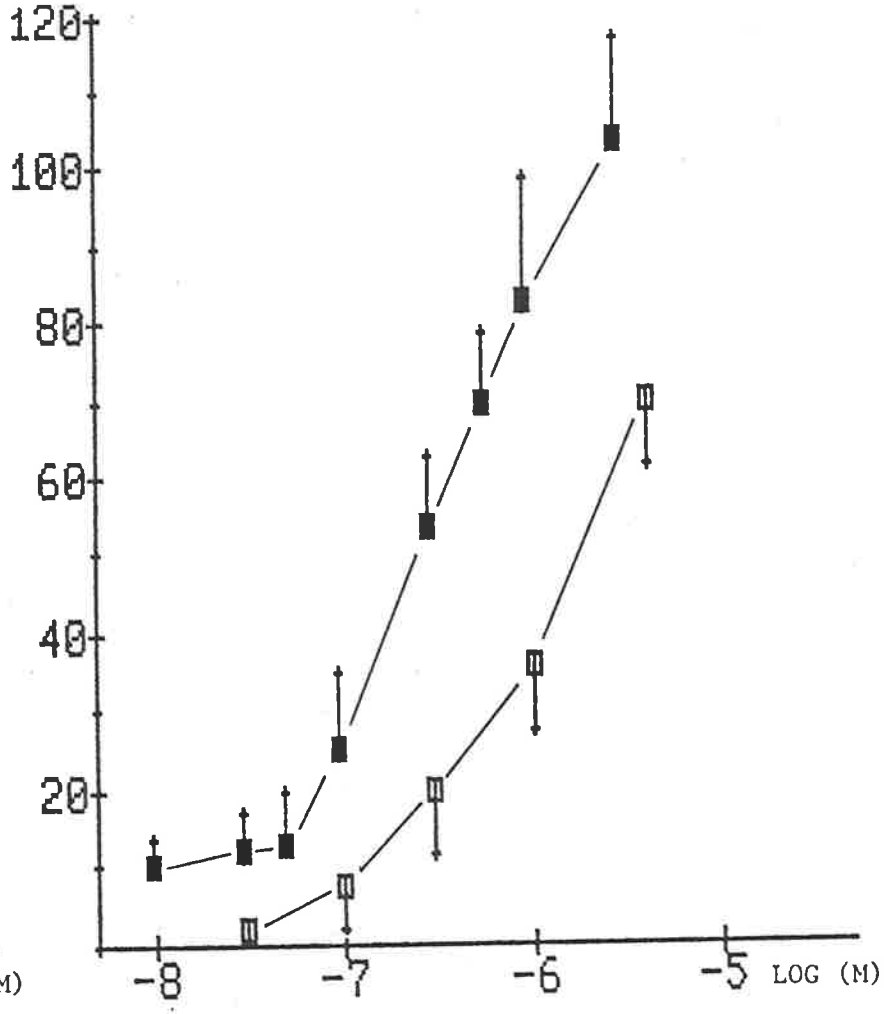


Fig. 34
Depression of ATP inhibition of the carbachol (3×10^{-8}M) response by PibF
(ATP)
(after PibF (10^{-5}M)

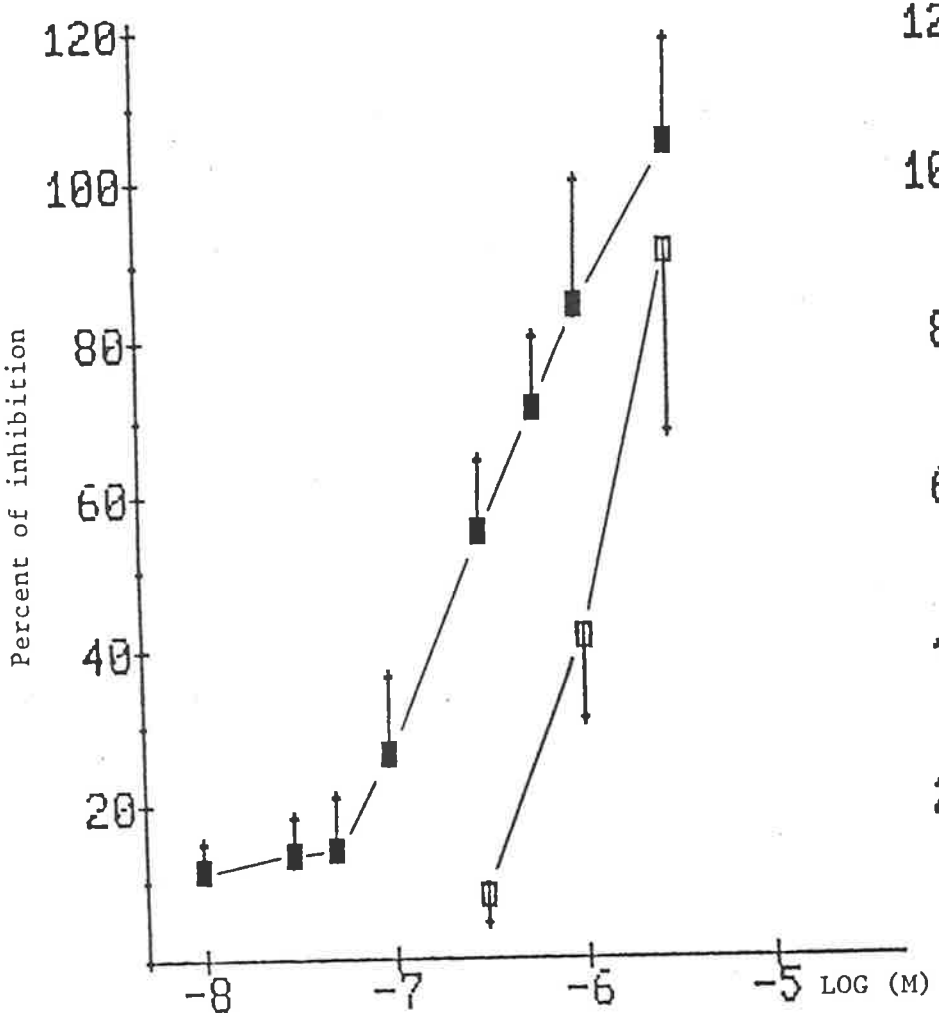


Fig. 35 a
Depression of ATP inhibition of the carbachol (3×10^{-8}M) response by PicF
(ATP)
(after PicF (10^{-5}M)

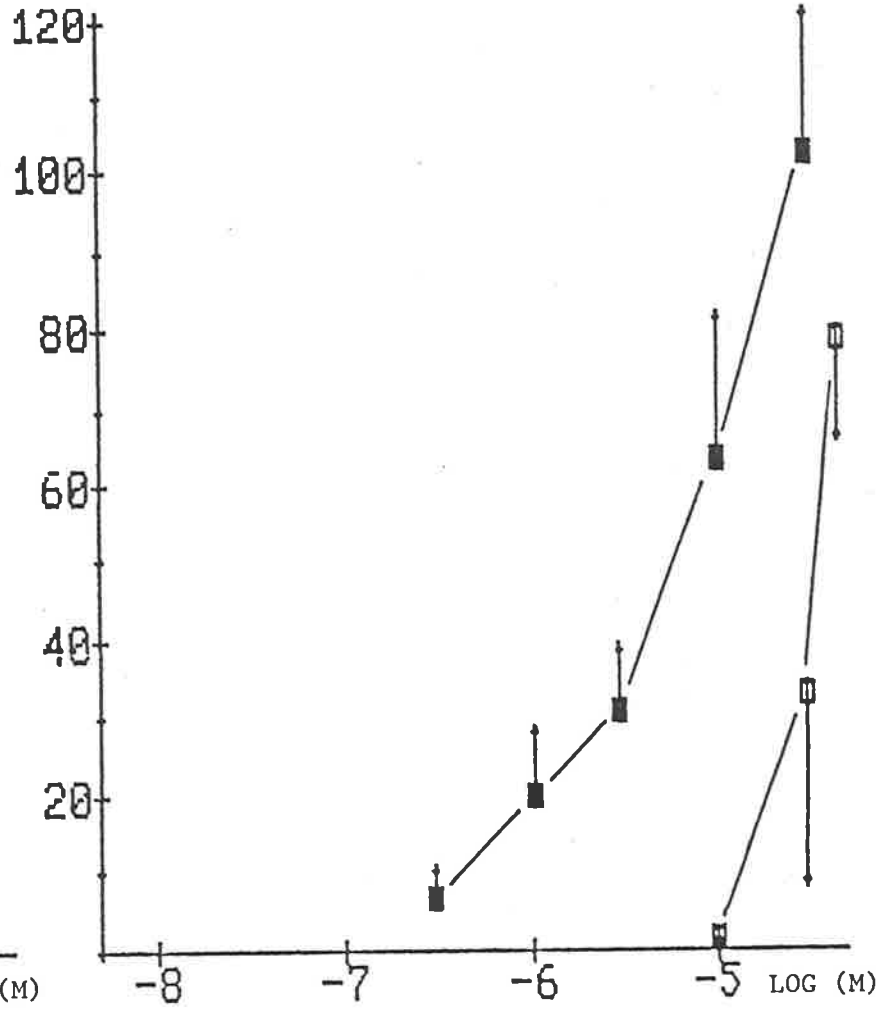


Fig. 36
Depression of ATP inhibition of the carbachol (3×10^{-8}M) response by PicF in the vas deferens
(ATP)
(after PicF (10^{-5}M)

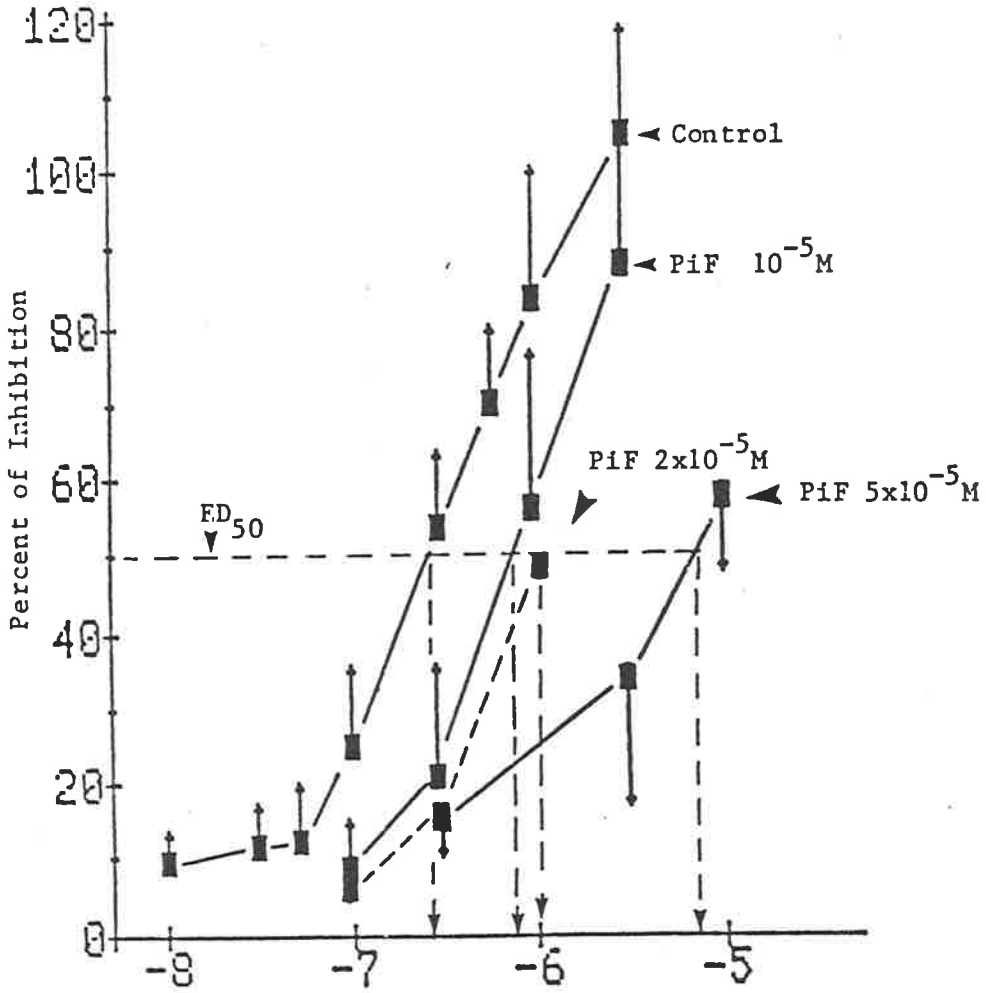


Fig. 35 b

The ED_{50} of three different PiF doses for the determination of the PA_2 value.

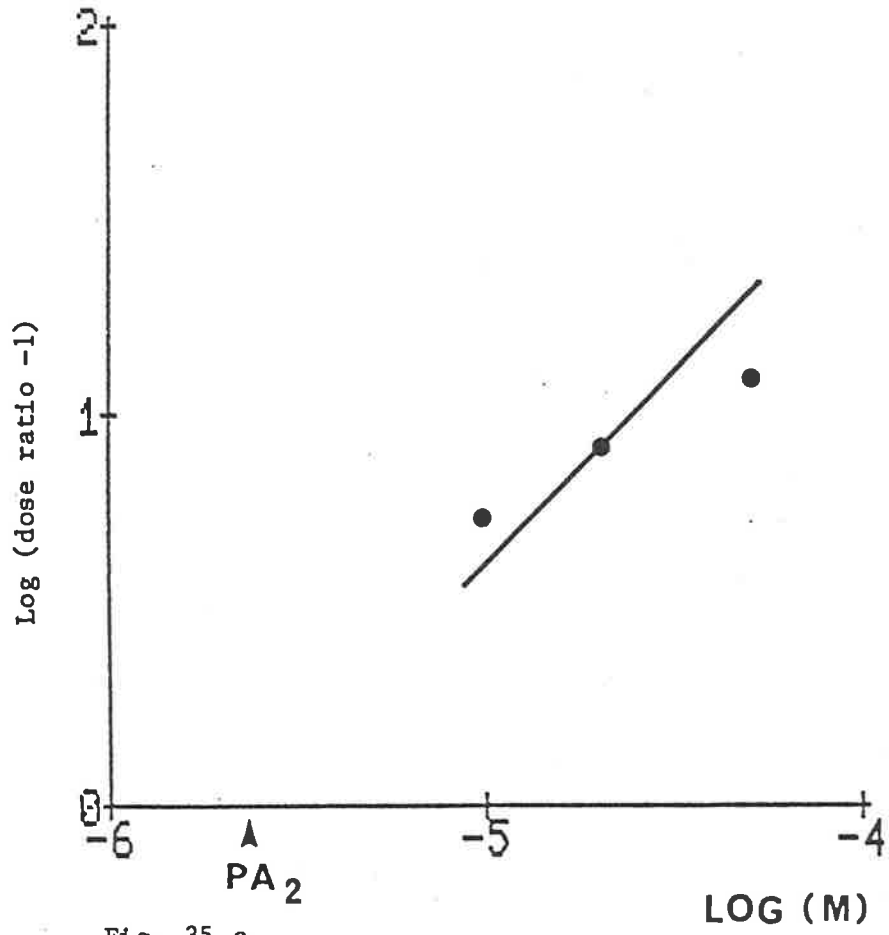


Fig. 35 c

Schild plot of PiF antagonism. Slope constrained to -1 (PA_2 = 2.45 x 10^{-6}).

Effects of PicF (10^{-5}M) on the ATP inhibition of cholinergically induced contractions of the guinea-pig vas deferens are shown in figure 36 (table XIX). It had a strong antagonising effect, but the antagonism did not shift the ATP dose-response curve in a parallel fashion. Tests with PicF (10^{-5}M) on guinea-pig taenia coli revealed its lack of a significant ATP-inhibition depressant effect on taenia coli (fig. 37, table XX).

The binding reversibility of PicF (10^{-5}M) acting against ATP (3×10^{-7}M) is shown in figure 38 (table XVIII). When the antagonist PicF was washed out, the relaxation of the colon induced by ATP (3×10^{-7}M) returned to control levels within 25 min.

Thus it was found that the most homogenous components purified, Pi .45 and PicF (bottom row, plate 7), had the most significant ATP antagonistic properties with the fewest unwanted side effects.

Since a very small amount of the crystallization-purified, paper-chromatography-separated Pi .45 was obtained, only PicF was tested at 10^{-5}M on balloon-induced descending inhibition in the colon. Results indicated that a fast and complete block of the inhibition occurred within three minutes after PicF application (fig. 39). This effect was reversed with a single wash, and the inhibition returned after seven minutes.

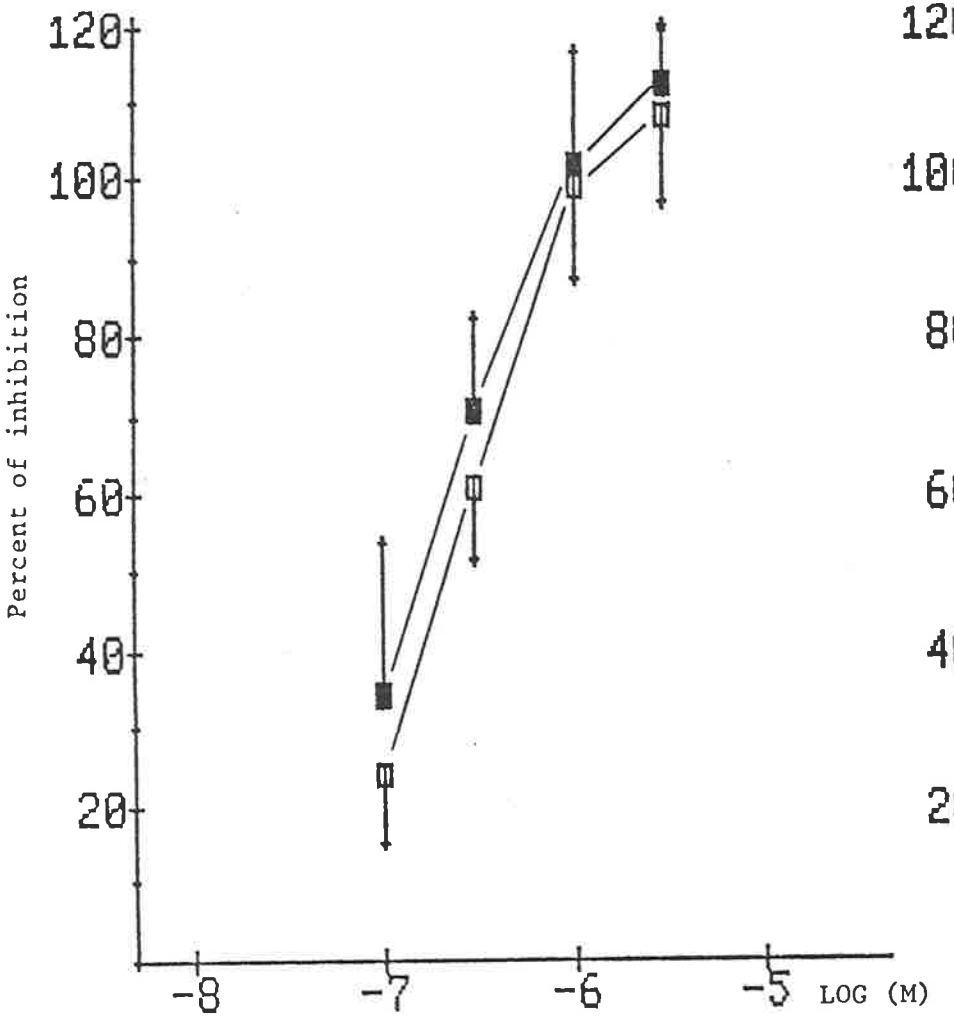


Fig. 37
Depression of ATP inhibition of the carbachol (3×10^{-8}M) response by PicF in the taenia coli

(ATP)
(after PicF (10^{-5}M)

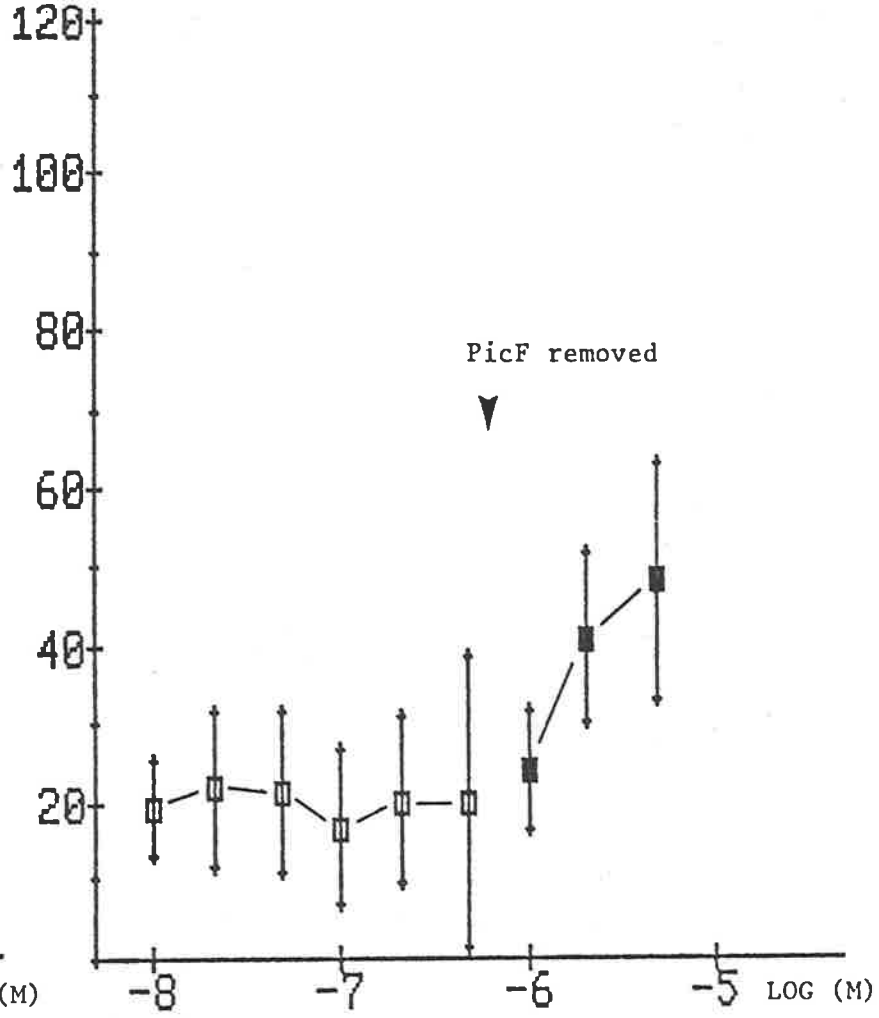
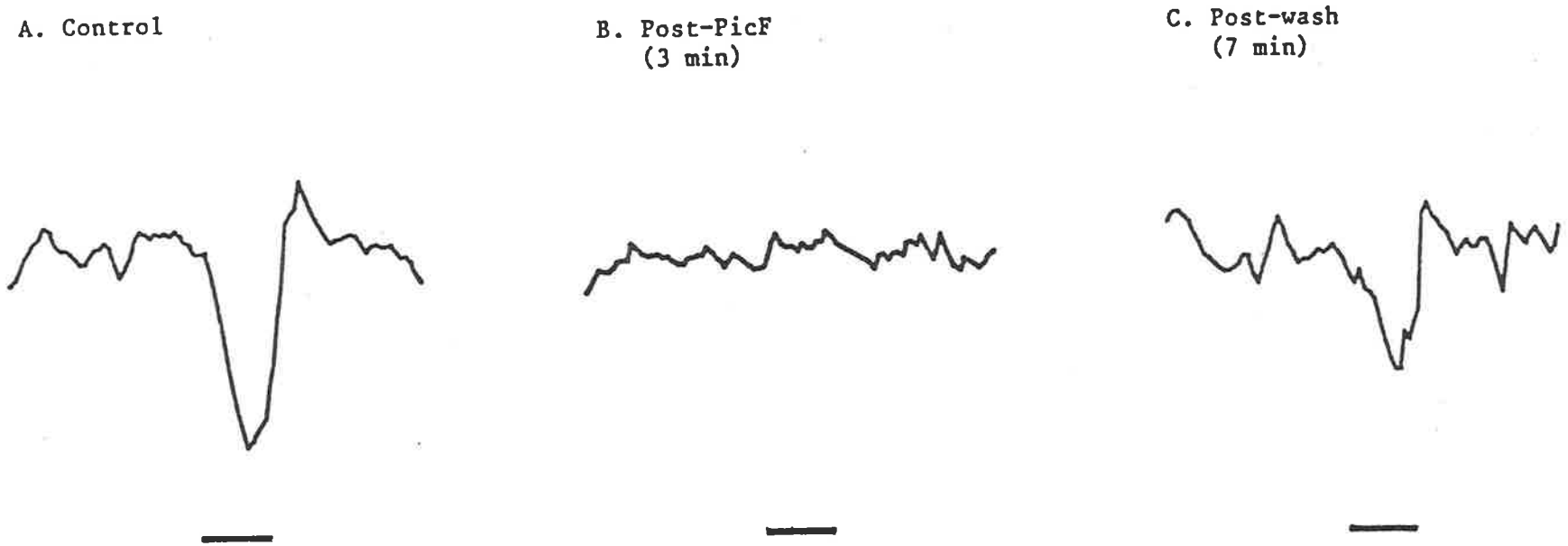


Fig. 38
Depression of ATP inhibition of the carbachol (3×10^{-8}M) response by binding reversibility of PicF (10^{-5}M)

(ATP plus PicF)
(after PicF (10^{-5}M) removal



Effect (n=8) of a cibacron blue fraction (PicF 10^{-5}M)
on the descending inhibition

Fig. 39

DISCUSSION

The idea of using Cibacron-Blue as an ATP-antagonist to examine a purinergic component, in the descending inhibitory peristaltic reflex, did not appear to be feasible when it was found that it also produced a strong cholinergic depression. But since Weber et al. (1979) had overcome non-specific features of the dyes by removal of contaminants using chromatography, this method was employed to separate, and subsequently compare, the individual components of the dye from several sources, in order to obtain a homogeneous compound which then, presumably, possessed a more specific anti-purinergic action. A second reason for purification was the observation by Weber et al. (1979) that their purified Cibacron-Blue showed, upon TLC examination after storage for several months, some components that corresponded to those fractions observed in the crude dye which had been previously removed. It appeared that some of the dye's heterogeneity had reappeared due to chemical instability after purification.

Paper chromatography separation of fractions from the dye was slow and resulted in only small quantities of the desired substances. When compared against their cholinergic depression, some components (Pi .50, Pi .45 and Ci .45) had a better ratio of purinergic antagonism than the parent compound (fig. 24), but this method was not satisfactory for purification of the various fractions which usually consisted of at least two components after the separation procedure.

However, subsequent column chromatography proved just as inaccurate. It was performed, using at first a cellulose substrate, and the same solvent that had been employed for paper chromatography, in order to obtain comparable relative fronts. Fronts were never well defined in the column and appeared to run much faster in some parts of the column than in others. These problems were diminished when silica gel was exchanged for the cellulose as the solid phase, and the solvent, butanol/alcohol/water mixture, was replaced with an ethylacetate/tetrahydrofuran/water combination. Under these conditions fronts were still not entirely clearly separated, and mixing occurred, although at a much improved ratio if compared with paper or cellulose-column chromatography, and the procedure was only suitable for purification of 2 or, at most, 3 closely related fractions.

Thus column chromatography was subsequently employed only when the main components had already been separated by the crystallization process, which made use of different solubility factors of the components contained in the crude Cibacron-Blue dye. The two, possibly identical, components obtained by that technique (purified Pi .45 and PicF) exhibited a high degree of homogeneity, and specificity of ATP antagonism specificity, which was obvious from the parallel shift to the right of the dose-response curve indicating competitive antagonism. With PiF there was an estimated pA_2 value of 6.2, there being no essential difference between any PiF fraction regarding their ATP-antagonism efficacy.

PicF was not only highly effective in the guinea-pig colon as an ATP-antagonist, but also blocked the ATP response in the guinea-pig vas

deferens, but the shift in the latter dose-response curve was not parallel. It is not clear why ATP-induced relaxations of the guinea-pig taenia coli could not be blocked by PicF, since crude Reactive-blue does exhibit such antagonism in the carbachol-toned taenia (Kerr and Jenkinson, unpublished), but could possibly be due to the presence of a co-transmission system (see review by Burnstock, 1982b) or the test dose of ATP was too high thus activating different subgroups of purinergic receptors, since agonist potency and analog stereospecificity differ in guinea-pig vas deferens and taenia coli (Burnstock and Kennedy, 1985).

The antagonism of the colonic relaxation induced by balloon inflation implicates a transmitter role for ATP in the colon's descending inhibition. The effective dose of PicF, 10 micromolar was based on the parent compound's molecular weight (MW 840). Future work on this Cibacron-Blue fraction should include the establishment of its molecular weight, research into its usefulness as a general ATP antagonist in various physiological, ATP-dependent systems in other animal species, and a means of preserving the compound in order to avoid the need for continual re-purification. Another avenue for further research would be PicF's ability to block the rise in tone induced by PGE_2 in fatigued colonic sections, a finding that was observed but neither reported in the results nor followed up, but which suggests some PG/ATP co-transmission interaction in the descending inhibition of the peristaltic reflex. A further avenue that should be pursued is the possibility that PicF also has antagonistic actions at the $GABA_A$ - receptor site, partly responsible for its antagonism of the reflex relaxation.

CHAPTER VI

SODIUM - MECLOFENAMATE

INTRODUCTION

Winder et al. (1965) described the nonsteroidal anti-inflammatory properties of Sodium-Meclofenamate (MEF) at low doses; it was subsequently found to be the most potent of these drugs, being 95% effective at 10^{-7}M (Flower et al., 1972) or five times as effective as the second most potent antipyritic drug Indomethacin, but a toxic gastrointestinal side-effect of MEF was also discovered (Winder et al., 1967 and Shen, 1979). In the present work, the application of MEF as a tool in the search for the initiating transmitter of the descending colonic inhibition was suggested, by the fact that MEF also interfers with or inhibits prostaglandin synthesis at lowest doses, compared with 23 other antipyritic drugs (Flower and Vane, 1974; Bray and Gordon, 1978; Bennett et al., 1980a and 1980b), especially in gastrointestinal tissue, combined with the discoveries of Bennett et al. (1968) that PGE_2 contracts human, rat and guinea pig ileal and colonic longitudinal muscles, but relaxes the circular muscles of the same tissues, and that these effects are not mediated cholinergically, adrenergically or serotonergically. In addition Bennett et al. (1980b) have also shown that MEF antagonizes PGE_2 induced ileal and colonic longitudinal muscle contractions of guinea pig and human tissue strips. Some other effects of MEF (10-7M) are that it blocks arachidonic acid (10-5 - 10-3M) induced

arterial contractions (Tulenko, 1981), abolishes the 'Portuguese Man-of-war' venom induced vasodilation (Loredo and Gonzales, 1983), and increases ammonia production by the kidneys 2.5 times (Kapoor et al., 1983). Moreover, Burch et al. (1983) found that Ca^{++} accumulation by mitochondria is blocked by MEF, but that it does not affect membrane Ca^{++} transport, while Northover (1973) reports that MEF (10^{-4}M) inhibits (25%) the ATP dependent Ca^{++} uptake by gastric smooth muscles membranes.

Another major side effect attributed to MEF by a number of researchers is its property to counteract the cytoprotective ability of the prostaglandins (Vane, 1971; Smyth and Bravo, 1975; Lacy and Ito, 1982; Rampton and Hawkey, 1984). However, Flower and Vane (1974) recognized that all aspirin-like drugs interfere with a variety of enzymes and therefore warned, "be aware of these other actions!"

Nevertheless, it was shown (Chapter III), using freeze substitution, that the inhibition in front of a pellet consisted of a longitudinal muscle contraction and a circular muscle relaxation, exactly the response claimed as the intestinal effect of PGE_2 by Bennett et al (1968). This information combined with results (Chapter IV) of Meclofenamate's ability to block the descending inhibition, made PGE_2 the prime candidate for the mediator of the descending colonic inhibition. But since involvement of GABA and ATP in the colonic relaxation had also been demonstrated (Chapters IV and V), the interaction between MEF and these drugs was mainly investigated. Because it was suspected that MEF had a blocking effect on other neuroactive substances, meclofenamate was checked for its effect on a number of known neurotransmitters.

MATERIALS AND METHODS

The interaction of Sodium-Meclofenamate with various neuroactive substances was tested in the four bath design (Fig. IIc, appendix). Longitudinal muscle strips (approx. 50 mm long, 2 mm wide) were suspended in the small tubular baths (20 ml volume) with the lower end attached to the aeration tube and the upper part of the strip tied to the isometric force transducer (0.5 g tension). The strips were cut from four equal segments, each obtained from a length of colon removed approximately 10 cm aboral from the caecum to 10 cm oral of the anus. Ileal longitudinal segments were taken successively 10 cm proximal from the caecum, each approximately 5 cm long.

Since spirally cut, thin, circular muscle strips (1 mm) were active for only relatively short periods, and responses of thick strips (3 mm) appeared to contain a longitudinal effect, circular muscle rings or zig-zag cut strips were used. When colonic segments, obtained as described above, were cut into 6 rings of approximately 5 mm diameter and tied with surgical cotton, considerable spontaneous activity resulted, possibly due to point stimulation where the cotton made contact with the tissue, and so causing a sharp invagination. This was eliminated by the replacement of the cotton with small rounded plastic rings as interconnectors.

For the preparation of zig-zag circular muscle strips, three cuts were made in a flap approximately 8 mm long from a colonic segment that had been sliced open near the mesenteric line. The first cut was made in the

middle of the flap, perpendicular to the gut axis, reaching to within 2 mm of the opposite side, subsequently two cuts, 2 mm on either side of the first cut, were made from that side to within 2 mm of the first side. This cutting procedure resulted in an approximately 2 mm thick and 4-6 cm long intestinal strip, consisting mainly of circular muscle oriented in the force effective direction between the two connection points. These colonic strips or rings were either chemically or electrically stimulated. During chemical stimulation the volume of the drug plus solvent vehicle added to the bath was never more than 1% of the total bath volume, and the solvent vehicle was always tested separately beforehand. Electrical stimulation of gut strips was effected by centrally located platinum ring electrodes placed 5 mm apart. Colonic rings were stimulated by plate electrodes (8 cm x 1 cm) 10 mm apart with the chain of rings placed in the middle between the two plates. Electrical pulse parameters were: 500 ms duration trains every 10 seconds, using 50 pulses per sec of 0.2 to 2 ms duration, 60 to 70 V (supramaximal).

Responses acting on the isometric force transducers were transmitted to the Grass Polygraph and printed, as previously described. Pen deflection was calibrated at 1 mm = 0.1 g displacement force at 2 mV/ cm, i.e. 2 mV = 1 g. During these experiments data were also entered into a Rockwell (AIM 65) Microcomputer, averaged by the Neurophysiology Data Acquisition System, Dept. of Biological Sciences, Smith College, Northampton MA (1983) and printed by a Hewlett Packard Autograph (2 D-2) X-Y Recorder.

RESULTS

The dose-response curve for ACH, acting on longitudinal muscle strips of the guinea pig colon, exhibited a gradual increase over a dose-range of 10^{-8}M to 10^{-6}M followed by a sharply increased contractile response with an onset at 10^{-5}M. This was observed and recorded up to 4 x 10^{-5}M (fig. 40; table XXIa).

Carba had a much greater effect at lower dosages. From 10^{-8}M to 10^{-6}M the contraction response increased from 0.6 mV to 2 mV, i.e. 0.3 g to 1 g displacement force. (fig. 40).

The effects of PGE_2 on longitudinal muscle strips consisted of a tension increase up to 10^{-7}M after which it declined, PGE_2 at 10^{-6}M having less than half the contractile effect as had been recorded at 10^{-8}M (Fig. 40), possibly due to a superimposed relaxation seen at doses in excess of 10^{-7}M, where a dose-dependent increasing relaxation to PGE_2 occurred, which preceded the contractions, but is not shown here. These dose-response curves were used only to establish doses of the three drugs to be considered acceptable as effective means of raising the longitudinal muscle tone (fig. 40).

Similar observations were made after testing the same drugs for their effective concentrations on circular colonic muscle strips. ACH again had little effect up to 10-6M, but its contractile effect rose substantially from 10^{-5}M, and although the ACH effectiveness on the circular muscle was initially more than a 100 times smaller than on the longitudinal muscle,

the responses of both muscle types nearly coincided at 10^{-5}M, ACH subsequently had a greater effect on the circular muscle than on the longitudinal muscle (figs. 40 and 41; table XXIb).

The dose-response curve of the contractile response of the circular muscle of Carba was extremely steep, rising from 0.42 mV at 10^{-8}M to 2.21 at 10^{-7}M (fig. 41).

PGE_2 had a similar but reduced effect on circular muscle strips when compared with its effect on longitudinal muscles, also rising to a peak at 5 x 10^{-8}M (fig. 41), and again exhibited a decline with higher doses.

Table 3 demonstrates the effect of MEF on the response of longitudinal and circular muscles as well as the response of whole colon segments to various doses of ACH, Carba and PGE_2. MEF was also tested at two doses (5 x 10^{-5}M, 10^{-4}) against the ATP relaxation at 10^{-6} and 2 x 10^{-6}M, using Carba (3 x 10^{-8}M) to induce a tone increase. Doses of ACH, Carba and PGE_2, that were thus shown to be least affected by MEF, were subsequently used for muscle tone raising purposes whenever needed. During the majority of experiments Carba (3 x 10^{-8}M) was successfully used to raise the muscle tone. Only when cholinergic transmission was blocked, was barium chloride ($BaCl_2$) employed in doses needed to restore the previous muscle tone level, which ranged from 5 x 10^{-6} to 5 x 10^{-5}M. MEF had no effect at all on the tone increase with barium chloride.

In order to keep responses similar, and within measurable levels, different doses of agonists were tested on the two different muscle

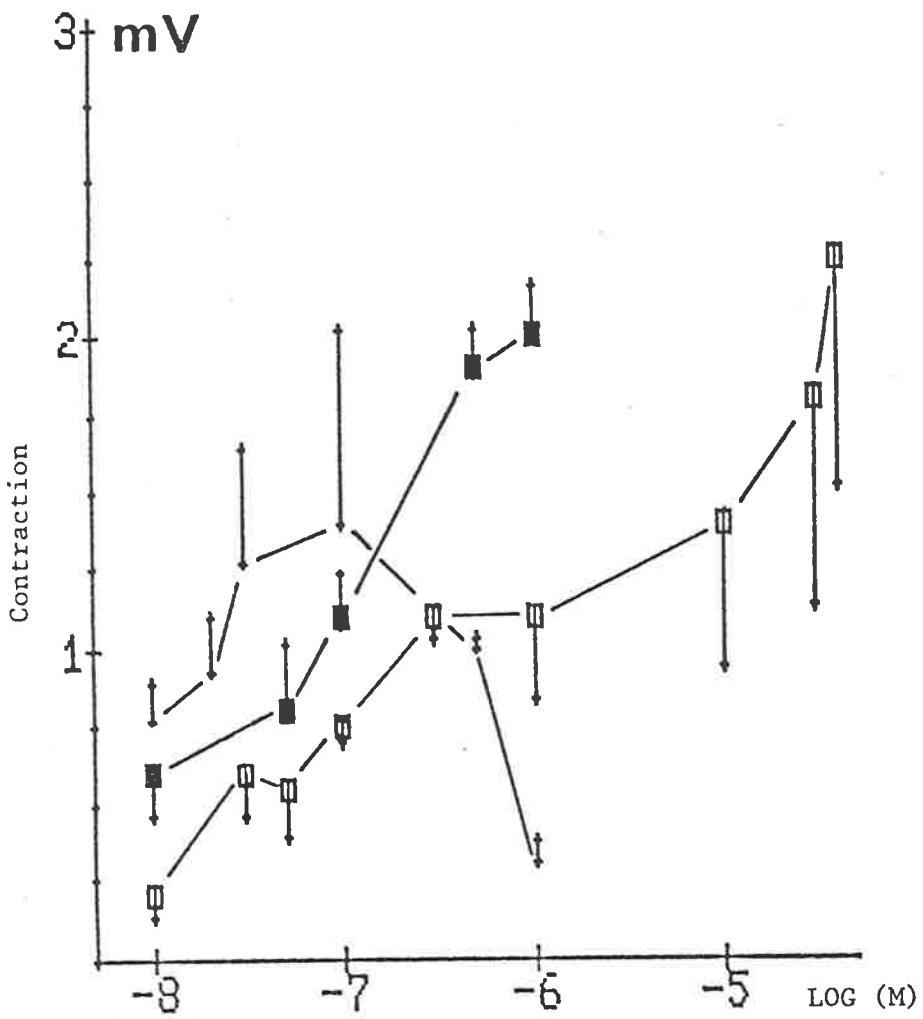


Fig. 40
Dose-dependent responses to chemical stimulation of longitudinal colonic muscle strips (n 8) with ACH (⊞——⊞), Carba (■——■) and PGE_2 (+——+)
(1 mV = 0.5g displacement force)

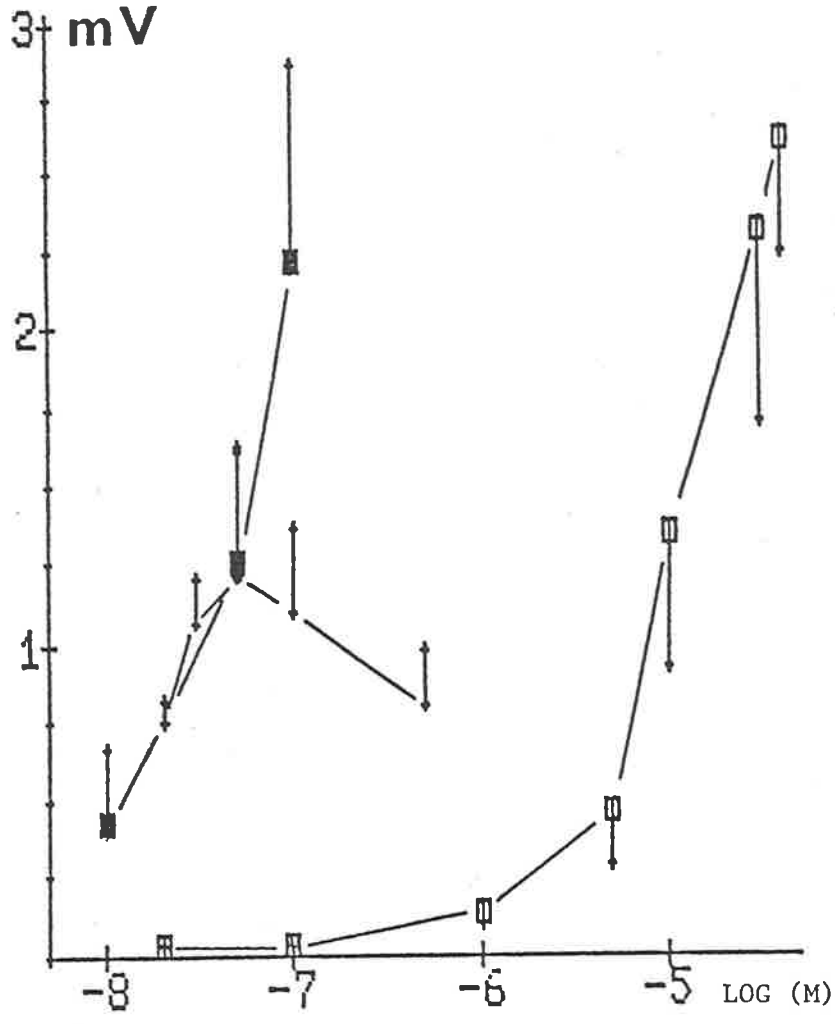


Fig. 41
Dose-dependent responses to chemical stimulation of circular colonic muscle strips (n 8) with ACH (⊞——⊞), Carba (■——■) and PGE_2 (+——+)
(1 mV = 0.5g displacement force)

Table 3

The effect of MEF on chemical stimulation of the whole colon segment (W.L.M.) and longitudinal (L.M.) as well as circular colonic muscle (C.M.) strips (n 8).

Muscle	Agonist	mol/l	MEF mol/l	Response % *
L.M.	ACH	3×10^{-6}	10^{-4}	86 ± 11.3
		10^{-7}	10^{-5}	107 ± 3.8
C.M.	ACH	10^{-5}	10^{-5}	134.5 ± 48.7
		3×10^{-6}	10^{-4}	80 ± 39.6
		3×10^{-6}	10^{-5}	95.5 ± 6.4
W.L.M.	ACH	3×10^{-7}	5×10^{-5}	74 ± 12.1
		3×10^{-7}	10^{-4}	69 ± 16.3
L.M.	CARBA	3×10^{-8}	10^{-4}	60.5 ± 9.1
		10^{-8}	10^{-4}	31.5 ± 16.1
C.M.	CARBA	10^{-7}	7.5×10^{-5}	76 ± 6.1
		10^{-7}	5×10^{-5}	77.5 ± 31.8
		3×10^{-8}	10^{-4}	71 ± 16.5
W.L.M.	CARBA	3×10^{-8}	5×10^{-5}	79 ± 8.6
		3×10^{-8}	10^{-4}	61 ± 18.3
L.M.	PGE_2	10^{-7}	10^{-4}	26 ± 8.3
		5×10^{-8}	10^{-4}	41 ± 16.9
		3×10^{-8}	10^{-4}	0
		3×10^{-8}	7×10^{-5}	65 ± 7
		3×10^{-8}	5×10^{-5}	97 ± 13.2
C.M.	PGE_2	10^{-7}	10^{-4}	37.5 ± 23.3
		5×10^{-8}	10^{-4}	58 ± 7.1
		3×10^{-8}	5×10^{-5}	0
W.L.M.	PGE_2	3×10^{-9}	5×10^{-5}	0
		3×10^{-8}	5×10^{-5}	65 ± 8.4
W.L.M.	ATP (post-Carba 3×10^{-8})	10^{-6}	10^{-4}	79 ± 16.1
		2×10^{-6}	10^{-4}	82.5 ± 8.9
		10^{-6}	5×10^{-5}	89 ± 7.9
		2×10^{-6}	5×10^{-5}	103 ± 12.8

* Percent of previous response retained after MEF application.

strips or on the whole colon segments. It was found that ACH was not effective in producing equivalent contractions at the same doses as Carba or PGE_2 but was less readily blocked by MEF. Longitudinal muscle strips were more sensitive than circular muscle strips, but both were less sensitive than whole colonic segments. The effect of ACH ($3 \times 10^{-6}M$) on longitudinal and circular muscle strips was reduced by MEF ($10^{-4}M$) to a similar degree, but was about 10% less than the reduction of the colon segment responses to ACH ($3 \times 10^{-7}M$) (table 3). While the medium response producing Carba dose of $3 \times 10^{-8}M$, which had a larger effect on whole colon pieces than it had on strips, was reduced similarly (29-39%) in all three preparations by MEF $10^{-4}M$ (table 3).

It was not found that PGE_2 relaxed the circular muscles and contracted longitudinal muscles as claimed by Bennett et al. (1968, 1975) and Horton (1979). At concentrations including and exceeding $3 \times 10^{-8}M$, an initial relaxation was immediately followed by a long lasting contraction. This relaxation, as well as the contraction, increased in a dose-dependent manner. At the same dosage of PGE_2 ($10^{-7}M$) the longitudinal muscle preparation yielded a larger response than that of the circular muscle strip, but was reduced by $10^{-4}M$ MEF to a greater extent, a similar observation was made for $5 \times 10^{-8}M$ of PGE_2. Another curious result was the effect of MEF on the two PGE_2 dose responses, where the higher PGE_2 ($10^{-7}M$) concentration was blocked to a greater degree than the lower one ($5 \times 10^{-8}M$) by the same antagonist concentration, possibly due to the 'turn over' of the control higher PGE2 dose (figs. 40, 41). The response to PGE_2 ($3 \times 10-8M$) could be totally blocked by MEF ($5 \times 10^{-5}M$) in circular muscle strips, while MEF at the same concentration reduced the

response to PGE_2 (3×10^{-8}M) in longitudinal preparations by only about 3%, but only reduced by a third the response in whole colon segments. A five-fold increase of the MEF dose to 10^{-4}M also blocked the PGE_2-response in longitudinal muscles completely (table 3).

The relaxant effect of ATP (10^{-6} and 2×10^{-6}M) on Carba (3×10^{-8}M)-primed whole colon segments was not significantly affected by MEF 5×10^{-5}M, but a slight depressant effect of MEF at 10^{-4}M was recorded (table 3).

The next set of results was obtained from circular muscle strips, which were stimulated electrically. The effect of MEF and trimethoquinol (TRIM) on responses induced by various neuroactive chemicals was examined, and is given in table 4. It was found that neither MEF nor TRIM had any effect on responses to adrenaline (N.A., 10^{-6}M), prostacyclin (PGI_2, 5×10^{-8}M), or 5-HT. Both antagonists had a slight depressant effect on the response to ATP. MEF had no effect on histamine (3×10^{-7}M) induced contractions, while TRIM (10^{-6}M) reduced the response to histamine in the colon circular muscle by 20%. TRIM (10^{-6}M) had no effect on responses to Carba (10^{-8} to 5×10^{-8}M), while MEF (5×10^{-5}M) reduced the response to Carba response to such a degree that the same response could subsequently only be obtained by a ten-fold concentration increase of Carba. MEF (5×10^{-5}M) had no effect on responses to dopamine (10^{-5}M), DNT (10^{-10}M), N.A. (5×10^{-7}M), or N.T. (5×10^{-11}M) but MEF (10^{-4}M) reduced N.T. (5×10^{-11}M) induced responses by 30%. TRIM had no effect on responses to GABA (5×10^{-5} to 10^{-4}M), while MEF (5×10^{-5}M) blocked GABA-responses (10^{-5} to 5×10^{-5}M) but had no effect on responses to GABA at 10^{-4}M. Responses

Table 4

The effect of sodium meclofenamate (MEF) and trimethoquinol (TRIM) on the response of guinea pig isolated colonic circular muscle strips to various drugs during electrical stimulation ($n > 6$).

Agonist	Dose:mol/l	Response	Antagonist	Dose:mol/l	Effect
Adrenaline	10^{-6}	relaxation	MEF	5×10^{-5}	none
	10^{-6}	relaxation	TRIM	10^{-6}	none
ATP	5×10^{-5}	relaxation	TRIM	10^{-6}	-15%
	5×10^{-5}	relaxation	MEF	5×10^{-5}	-12%
CARBA	10^{-8} to 5×10^{-8}	contraction	MEF	5×10^{-5}	10-fold decrease
	10^{-8} to 5×10^{-8}	contraction	TRIM	10^{-6}	none
DOPA	10^{-5}	relaxation	MEF	5×10^{-5}	none
DNT	10^{-10}	relaxation	MEF	5×10^{-5}	none
GABA	10^{-5} to 5×10^{-5}	relaxation	MEF	5×10^{-5}	blocked
	10^{-4}	relaxation	MEF	5×10^{-5}	none
	10^{-5} 10^{-4}	relaxation	TRIM	10^{-6}	none
HIST	3×10^{-7}	contraction	MEF	5×10^{-5}	none
	3×10^{-7}	contraction	TRIM	10^{-6}	20% reduction col.not ileum
N.A.	5×10^{-7}	relaxation	MEF	5×10^{-5}	none
N.T.	5×10^{-11}	relaxation	MEF	5×10^{-5}	none
	5×10^{-11}	relaxation	MEF	10^{-4}	-30%
PGE_2	5×10^{-9} to 10^{-7}	contraction	MEF	5×10^{-5}	-15%
	5×10^{-9} to 10^{-7}	contraction	TRIM	10^{-6}	blocked
PGI_2	5×10^{-8}	contraction	TRIM	10^{-6}	none
	5×10^{-8}	contraction	MEF	5×10^{-5}	none
5-HT	10^{-5}	contraction	MEF	5×10^{-5}	none
	10^{-5}	contraction	TRIM	10^{-6}	none

to PGE_2 (5×10^{-9} to 10^{-7}M) reduced by only 15% using MEF (5×10^{-5}), but completely blocked by TRIM (10^{-6}M).

Figure 42 shows typical responses of electrically stimulated colonic circular muscle strips during GABA/MEF interaction experiments. In the first section of trace 1, the depression of "twitch" by GABA (5×10^{-5}M) is shown, the following part of trace 1 shows that this GABA (5×10^{-5}M) induced depression was not completely blocked by MEF (5×10^{-5}M) within 4 min, but was totally antagonized after $11\frac{1}{2}$ min despite an intermediary wash. Trace 2 shows a total block of this GABA induced depression (5×10^{-5}M) by MEF (5×10^{-5}M) within 8 min, and it was demonstrated by the addition of 10^{-6}M adrenaline that the circular muscles still retained their relaxing ability. In trace 3 depression of the twitch by GABA (5×10^{-5}M) was antagonised by the application of DAVA (10^{-4}M). Fig. 43 depicts some interactions of PGE_2 with MEF and TRIM. In the first section of trace 1, a control contractile response to PGE_2 (2×10^{-7}M) was not significantly affected by MEF (5×10^{-5}M). The first half of trace 2 demonstrates again a control contraction to PGE_2 (2×10^{-7}M) while the second part shows a significant reduction of the PGE_2 induced contraction using TRIM (10^{-6}M). The retention of contractile properties of the tissue was obvious when Carba (3×10^{-8}M) was added. Trace 3 depicts a smaller contraction due to PGE_2 (10^{-7}M), which was completely blocked by TRIM (10^{-6}M), as can be seen in the subsequent part of the record.

Possible antagonistic effects of MEF ($5 \times 10-5$M) to various agonists, were also tested on electrically stimulated, isolated, longitudinal colon muscle responses, as recorded in table 5. MEF had either an insignificant

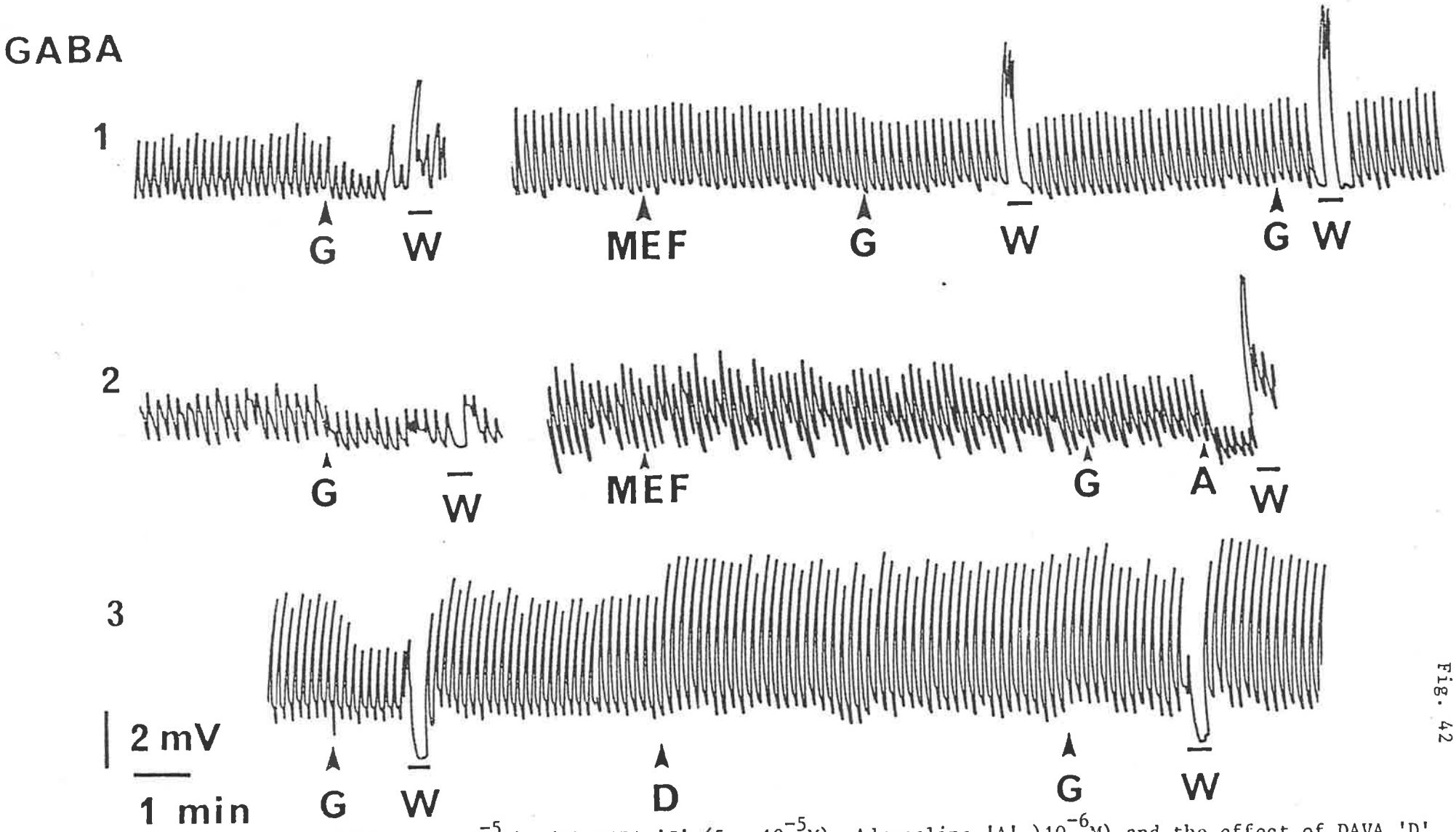


Fig. 42

The interaction of MEF (5×10^{-5}M) with GABA 'G' (5×10^{-5}M), Adrenaline 'A')10^{-6}M) and the effect of DAVA 'D' (10^{-4}M) on the GABA response in colonic circular muscle strip experiments.

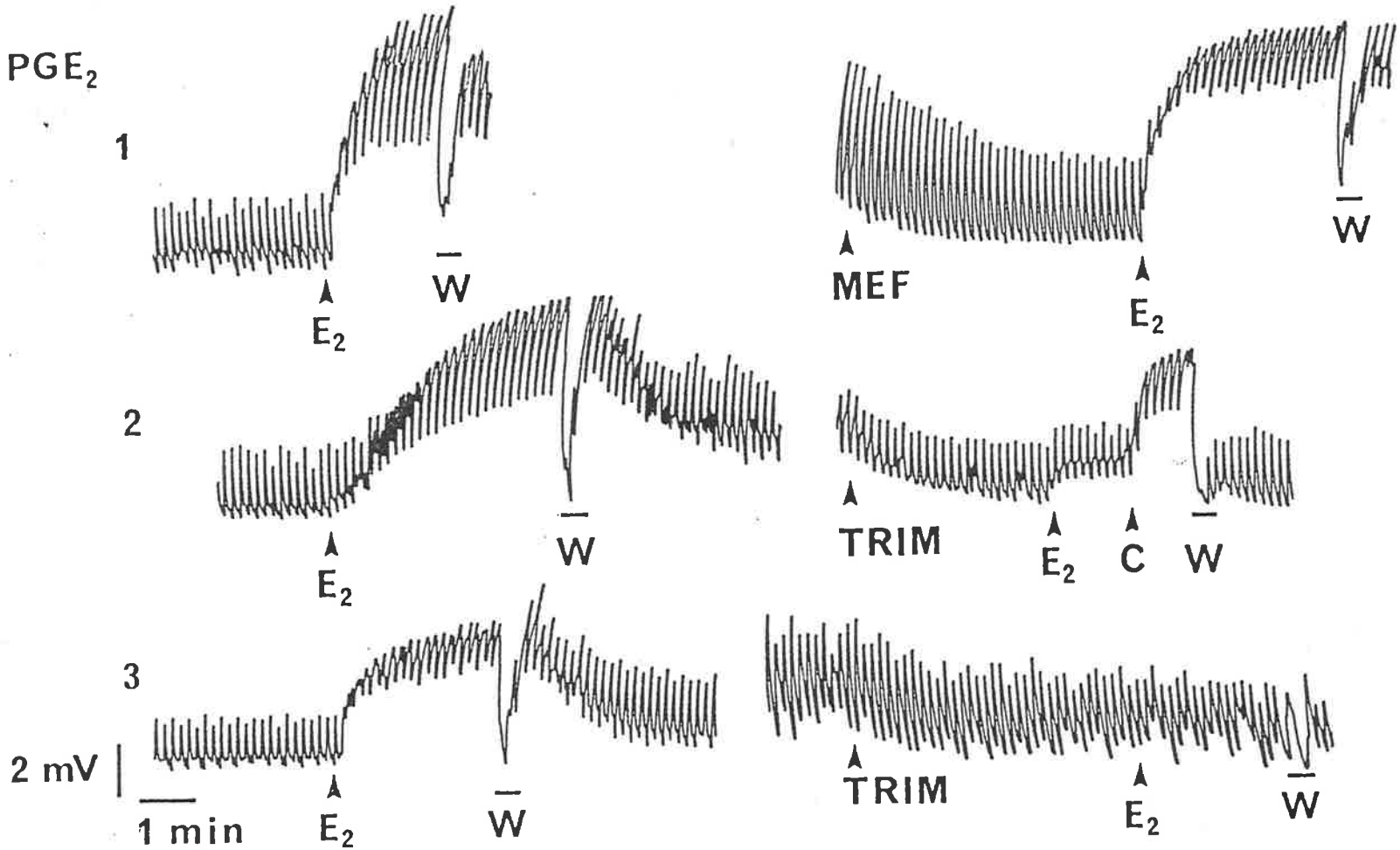


Fig. 43

MEF (5×10^{-5}M, trace 1) had no effect on PGE_2 (2×10^{-7}M). TRIM (10^{-6}M, trace 2) blocked approx 90% of the PGE_2 (2×10^{-7}M) response. TRIM (10^{-6}M, trace 3) blocked PGE_2 (10^{-7}M). Colonic circular muscle strip experiments.

or no effect on the actions of adrenaline (10^{-6} to 5 x 10^{-6}M), ATP (5 x 10^{-6} to 10^{-5}M), dopamine (5 x 10^{-6} to 10^{-5}M), histamine (10^{-7} to 3 x 10^{-5}M), N.A. (10^{-7} to 10^{-5}M), 5-HT (10^{-6} to 3 x 10^{-5}M), or PGF_2 (10^{-9} to 10^{-8}M). MEF substantially (85%) reduced responses to a low Carba (10^{-9}M) dose, but did not significantly alter those to the most effective and most often used Carba range from 10^{-8} to 10^{-7}M. MEF (5 x 10^{-5}M) blocked the morphine (10^{-6} to 5 x 10^{-6}M) induced relaxation, and the PGE_2 (10^{-9} to 10^{-8}M) contractile effect; it also reduced (24-40%) the depression of the twitch by GABA (5 x 10^{-6} to 10^{-4}M). During the same experiment DAVA (10^{-4}M) reduced the GABA (5 x 10^{-5}M) induced depression by 82% (table 5).

DISCUSSION

The effects on longitudinal and circular muscle strips of PGE_2, the main putative target chemical of MEF antagonism, and the two cholinergic agonists ACH and Carba have been examined (figs. 40 and 41). Muscle strips were used to separate clearly the longitudinal and circular muscle effects. It was occasionally observed in experiments using whole gut segments, that tetanic circular muscle contractions extended over the whole length of the gut and could have been mistaken for a longitudinal muscle relaxation. Conversely, massive longitudinal contractions were noticed to result in bulging, which might have mimicked circular muscle relaxations. It was expected that these complications could be avoided by the use of muscle strips. However, the circular muscle 'zig-zag' preparation might have contained a small longitudinal component, because 3 sections consisting of approximately 2 mm of longitudinal tissue, the

Table 5

The effect of sodium-meclofenamate (MEF) on the responses of guinea pig isolated longitudinal muscle strips to various drugs during electrical stimulation.

Agonist	Dose:mol/l	Response	Antagonist	Dose:mol/l	Effect
Adrenaline	10^{-9}	contraction	MEF	5×10^{-5}	none
ATP	5×10^{-6} to 10^{-5}	relaxation	MEF	5×10^{-5}	-15%
CARBA	10^{-9}	contraction	MEF	10^{-5}	-85%
	5×10^{-9}	contraction	MEF	10^{-5}	-9%
	10^{-8} to 10^{-7}	contraction	MEF	5×10^{-5}	none
DOPA	5×10^{-6} to 10^{-5}	relaxation	MEF	5×10^{-5}	none
GABA	5×10^{-6} to 10^{-4}	relaxation	MEF	5×10^{-5}	-24% to -40%
	5×10^{-5}	relaxation	DAVA	10^{-4}	-82%
HIST	10^{-7} to 5×10^{-7}	contraction	MEF	5×10^{-5}	none
	3×10^{-5}	contraction	MEF	5×10^{-5}	+15%
MORPH.	10^{-6} to 5×10^{-6}	relaxation	MEF	5×10^{-5}	blocked
N.A.	10^{-7} to 10^{-5}	relaxation	MEF	5×10^{-5}	none
PGE_2	10^{-9} to 10^{-8}	contraction	MEF	5×10^{-5}	blocked
PGF_2	10^{-9} to 10^{-8}	contraction	MEF	5×10^{-5}	-10%
5-HT	10^{-6} to 3×10^{-5}	contraction	MEF	5×10^{-5}	+10%

apex of each cut, had to be included in each of the 40 to 50 mm circular muscle strips.

The contractile response to PGE_2 was similar in longitudinal and circular muscle experiments (figs. 40 and 41), although slightly reduced with circular muscle strips. The contractile response to PGE_2 appeared to reach a peak between 5×10^{-8} and 10^{-7}M, after which it declined. Since each dose level was used on a fresh preparation, this result suggested progressive receptor desensitization at high doses. Similar desensitizing effects of PGE in rat uteri were reported by Sanner (1982). It is unlikely that this decrease in response was due to a PGE/adrenergic interaction (Binder et al., 1983; Greenberg et al., 1978), since it was still observed during experiments after cholinergic and adrenergic systems had been blocked (Coleman et al., 1979). There was no 'turnover' of the response to PGE_2 using a concentration in excess of 10^{-7}M (c.f. figs. 40 and 41) during electrical stimulation of muscle strips.

ACH was much more effective on longitudinal than on circular muscle strips in doses below 10^{-5}M. This suggests that either cholinergic receptors were much more accessible to ACH in the former than in the latter, or that the tissue had been more severely disrupted during dissecting procedures in circular muscles. The rapidly increasing contractile effect in both muscle systems at concentrations exceeding 10^{-5}M could possibly be attributed to a different ratio of high and low affinity receptors or to direct activation of more or all available cholinergic receptors, even on muscle cell surfaces of inner cell layers. ACH was not used for muscle tone raising purposes, because of its

differential effect at low concentrations, and the interfering effects of ACH-removal by choline-esterases.

The Carba response was similar in both muscle systems, although ten times more effective on the circular muscle strips at the respective maximal doses (Fig. 40 and 41). The dose of 3×10^{-8}M resulted in a lasting, distinct and comparable response in both muscle types and was therefore chosen as the dose for muscle tonus elevation in experiments when a decrease in muscle tone had to be counteracted; Carba had the additional advantage of not being bydrolysed by esterase activity.

In the next set of experiments, the blocking effect of MEF (5×10^{-5} to 10^{-4}M) on dose-responses of tested drugs, as well as its interaction with the ATP inhibition, was examined, using colonic longitudinal and circular muscles as well as the longitudinal response of whole colon segments. The ACH response varied greatly when the same doses were used in the three different muscle systems. A dose of 3×10^{-6}M elicited only a small response in circular muscles, and a medium response in longitudinal strips, but the response was too large to be measured (off scale) when this dose was applied to the whole colon segment. A dose of 3×10^{-7}M had to be used to give responses of intermediate size, which in turn was more readily blocked by MEF (10^{-4}M). Due to this variability of response, ACH was considered unsuitable, although it had a higher resistance to MEF (10^{-4}M).

Carba displayed comparable responses at 3×10^{-8}M in all three systems. Its effect was considerably reduced by the maximal MEF (10^{-4}M) dose

(table 3), but the most commonly applied MEF concentration of 5×10^{-5}M had only a less than 20% blocking effect , and Carba was still considered the most suitable tone increasing drug.

Several significant observations were made regarding the MEF/PGE_2 interaction. The higher (10^{-7}M) and the lower (3×10^{-8}M) PGE_2 concentrations were blocked to a greater degree than the medium dose (5×10^{-8}M) by MEF (10^{-4}M). This phenomenon could have possibly been due to the previously mentioned, smaller responses to PGE_2 at both lower and higher concentrations. Another remarkable difference was that the circular muscle response to PGE_2 (3×10^{-8}M) could be blocked with MEF at 5×10^{-5}M, whilst 97% of the longitudinal muscle response remained at the same dose ratio, although a twofold increase in MEF concentration reduced the response to 65% and a fivefold MEF dose to 10^{-4}M abolished it. It became obvious that 5×10^{-5}M of MEF was sufficient to block PGE_2 effects in colonic circular muscle strips.

An ATP/PGE_2 involvement in the colon, similar to that in the rabbit vas deferens (Binder et al., 1983) was examined using MEF (5×10^{-5} and 10^{-4}M) on the relaxation induced by ATP (10^{-6} and 2×10^{-6}M). Only a small reduction of the relaxation could be observed, possibly due to some interaction between the tone elevation with Carba and the ATP induced relaxations which the former counteracts.

In the next set of experiments the interaction of MEF with various neuroactive drugs was examined on responses of the colon muscle systems to electrical stimulation. MEF ($5 \times 10-5$M) did not appear to have had any

effect at all in the circular muscle preparation, on the depression of the responses by adrenergic drugs nor on the effects of dopamine, N.T., DNT, histamine, prostacylin or 5-HT responses. Only a small depression of responses was noticed for ATP (5×10^{-5}M), PGE_2 (5×10^{-9} to 10^{-7}M) and N.T. (5×10^{-11}M). Responses to various Carba doses were reduced by 30% to 40%, which usually required a tenfold increase in the Carba concentration to restore it to pre-MEF levels. The most important finding was that MEF (5×10^{-5}M) blocked the GABA (10^{-5} to 5×10^{-5}M) inhibition of the contractile response evoked by electric-stimulation. The inhibition caused by higher GABA (10^{-4}M) concentrations could not be antagonized by MEF, suggestive of competitive antagonism by MEF. However, the blockade of this GABA action by MEF was very similar to its blockade by DAVA. Adrenaline was used to show that the relaxing potential of the preparation had not been impaired (Fig. 42, traces 1-3). DAVA had a 4-8 min onset delay, which also applied to the MEF antagonism. Since DAVA has been shown to be a $GABA_B$ - receptor antagonist, and since GABA induced depression of transmural stimulation (T.S.) is also attributed to $GABA_B$ - receptors (Bowery et al., 1981; Ong and Kerr,1983a and 1984), it appeared that MEF had acted by blocking $GABA_B$ - receptors in the colonic circular muscles. This GABA antagonism was a major neurophysiological MEF effect observed in colonic circular muscle preparations.

MEF did not exhibit any antagonism to PGE_2-, PGI_2- or PGF_2-induced contractions of colonic circular muscles, whilst TRIM (10^{-6}M) completely blocked the PGE2 contractions (fig. 43). However, when relaxations were observed just preceding the contractions in response to PGE_2, these relaxations were blocked by MEF but not by TRIM. Unfortunately, these

(pre-contraction) relaxations were irregular and occurred only in preparations with very high muscle tone and although, a muscle tone decrease by MEF or TRIM was always counteracted by Carba or barium chloride, the preparation had usually lost all spontaneous activity and responses appeared somewhat modified. This effect of MEF on PGE_2-induced circular muscle relaxations, which were not affected by TRIM, was therefore not regarded as suitable for inclusion in the results. TRIM did not have any effect on the $GABA_B$ - receptor mediated inhibition of cholinergic transmission in colonic circular muscle, but it completely blocked the PGE_2 induced contraction (fig. 42). A direct PGE_2/GABA interaction, the existence of which was suggested in the central regulation of food intake (Morley and Levine, 1985), appeared likely but was not found, GABA had no effect on the PGE_2 response and PGE_2 did not interfere with the GABA-induced inhibition in circular muscle of the colon (table 4).

When MEF interaction with various drugs was investigated on longitudinal muscle preparations activated by T.S., results similar to those with circular muscle were obtained. The main differences were that MEF blocked PGE_2 contractions completely but antagonised the GABA-induced depression of responses to T.S. only by about a third. The similar depression of morphine was also blocked, but this was not pursued further (table 5).

Concluding this chapter, the main findings were: firstly, the establishment of the most suitable stimulatory doses of ACH, Carba and PGE_2, as well as their interaction with effective MEF concentrations in circular, longitudinal and whole colon segments. Secondly, the MEF

antagonism of $GABA_B$ - receptor mediated depression of responses to electrical transmural stimulation in colonic circular muscle, whereas most of the major neuroactive inhibitory drugs were not significantly influenced. Thus the correlation of the above with observations regarding the blockade of the reflex descending inhibition blockage by MEF (Chapter IV) led to the tenet that GABA plays an essential role in colonic motility.

CHAPTER VII

GENERAL DISCUSSION

The first aim of this research project had been the development of a reliable technique, which could be used to evoke peristaltic motility of the colon *in vitro*. It was intended to develop a method, which specifically allowed the examination of the descending inhibition, the forces acting upon it, and the neurotransmitters responsible for it.

Fluid infusion (Trendelenburg), electrical transmural stimulation, various stretch techniques, bolus distensions, artificial pellet chain, single pellet force transducer arrangements and balloon distension methods were exhaustively tested. Four of these methods were found to produce reliably repeatable responses, but the only reliably reproducible result, using the Trendelenburg fluid distension method, was a bicuculline-induced alteration in the descending reflex response (fig. 1d).

Of the four methods selected the first two, which were very closely examined, were the pellet chain technique, which permitted determination and modification of peristaltic velocity, and the single pellet/force transducer combination, which recorded the peristaltic force exerted on pellets, the peristaltic wave frequency, and the circular and longitudinal muscle contractions of the gut segment. The technique facilitated measurement, observation and modulation of these effects by

various drugs. Intestinal motility proved to be a very complex, not easily initiated process, involving a number of interlocking events, controlled and regulated by a number of intrinsic and extrinsic factors (different neurotransmitters, temperature, pressure, pH, state of fatigue), and could be inhibited by a diverse range of agents. Most of the drugs screened would stop peristalsis at suitably high concentrations, whilst Carba increased chain transport velocity even at high concentrations. GABA and naloxone initially increased colonic motility at low concentrations but reduced it considerably at high concentrations (table 1). Pellet chain velocity experiments also proved to be a useful and sensitive technique for testing agents affecting motility, especially since it imitated natural events very closely. It is proposed to be potentially a valuable tool for future work, when assessing colonic effects of new drugs suspected of possessing gastrointestinal modulatory properties.

But as the pellet-chain method did not specifically show effects on the descending inhibition, an arrangement was employed, whereby a single artificial pellet was attached to a force transducer with torque transducers recording longitudinal and circular muscle events at three different points on the gut segment. A correlation was attempted between the change of force and frequency of the peristaltic wave, acting on the pellet, and the longitudinal and circular muscle responses. Although significant results were obtained and recorded (table 2), unfortunately the initial anal circular muscle relaxation was immediately obscured and subsequently obliterated, by the longitudinal muscle contractions which pulled intestinal tissue in an oral direction over the positionally fixed

pellet. This resulted in the anal part of the colonic segment being stretched tautly between the pellet and the anally positioned anchoring hook, thus completely eliminating and falsifying relaxation recordings from the anally attached torque transducer. This effect considerably reduced the method's usefulness in the present studies.

Two of the most important results that could be attributed to the single pellet/force-transducer combination were: firstly, the often overlooked importance of the longitudinal muscle, which appeared to be a most prominent factor in the development of the propulsive force (fig. 4d); secondly, the appearance of 'bunching' or 'bulging' of intestinal muscle tissue oral of the pellet, which defied explanation but led to the decision to apply freeze substitution techniques in the actively peristalting gut.

However, the methods used most extensively in the subsequent experiments were balloon distension and electrical stimulation techniques. Balloon distension had the advantage of allowing regulation of stimulus intensity by volume control, stimulus and interval duration as well as automation of repetitive stimuli. Using this method, continuously reproducible descending-inhibition responses were successfully subjected to modulation by various neuroactive drugs, the results of which form a major portion of this work, the most significant ones being dealt with in relevant chapters. Electrical transmural stimulation was relied upon when responses of gut strips were examined in order to separate and classify the different effects that various drugs had on circular and longitudinal muscles.

The freeze substitution technique, applied to the peristalting colon - with arrest of muscle activity during propulsive motility - was initially employed to explain 'bulging', and to elucidate the different roles of the two main muscle layers of the lamina muscularis. It became obvious that the descending inhibition did not simply consist of a relaxation of both muscle layers, but that, whenever a widening of the gut lumen occurred, an active contraction of the longitudinal muscle layer, usually in front of a pellet or directly behind a pellet, was associated with it. Opening the intestine in front of a pellet effected facilitation of the pellet passage, and opening behind the pellet accommodated a certain volume of mucosa. 'Bulging' was shown to have been due to large accumulations of circularly folded mucosa, possibly for the purpose of containing excess mucosal slack.

The most important discovery made during the freeze substitution experiments, however, was that a compressed amount of circularly folded mucosa could be observed behind every pellet. This phenomenon explained the orally travelling inhibition, the' anti-peristaltic' wave, which never transported anything orally, but in reality was the bunched mucosal slack being pulled back towards the caecal end of the colon in readiness to travel anally again with further pellets. Although it must be made clear that it was "the slack" of the mucosa, that was used to push the pellets and accompany them anally. This action of the mucosal slack needs a well developed flexible muscular system, the muscularis mucosae, which consists of an elaborately arranged longitudinal and circular muscle layer, the purpose of which had been previously unclear and subject to

questioning (Angel et al., 1982; Mathias and Sninsky, 1985). This also explains why a peristalting colon segment, which had had its mucosal layers removed, could not transport any solid or fluid material at all (Frigo and Lecchini, 1970). When this complexity, and more importantly the necessity for plicae folding during faecal movement, was considered it became obvious why so many drugs could interfere with, or stop, pellet chain motility, as well as impede propulsive activity exerted on the single-pellet/force-transducer combination.

These freeze substitution results naturally led to the search for a neurotransmitter capable of either affecting the two muscle layers differently, or having a dual effect. Since GABA exhibited dual activity in the ileum, a contraction preceding a relaxation, and because of claims of its functions as the intestinal inhibitory neurotransmitter, its possible role in the balloon distension-initiated descending inhibition was examined. It was found that the $GABA_B$ - receptor antagonist DAVA indeed blocked the descending inhibition. However, DAVA was not always effective on all colonic sections, and sometimes did not block the inhibition fully but only partially; also, very high concentrations of DAVA had to be applied since it has only low potency. These results suggested that perhaps a second controlling transmitter is involved, which had been deficient in animals where DAVA had been successful. The other possibility is that GABA regulates only one of the two muscle effects responsible for the relaxation, and that in successful experiments, where relaxation was blocked by DAVA, the other muscle layer had been activated by other unknown influences, perhaps due to vitamin deficiency, enzymatic changes due to stress, or age differences. That

DAVA blocked only the latter part of the inhibition at low doses, when it did not fully antagonise the relaxation evoked by balloon distension, could also indicate the presence of a second transmitter system responsible for extending the intestinal lumen (fig. 14b, A2).

Thus the search widened, after it had been ascertained that the observed relaxation in response to the balloon distension stimulation was neurogenic, after its total block by TTX application, and also that it was not due to adrenergic or cholingergic stimulation. Peptides, which had been tested, could also be eliminated from the list of putative transmitters responsible for the inhibition. Naloxone had no major effect on the inhibition at normally effective doses and although it blocked the decending inhibition at high doses, this is likely due to non-specific actions of naloxone at these doses.

Since prostaglandins had been reported to have diametrically opposed effects on circular and longitudinal muscles in the colon, four of these (PGE_1, PGE_2, PGF_2 and PGI_2) were examined. PGE_2 was found to produce the most substantial responses at lowest dosages, when tested in whole colon segments; low to medium doses evoked a relaxation followed by a contraction in both muscle systems. But in the longitudinal muscle, higher doses caused only a contraction, while the circular muscle remained in a relaxed state at the same dosage (fig. 10; C_1 and C_2). The 'revitalization' of the relaxation in fatigued colon segments, by the addition of arachidonic acid, was a strong indication of prostaglandin involvement in the descending inhibition. Further proof was presented when the two prostaglandin blockers MEF and PPP, which had antagonized

the relaxation evoked by balloon distension, also blocked the PGE_2 induced circular muscle relaxation in whole colon segments. When the two low dosage effects of DAVA and MEF (fig. 14b; A_1 and A_2) were examined, it was noticed that MEF blocked the first, and DAVA the second part of the descending inhibition. It thus appeared that DAVA had antagonised a GABA induced circular muscle relaxation but had left intact the longitudinal muscle contraction necessary for a gut lumen distension (fig. 5). Since this in turn was blocked by MEF, the first part of the relaxation was most likely a prostaglandin effect. However, when low dosages of DAVA and MEF were combined, in sequence, or simultaneously applied, the relaxation was only reduced, usually to less than half, but never abolished. This unexpected result could not be explained.

When the possible involvement of 5-HT in the descending inhibition was examined, it was found that the 5-HT antagonists methysergide and mianserin had no significant effect on the relaxation, but it could be shown that both blocked oral reflex contractions. Another peripheral finding was that DAVA enhanced oral contractions, possibly by blockade of a GABA suppression mechanism acting on the serotonergic system.

When it was found that Cibacron-Blue also antagonised the relaxation evoked by balloon distension, but that theophylline did not, attempts were made to separate and purify the active component in the dye and analyze its ATP-blocking efficacy in the colon. The 'pure' Cibacron-Blue had to be re-purified because of its short homogeneous lifespan.

Separation of components was also carried out to find the ATP-specific antagonist, which was effective at the lowest level of anti- cholinergic activity.

Results of paper-chromatography-separated compounds indicated that the most effective constituent was present in highest concentrations in Cibacron-Blue, manufactured by Pierce Chemical Co. Purification and separation efforts were subsequently concentrated on this product, and, with the aid of Professor Prager, a method was developed to produce the most active ATP-blocker in quantities of several gramms, by combining crystallization and column-chromatography techniques. A Pierce Cibacron-Blue component, which was called PicF (Pierce crystallization fluid), and which closely resembled Pi 0.45 (Pierce, paper chromatography Rf. .45), was the most potent ATP antagonist, with greatest specificity against ATP, and extremely low cholinergic antagonism in colonic preparations. This component was also effective as an ATP- blocker in the guinea-pig vas deferens, but not in taenia coli preparations. PicF also blocked the balloon distension-initiated descending colonic inhibition at 10^{-5}M (based on MW 840), which implicates ATP in the inhibitory neurotransmission of the colon. A possible interference with PGE_2 contractile properties by this new ATP antagonist would need testing, and could lead to further proof of a co-transmission system of ATP and PGE_2, possibly working on that part of the descending inhibition which relies on longitudinal muscle contractions. Alternatively ATP may relax some PG that reinforces its action on the smooth muscle.

It has become evident that no single transmitter was responsible for the descending colonic inhibition, because although MEF regularly blocked the inhibition completely, in contrast most prostaglandin blockers did not do so. It is, therefore, assumed that MEF also blocked some second transmitter involved. A search for such a second transmitter, using MEF antagonism, was initiated. In order to avoid compounded effects,circular and longitudinal muscle strips were employed.

Dose-response curves of tone-raising drugs were obtained separately for circular and longitudinal muscle strips. Concentrations of tone-raising drugs were kept below plateau levels when used during experiments. The next step was to check the effect of MEF on the tone-raising drugs as well as on PGE and ATP to establish the MEF doses to be used during the search. This agonist/antagonist interaction was tested purely on a chemical stimulation basis, which allowed necessary adjustments to be made to drug concentrations for subsequent electrical transmural stimulation experiments. MEF antagonism to a number of neuroactive substances was investigated. The most notable results were obtained for antagonism of prostaglandin E_2 and GABA by MEF.

It was observed that MEF did not block the contractile effect of PGE_2 on the circular muscle, which TRIM did. The irregularly occurring relaxation, preceding a PGE_2 induced contraction, was always blocked by MEF but never by TRIM. However, MEF always blocked PGE_2-evoked longitudinal muscle contractions, but only decreased GABA-induced longitudinal muscle relaxations. A curious effect of MEF antagonism to PGE_2 was that it blocked responses to low and high doses more readily

than medium dose effects, doses which had given the best contraction results during dose-response trials. However, the most significant finding was that MEF blocked the GABA induced depression of electrically stimulated, colonic, circular muscle contractions. The efficacy of GABA antagonism by MEF in this experimental design was comparable to that of DAVA at similar concentrations, while TRIM had no blocking effect at all on the GABA inhibition. Thus it could be shown that MEF blocked PGE_2-evoked longitudinal muscle contractions, as well as GABA induced relaxations of circular muscles, of electrically stimulated colonic strips. These results suggest that the phenomenon of reflex descending inhibition requires the independent action of two neurotransmitters on the two different colonic muscle layers, which makes it so difficult to block with only one antagonist. MEF was successful, because it blocked both transmitters involved. That GABA and prostaglandin cooperate during intestinal activity correlates well with the suggestion (Minano et al., 1985) that GABA is also involved in gastrointestinal cytoprotection, which previously has been mainly postulated as a prostaglandin effect. In cases where DAVA counteracted the descending inhibition, prostaglandin/GABA interaction must have been disturbed, possibly due to either some intrinsic anomalies, vitamin deficiencies or the age of the animals, which had been reported to affect GABA efficacy in the brain (Ciesielski et al., 1985).

CONCLUDING REMARKS

Important results were obtained with the development of the artificial pellet chain technique allowing measurement of changes in propulsive velocity due to drug interference or fatigue. The artificial-pellet/force -transducer combination permitted the recording of changes in propulsive force due to chemical influences, whilst the automated stimulation by balloon distension resulted in accurately reproducible descending inhibition, which could be maintained for hours and manipulated by neuroactive agents.

Freeze substitution disclosed the active involvement of the mucosal layer in colonic peristalsis, this has suggested the reasons for the existence of the excessive length of the mucosal layer, and its extremely well developed muscle system. In addition, a double and opposite action of the two muscle layers of the lamina muscularis during the descending inhibition could be seen using the freeze substitution technique.

The search for an effective ATP antagonist in the intestine led to the separation and purification of a component of Cibacron-Blue, which displayed remarkably specific ATP antagonism. Although Grider et al. (1985) showed, using the ATP analogue B_2-ATP, that ATP was the relaxant neurotransmitter in the guinea-pig taenia coli our results did not confirm it.

However, the most significant results were the findings that MEF blocked $GABA_B$ -receptor-mediated depression of cholinergic transmission of

circular colonic muscles, as well as PGE_2 induced contractions of longitudinal colonic muscles. Since MEF blocked the descending inhibition reliably and effectively, this suggests that GABA may be the mediator of the inhibitory effect acting on the circular muscle, whilst PGE_2 may be the main effector of the excitatory pathway contracting the longitudinal muscle. Although it has been claimed that longitudinal muscles are not needed for intestinal emptying (Kosterlitz et al., 1956; Innes et al., 1956), the latter observed fluid transport only, which might readily pass through the lumen without its active opening. It is therefore interesting to note that more than fifty years ago Raiford and Mulinos (1934b) already claimed that they nearly always observed longitudinal contractions below the anally propelled faecal mass.

Thus the screening of putative intestinal inhibitory neurotransmitters suggests that both PGE and GABA, may be involved in the colonic relaxation associated with the peristaltic reflex.

DIRECTIONS OF FUTURE RESEARCH:

The artificial pellet chain, and the single-pellet/force-transducer, arrangements could become a valuable tool during drug dependency studies or for assessment of gastrointestinal effects of new pharmaceutica.

Freeze substitution could be employed to elucidate mucosal folding and the transmitters responsible. It could also be useful in examining

different muscle interactions in the tissue, for instance in cardiovascular or myometrial systems.

The use of MEF and DAVA might clarify whether gastrointestinal ulcerogenicity due to PGE paucity is a direct result of a deficient descending inhibition in the G.-I. tract.

The new ATP antagonist, purified from Cibacron-Blue, needs to be thoroughly examined. Its molecular weight must be established, and storage parameters preventing its degradation into a heterogenous compound should be found. Its efficacy as an ATP antagonist should be investigated on a wide variety of physiological systems, and a possible $GABA_B$ - receptor antagonism further explored.

The role of MEF as a dual antagonist against GABA and PGE_2, the mechanism involved, and its antagonism of the GABA inhibitory effect should be tested in other biological systems.

A P P E N D I X

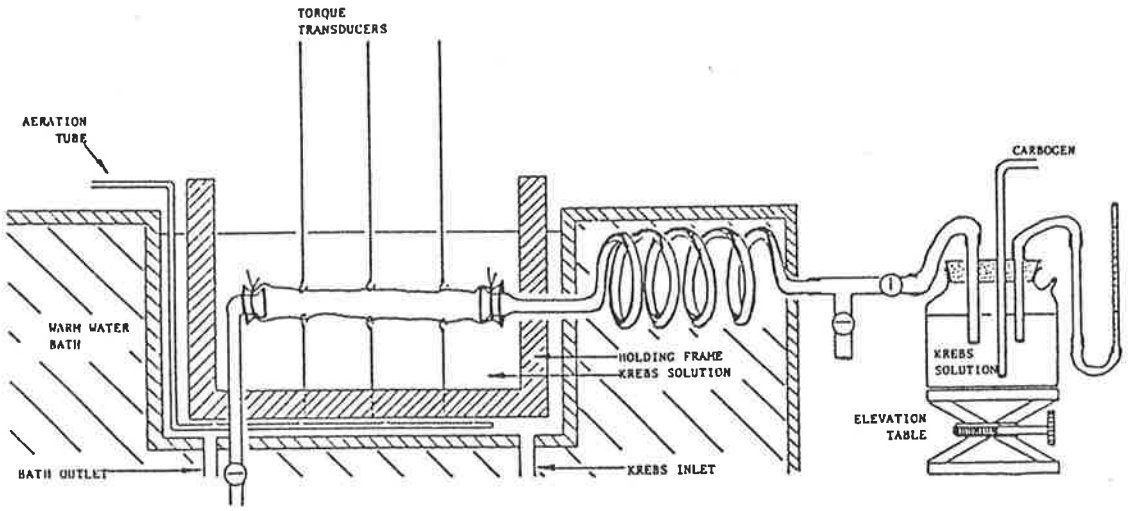


Fig. I
Modified Trendelenburg's apparatus

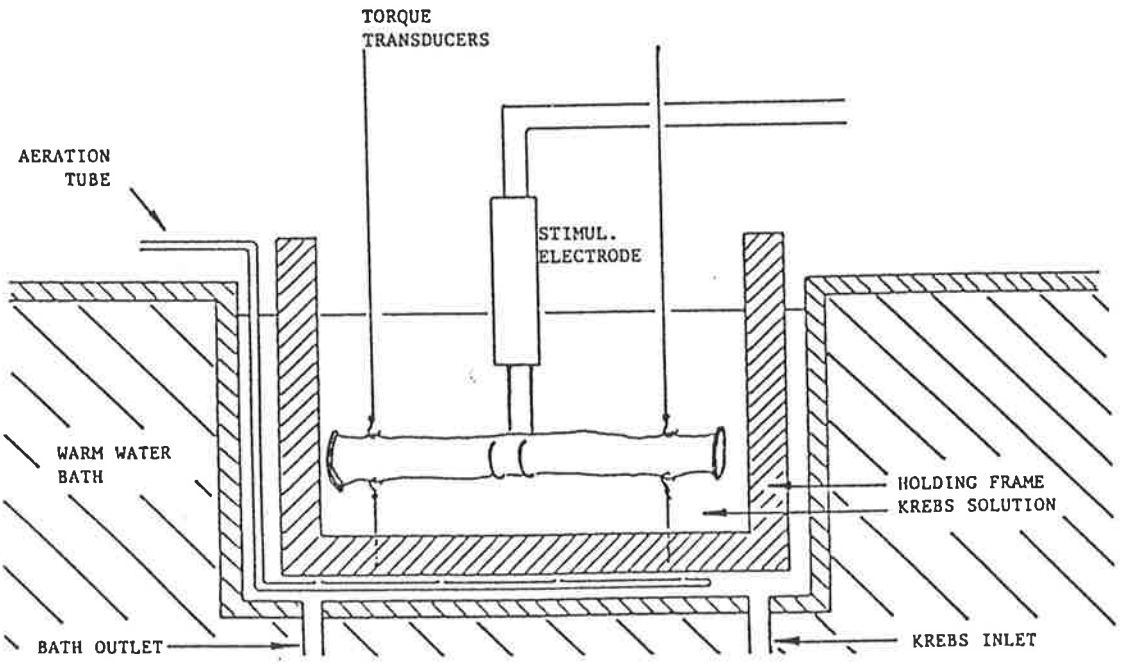


Fig. IIa
Electrical stimulation of colon segment

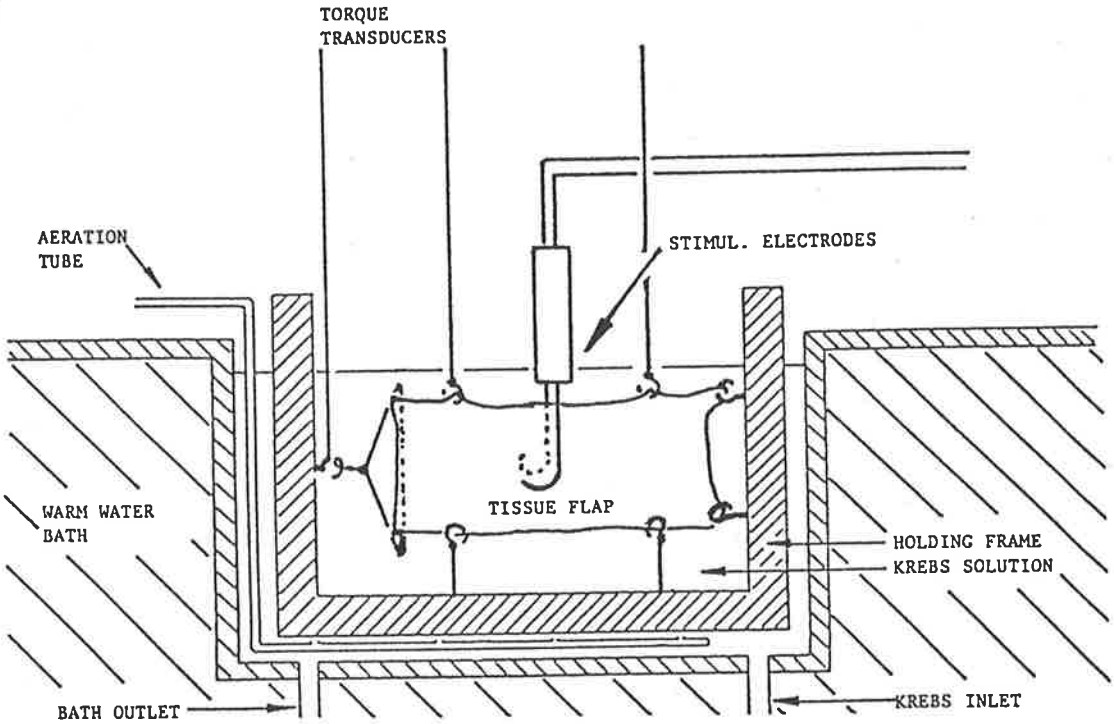


Fig. IIb
Electrical stimulation of colonic tissue

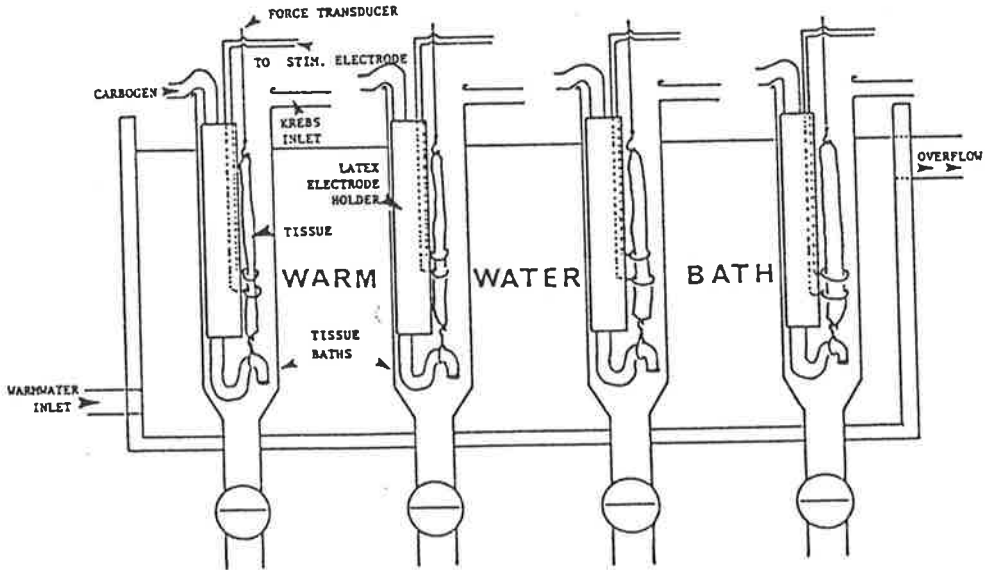


Fig. IIc
The four chamber stimulation design

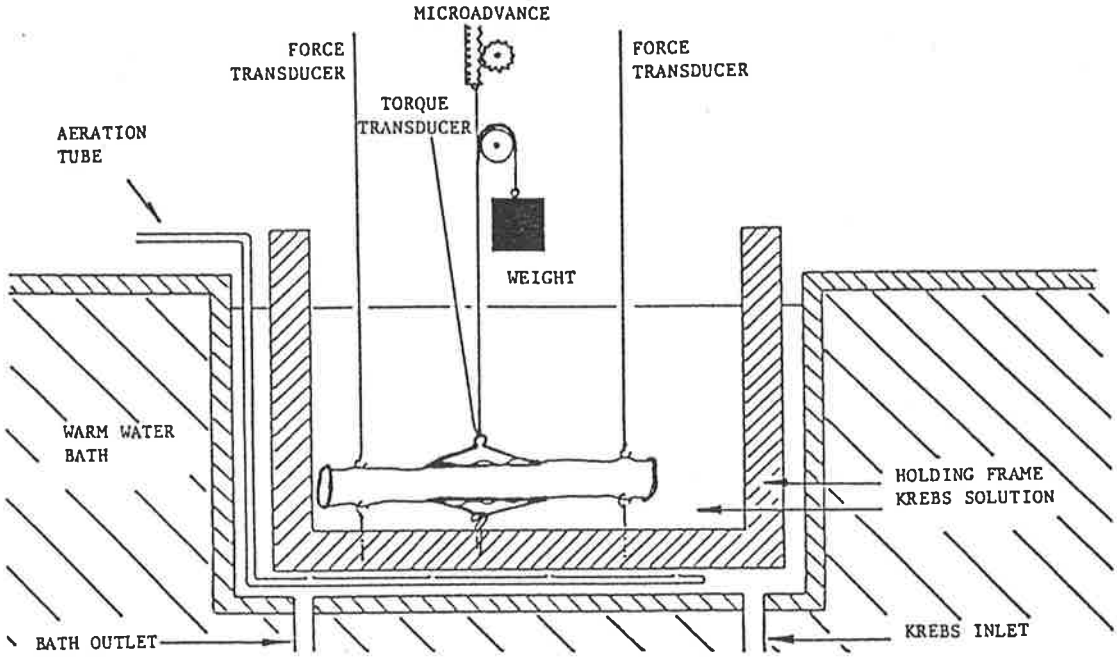


Fig. IIIa
Weight distension stimulation of colonic section

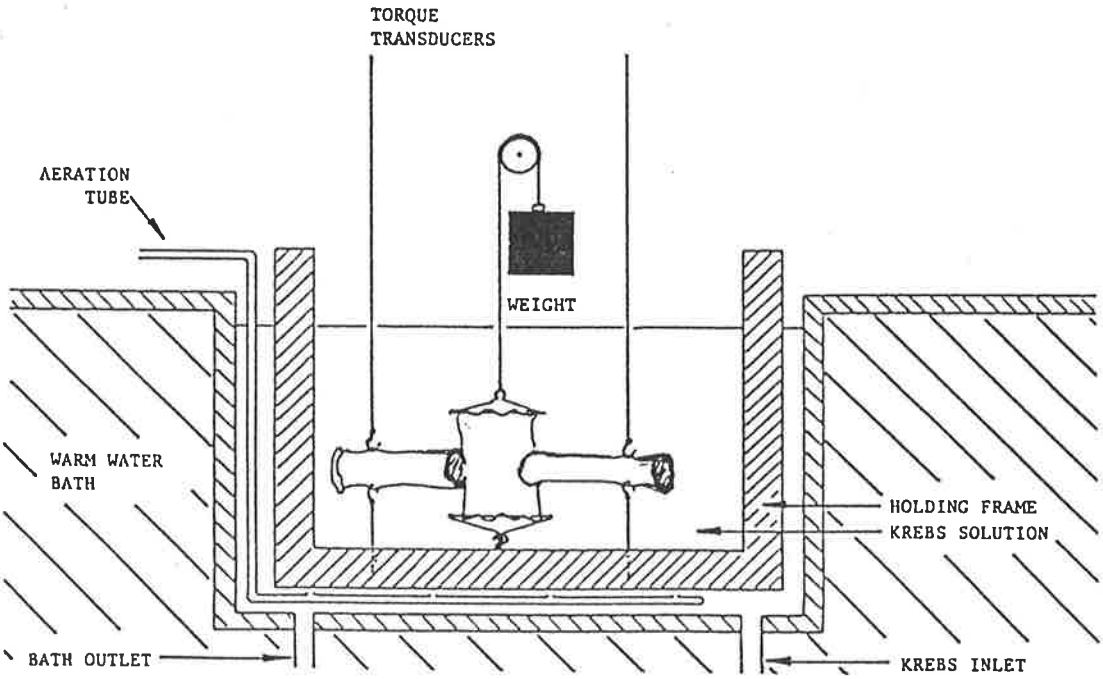


Fig. IIIb
Weight distension stimulation of colonic tissue flap

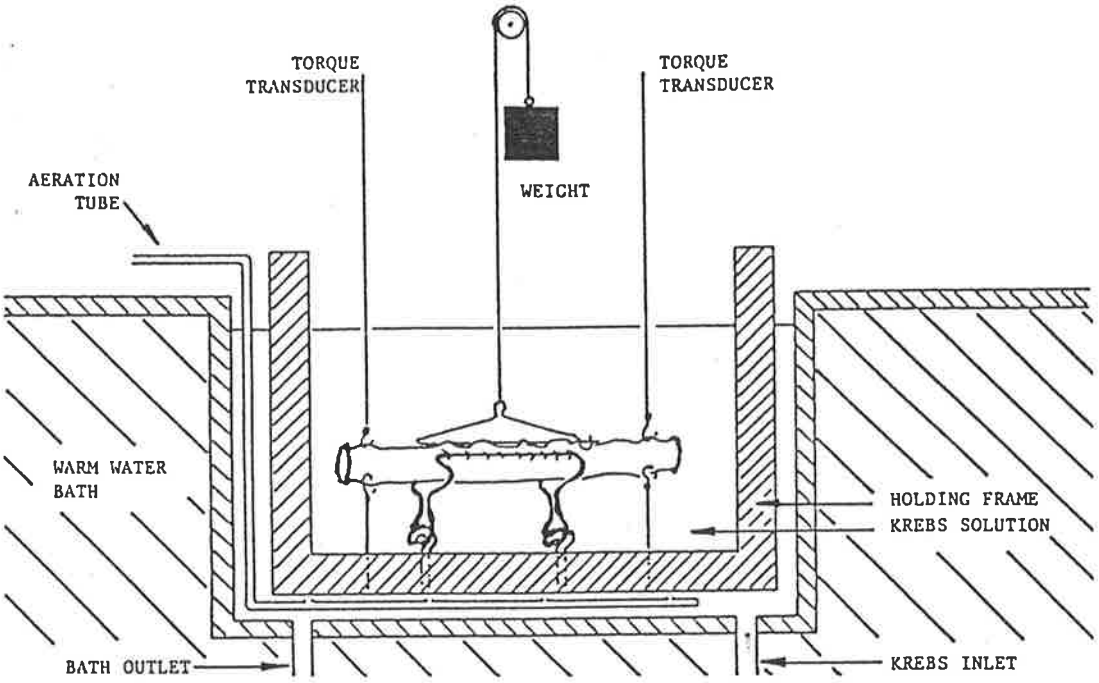


Fig. IIIc
Weight distension point stimulation

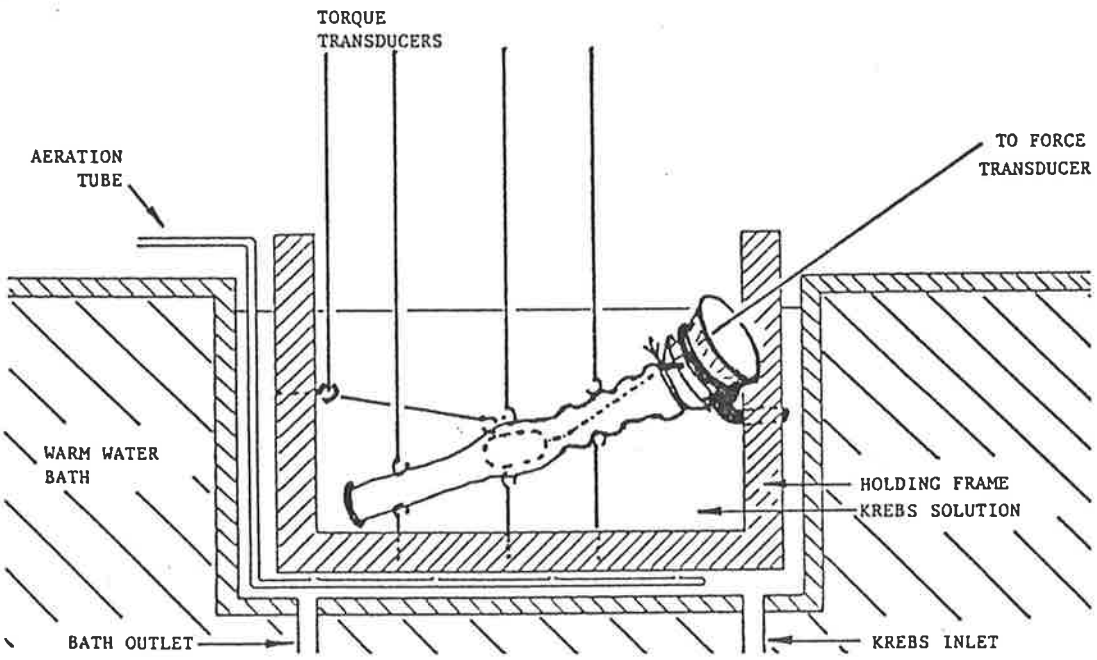


Fig. IVa
Artificial pellet - force transducer design

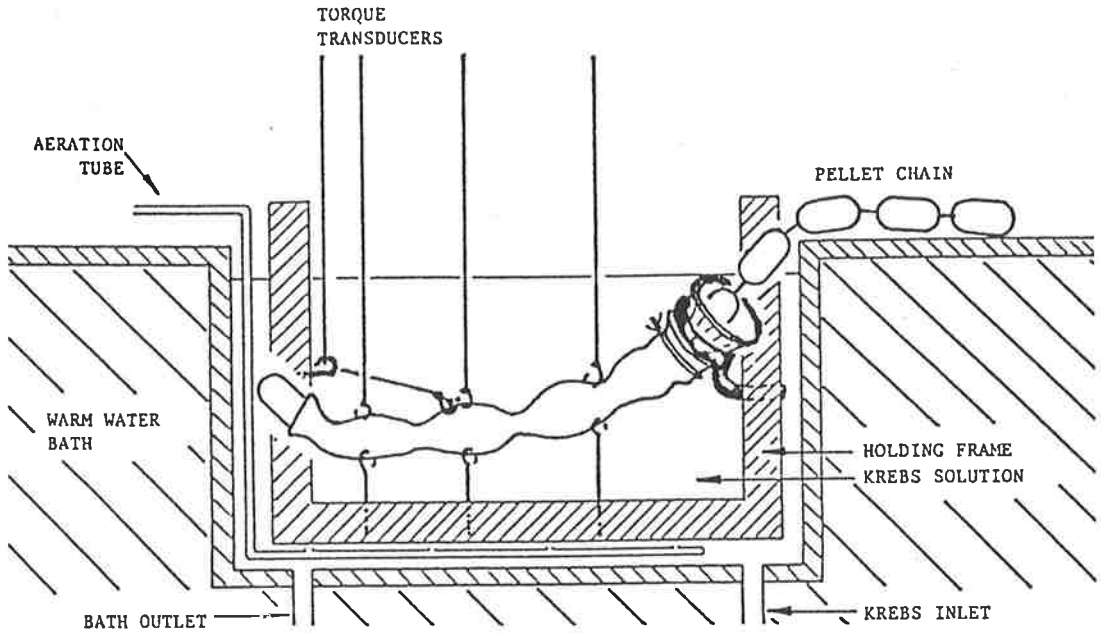


Fig. IVb
Artificial pellet chain arrangement

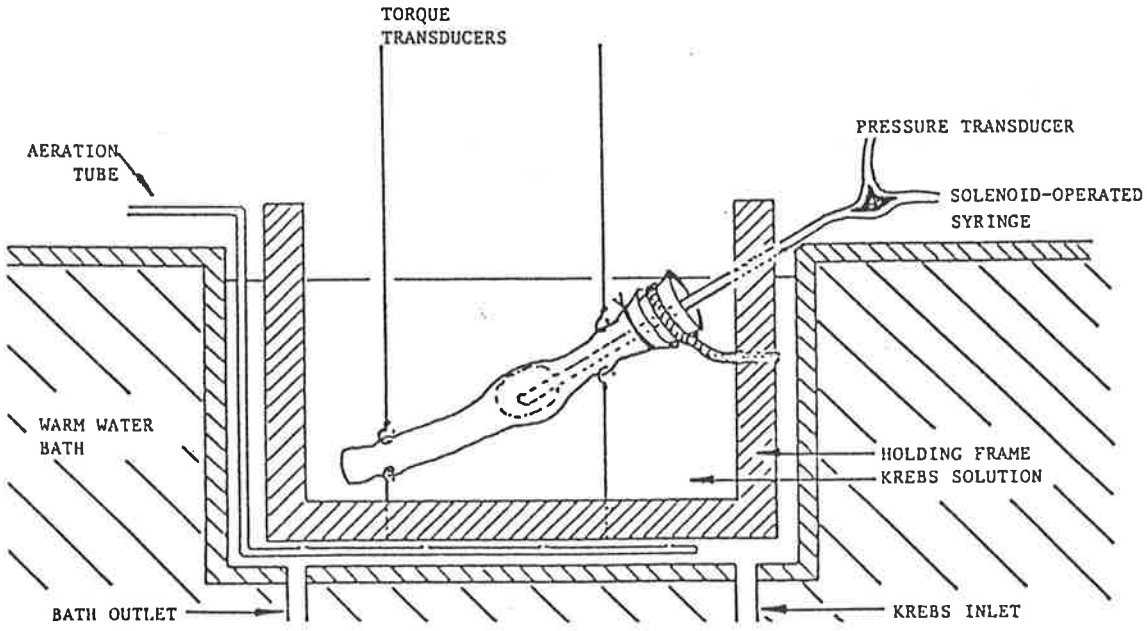


Fig. V
Balloon inflation distension stimulation

A

Preparation of Krebs solution (modified)

Chemical	g l^{-1}	Chemical	mM
NaCl	7.29	Na^+	151
KCl	0.34	K^+	4.6
$NaHCO_3$	2.09	Mg^{++}	0.6
$NaH_2PO_4.2H_2O$	0.20	Ca^{++}	2.8
$MgSO_4.7H_2O$	0.15	Cl^-	134.9
$C_6H_{12}O_6$	1.39	HCO_3	24.9
		SO_4	1.3
		H_2PO_4	0.6
		Glucose	7.7

Add 2.8 ml of 1 M $CaCl_2$ l^{-1} or 0.41g $CaCl_2$ l^{-1}

Table I

The averaged (n4) mean (± S.E.M.) degree of descending inhibition in response to weight stimulation of the whole colon section expressed as mV change from baseline (10 mV/cm)

Weight (g)	1	1.5	2	4	6
mV	10.4±1.6	13.6±.6	16±.6	25.6±.6	30.4±1.6

Weight (g)	10.5	15.5	20	30	40
mV	39±1.8	46.6±1.2	55.6±1.6	59.6±2.6	62±2.1

Table II

The averaged (n4) mean (± S.E.M.) degree of descending inhibition in response to weight point stimulation expressed as mV change from baseline (5 mV/cm)

Weight (g)	1	3	4	6	8
mV	0	4.8±1	5±1.3	7.5±1.1	7.3±.4

Weight (g)	10	15	18
mV	8.5±1.5	7±.9	6.5±2

Table III

The averaged mean (± S.E.M.) velocities of several continual artificial pellet chain passages through a colonic section (v = mm/s)

Chain passage	1	2	3	4	5	6
V = mm/s	.23±.01	.33±.06	.32±.06	.36±.08	.37±.08	.26
n	5	4	3	2	2	1

Table IV

A typical record of drug interference with peristaltic velocity of an artificial pellet chain. A 5 min pause was allowed between each passage (N = naloxone, W = wash)

Chain passage	1	2	3	4	5	6	7	8	9	10	11	12
V = mm/s	.24	.27	.32	.30	.31	.26	.28	.28	.1	.03	.18	.20
Drug						N $5x10^{-8}$		N $5x10^{-7}$			W	

Table V

Fogarty arterial embolectomy catheter volume/distension ratios.

Type: AE 3F (green) volume (ul)	dimensions (mm)
230	7 x 10
200	5.8 x 6.5
180	5.4 x 6.5

Type: AE 3F (red) volume (ul)	dimensions (mm)
750	9.8 x 14
580	9 x 12.5
430	8 x 11.5
290	8 x 10
145	6 x 8.5
100	5.8 x 8

Type: AE 3F (white) volume (ul)	dimensions (mm)
1500	11 x 18
1000	10 x 15
750	9.8 x 13
500	9 x 12
300	7.5 x 11
250	6.5 x 10

Type: AE 3F (blue) volume (ul)	dimensions (mm)
2000	13 x 18
1500	11.8 x 14
1000	10.5 x 12
500	8.5 x 10
300	7.5 x 10
200	6.5 x 9

Table VI

Measurements* of intestinal muscle layers from micrographs of freeze-stubstituted and chromium trioxide fixed colon sections.

distance mm	longitudinal muscle	circular muscle	muscularis mucosa	outside diameter
1	.035	.150	.040	4.50
2	.035	.230	.040	3.75
3	.030	.200	.040	3.50
4	.030	.050	.040	3.45
5	.030	.100	.040	3.20
6	.100	.130	.040	2.80
7	.090	.150	.050	2.40
8	.045	.120	.050	2.10
9	.060	.150	.030	2.30
10	.090	.270	.035	2.50
11	.045	.360	.035	2.30
12	.040	.290	.030	2.65
13	.035	.240	.035	3.10
14	.030	.220	.030	3.35
15	.030	.230	.035	4.00
16	.025	.110	.035	3.75
17	.100	.100	.030	3.45
18	.120	.100	.035	3.35
19	.080	.120	.030	3.55
20	.060	.110	.040	3.85

*all measurements in millimetres

Table VII

Averaged response of ATP and Carbachol to Cibacron-Blue and its components (n 16).

CIBA-GEIGY	Rfs.	.27	.36	.48	.77
ATP-Reduction	89 *	90	85	75	19
Carbachol-Reduction	32 *	44	60	75	+11
PIERCE	Rfs.	.24	.39	.45	.52
ATP-Reduction	94 *	29	99	77	95
Charbachol-Reduction	73 *	15	78	16	74
Polyscience	Rfs.	.26	.33	.48	.74
ATP-Reduction	94 *	33	70	63	3
Carbachol-Reduction	62 *	17	71	33	+2

* all numbers represent percent of response reduction.

Table VIII.

Varying ATP dose-responses (n12) after carbachol (3×10^{-8}M) application

Dosage *	Response % **	± S.E.M.
10^{-8}	3.9	3.4
2×10^{-8}	4.7	4.5
5×10^{-8}	10.6	10
10^{-7}	24.8	11
5×10^{-7}	43.8	11
10^{-6}	51.5	7
2×10^{-6}	62.5	11
3×10^{-6}	74.0	12
7.5×10^{-6}	80.7	10
10^{-5}	84.0	8

* dosage in molar/litre
** percent inhibition of the carbachol response

Table IX.

Varying ATP dose-responses (n12) after carbachol (10^{-8}M) application

Dosage	Response %	± S.E.M.
10^{-8}	1.9	3.6
2×10^{-8}	6.5	4.4
3×10^{-8}	19.8	7.7
4×10^{-8}	30.8	9.1
5×10^{-8}	53.1	29
10^{-7}	86.0	16.5

Table X.

ATP dose-response (n8) on the carbachol (3×10^{-8}M) effect and its reduction by PiF (5×10^{-5}M) (n12)

Control		
Dosage	Response %	± S.E.M.
5×10^{-8}	11	.7
3×10^{-7}	56.6	8.8
6×10^{-7}	69.8	10.7
3×10^{-6}	112	23.8

PiF (5×10^{-5}M)		
Dosage	Response %	± S.E.M.
5×10^{-8}	9.4	4.6
3×10^{-7}	15.3	4.2
3×10^{-6}	33.4	16.5
10^{-5}	57.3	9.4
2×10^{-5}	78.2	11.0
3×10^{-5}	94.6	7.3

Table XI.
Control ATP dose-response (n8) on the carbachol effect and its reduction (n12) by PiF (2 x 10^{-5}M)

Control		
Dosage	Response %	± S.E.M.
10^{-8}	12.5	7.5
10^{-7}	16	7.9
3 x 10^{-7}	51.6	19.9
10^{-6}	93.2	27
3 x 10^{-6}	99.5	20

PiF (2 x 10^{-5}M)		
Dosage	Response %	± S.E.M.
10^{-7}	8.2	5.4
3 x 10^{-7}	20.3	13.6
10^{-6}	54.9	19.9
3 x 10^{-6}	86.6	17.3

Table XII.
Control ATP dose-response (n12) on the carbachol effect and its reduction (n12) by PiF (10^{-5}M)

Control		
Dosage	Response %	± S.E.M.
10^{-7}	15.3	3.3
3 x 10^{-7}	44.4	8.8
10^{-6}	71.1	8.9

PiF (2 x 10^{-5}M)		
Dosage	Response %	± S.E.M.
10^{-7}	6.3	4.1
3 x 10^{-7}	15.8	4.7
10^{-6}	49.0	12.6

Table XIII.
Control ATP dose-response (n8) on the carbachol effect and its reduction (n12) by Pi .45 (5 x 10^{-5}M)

Control		
Dosage	Response %	± S.E.M.
10^{-7}	22.8	6.3
3 x 10^{-7}	37.8	10.9
10^{-6}	68.0	5.9

Pi .45 (5 x 10^{-5}M)		
Dosage	Response %	± S.E.M.
3 x 10^{-7}	5.5	4.2
10^{-6}	20.8	9.2
3 x 10^{-6}	34.5	8.7

Table XIV.
Control ATP dose-response (n8) on the carbachol effect and its reduction (n12) by Ci .45 (5 x 10^{-5}M)

Control		
dosage	response %	S.E.M.
3 x 10^{-8}	43.7	8.6
10^{-7}	53.4	9.2
3 x 10^{-7}	57.6	14.3
10^{-6}	78.2	5.8

Ci .45 (5 x 10^{-5}M)		
dosage	response %	S.E.M.
5 x 10^{-8}	14.9	5.4
3 x 10^{-7}	27.7	5.7
10^{-6}	52.6	11
3 x 10^{-6}	74.2	14.1

Table XV.

Control ATP dose-response (n12) on the carbachol effect and its reduction (n12) by Pi .50 (5×10^{-5}M)

Control		
Dosage	Response %	± S.E.M.
5×10^{-8}	22.3	19.1
10^{-7}	27.4	5.8
3×10^{-7}	51.8	7.6
10^{-6}	71.8	14
3×10^{-6}	88.1	16.1

Pi .50 (5×10^{-5}M)		
Dosage	Response %	± S.E.M.
5×10^{-8}	10	8.7
3×10^{-7}	16.7	7.6
10^{-6}	46.0	3.1
3×10^{-6}	69.3	10.7
10^{-5}	97.0	15.7

Table XVI.

Control ATP dose-response (n8) on the carbachol effect and its reduction (n8) by PiS (5×10^{-5}M), PiaF (10^{-5}M), PibF (10^{-5}M)

Control		
Dosage	Resp.%	± S.E.M.
3×10^{-8}	10.9	7.5
10^{-7}	42.6	7.4
3×10^{-7}	65.3	10.3
10^{-6}	84.1	4.1

	PiS(5×10^{-5}M)		PiaF(10^{-5}M)		PibF(10^{-5}M)	
Dosage	Resp.%	± SEM	Resp.%	± SEM	Resp.%	± SEM
3×10^{-8}	4.2	8.3	2	4	1.6	1.5
10^{-7}	31.6	12.2	18.6	13.3	7.5	6
3×10^{-7}	51.0	21.2	32.3	8.5	19.5	18
10^{-6}	78.9	5.9	65.6	12.0	35.6	7.9
4×10^{-6}	--	--	--	--	69.8	8.2

Table XVII.

Control ATP dose-response (n8) on the carbachol effect and its reduction (n8) by PicF (10^{-5}M)

Control		
Dosage	Response %	± S.E.M.
3×10^{-8}	3.3	3.6
10^{-7}	22.8	7.2
3×10^{-7}	60.5	16.9
10^{-6}	110.2	31
3×10^{-6}	117.0	37.5

PicF (10^{-5}M)		
Dosage	Response %	± S.E.M.
3×10^{-7}	7.7	2.9
10^{-6}	41.0	10.1
3×10^{-6}	90.3	23.0

Table XVIII.

Receptor binding reversibility: ATP (3×10^{-7}M) and PicF (10^{-5}M)

Time (min)	Response %	± S.E.M.
0	19.0	6.4
5	21.6	10.0
10	21.0	10.4
15	16.5	10.2
20	20.1	10.5
25	20.0	18.7
30	24.0	7.6
35	40.7	10.8
40	47.9	14.9

Table XIX.

Control ATP dose-response (n8) on the carbachol effect and its reduction (n12) by PicF (10^{-5}M) in the vas deferens

Control		
Dosage	Response %	± S.E.M.
3×10^{-7}	6.9	3.2
10^{-6}	19.5	8.7
3×10^{-6}	31.2	7.6
10^{-5}	62.7	17.8
3×10^{-5}	101.8	30.8

PicF (10^{-5}M)		
Dosage	Response %	± S.E.M.
10^{-5}	1.9	.6
3×10^{-5}	33	24
5×10^{-5}	78	12.4

Table XX.

Control ATP dose-response (n8) on the carbachol effect and its reduction (n8) by PicF (10^{-5}M) in taenia coli

Control		
Dosage	Response %	± S.E.M.
10^{-7}	34.3	19.2
3×10^{-7}	70.2	12.1
10^{-6}	101.5	14.6
3×10^{-6}	112.0	11.7

PicF (10^{-5}M)		
Dosage	Response %	± S.E.M.
10^{-7}	24.1	11.1
3×10^{-7}	60.7	9.2
10^{-6}	98.7	8.7
3×10^{-6}	108.2	11.4

Table XXIa

Dose-dependent responses (n 8) due to chemical stimulation of longitudinal muscle strips with Acetylcholine (ACH), Carbachol (Carba) and Prostaglandin E_2 (PGE_2).

colonic longitudinal muscle strip			
Dose mol/l	ACH mV* ± S.E.M.	Carba mV* ± S.E.M.	PGE_2 mV* ± S.E.M.
10^{-8}	0.2 ± 0.07	0.6 ± 0.14	0.77 ± 0.13
2×10^{-8}			0.92 ± 0.18
3×10^{-8}	0.6 ± 0.14		1.27 ± 0.37
5×10^{-8}	0.56 ± 0.07	0.8 ± 0.21	
8×10^{-8}	0.54 ± 0.15		
10^{-7}	0.75 ± 0.05	1.1 ± 0.14	1.4 ± 0.62
3×10^{-7}	1.1 ± 0.06		
5×10^{-7}		1.9 ± 0.12	1.0 ± 0.04
10^{-6}	1.1 ± 0.26	2.0 ± 0.16	0.3 ± 0.07
5×10^{-6}			
10^{-5}	1.4 ± 0.46		
3×10^{-5}	1.8 ± 0.67		
4×10^{-5}	2.25 ± 0.74		

* mean pen deflection from baseline (=0) at 2mV/cm, where 2mV=1g displacement force.

Table XXIb

Dose-dependent responses (n 8) due to chemical stimulation of circular colonic muscle strips with Acetylcholine (ACH), Carbachol (Carba) and Prostaglandin E_2 (PGE_2).

colonic circular muscle strip			
Dose mol/l	ACH mV* ± S.E.M.	Carba mV* ± S.E.M.	PGE_2 mV* ± S.E.M.
10^{-8}		0.42 ± 0.25	
2×10^{-8}	0		0.75 ± 0.07
3×10^{-8}			1.06 ± 0.15
5×10^{-8}		1.26 ± 0.34	1.21 ± 0.41
8×10^{-8}			
10^{-7}	0.03 ± 0.02	2.21 ± 0.65	1.1 ± 0.26
3×10^{-7}			
5×10^{-7}			0.8 ± 0.18
10^{-6}	0.13 ± 0.04		
5×10^{-6}	0.46 ± 0.17		
10^{-5}	1.35 ± 0.42		
3×10^{-5}	2.3 ± 0.61		
4×10^{-5}	2.6 ± 0.37		

* mean pen deflection from baseline (=0) at 2mV/cm, where 2mV=1g displacement force.

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