# THE NEUROMUSCULAR CONTROL OF HUMAN INTERNAL ANAL SPHINCTER SMOOTH MUSCLE: THE ROLE OF NITRIC OXIDE

by

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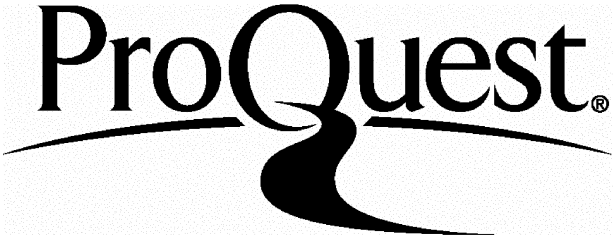

# *Abstract*

The internal anal sphincter is a smooth muscle collar which encircles the anal canal. It plays a crucial role in continence and augmenting its activity by pharmacological means could diminish faecal leakage in patients suffering from anorectal incontinence. Such advances however, are only likely if we understand its behaviour, particularly the mechanisms which sub serve contraction and relaxation. The experiments described here examine the role of nitric oxide, a novel neurotransmitter, in human internal anal sphincter smooth muscle relaxation.

Isometric tension recording of isolated strips of internal anal sphincter smooth muscle *in vitro*, reveals that nitric oxide is involved in neurogenic relaxation in this tissue because such relaxations are *(i)* mimicked by application of exogenous nitric oxide; *(ii)* inhibited by antagonists of nitric oxide synthase, the enzyme which catalyses the synthesis of nitric oxide and; *(iii)* inhibited by oxyhaemoglobin, which scavenges extracellular nitric oxide.

A neuronal source for nitric oxide within the human internal anal sphincter is suggested by the presence in this tissue of nerves which contain nitric oxide synthase. It is likely that these play a key role in anorectal function because their distribution and morphology is consistent with them mediating the rectoanal inhibitory reflex. They descend from cell bodies which lie within rectal myenteric ganglia and their appearance suggests that they are inhibitory motor neurones within the gut. Such nerves are absent in Hirschsprung's disease, a condition in which the reflex can not be demonstrated.

The importance of nitric oxide in human internal anal sphincter smooth muscle activity is further emphasised by the demonstration that *in vitro*, it mediates relaxation of this tissue in response to muscarinic cholinoceptor stimulation.

These results indicate that nitric oxide is an important mediator in the control of human internal anal sphincter smooth muscle.

# *Contents*

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# *Figure and table legends*

**Chapter 1**



**Chapter 2**

## Chapter 3

Figure 1.

The activity of pig internal anal sphincter smooth muscle strips when they are first set up for isometric tension recording is illustrated by this characteristic trace. A 1gm weight is attached to the tension transducer at point A. This produces an upward deflection. The weight is then removed and the muscle strip is attached- point B. The strip is stretched with a force equivalent to that provided by the 1gm weight. After a period of relative inactivity, the strip then contracts spontaneously (point C) producing further upward deflection of the trace. Eventually a steady state of tension is achieved (point D).

Figure 2.

The response of isolated strips of pig internal anal sphincter smooth muscle to electrical field stimulation are illustrated in the form of characteristic traces.

(*i.*) Each . represents an episode of electrical field stimulation using an impulse strength of 10V and a stimulus duration of 0.5ms. The frequency of stimulation increases from 0-30 Hz. Relaxant responses are produced (downward deflections of the trace). Atropine ($10^{-6}$M) and guanethidine ($3x10^{-6}$M) are present in the superfusate throughout.

(*ii.*) Repeatable relaxations are produced if electrical field stimulation (.= 10V, 0.5ms duration, 8Hz, for 1 sec) occurs every 180 seconds. These responses are abolished by tetrodotoxin ($3x10^{-6}$M). (Zero tension in this and subsequent figures is the level of tension remaining in the strip when it was superfused with calcium free solution at the end of the experiment. Superfusion with calcium free solution produces maximal relaxation in the smooth muscle strip.)

Figure 3.

This characteristic trace illustrates the effect of sodium nitroprusside on isolated strips of pig internal anal sphincter smooth muscle. Sodium nitroprusside was added to the superfusate for periods of 60 seconds and its concentration was increased from $10^{-7}$ M to $5x10^{-5}$M. Downward deflections of the trace are produced which represent relaxation.

Figure 4.

The concentration dependent effect of L-nitroarginine (NOARG) on nerve mediated relaxation of pig internal anal sphincter smooth muscle. Neurogenic relaxation occurs in response to electrical field stimulation (.= pulses of 10V, 0.5ms duration, 8Hz, for 1 sec) and the size of the relaxant response observed decreases as the concentration of NOARG in the superfusate increases. The action of NOARG is antagonised by the subsequent addition of L-arginine ($10^{-3}$ M) to the superfusate.

**Chapter 4**

Figure 1.

Two characteristic traces which illustrate the behaviour of smooth muscle strips from the internal anal sphincter and the conjoint longitudinal coat during the equilibration period at the start of experiments. At point A, both strips were stretched with a force equivalent to 1gm. Subsequently, the strip from the internal anal sphincter contracts (B- upward deflection) whereas that from the conjoint longitudinal coat relaxes (C- downward deflection)

Figure 2.

*i.* The response of internal anal sphincter smooth muscle to carbachol, illustrated in the form of a characteristic trace (above) and a cumulative dose response curve (below). Carbachol produced downward deflection of the trace indicating relaxation. In the cumulative dose response curve,

the degree of relaxation produced is calculated in terms of the residual tension left in the strips after application of the drug.

*ii.* The response of smooth muscle from the conjoint longitudinal coat to carbachol, illustrated in the form of a characteristic trace (above) and a cumulative dose response curve (below). Carbachol produced upward deflection of the trace indicating contraction.

*iii.* The response of rectal smooth muscle to carbachol. Both the circular and longitudinal muscle layers contracted in a dose dependent manner in response to this agent, as illustrated in this dose response curve.

Figure 3.

The response of the internal anal sphincter and conjoint longitudinal coat smooth muscle to noradrenaline (i.), and the response of the internal anal sphincter to isoprenaline (ii.).

Figure 4.

The response of rectal smooth muscle to adrenergic stimulation. (P/E- phenylephrine; Iso- isoprenaline)

Figure 5.

Analysis of the actions of carbachol and noradrenaline on isolated strips of human internal anal sphincter (*i.*), and conjoint longitudinal coat (*ii.*), illustrated in the form characteristic traces. Control responses to agonists are shown on the left, and the responses of the agonists in the presence of inhibitors is shown on the right.

Agonists: C- carbachol $10^{-4}$M NA- noradrenaline $5x10^{-5}$M

Antagonists: Atropine $10^{-6}$M (muscarinic cholinoceptors)

Hex- hexamethonium $5x10^{-6}$M (nicotinic cholinoceptors)

Phent- phentolamine $10^{-6}$M ($\alpha$-adrenoceptors)

Prop- propranolol $5x10^{-6}$M ($\beta$-adrenoceptors)

Figure 6

Characteristic responses of internal anal sphincter smooth muscle strips illustrating the effect of muscarinic and nicotinic cholinoceptor stimulation. *i.* Carbachol ($10^{-4}$) produces relaxation, a response which is abolished by atropine ($10^{-6}$M- a muscarinic cholinoceptor antagonist). *ii. and iv.* The response to carbachol is affected by neither hexamethonium ($5x10^{-6}$M- a nicotinic cholinoceptor antagonist) nor propranolol ($5x10^{-6}$M- a β-adrenoceptor antagonist). *iii. and v.* Relaxation produced by nicotine ($10^{-4}$), which stimulates nicotinic cholinoceptors, is abolished by both hexamethonium and propranolol at the same concentrations. Control responses to agonists are shown on the left and the corresponding response in the presence of an antagonist is shown on the right.

**Chapter 5**

Figure 1.

The response of isolated strips of internal anal sphincter smooth muscle to electrical field stimulation is illustrated in the form of a characteristic trace *(i.)* and a frequency response curve *(ii.)*. At each ., impulses of 10V are applied for 0.5ms duration, over a range of frequencies.

Figure 2.

*i.* A characteristic trace which illustrates the response of a muscle strip to cholinergic and adrenergic agonists, and to repetitive electrical field stimulation (.= 10V, 0.5ms duration, 10Hz, for 1 sec). In the presence of muscarinic (atropine), α-adrenergic (phentolamine) and β-adrenergic (propranolol) receptor antagonists, the response to electrical field stimulation is unchanged but, the action of the agonists is abolished.

*ii.* The frequency response curve of internal anal sphincter smooth muscle strips in the presence and absence of atropine and guanethidine, which inhibit adrenergic and cholinergic neurotransmission.

Figure 3.

A characteristic trace to illustrate the neurogenic nature of the response of internal anal sphincter smooth muscle to electrical field stimulation (.= 10V, 0.5ms duration, 10Hz, for 1 sec) . The response is abolished following addition of tetrodotoxin to the superfusate.

Figure 4.

The effect of sodium nitroprusside shown in the form of a characteristic trace and a cumulative dose response curve.

Figure 5.

The effect of N-monomethyl-L-arginine (L-NMMA) and N-nitro-L-arginine (L-NOARG, which are both inhibitors of nitric oxide synthase, on nerve mediated relaxation of internal anal sphincter smooth muscle induced by electrical field stimulation (10V, 0.5ms duration, 10Hz, for 1 sec).

Figure 6.

Characteristic traces illustrating the effects of *(i.)* N-monomethyl-L-arginine (L-NMMA) and *(ii.)* N-monomethyl-D-arginine (D-NMMA) on nerve mediated relaxation of human internal anal sphincter smooth muscle (.= 10V, 0.5ms duration, 10Hz, for 1 sec). The reversing action of L-arginine (L-arg $5x10^{-4}$ M), but not D-arginine (D-arg $5x10^{-4}$ M) is evident.

Figure 7.

A characteristic trace which illustrates the effect of $10^{-5}$M N-nitro-L-arginine (L-NOARG) on nerve mediated relaxation of internal anal sphincter smooth muscle (.= 10V, 0.5ms duration, 10Hz, for 1 sec). In the presence of L-NOARG, neurogenic relaxation disappears. Its action is antagonised by L-arginine (L-arg $5x10^{-4}$ M), but not D-arginine (D-arg $5x10^{-4}$ M).

Figure 8.

*(i.)*. Oxyhaemoglobin inhibits nerve mediated relaxation of human internal anal smooth muscle in a concentration dependent manner. *(ii.)* A characteristic trace which illustrates that neurogenic relaxation (.= 10V, 0.5ms duration, 10Hz, for 1 sec) is reversibly abolished by oxyhaemoglobin (oxy) at a concentration of $5x10^{-5}$M Methaemoglobin (met) has no such action at the same concentration.

**Chapter 6**

Figure 1.

A cryostat section through rectal myenteric ganglia (a) stained first for nitric oxide synthase immunoreactivity (top) and then for NADPH diaphorase activity (bottom). It is seen that the same population of enteric neurones (b) stains positive for the two enzyme activities. Positively stained nerve fibres are also seen in the circular smooth muscle layer here (c). (bar=100μm)

Figure 2.

A cryostat section of a rectal myenteric ganglion showing nerve cell bodies stained for NADPH diaphorase activity (blue; a) and SMI 311/312 (pan-axonal/pan-neuronal) immunoreactivity: brown; b). It is apparent that nitric oxide synthase containing neurones form a sub-population of the total number of enteric neurones present. (Circular muscle layer; c: bar=100μm)

Figure 3.

A whole-mount of the rectal myenteric plexus stained using NADPH diaphorase histochemistry. This illustrates the appearance of individual nitric oxide synthase containing neurones. These have a single axonal process (a) and broad dendrites (b). This is typical of Dogiel type 1 enteric neurone morphology. (bar=25μm)

Figure 4.

*i.* The appearance of the myenteric plexus in the proximal two-thirds of the rectum as stained by NADPH diaphorase histochemistry. Nitric oxide synthase containing neurones aggregate together in ganglia (a) which are joined by internodal strands (b) to form a meshwork (primary plexus) which does not have apparent polarity. A few nerve cell bodies lie outside the confines of the primary plexus (c). (Cranial: top; bar=1mm)

*ii.* An individual ganglion from the proximal two-thirds of the rectum stained by NADPH diaphorase histochemistry. Note that individual nerve cell bodies are scattered throughout the structure. (bar= 100μm)

Figure 5.

*i.* The appearance of the distal rectal myenteric plexus stained by NADPH diaphorase histochemistry. Nitric oxide synthase containing neurones are again clustered in ganglia (a). Some of their axons traverse shunt fascicles (b) to descend to the anal canal. Note the cranio-caudal polarity here. (Cranial: top; bar=1mm)

*ii.* A ganglion in the distal rectal myenteric plexus stained by NADPH diaphorase histochemistry. Note that nitric oxide synthase containing neurones (a) are densely packed together with little room for other neurone types. (bar=100μm)

Figure 6.

The submucosal plexus in the proximal rectum stained by NADPH diaphorase histochemistry. Nitric oxide synthase containing neurones (a) are present but they are sparse. (Cranial: top; bar=1mm))

Figure 7.

The appearance of the myenteric plexus in the upper anal canal stained by NADPH diaphorase histochemistry. The plexus is seen here passing caudally just outside the internal anal sphincter at the junction of its

proximal and middle thirds. There are few ganglia here and those that are present are densely packed with nitric oxide synthase containing nerve cell bodies (a). Stained nerves disappear as they branch and ramify into the internal anal sphincter itself (b). (Cranial: top; bar= 1mm)

Figure 8.

Cryostat sections of the distal internal anal sphincter stained by NADPH diaphorase histochemistry, with a light neutral red counter stain;

(i) nitric oxide synthase containing nerve trunk (a; bar=100μm)

(ii) nitric oxide synthase containing axon (b). This is seen passing between smooth muscle cells whose nuclei are stained red (c). Note the distribution of stain here which gives the nerve a beaded appearance. (bar=10μm)

**Chapter 7**

Figure 1

*(i)* A cryostat section of normally ganglionated sigmoid colon in Hirschsprung's disease stained to reveal NADPH diaphorase activity and hence the presence of nitric oxide synthase. Positively stained nerve cell bodies (blue; a) are seen in the myenteric plexus and their processes (b) traverse the muscularis externa (circular muscle-c; longitudinal muscle-d). (Counter stain: haematoxylin; bar=100μm)

*(ii)* A cryostat section of normally ganglionated sigmoid colon in Hirschsprung's disease stained to reveal NADPH diaphorase activity and hence the presence of nitric oxide synthase. Positively stained nerve processes are seen in the circular muscle layer (blue; a) while nerve cell bodies (b) are present along its submucosal border. Some very fine nerves extend from the submucous plexus into the mucosa but no other structures are stained positive. (Counter stain: haematoxylin; bar=100μm)

Figure 2.

Cryostat sections of the junction between the circular (a) and longitudinal muscle layers (b) of the ganglionated and aganglionic segments in Hirschsprung's disease stained to reveal NADPH diaphorase activity and hence the presence of nitric oxide synthase. Note the presence of the positive staining (blue) in the former and its absence in the latter. (Counter stain: haematoxylin; bar=100μm)

Figure 3.

Adjacent cryostat sections of a nerve trunk in the aganglionic segment of gut in Hirschsprung's disease, stained for;
(i) SMI 311/312 (pan-axonal/pan-neuronal) immunoreactivity (brown) and, (ii) NADPH diaphorase activity. Note the absence of positive staining (blue). (Counter stain: neutral red; bar=100μm)

Figure 4.

*(i)* A whole mount of the myenteric plexus in the normally ganglionated colon of a child suffering from Hirschsprung's disease, stained to reveal NADPH diaphorase activity and hence the presence of nitric oxide synthase. The plexus here lacks polarity and consists of ganglia (a) joined by internodal strands (b). (Cranial: top; bar=100μm)
*(ii)* A whole mount of the myenteric plexus in the normally ganglionated segment of gut in Hirschsprung's disease stained to reveal NADPH diaphorase activity and hence the presence of nitric oxide synthase. The appearance of individual positively stained neurones is shown. These have several dendrites (a) and a single axon (b). This is typical of Dogiel type 1 enteric neurones. (bar= 50μm)

Figure 5.

A sequence of whole mounts of the transition zone in Hirschsprung's disease, stained to reveal NADPH diaphorase activity and hence the presence of nitric oxide synthase.

*(i)* Initially, ganglia (a) and internodal strands (b) become aligned linearly along the cranio-caudal axis of the colon. (Cranial: top; bar= 100μm)

*(ii)* Further distally in the transition zone, discrete ganglia disappear and positively stained neurones (a) lie in series along nerve trunks (b). (Cranial: top; bar= 100μm)

*(iii)* Further distal still, positively stained nerve cell bodies are absent. Stained axons (a) can be traced distally for a short distance but these too disappear. (Cranial: top; bar= 100μm)

**Chapter 8**

Figure 1

*i.* The effect of N-nitro-L-arginine (NOARG) and L-arginine on neurogenic relaxation (.= 10V, 0.5ms. duration, 10Hz, for 1 sec) and the response to carbachol (c: $10^{-4}$M). The effect of N-nitro-L-arginine is antagonised by L-arginine (L-arg). Test strips were run in parallel with control strips which were not incubated with the antagonist.

*ii.* The effect of oxyhaemoglobin (Oxy : $5x10^{-5}$M) on the response to carbachol (c: $10^{-4}$M) and electrical field stimulation (.= 10V, 0.5ms.duration, 10Hz, for 1 sec).

*iii.* The effect of oxyhaemoglobin (Oxy: $5x10^{-5}$M) and N-nitro-L-arginine (L-NOARG: $10^{-5}$M) on the dose dependent response of internal anal sphincter smooth muscle to carbachol.

Figure 2.

The effect of *i.* tetrodotoxin ($3x10^{-6}$M) and *ii.* scorpion venom (20μm/ml) on the response of internal anal sphincter smooth muscle to carbachol $10^{-4}$M (c). At this concentration, both neurotoxins abolish electrical field stimulated relaxation (.= 10V, 0.5ms.duration, 10Hz, for 1 sec), but the effect of carbachol is unchanged.

Figure 3.

The effect of putative antagonists on the response of internal anal sphincter smooth muscle to carbachol ($10^{-4}$M). This figure includes data from chapter 4.

Figure 4.

The initial contraction seen in muscle strips in response to carbachol, before and after administration of N-nitro-L-arginine (L-NOARG 10-5M).

Figure 5.

A possible arrangement of muscarinic receptors which would explain the responses of internal anal sphincter smooth muscle to carbachol.

# *Foreword*

Of the functional disorders that affect the anal canal, anorectal incontinence is perhaps the most distressing. It is not only unpleasant and embarrassing for the suffer, but it can also have profound social consequences due to its perceived association with uncleanness. Indeed, any feeling of hopelessness may be compounded by current medical management because the outcome of treatment is often unpredictable and can be disappointing. One reason for this is that our understanding of normal continence and the neuromuscular mechanisms which subserve it is far from complete. In the past, attention has primarily focused on the striated muscles which contribute towards continence and significant advances in research into the pathophysiology of incontinence and its treatment have been made. However, this approach has left many issues unresolved and surgery directed at repairing lax muscles has not proved to be a universal panacea.

Recently, there is a growing appreciation of the important contribution that the smooth muscle components of the anal canal, particularly the internal anal sphincter, make towards continence. It is clear that the internal anal sphincter is abnormal in many incontinent patients although the cause of this is often obscure.

Therapeutic modulation of internal anal sphincter function is an attractive proposition. Before this is possible however, we must understand how it works. We must determine the processes which normally produce contraction and those which mediate relaxation. The research described in this thesis is directed principally at determining the neuromuscular mechanisms which subserve the latter.

*Chapter 1*

# GENERAL INTRODUCTION

# ANORECTAL INCONTINENCE AND THE ROLE OF THE INTERNAL ANAL SPHINCTER

## 1. Definition

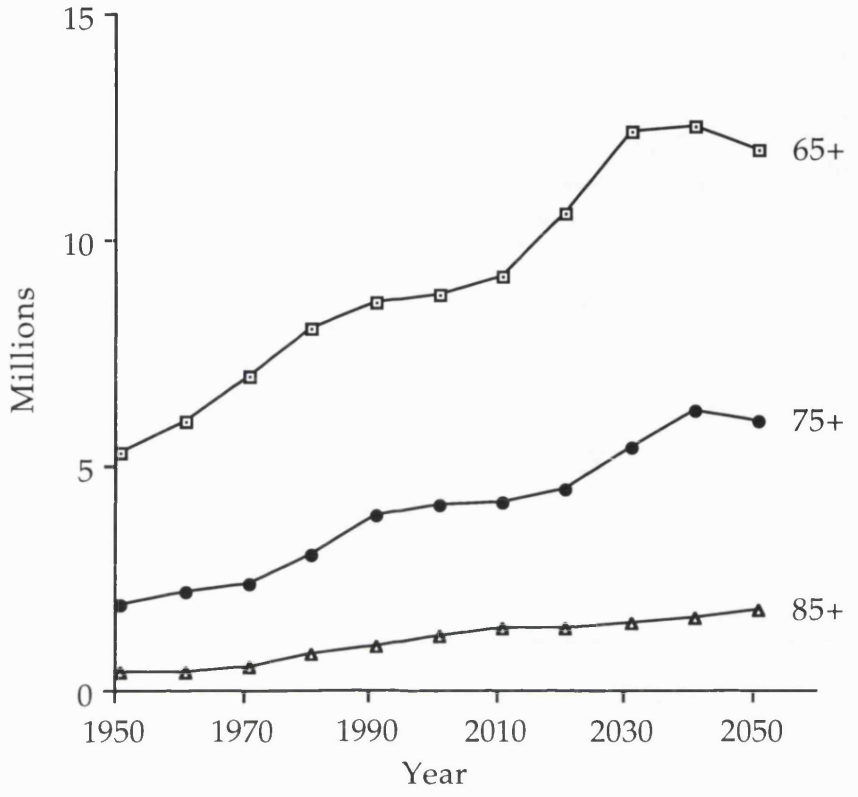
Anorectal incontinence can defined as the inability to prevent inadvertent discharge from the anal canal. Both the type (flatus, fluid or solid) and the amount of leakage can vary but, even small amounts can cause embarrassment and social disability.

## 2. Epidemiology

Such is the shame experienced by patients with anorectal incontinence that many deny their symptoms. Indeed anorectal incontinence was called the 'unvoiced symptom' by Leigh and Turnberg (1982) who found that 39 out of 76 patients referred for investigation of diarrhoea were, in reality, incontinent of faeces and fewer than half (19) volunteered the information spontaneously. The best estimate of the community prevalence of anorectal incontinence is derived from the results of an anonymous postal survey carried out by Thomas *et al.* (1984). This suggests that prevalence increases with age such that 0.4% of men and 0.2% women aged 15-64 years are incontinent of faeces whereas, 1.1% of men and 1.3% of women over 65 years are affected. In some groups, notably long stay geriatric and psychogeriatric patients, the prevalence is even higher with up to 25% leaking faeces each day (Rands and Malone-Lee, 1991). As our population becomes progressively elderly, as it is expected to (see figure 1.), so numerically, anorectal incontinence will become an even greater problem than it is at present, adding to the burden of suffering in society and imposing huge, additional demands on social and medical services.

Figure 1.

The elderly population of the United Kingdom: past, present and future (source: OPCS 1989 PP2 No. 17).

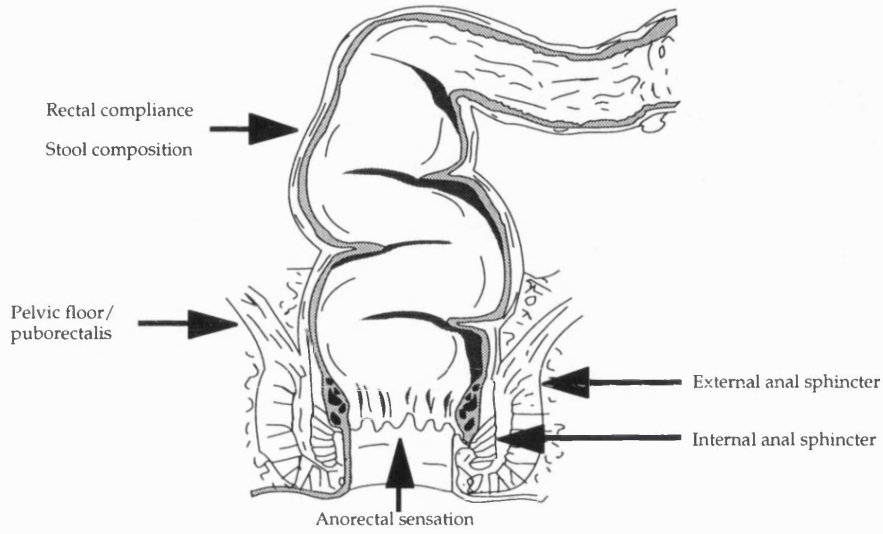



## 3. Mechanism of normal anorectal continence

As illustrated in figure 2, anorectal continence is determined by a number of factors which include the anal sphincter muscles, anorectal sensitivity, rectal compliance and stool composition. Of these, the anal sphincter muscles are of prime importance and together they generate a high pressure zone which opposes the passage of gastrointestinal contents. Loss of this invariably results in incontinence.

Figure 2.

A diagram of the human anorectum indicating the factors which play an important role in anorectal continence.




## The anal sphincter muscles

### *The external anal sphincter*

*Morphology* This is a complex of striated muscles which is effectively inseparable above from puborectalis. The external anal sphincter itself is most commonly described in three parts which all encircle the internal anal sphincter; *subcutaneous, superficial* and *deep* (Warwick and Williams, 1973). The *subcutaneous* part is a flat band which surrounds the lowermost extremity of the anal canal and passes circumferentially around the distal border of the internal anal sphincter. Cranial to this lies the *superficial* component which is attached posteriorly to the coccyx and anteriorly to the perineal body. The *deep* part of the sphincter constitutes a thicker band of muscle around the cranial portion of the internal anal sphincter and is effectively inseparable from puborectalis.

Some authors have dispensed with the tri-laminar classification and instead describe two components only; 'superficial', which combines *subcutaneous* and *superficial* (above), and 'deep' which amalgamates *deep* (above) with puborectalis (Lawson, 1974). Goligher (1984) suggests that such divisions are artificial and instead the muscle should be considered as one continuous sheet.

Puborectalis arises from the posterior aspect of the pubis and passes infero-posteriorly around the anorectal junction, pulling it forward and upward, so generating acute angulation between the rectum above and the anal canal below. Lawson (1974) divided puborectalis into two parts; that which is intimately associated with the anorectal junction, and that which passes behind it in close contact with levator ani

*Innervation* The external anal sphincter and puborectalis are innervated by nerves whose cell bodies are situated in Onuf's nucleus in the ventral horn of the second and third sacral segments of the spinal cord (Schroder, 1981). Motor efferents to the muscles pass in the pudendal nerve, but those fibres of puborectalis which are not attached to the external anal sphincter may be innervated by direct branches from the sacral plexus (Lawson, 1974).

*Function* Physiologically, the external anal sphincter and puborectalis appear to function together, although it is the former which has attracted most research attention. The external anal sphincter does exhibit activity at 'rest' but contributes only 15% to anal canal pressure in this state (Frenckner and von Euler, 1975). Its activity is recruited when intra-abdominal pressure rises, for instance during coughing or lifting weights and this response is mediated via a spinal reflex as it is unaffected by spinal cord transection above the third lumbar segment (Frenckner, 1975). In addition, the external anal sphincter contracts in response to rectal distension (Gaston, 1948, Schuster et al, 1965). This also

appears to be a spinal reflex because it is unaffected by cord transection (Frenckner, 1975) but it is abolished by pudendal nerve blockade (Frenckner and von Euler, 1975). The exact neural pathway involved however, is unknown.

The behaviour of the external anal sphincter and puborectalis during defaecation has also not been fully established. The most plausible evidence is derived from electrophysiological experiments which suggests that upon straining at stool there is a momentary burst of electromyographic activity in the sphincter, followed by complete inhibition of tonic activity (Parks et al., 1962; Kerremans, 1969; Ihre, 1974) Other patterns have also been recorded, such as increased electrical activity throughout (Kerremans, 1969) but these may be artefactual and reflect the abnormal environment in which such studies are performed.

Finally, the external anal sphincter relaxes in response to anal canal distension but contracts once the distending force disappears. This has been called the 'closing reflex' (Porter, 1962).

*The internal anal sphincter*

*Morphology* The internal anal sphincter is the thickened terminal part of the circular smooth muscle coat of the gut. It is composed of elliptical fascicles of smooth muscle fibres ensheathed by connective tissue and surrounds the anal canal for up to 4cm, stopping short of the anal verge (Goligher, 1984). In the cranial portion of the sphincter, fascicles are aligned obliquely and pass downwards towards the lumen. Caudally, they are more horizontal and the lower most fascicles may be inclined upwards (Goligher, 1984). At the distal margin of the sphincter lies the subcutaneous part of the external anal sphincter. It has been suggested that the internal anal sphincter can be divided in to two parts by a fibromuscular septum which traverses the junction between the oblique and horizontal fascicles (Lawson 1974). However, I have not noted this

and agree with most other authors that the internal anal sphincter is essentially homogeneous (Wood and Kelly, 1992).

*Innervation* The internal anal sphincter, like other gut smooth muscles, is innervated by both *extrinsic* and *intrinsic* nerves. Its extrinsic innervation is derived from the sympathetic and parasympathetic divisions of the autonomic nervous system. Sympathetic fibres pass to the sphincter via the hypogastric and presacral nerves from the fifth lumbar segment and parasympathetic fibres reach the sphincter via pelvic nerves from the first, second and third sacral segments. In addition, the sphincter also receives *intrinsic* autonomic nerves from the enteric nervous system.

To appreciate the influence these neural elements have on sphincter function, it is important first to consider the inherent behaviour of the sphincter itself. Unlike striated muscle which forms the external anal sphincter, smooth muscle in the internal anal sphincter can generate spontaneous myogenic tone and is not therefore dependent upon neural stimulation for its activity. This has been most thoroughly investigated in lesser mammals but available evidence suggests the same is also true in humans. Culver and Rattan (1986) studied the effect of tetrodotoxin on internal anal sphincter activity *in vivo* in anaesthetised opossums whose striated muscle was paralysed by pancuronium. In these animals, anal canal pressure reflected internal anal sphincter smooth muscle contraction and this was unaffected by tetrodotoxin, a neurotoxin, administered at a dose sufficient to abolish visceral neural activity. Such experiments are not possible in man but study of isolated smooth muscle strips from the human internal anal sphincter *in vitro* suggests that these too contract spontaneously and that the resulting tension is also unaffected by tetrodotoxin (Burleigh *et al.*, 1979). In the presence of this spontaneous myogenic contraction, extrinsic and

intrinsic neurones serve to modulate sphincter activity but in man, the true nature of these influences is not fully understood.

Stimulation of sympathetic fibres running in the presacral nerves in patients during operations has been shown to both increase (Rankin and Learmonth, 1930, Carlstedt *et al.*, 1988) and decrease internal anal sphincter tone (Shepherd and Wright, 1968; Lubowski *et al.*, 1987; Horgan *et al.*, 1989). The precise explanation for this discrepancy is unclear but may reflect differences in the frequency of stimulation used and the influence of background sympathetic activity in each case. An excitatory role for sympathetic nerves is supported by the action of noradrenaline on sphincter tissue *in vitro* and by animal studies. Noradrenaline, the principal neurotransmitter released by postganglionic sympathetic nerves, has been demonstrated to contract human internal anal sphincter smooth muscle *in vitro* through activation of α-adrenoceptors (Parks *et al.*, 1969; Friedmann, 1968; Burleigh *et al.*, 1979; Speakman *et al.*, 1990). This effect has also been demonstrated in the vervet monkey where, in addition, hypogastric nerve stimulation *in vivo* resulted in sphincter contraction (Rayner, 1979). Similar responses have been recorded in the cat (Garrett *et al.*, 1974; Bouvier and Gonella, 1981).

An excitatory role for sympathetic nerves has also been inferred by studies of sympathetic 'blockade' in man *in vivo*. Infusion of phentolamine, an α-adrenoceptor antagonist, has been shown to produce a significant fall in internal anal sphincter tone (Gutierrez and Shah, 1975), as has blockade of lumbar sympathetic outflow by high (T6-T12) spinal anaesthesia (Frenckner and Ihre, 1976).

The role of the parasympathetic innervation is similarly confusing. Stimulation of muscarinic cholinoceptors by application of cholinergic agonists *in vitro*

(Burleigh *et al*, 1979; Speakman *et al., 1992*) and *in vivo* (Gutierrez and Shah, 1975) causes sphincter smooth muscle relaxation but studies involving manipulation of parasympathetic nerves *in vivo* either show them to have no effect on sphincter function (Frenckner and Ihre, 1976) or an excitatory action (Meunier and Mollard, 1977).

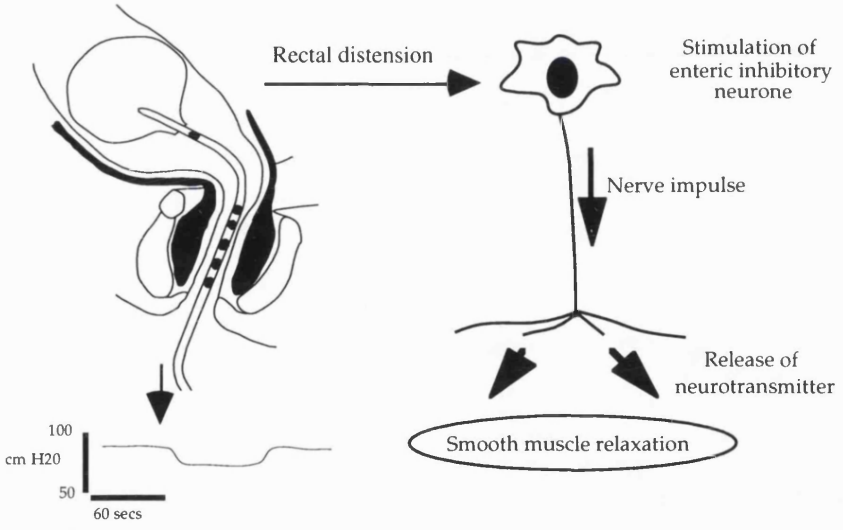
The principal action of enteric nerves on the activity of the sphincter is relaxation, mediated through release of a non-adrenergic, non-cholinergic (NANC) neurotransmitter. The role of excitatory enteric nerves in the sphincter is unknown.

*Function* Internal anal sphincter contraction is principally responsible for high pressure in the anal canal at rest (Duthie and Watts, 1965). In lesser mammals it is clear that this can result from spontaneous myogenic activity alone (Culver and Rattan, 1986) but in man some evidence suggests that intrinsic contraction is augmented by excitatory input from sympathetic nerves. Gutierrez and Shah (1975) noted a reduction in anal canal pressure of approximately 50% following infusion of phentolamine into volunteers and Frenckner and Ihre (1976) recorded a similar fall in those undergoing high spinal anaesthesia. Other data suggests that sympathetic nerves play a less important role. Carlstedt *et al.*, (1988) found the reduction in anal canal pressure following thoraco-lumbar epidural anaesthesia to be only 28% and Lubowski *et al.*, ( 1987) found that local anaesthetic blockade of presacral nerves did not have any effect on resting anal canal pressure.

A fundamental feature of internal anal sphincter function is relaxation in response to rectal distension. This phenomenon is mediated by enteric inhibitory motor neurones which lie wholly within the wall of the gut and it is called the rectoanal inhibitory reflex (Gowers, 1877; Denny-Brown and

Robertson, 1935; Lubowski *et al.*, 1987; Kamm *et al.*, 1989). The reflex facilitates defaecation and in a modified form it equalises rectal and upper anal canal pressure thus allowing sensory nerve endings in the upper anal canal to 'sample' the contents of the rectum (Duthie and Bennett, 1963; Miller et al, 1988). Sampling can be associated with a small increase in intra-rectal pressure (Miller *et al*, 1988, Farouk *et al.*, 1993). Recruitment of external anal sphincter activity coincides with internal anal sphincter relaxation (Sun et al., 1990; Farouk et al., 1993).

Figure 3.
The rectoanal inhibitory reflex




*The conjoint longitudinal coat*

*Morphology* The anatomy of the conjoint longitudinal coat has been the subject of a number of different interpretations. Recently, these have been comprehensively reviewed (Lunniss and Phillips, 1992). The description given here is that provided by Goligher (1984) and this concurs with my own observations made during dissections of the anal canal for these investigations.

The conjoint longitudinal coat is the most caudal extension of the longitudinal smooth muscle layer of the gut. At the level of the pelvic floor it is joined by some (scant) striated muscle fibres from levator ani (pubococcygeus) and then traverses the anal canal in the intersphincteric plane between the internal and external anal sphincters. As it descends it becomes progressively fibroelastic and gives off several septa which ramify both the internal and external anal sphincters, to gain attachment to the dermis of the anal and circumanal skin. In addition, other septa pass outward, away from the anal canal, to blend with the connective tissue of the ischiorectal fossa and peri-anal space.

*Innervation* The innervation of the conjoint longitudinal coat is unknown but, like other gastrointestinal smooth muscles it presumably receives nerves from the enteric, sympathetic and parasympathetic nervous systems. The influence these have on the mechanical activity of this muscle has not been determined.

*Function* The function of the conjoint longitudinal coat is also uncertain. It is suggested that it helps to maintain the structural integrity of the anal canal throughout the defaecation cycle, and contracts during the passage of faeces to shorten the anal canal and evert the anal margin (Courtney, 1950; Shafik, 1976). However, it has also been suggested to play a role in the pathogenesis of haemorrhoids and in the containment of peri-anal sepsis (Haas and Fox, 1977).

## Anorectal sensitivity

### *Rectal sensation*

Distension of the rectum is associated with a sensation of rectal fullness and a desire to defaecate (Goligher and Hughes, 1951). The receptors which detect

distension do not appear to reside within the rectum itself as the sensation is maintained after rectal resection and colo-anal anastomosis (Lane and Parks, 1977). It is thought instead that they lie within the pelvic floor musculature (Lane and Parks, 1977).

*Anal sensation*

Touch, pain, change in temperature and movement are all perceived within the anal canal and their perception is maximal in the anal transition zone, where rectal columnar epithelium (above) changes to anal squamous epithelium (below) (Duthie and Gairns, 1960). There is a high concentration of sensory nerve endings here (Duthie and Gairns, 1960) which are exposed to the contents of the rectum when anal and rectal pressures equalise (Duthie and Bennett, 1963). This occurs subconsciously several times each hour as a normal physiological process and results from transient relaxations of the internal anal sphincter (see above-Miller *et al.*, 1988). It is called 'anorectal sampling' and is believed to be pivotal in allowing discrimination between faeces, fluid and flatus.

Rectal compliance

Like the stomach and the urinary bladder, the rectum undergoes receptive relaxation. This means that it can accommodate large volumes of faeces, fluid or flatus without an associated rise in intraluminal pressure. Loss of this property in inflammatory conditions such as Crohn's disease (Buchman *et al.*, 1980) and irradiation proctitis (Varma *et al.*, 1985) leads to frequency and urgency of defaecation.

Stool composition

Personal experience suggests that, even if the motor and sensory components of the anal canal function normally, continence can still be threatened by the arrival of copious amounts of fluid stool. Thus anorectal function is dependent to an extent upon the normal digestive and absorptive capacity of the more proximal gastrointestinal tract.

Other factors

*The anal cushions*

Gibbons *et al.* (1986) and Lestar *et al.* (1992) have suggested that the anal sphincters might not occlude the anal canal completely and that fine tuning of closure could result from the complementary effects of the anal cushions. Given the dynamic properties of the cushions, this seems very reasonable.

*The flap valve*

Parks (1975) proposed that acute anorectal angulation, produced by the forward pull of puborectalis, was essential for normal continence as it allowed the mucosa of the anterior rectal wall to lie across the lumen of the anal canal so occluding it. For this theory to have credence, increases in intra-abdominal pressure should enhance the occlusive effect of the anterior rectal mucosa and for occlusion to occur, pressure in the rectum should exceed that in the anal canal. In fact, these prerequisites are not fulfilled. Bartolo *et al.* (1986) imaged the rectum radiologically during Valsalva manoeuvres and at the same time recorded both anorectal pressures and electromyographic activity in the external anal sphincter and pelvic floor. Contact between the anterior rectal wall and the anal canal was not seen and anal canal pressure consistently exceeded rectal pressure. Increases in intra-abdominal pressure were associated with increased

activity in the external anal sphincter and pelvic floor. These results are supported by the findings of a manometric study (Bannister *et al.*, 1987) and suggest strongly that a flap valve does not contribute to continence.

## 4. The causes of anorectal incontinence

Anorectal incontinence can occur when any facet of the normal continence mechanism is defective. The causes of such abnormalities are outlined in Table 1. In some cases internal anal sphincter dysfunction plays an important role in the pathophysiology of faecal leakage and these are described detail.

Table 1.

The causes of anorectal incontinence.

*Incontinence with normal sphincters*

- Severe diarrhoea
- Inflammatory bowel disease
- Faecal impaction
- Fistula
- Dementia or mental retardation
- Anorectal carcinoma

*Incontinence with abnormal sphincters*

- Direct sphincter injury
  - surgical
  - trauma
  - obstetric
- Sphincter neuropathy
  - upper motor neurone lesions
    - cerebral- tumour, stroke, trauma
    - spinal- demyelination, tumours
  - lower motor neurone lesions
    - idiopathic
    - cauda equina lesions
    - pelvic tumours
    - demyelination
    - diabetes
- Congenital abnormalities
- Rectal prolapse
- Ageing

Idiopathic anorectal incontinence

This is the most common cause of primary anorectal incontinence seen in adult surgical practice and it particularly affects middle aged women. One of the main features of this condition is weakness of the striated muscles of the sphincter apparatus and the pelvic floor. Histological (Parks *et al.*, 1977; Beesierk *et al.*, 1979) and electrophysiological (Neill and Swash, 1980; Bartolo *et al.*, 1983; Kiff and Swash, 1984) evidence suggests that muscle weakness is a consequence of denervation and subsequent reinervation and it is thought this is caused by chronic stretch injury to the pelvic and pudendal nerves.

In most instances the primary neuronal insult (stretch or compression) is thought to occur during childbirth, particularly when the second stage of labour is prolonged, if delivery is assisted by forceps or if the birth weight is high (Snooks *et al.*, 1984; Snooks *et al.*, 1985). It is believed that nerve injury results in weakness of the pelvic floor muscles with the consequence that they are unable to withstand rises in intra-abdominal pressure without bulging caudally. Such displacement then leads to stretching of the pudendal nerves which are particularly vulnerable to this form of insult as they leave Alcock's canal. Over time, stretch-injury produces denervation and a viscious cycle is established producing more muscle weakness, greater stretch injury and so progressive denervation. This can eventually culminate in incontinence.

It is clear that in idiopathic anorectal incontinence, faecal leakage occurs in the presence of pelvic floor and external anal sphincter weakness. Indeed, one might expect a direct relationship between the two but, this does not in fact seem to be the case. In an elegant study Womack *et al.* (1986) demonstrated that neurogenic pelvic floor weakness, as measured by perineal descent, can be present to a greater degree in normally continent individuals when compared

with those who are incontinent. Womack *et al.* (1986) also noted that external anal sphincter weakness is not predictive of incontinence as this can be present to a similar degree in continent and incontinent individuals. Other factors must therefore be involved and those which have been implicated include internal anal sphincter weakness, abnormal anorectal sampling, diminished rectal compliance (Womack *et al.*, 1986) and impaired anorectal sensation (Roe *et al.*, 1986; Miller *et al.*, 1987, Miller *et al.*, 1988).

Internal anal sphincter weakness is a common finding in idiopathic anorectal incontinence, affecting one quarter to two thirds of sufferers (Neill *et al.*, 1981; Sun *et al.*, 1989) but its cause is unknown. Morphological abnormalities demonstrated in biopsy material from the distal part of the sphincter in this condition include; loss of smooth muscle cells, disruption of normal cell-cell relationships, stretching of elastic tissue and increased collagen fibril content (Swash *et al.*, 1988). Similar but less marked changes have been observed in ageing. Abnormalities in the pharmacological behaviour of sphincter tissue *in vitro* have also been noted and include; reduced sensitivity to exogenous α-adrenoceptor agonists (Speakman *et al.*, 1990), β-adrenoceptor agonists (Lubowski et al., 1988), nicotinic cholinoceptor agonists (Lubowski et al., 1988) and muscarinic cholinoceptor agonists (Speakman *et al.*, 1992). In addition, functional impairment of intramural adrenergic nerves has also been suggested, but this may in fact reflect insensitivity of this tissue to α-adrenoceptor stimulation (Speakman *et al.*, 1990). The results of these *in vitro* studies require verification and further examination.

Abnormalities of anorectal sampling have recently been discovered in patients suffering from idiopathic anorectal incontinence. Using static and ambulatory monitoring techniques, it has been demonstrated that sampling episodes (equalisation of rectal and upper anal canal pressures) occur more frequently in

those afflicted by this condition compared with controls. Also, in idiopathic anorectal incontinence, sampling is associated with a more pronounced fall in anal canal pressure and a greater rise in rectal pressure than is recorded in controls (Miller *et al.*, 1988; Sun *et al.*, 1990; Farouk *et al., 1993*). Finally, in incontinent patients internal anal sphincter relaxation is associated with poor recruitment of external anal sphincter activity (Sun *et al.*, 1990; Farouk *et al., 1993*). The cause of these abnormalities is unknown but it seems reasonable to suggest that their occurrence makes faecal leakage more likely.

Direct sphincter injury

The sphincters, both external and internal, can be divided or disrupted erroneously at the time of anorectal surgery (Speakman *et al.*, 1991). In addition, they are sometimes damaged by accidental injuries. Direct sphincter injury also occurs during childbirth, making this a numerically important and potentially preventable cause of incontinence.

Diabetes mellitus

Anorectal incontinence can affect up to one fifth of patients suffering from diabetes mellitus (Aitchison *et al.*, 1991). Here, incontinence is multifactorial in origin, and results from malabsorption and colonic dysmotility as well as anorectal dysfunction (Schiller *et al.*, 1982; Ogbonnaya and Arem, 1990). When compared with controls, incontinent diabetic patients have blunted rectal (Wald and Tunuguntla, 1984; Caruana *et al.*, 1991) and anal sensation (Rogers *et al.*, 1988; Aitchison *et al.*, 1991). In addition, both the internal and external sphincters can be weak (Schiller *et al.*, 1982; Caruana *et al.*, 1991). These abnormalities are a likely consequence of somatic and autonomic neuropathies induced by diabetes (Rogers *et al.*, 1988; Belai and Burnstock, 1992).

Rectal prolapse

Rectal prolapse can be defined as full thickness rectal intussusception associated with descent of the intussusceptum through the anal canal so that the apex emerges at the anal verge (Keighley, 1992). This is frequently associated with faecal incontinence, but the precise incidence of this complication is uncertain as the reported occurrence varies from 22% (Watts *et al.*, 1985) to 100% (Prasad *et al.*, 1986). An approximate incidence of 70-75% has been suggested (Keighley *et al.*, 1992; Farouk *et al.*, 1992). Internal anal sphincter weakness is a contributory factor in the pathogenesis of incontinence in these patients and improvement in its function has been implicated as an important reason why patients regain continence following repair of their prolapse (Broden *et al.*, 1988; Sainio *et al.*, 1991; Duthie and Bartolo, 1992; Farouk *et al.*, 1992; Hiltunen and Matikainen, 1992). Farouk *et al.* (1992) have suggested that rectal prolapse produces persistent rectoanal inhibition which results in reduced electromyographic activity within the internal anal sphincter and impaired smooth muscle contraction. The processes and mechanisms responsible for this are unclear but it can be reversed by rectopexy (Farouk *et al.*, 1992). Failure of internal anal sphincter function to recover after surgery is noted in those in whom incontinence persists (Broden *et al.*, 1988; Hiltunen and Matikainen, 1992).

Incontinence in geriatric patients

Elderly people can develop anorectal incontinence for most of the reasons outlined in table 1. There are however, a number of factors which make this group particularly prone to faecal leakage. Dementia and impaired consciousness, perhaps as a result of a cerebro-vascular accident, may result in indifference to the 'call to stool' and immobility can delay appropriate responses sufficiently to

make leakage inevitable. Other important factors include sphincter weakness and faecal impaction.

The external anal sphincter is weaker in geriatric patients when compared with younger controls (Bannister *et al.*, 1987; Laurberg and Swash, 1989) but this does not appear to be a direct cause of faecal leakage here because external sphincter laxity and incontinence do not appear to be related (Barrett *et al.*, 1989). The cause of striated muscle weakness is unclear. It may result from distal pudendal nerve neuropathy (Laurberg and Swash, 1989) but this is not a consistent finding (Barrett *et al.*, 1989). Immobility is a factor though and external anal sphincter weakness is most pronounced in those who are least mobile (Barrett *et al.*, 1989).

Age related weakness of the internal anal sphincter has been reported (Bannister *et al.*, 1987) but again, this is not a universal finding (Laurberg and Swash, 1989; Barrett *et al.*, 1989). Impaired internal anal sphincter tone is however a prominent feature in elderly incontinent patients and this is thought to be an important contributory factor in the development of faecal leakage in this group (Barrett *et al.*, 1989; Barrett and Kiff, 1992). The cause of internal anal sphincter weakness here is unknown.

The elderly are prone to faecal impaction due to deficient colonic propulsion, defaecatory difficulties and impaired rectal sensation (Barrett and Kiff, 1992). They commonly develop faecal incontinence as a result (Barrett and Kiff, 1992). The cause of this is multifactorial and results from abnormal rectal sensation, compounded by sphincter laxity as described above (Read and Abouzekry, 1986). The degree of rectal distension required to stimulate the desire to defaecate is greater in impacted incontinent patients than in controls of a similar age but, the rectal volume required to initiate rectoanal inhibition is less (Read and Abouzekry, 1986).

Faecal incontinence after restorative entero-anal surgery

Imperfections of continence can occur following resection of the rectum for either malignant or inflammatory bowel disease. Potential contributory factors include poor neo-rectal compliance (Batignani *et al.*, 1991; Lewis *et al.*, 1992), liquid stool (Horgan *et al.*, 1989), diminished sensation (Johnston *et al.*, 1987; Holdsworth and Johnston, 1988; Batignani *et al.*, 1991) and internal anal sphincter weakness (Williams *et al.*, 1980; Neal *et al.*, 1982; Becker, 1984; Lazorthes *et al.*, 1986; Luukkonen, 1988; Horgan *et al.*, 1989; Williams *et al.*, 1989; Lewis *et al.*, 1992). A significant correlation between internal anal sphincter weakness and incontinence has been demonstrated following restorative proctocolectomy with ileal pouch-anal anastomosis (Taylor *et al.*, 1983; O'Connell *et al.*, 1988). The precise reason why internal anal sphincter function is impaired in this way is unknown. Direct injury to the sphincter might result from excessive retraction during construction of hand sewn per-anal anastomoses or, it could emanate from over-stretching of the sphincter ring during instrumentation of the anal canal with a stapling device. Incorporation and inadvertent amputation of the proximal part of the internal anal sphincter during stapling has also been suggested (Williams *et al.*, 1989). Such an injury would not only damage the sphincter directly but would also disrupt its innervation. Damage to extrinsic autonomic nerve fibres might occur during rectal mobilisation (Horgan *et al.*, 1989; Williams *et al.*, 1989).

## 5. Treatment of incontinence

Conservative (non-operative) and operative measures are currently available for the management of those with anorectal incontinence.

Conservative measures

Conservative measures should be tried in all patients except when the clinical features and results of investigations suggest an underlying pathology such as inflammatory bowel disease, carcinoma or rectal prolapse which require alternative, appropriate treatment. Patients should be counselled and given advice with the aim of producing a solid stool once each day. Opiates, such as loperamide can be helpful. They have been demonstrated to increase internal anal sphincter pressure and to inhibit the recto-anal inhibitory reflex via activation of opiate receptors (Read *et al.*, 1982; Rattan and Culver, 1987). A low fibre diet is recommended. Such measures will be most beneficial in patients with mild symptoms, especially if their problem is either transient or intermittent. A successful outcome has been reported in 40% of patients treated by diet and drugs alone (Miller *et al.*, 1988). Others however, have reported acceptable continence in only 16% of those treated similarly (Keighley and Fielding, 1983).

Other non-operative therapies include pelvic floor physiotherapy, biofeedback conditioning and electrical stimulation. Promising early results have been reported for the latter with repeated stimulation of the pudendo-anal reflex arc (Binnie et al., 1990). Biofeedback conditioning has been used successfully in some centres, particularly in North America (Macleod, 1987) and improved rectal sensation is an important determinant of outcome (Wald, 1983; Miner *et al.*, 1990). However, these techniques have not yet been shown to be of long term benefit and studies claiming their success are flawed by lack of controls and imprecise definitions of how continence was assessed and graded. The only controlled study that has been performed did not show any difference between patients having biofeedback and controls (McHugh et al., 1986). This finding was supported by Loening-Baucke (1990), who found that biofeedback was

ineffective and suggested that improvements noted in anorectal function may in fact be due to medication or to regression of symptoms with time.

Operative measures

*Principles of surgery in anorectal incontinence*

Surgical intervention in anorectal incontinence is considered if conservative measures fail to ameliorate the problem. Several techniques are available and their selection is determined by the aetiology of incontinence and the outcome of previous treatments.

*Direct sphincter injuries:* These are explored through a peri-anal incision in the area of the previously defined defect. The abrupted ends of the external sphincter are isolated by dissection and with the aid of per-operative nerve stimulation. Enough muscle is mobilised to allow an overlapping repair over a reasonable distance cranially (3-4cm) so that an anal canal of adequate length can be reconstructed. The fibrous scar at the abrupted ends of the injured muscle can be used to secure the overlap. The repair is performed using non-absorbable sutures and, where possible, the internal anal sphincter is also tightened by imbrication.

*Post anal repair:* This is the most commonly performed operation in cases where there is pelvic floor and external sphincter weakness due to neuropathy. It was conceived by Parks (1975) on the premise that a 'flap valve' (above) is essential for normal continence and that this is missing in neurogenic faecal incontinence. Post anal repair attempts to recreate acute anorectal angulation by moving the anorectal junction upwards and forwards.

The sphincters are first exposed through a curvi-linear or 'V' shaped post anal incision and the intersphincteric plane is sought. This is the bloodless anatomical key to the operation which, when entered and followed superiorly, leads up to pelvic floor where Waldeyer's fascia is incised to gain access to the pre-sacral space above. The levators (ileococcygeus, pubococcygeus and puborectalis) are approximated in turn using interrupted non-absorbable sutures. The external sphincter is then imbricated with a similar suture.

Results of surgery

Following the repair of a direct sphincter injury, 70-90% of patients can expect to have full continence restored with less than 10% achieving no benefit at all (Browning and Motson, 1983. Fang *et al*, 1984).

The results of postanal repair are outlined in Table 2.

Table 2.

The outcome of postanal repair for anorectal incontinence.

| | *Fully continent* | *Improved but not f ully continent* | *Not improved* |
|---|---|---|---|
| Keighley and Fielding 1983. n=39 | 27 (69%) | 6 (15%) | 6(15%) |
| Browning and Parks 1983. n=42 | 8 (19%) | 26 (62%) | 8 (19%) |
| Womack *et al.*, 1988. n=16 | 6 (37%) | 8 (50%) | 2(12.5) |
| Miller *et al.*, 1988. n=17 | 10 (59%) | 4 (23%) | 3 (18%) |
| Scott *et al.*, 1990. n=62 | 28 (45%) | 23 (37%) | 11(18%) |

It is clear that although most patients gain some benefit from postanal repair, the quality of continence achieved is often imperfect. In addition, long term follow-up indicates that the functional outcome deteriorates with time such that by three years after surgery, 76% have soiling and 52% require the protection of pads (Yoshioka and Keighley, 1989). Failures occur due to technical problems, the presence of concomitant proximal disease such as the irritable bowel syndrome, or because of continuing neuropathy which might result from the operation itself (Laurberg *et al.*, 1990).

The factors associated with a successful outcome after post anal repair remain uncertain. Restoration of acute anorectal angulation is not correlated with restitution of continence (Miller *et al.*, 1988; Womack *et al.*, 1988; Yoshioka *et al.*, 1988). Instead, operative success has been linked with other parameters, but with none unequivocally; increased sphincter pressures (Browning and Parks, 1983; Miller *et al.*, 1988), lengthening of the anal canal (Browning and Parks, 1983; Womack *et al.*, 1988) and enhanced anal canal sensation (Miller *et al.*, 1988). Interestingly, results similar to those following post anal repair are achieved with anterior sphincter plication and levatorplasty (Miller *et al.*, 1989).

Patients who fail to benefit from these procedures form a difficult group to manage. A repair can be repeated or, where there has already been a posterior repair, an anterior repair can be performed. For patients with very weak sphincters, anterior and posterior repairs can be undertaken simultaneously. These procedures can be technically difficult and may again be unsuccessful. Alternative approaches have been suggested. These include the construction of a neo-sphincter by transposition of the gracilis muscle (Corman, 1985; Williams *et al.*, 1990) and the implantation of an artificial sphincter based on a device used in urinary incontinence (Christiansen and Lorentzen, 1987). Gracilis transposition has been combined with prolonged neuro-stimulation with the

effect of converting it from a fast twitch to a slow twitch muscle (Williams *et al.*, 1990). These techniques are still in their evolutionary phases but hold promise for the future.

## WHY STUDY INTERNAL ANAL SPHINCTER SMOOTH MUSCLE RELAXATION AND ITS NEUROMUSCULAR CONTROL?

It is evident that anorectal incontinence is a major problem in our society which is incompletely understood and, perhaps as a consequence, it is often inadequately treated. Attention has focused previously on the striated muscles of continence as the primary force which prevents faecal leakage but it is clear that the smooth muscle internal anal sphincter also plays a vital role in this respect. Therapeutic measures which could augment the activity of the internal anal sphincter are attractive and may make a significant contribution in the relief of symptoms in incontinent patients. Before such advances are possible however, we must appreciate better the mechanisms and processes which subserve normal sphincter activity and how these are disturbed in abnormal states. We need to understand how the internal anal sphincter generates and maintains tonic active contraction as well as the neuromuscular responses which mediate co-ordinated and appropriate relaxation.

Internal anal sphincter relaxation, such as occurs in the rectoanal inhibitory reflex, is a fundamental component of normal anorectal function as it permits defaecation and sampling. Little is known though of the underlying neuromuscular processes and one of the main reasons for this is that the neurotransmitter(s) involved have not been identified, except that they are neither adrenergic nor cholinergic in nature (Garrett *et al.*, 1974; Meunier and Mollard, 1977; Rayner, 1979; Bouvier and Gonella, 1981). Such messenger molecules have come to be known as nonadrenergic, noncholinergic (NANC)

neurotransmitters and the nerves which release them, as NANC nerves (Burnstock, 1986). It is believed that NANC nerves mediate the majority of inhibitory responses in the gut and in many instances, as with the rectoanal inhibitory reflex, the identity of the neurotransmitter(s) involved has not been established.

Recently, nitric oxide (NO) has been discovered to be an endogenous bioactive substance which has been proposed as a putative inhibitory NANC neurotransmitter within the mammalian gastrointestinal tract. Could this agent play a role in the neuromuscular control of human internal anal sphincter smooth muscle relaxation?

## NITRIC OXIDE: A NOVEL INHIBITORY NEUROTRANSMITTER

### 1. Discovery that nitric oxide is an endogenous messenger molecule

In 1980 Furchgott and Zawadzki demonstrated that relaxation of vascular smooth muscle which occurs in response to application of acetylcholine, is dependent upon the presence of endothelial cells. If endothelium is present, then acetylcholine stimulates smooth muscle relaxation but if it is removed then a contractile response is seen. Furchgott and Zawadzki concluded that the relaxant effect of acetyl choline must be mediated by a humoral agent which is released from endothelial cells. They called this *endothelium derived relaxing factor* (EDRF).

The importance of EDRF and the need to characterise it further, stimulated a great deal of research interest in the 1980's. It was demonstrated that in addition to acetylcholine, release of EDRF occurs in response to a variety of other agents,

for instance; adenine nucleotides, substance P, bradykinin, and hypoxia (Moncada *et al.*, 1991). EDRF was found to be very labile with a half-life of only a few seconds in physiological solutions (Griffith *et al.*, 1984). Its effect on smooth muscle was associated with a rise in cyclic guanosine 3',5' monophosphate (cGMP) levels, mediated through stimulation of cytosolic guanylate cyclase (Rappoport and Murad, 1983). In addition, agents which either antagonise or potentiate the action of EDRF were discovered. Antagonists include methylene blue, an inhibitor of guanylate cyclase (Martin et al., 1985); oxyhaemoglobin, which binds EDRF (Martin et al., 1985) and hydroquinone, which generates superoxide anions ($O_2^-$) (Griffith et al., 1984). Superoxide dismutase potentiates the effect of EDRF by reducing the concentration of $O_2^-$ (Grygleski *et al.*, 1986).

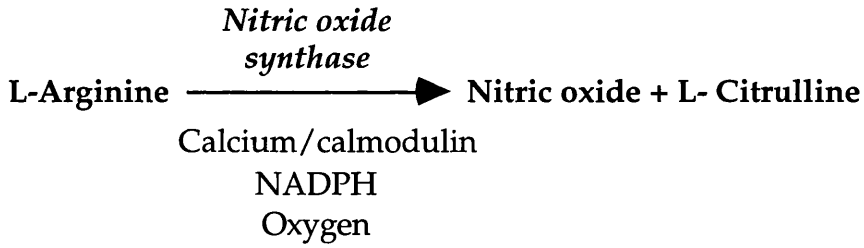
The suggestion that EDRF is nitric oxide (NO) or a NO-like substance was first made in 1986, appropriately by Furchgott (Furchgott, 1988). He based his proposal on the similarity between the pharmacological behaviour of EDRF and that of NO generated from inorganic nitrite ions ($NO_2^-$). The following year Palmer *et al.* (1987) demonstrated that NO could be produced by mammalian cells and its release accounted for the actions of EDRF. They based their conclusions on the results of experiments which utilised chemiluminescent and bioassay techniques to measure NO production by cultured porcine endothelial cells in response to application of bradykinin. The amount of NO released by the endothelial cells (as measured by chemiluminescence), was sufficient to account for the effect of EDRF (as measured by bioassay). Furthermore, the biological actions of EDRF and NO decayed at the same rate during passage down bioassay cascades and their effects were altered in an identical fashion by agents previously characterised as antagonists and potentiators of EDRF.

## 2. Biosynthesis of nitric oxide

Although endogenous production of NO has only been recognised recently, a great deal is already known about the processes responsible for its biosynthesis (Moncada *et al.*, 1991). Nitric oxide is formed from L-arginine, in a reaction catalysed by an enzyme, nitric oxide synthase . Nitric oxide synthase exhibits a high degree of substrate specificity (NO is not produced from D-arginine) and its activity can be competitively inhibited by arginine analogues such as N-monomethyl-L-arginine (L-NMMA) and N-nitro-L-arginine (L-NOARG). In endothelial cells nitric oxide synthase is constitutive, cytosolic, $Ca^{2+}$/ calmodulin dependent and produces NO for short periods in response to receptor or physical stimulation.

Figure 4.

The pathway for nitric oxide biosynthesis.




Following the demonstration that NO is released by endothelial cells, it was also reported that NO is produced by macrophages and that it can act as an effector molecule in immunological reactions. Here too NO is synthesised from L-arginine but the enzyme involved, also called nitric oxide synthase, is different from the one described above. In macrophages, synthesis of nitric oxide synthase is induced by cell activation and once expressed, the enzyme produces NO for long periods. This form of nitric oxide synthase is $Ca^{2+}$/ calmodulin independent and its activity can be inhibited by glucocorticoids.

## 3. Discovery that nitric oxide is a neurotransmitter

Nitric oxide was first implicated as a neuronal messenger in the central nervous system. It was noted that stimulation of rat cerebellar cells with N-methyl-D-aspartate (NMDA) induced an increase in cyclic GMP levels and release of a substance which resembled EDRF (Garthwaite *et al.*, 1988). Importantly, the cells that produced EDRF in response to stimulation with NMDA were not the same cells in which elevated levels of cyclic GMP were found. Later experiments revealed that the stimulation of cyclic GMP production was enhanced by L-arginine and inhibited by N-monomethyl-L-arginine, so implicating involvement of the L-arginine:NO pathway (Bredt and Snyder, 1989; Garthwaite *et al.*, 1989) .

Further evidence that nerves in the central nervous system can produce NO was provided by studies of rat synaptosomal cytosol in which addition of L-arginine resulted in formation of both NO and L-citrulline as well as stimulation of soluble guanylate cyclase (Knowles *et al.*, 1989). These findings indicated the existence of a neuronal nitric oxide synthase which was shown to have similar properties to the constitutive enzyme present in endothelial cells. In 1990 Bredt and Snyder isolated neuronal nitric oxide synthase from rat cerebellar homogenate and determined its distribution within the central nervous system immunocytochemically (Bredt and Snyder, 1990; Bredt *et al.*, 1990). They showed that in the rat brain, neuronal nitric oxide synthase immunoreactivity is exclusively associated with discrete populations of nerves, so adding to the evidence that there is a neuronal source for NO.

## 4. Discovery that nitric oxide is an inhibitory neurotransmitter in the gastrointestinal tract

In the light of results described above, attention focused on the possibility that NO is a neural messenger in the autonomic nervous system and in particular that it is the neurotransmitter released by NANC inhibitory nerves in the gut and related structures. The first indication that this might indeed be the case came from the study of NANC nerve mediated relaxation of the anococcygeus muscle in the rat (Gillespie *et al.*, 1989; Li and Rand, 1989). Neurogenic relaxation in this tissue was mimicked in a dose dependent fashion by sodium nitroprusside (a donor of NO) and inhibited by N-monomethyl-L-arginine, again in a dose dependent manner. The inhibitory action of N-monomethyl-L-arginine was antagonised by L-arginine. N-monomethyl-L-arginine had no effect on the response to sodium nitroprusside.

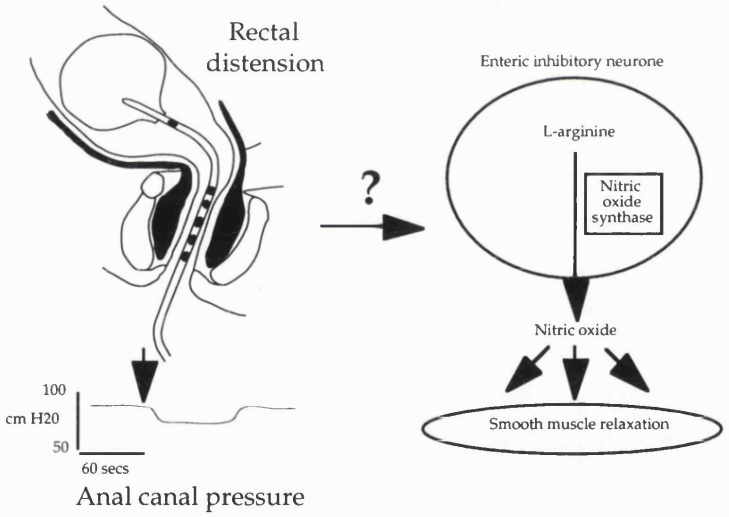
Following on the heels of these reports came investigations which suggested involvement of NO in NANC neurogenic relaxation in wholly gastrointestinal smooth muscles (stomach-Boeckxstaens *et al.*, 1990; duodenum-Toda *et al.*, 1990; ileocolonic junction-Bult *et al.*, 1990; Hata *et al.*, 1990). Perhaps most important was the study by Bult *et al.* (1990). Using a superfusion bioassay, they demonstrated that a NANC factor was released by canine ileocolonic smooth muscle in response to electrical field stimulation or activation of nicotinic receptors, which relaxed de-endothelialised rings of rabbit aorta. This factor was shown to be equally as unstable as authentic NO and its production was inhibited by N-nitro-L-arginine. In addition the effect of the factor was diminished by oxyhaemoglobin but enhanced by super oxide dismutase. The authors concluded that NO is released in response to stimulation of NANC inhibitory nerves in ileocolonic smooth muscle strips.

## THESIS

In the light of reports implicating NO as a NANC inhibitory neurotransmitter in the gastrointestinal tract, it seemed reasonable to propose that it might be involved in neurogenic relaxation of human internal anal sphincter smooth muscle. If this proved to be the case, then NO could also be the hitherto undiscovered agent which mediates the rectoanal inhibitory reflex in man. The studies described here were designed to test this proposition.

Figure 5.

Hypothesis: Nitric oxide has a role in the neuromuscular control of human internal anal sphincter relaxation and could therefore mediate the rectoanal inhibitory reflex in man.




## PLAN OF THESIS

I have attempted to chronicle my investigations as they evolved in what was a period (1991-1993) of feverish interest in NO in laboratories around the world. The role of NO as an enteric neurotransmitter unfolded during this time and the

pattern of my investigations reflects this. Contained here are a series of experimental chapters, the first of which describes some preliminary investigations I undertook in the pig, prior to embarking upon the study of human tissues. These initial experiments provided an important insight into the role that NO plays in the neuromuscular control of internal anal sphincter smooth muscle activity. This chapter is followed by a description of some aspects of the behaviour of human internal anal sphincter smooth muscle in *vitro*. Each of the succeeding experimental chapters describes investigations that focus upon a separate facet of the role of NO in the neuromuscular control of this tissue. In each I present and examine a hypothesis which was formulated from information available at the time, rather than at the completion of my studies. The experimental chapters are preceded by an outline of the methods used and are followed by a concluding chapter in which results are drawn together and examined in the light of currently available published data. The following are the essential questions asked;

*Chapter 4* **How does the human internal anal sphincter behave *in vitro* and how does this compare with other anorectal smooth muscles?**

*Chapter 5* **Is NO involved in neurogenic relaxation of human internal anal sphincter smooth muscle *in vitro*?**

*Chapter 6* **Is there a neuronal source for NO in the human internal anal sphincter? If so, where are the cell bodies of these nerves and could these neurones mediate the motor component of the human rectoanal inhibitory reflex?**

*Chapter 7* **Are NO producing neurones present in Hirschprung's disease, a condition in which the rectoanal inhibitory reflex is absent?**

*Chapter 8* **Does NO mediate relaxation of human internal anal sphincter smooth muscle induced by stimulation of muscarinic cholinoceptors *in vitro*?**

# *Chapter 2*

## PATIENTS AND METHODS

# INTRODUCTION

This chapter details the various techniques used in the investigations described in this thesis. I have attempted to make my descriptions as clear and as complete as possible . Techniques are e xplained under the following headings;

**Patients and tissue collection**

***In vitro* physiological and pharmacological studies**

1. Preparation of tissue for tension studies
2. Administration of drugs
3. Drugs and chemicals used
4. Electrical field stimulation of muscle strips

**Morphological studies**

1. Preparation of whole mounts of the enteric nerve plexuses
2. Preparation of tissue for histology of cryostat sections
3. Chemicals and solutions used in morphological studies

**Staining techniques**

1. Nitric oxide synthase immunocytochemistry
2. NADPH diaphorase activity
3. Immunocytochemical nerve stain

**Quantitation in morphological studies**

**Photography**

**Statistics**

## PATIENTS AND TISSUE COLLECTION

With the exception of pig material used in preliminary studies, all tissue for the experiments described in this thesis was obtained from adult patients undergoing resectional surgery for anorectal cancer and from infants undergoing definitive surgery for Hirschsprung's disease. In addition paediatric 'control' tissue was obtained from age matched infants with anorectal agenesis. The number and type of specimens used in each series of experiments is out-lined in the methods section of the relevant chapters.

Specimens were made available for study by General Surgeons throughout the Oxfordshire Health Region (John Radcliffe Hospital, Oxford; Battle and Royal Berkshire Hospitals, Reading; Milton Keynes General Hospital, Milton Keynes; Horton General Hospital, Banbury; Northampton General Hospital, Northampton). All paediatric tissue was made available by Paediatric Surgeons at the John Radcliffe Hospital, Oxford. Appropriate ethical committee approval was obtained for these investigations (Central Oxford Research Ethics Committee project number 2075).

Tissue was collected at the time of surgery by the author in person. Upon removal, the surgical specimen was rinsed and cleaned in ice-cold (4$^{o}$C) normal saline. The mesorectum was excised and, if necessary, striated muscle from the pelvic floor was trimmed. The remaining tissue was then immersed in ice-cold (4$^{o}$C) Kreb's solution and transported to the laboratory where it was processed as soon as possible after collection.

## *IN VITRO* PHYSIOLOGICAL AND PHARMACOLOGICAL STUDIES

### 1. Preparation of tissue for tension studies

With the aid of a dissecting microscope, the anorectal mucosa was removed together with the submucosa. Parallel strips of the appropriate smooth muscle were cut, each with approximate dimensions 10 x 1 x 1mm. All strips contained parallel bundles of smooth muscle fibres. Fine silk ligatures were tied to each end of the strips to allow them to be mounted in organ baths. One end was fixed and the other was attached to an isometric tension transducer.

The muscle strips were mounted in 0.2ml capacity, perspex organ baths (Brading and Sibley, 1983). They were placed between recessed platinum electrodes which permitted electrical field stimulation. The strips were continuously superfused with Krebs solution (37$^{o}$C) at a rate of 1ml per minute. By mounting six organ baths in parallel, it was possible to study six muscle strips simultaneously. The apparatus used in these studies is illustrated in figures 1 and 2.

Figure 1

Superfusion apparatus used in *in vitro* physiology studies.

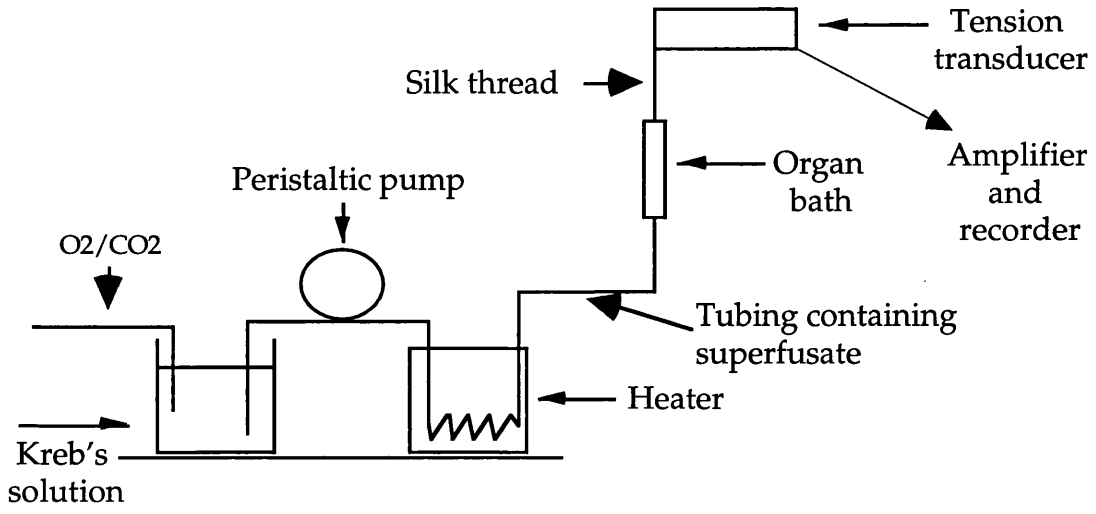
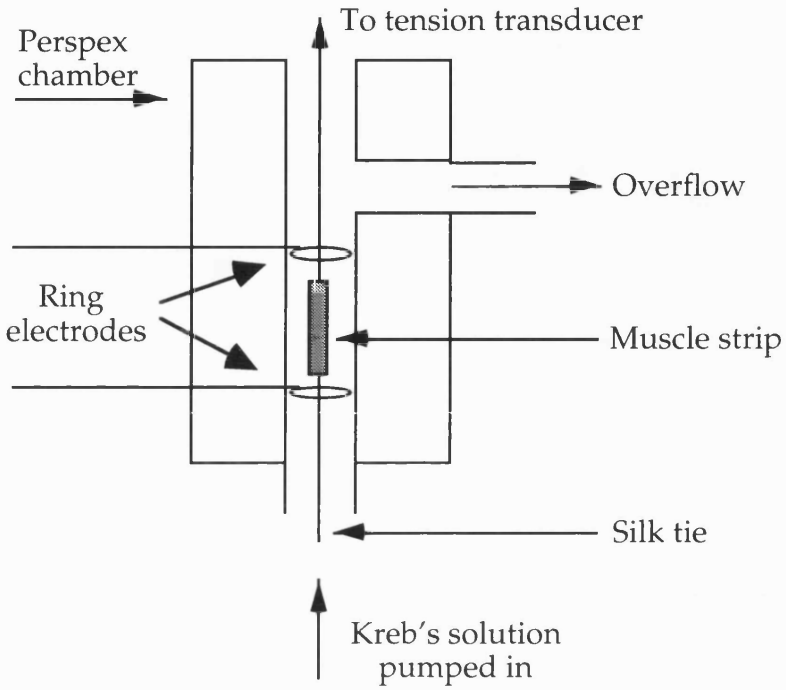


Figure 2.

Superfusion organ bath




The contractile force generated by smooth muscle strips was measured by Pioden Dynamometer UF1 transducers (Pioden Controls Ltd., Canterbury, Kent, UK), amplified (Harvard Transducer/Amplifier, Harvard Apparatus Ltd., Edenbridge, Kent) and recorded by a six channel Teckman 900 pen recorder (Teckman Electronics Ltd., Leamington Spa, Warwickshire, UK).

Tension was initially established in the muscle strips by stretching them with a force equivalent to that provided by a 1.0gm weight. Strips were then allowed to equilibrate for at least an hour prior to the start of experiments. At the end of experiments the strips were superfused with calcium free solution in order to fully relax the strips so that baseline (zero) tension could be determined. The strips were then weighed

## 2. Administration of drugs

Drugs were administered by immersing the superfusate-delivery tubing in a solution containing the test drug and each drug was applied for the minimum time which produced the maximum response. Introduction of a small bubble of air during this procedure and the narrow calibre of the tubing ensured that there was minimal mixing and consequent dilution of the test solution with the normal superfusate.

## 3. Drugs and chemicals used

The following agents were obtained from Aldrich Chemical Company Inc., Gillingham, Dorset, UK:

D-arginine

L-arginine

The following agents were obtained from BDH Chemicals Ltd., Atherstone, Warwickshire, UK:

Atropine sulphate

Noradrenaline

Sodium nitroprusside

Tetraethylammonium chloride (TEA)

The following agent was obtained from ICI Ltd., Macclesfield, Cheshire, UK.

Propranolol hydrochloride

The following agents were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, UK:

Bovine haemoglobin

Carbamylcholine chloride (carbachol)

Guanethidine monosulphate

Hexamethonium bromide

Isoprenaline sulphate

N-monomethyl-D-arginine acetate

N-monomethyl-L-arginine acetate

N-nitro-L-arginine

Phentolamine hydrochloride

Phenylephrine hydrochloride

Tetrodotoxin

Bovine haemoglobin as supplied by Sigma Chemical Co. Ltd. (Poole, Dorset, UK), contains 75% methaemoglobin and 25% oxyhaemoglobin. Oxyhaemoglobin of greater purity was prepared by reduction of bovine haemoglobin with sodium hydrosulphite (10 fold molar excess) followed by gel filtration through a pre-packed disposable ion exchange column (PD-10, Pharmacia LKB, Uppsala, Sweden), previously equilibrated with buffered Krebs solution (Salvemini *et al.*, 1989). The resulting oxyhaemoglobin concentration was then determined spectrophotometrically ($E_{576nm}$=15.99 mM $^{-1}cm^{-1}$ ). Methaemoglobin was made in a similar fashion, except a 2 fold molar excess of potassium ferricyanide was used instead of sodium hydrosulphite (Martin *et al.*, 1986). Methaemoglobin concentrations were calculated on a weight:volume basis.

Krebs solution contained (mM.): NaCl 120. KCl 5.9, $NaHCO_3$ 15.4, $NaH_2PO_4$ 1, $CaCl_2$ 2.5, $MgCl_2$ 1.2 and glucose 11. Solutions were equilibrated with 97% $O_2$, 3% $CO_2$, producing pH7.4 at 37°C±1°C. In *calcium free* Krebs solution, $CaCl_2$ was replaced by an equimolar quantity of $MgCl_2$ and the $Ca^{2+}$ chelator, EGTA (0.5mM) was added.

## 4. Electrical field stimulation of muscle strips

Impulses for electrical field stimulation were generated by, and delivered from, a Grass S48 stimulator (Grass Medical Instruments, Quincy, Massachusetts USA). The stimulation parameters used in these experiments were modified from those previously described by Speakman *et al.* (1990); impulse strength- 10V, impulse duration- 0.5ms, frequency- 1-30Hz, stimulation time- 1 second.

## MORPHOLOGICAL STUDIES

## 1. Preparation of whole mounts of the human enteric nerve plexuses

Whole mounts of the human colon, rectum and anal canal were prepared by a modification of the procedure described by Ward *et al.*, 1992.

*i.* The bowel was slit along its cranio-caudal axis and converted into a rectangular sheet. It was washed in phosphate buffered saline (PBS, pH 7.4) and the serosa was removed.

*ii.* The bowel was then pinned out, slightly stretched, on silicone elastomer (Sylgard, Dow Corning, Wiesbaden, Germany) in an appropriate dish and fixed overnight in 4% paraformaldehyde in PBS (pH 7.4) at 4$^{o}$C.

*iii.* After washing (x2) in PBS, the tissue was again pinned out on silicone elastomer and continually bathed in PBS, pH 7.4. The tissue was visualised and magnified with the aid of a binocular microscope (Wild Leitz M3Z, Heerbrugg, Switzerland). The mucosa and muscularis mucosa were removed. The circular and longitudinal muscle layers were then gently teased apart using fine forceps.

Limited sharp dissection was also used if required. Tissue laminae were then stained.

*iv.* Final dissection of the enteric nerve plexuses was possible once they had been revealed by staining.

*v.* In addition to viewing tissue under the dissecting microscope, thin whole mount preparations were also viewed by standard light microscopy using either Leitz Laborlux D or Leitz Diaplan 22 microscopes. For this, tissue was placed on standard microscope slides and 'mounted' in 0.3% hypromellose before being covered with a cover-slip.

*vi.* Tissue was preserved in PBS ( pH 7.4), containing 0.02% thimerosal and stored in the fridge at 4°C.

## 2. Preparation of tissue for histology of cryostat sections

*i.* The bowel was slit along its cranio-caudal axis and converted into a rectangular sheet. It was washed in phosphate buffered saline (PBS, pH 7.4) and the serosa was removed.

*ii.* Small pieces of tissue from the area of interest were excised and embedded in Tissue-Tek O.C.T. compound on small cork blocks. Tissue thus mounted was snap-frozen, first in isopentane cooled in liquid nitrogen, and then liquid nitrogen itself. Frozen tissue was stored in the -70°C freezer until required for sectioning.

*iii.* Sections were cut in a cryostat (Microtome 5030, Bright instrument Co. Ltd, Huntingdon, UK). The cryostat chamber was maintained at -23°C,

corresponding to a block temperature of -18°C. This was found to be the optimum cutting temperature for most specimens, and after trimming through to the level of the tissue, sections 10μm thick were cut and mounted separately on to on poly-L-lysine coated slides glass slides. These were dried in air at room temperature for 30-60 minutes prior to staining. If sections were not to be used the same day, then they were dried as above and wrapped in foil before being stored in the -70°C freezer.

*iv.* Un-sectioned tissue was re-coated in Tissue-Tek O.C.T. compound and returned to the -70°C freezer.

*v.* Stained sections were viewed by standard light microscopy using either Leitz Laborlux D or Leitz Diaplan 22 microscopes.

## 3. Chemicals and solutions used in morphological studies

The following agents were obtained from BDH Chemicals Ltd., Atherstone, Warwickshire, UK:

DPX mountant

Triton X100

The following agent was obtained from Dako Corp., Carpinteria, California, USA:

Aminoethyl carbazole substrate system

The following agent was obtained from Miles Inc., Diagnostics Division, Elkhart, IN, USA:

Tissue-Tek O.C.T. compound

The following agent was obtained from Schering- Plough Ltd., Mildenhall, Suffolk, UK:

0.3% Hypromellose

The following agents were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, UK:

3,3 Diaminobenzidine (DAB)

Nitroblue tetrazolium

Paraformaldehyde

Thimerosal

The following agents were obtained from Sternberger Monoclonals Inc., Baltimore, Maryland, USA:

SMI 311 pan-neuronal marker

SMI 312 panaxonal marker

The following agents were obtained from Vector Laboratories, Peterborough, UK:

Biotinylated goat-anti-rabbit IgG antibody

Biotinylated goat-anti-mouse IgG antibody

Normal goat serum

Vectastain avidin/biotin complex (ABC) system reagents (standard and elite kits)

Nitric oxide synthase antibody was a generous gift from Dr. P. Emson, MRC Neuroendocrinology Unit, Institute of Animal Physiology, Babraham, Cambridge. This is a polyclonal antibody raised in rabbits inoculated with rat cerebellar nitric oxide synthase.

Phosphate buffered saline (PBS) contained 50mls 0.2M phosphate buffer, 8.76gms NaCl and 0.2gms KCl made up to 1000mls with distilled $H_2O$, pH 7.4-7.6.

0.2M phosphate buffer, pH 7.4, contained 4 parts 0.2M $Na_2HPO_4$ (35.6gms $Na_2HPO_4$/1000mls distilled $H_2O$) and 1 part 0.2M $NaH_2PO_4$ (31.2gms $NaH_2PO_4$ /1000mls distilled $H_2O$)

Tris buffered saline (TBS) contained 7.8gms Tris-HCl and 15gms NaCl in 1000mls distilled $H_2O$, pH 7.4-7.6 (adjusted with NaOH).

## STAINING TECHNIQUES

### 1. Nitric oxide synthase immunocytochemistry

*Method*

*i.* Sections were post-fixed in, buffered 4% paraformaldehyde (pH7.4) containing 0.005% glutaraldehyde, for 5 minutes and then washed in Tris-buffered saline (pH7.4) (TBS), 2 x 5 minutes.

*ii.* Endogenous peroxidase activity was blocked by incubation in 1% $H_2O_2$ in TBS, for 30 minutes. Sections were then washed in TBS for 5 minutes.

*iii.* Non-specific binding of antibody was blocked by incubating in the sections in dilute normal goat serum (from species in which second-layer antibody was raised: 1.5% in TBS containing 0.3% Triton X100), for 20 minutes.

*iv.* Sections were then incubated overnight in 1:2500 anti-nitric oxide synthase antibody in TBS containing normal serum and 0.3% Triton X100, 4 °C, .

*v.* Sections were then washed in TBS, 3 x 10 minutes.

*vi.* Sections were incubated in 0.5% biotinylated goat-anti-rabbit IgG, in TBS/Tween 20 for 30 minutes. Following this they were washed in TBS for 10 minutes.

*vii* Sections were incubated in ABC (avidin-biotin complex) reagent (in TBS, as described by manufacturers), for 30 minutes and washed again in TBS for 10 minutes.

*viii.* Peroxidase activity was visualised by incubating in AEC/$H_2O_2$ solution (as described by manufacturers) for approximately 4 min. The level of staining was checked visually. If sections were being used in co-localisation experiments, then they were mounted in 0.3% hypromellose and photographed as appropriate. Those sections not used in such experiments were dried through ethanol, cleared in xylene and mounted in DPX mountant before being covered with cover-slips.

## 2. NADPH diaphorase activity

The method used for demonstrating NADPH diaphorase activity was modified from that previously described by Scherer-Singler *et al.* (1983), Ward *et al.* (1992), and Young *et al.* (1992). Cells which contain NADPH- diaphorase activity stain blue .

*Method*

*i.* Slide mounted sections and tissue laminae were washed in 0.05M Tris buffer (pH 7.4-7.6) and then incubated in 0.05M Tris buffer (pH 7.4-7.6), containing;

0.25 mg/ml nitroblue tetrazolium,

1 mg/ml β- NADPH and

0.3% Triton X100.

The incubation solution was agitated and maintained at 37°C..

*ii.* The degree of staining was controlled visually and was terminated by rinsing the tissue in 0.1M Tris buffer (pH 7.4-7.6). For slide mounted sections an incubation period of 10-15 minutes was generally sufficient to produce the desired level of staining, but for whole mount material 25-45 minutes was needed. Longer incubations were required for whole mounts from the lower rectum and proximal anal canal, where connective tissue was particularly thick.

*iii.* Cryostat sections were dried through ethanol, cleared in xylene and mounted in DPX mountant and covered with cover-slips. Tissue laminae were preserved in PBS ( pH 7.4), containing 0.02% thimerosal and stored in the fridge at 4°C.

## 3. Immunocytochemical nerve stain

SMI (Sternberger Monoclonals Incorporated) 311 is a pan-neuronal marker directed against non-phosphorylated neurofilaments; SMI 312 is a pan-axonal marker directed against phosphorylated neurofilaments. Both are monoclonal antibodies raised in mice and they were used here in combination as a 'cocktail' to stain neurones and their processes. The immunocytochemical technique used to demonstrate SMI 311 and 312 immunoreactivity was the same as that described for nitric oxide synthase except;

*i.* 0.005% glutaraldehyde was not added to the fixative;

*ii.* the primary antibodies were applied at the following concentrations: SMI 311 at a dilution of 1:30,000 and SMI 312 at 1: 8000;

*iii.* the secondary antibody was 0.5% biotinylated goat-anti-mouse IgG used at a dilution of 1:400;

*iv.* peroxidase activity was visualised by incubating in DAB/$H_2O_2$/imidazole solution in TBS for approximately 3 minutes. Peroxidase visualising solution (pH 7.4) contained 0.5 ml stock DAB, 25 ml TBS, 62.5 µl 3% $H_2O_2$ and 17.5 mg imidazole. Stock DAB solution contained 0.5 g DAB dissolved in 13 ml TBS.

## QUANTITATION IN MORPHOLOGICAL STUDIES

Cell parameters were measured using a graticule calibrated against an appropriate stage micrometer (Graticules Ltd., Tunbridge, Kent, UK). The area occupied by individual enteric ganglia was measured by first drawing them with the aid of a Leitz Wetzlar drawing tube attached to a Leitz Diaplan 22 microscope. This data was then digitised and analysed using a Kurta 15/ ADB digitising board linked to a computer based MacStereology program. The number of stained neurones within each ganglion was also recorded.

## PHOTOGRAPHY

Photomicrographs were taken using Olympus single lens reflex cameras (OM10 and OM 4Ti) with appropriate microscope/camera attachments or a Wild Leitz

MPS 52 camera dedicated for use with the Leitz Diaplan 22 microscope. The film used was:

Monochrome- Kodak TMX 100 (Kodak Ltd., HemelHempstead, Herts, UK)

Colour- Fugichrome 64T (Fugi Film Co. Ltd., Tokyo, Japan).

Photographs were taken using condensed bright field illumination. Images of a stage micrometer 100x 0.01mm (Graticules Ltd., Tunbridge, Kent, UK) were taken at each magnification used on each roll of film to facilitate subsequent, accurate measurement of distances. Monochrome film was used when contrast enhancement was required. Monochrome images were printed on to Ilford RC Delux Multigrade III MGX. 1M (Ilford Ltd., Mobberley, Cheshire, UK). Colour images were printed by the Cibachrome process from transparencies.

## STATISTICS

In *in vitro* studies, the number of muscle strips and patients in which individual experiments were conducted is denoted by $n=x(y)$, where $x$ =number of strips and $y$ = number of patients. In morphological studies, the number of specimens in which a phenomenon was noted is described in the relevant section of the text.

Results are expressed here as mean $\pm$ standard error of the mean. Statistical analysis of the data was performed using a computer-based statistical processing programme ['Statworks' (Cricket Software Inc.)] and where appropriate, statistical differences were assessed with the unpaired t test. A value of $p<0.05$ was considered to be significant.

# *Chapter 3*

## A PRELIMINARY STUDY OF NEUROGENIC RELAXATION IN PIG INTERNAL ANAL SPHINCTER SMOOTH MUSCLE *IN VITRO* .

## INTRODUCTION

In the light of early reports which implicated nitric oxide (NO) as a NANC inhibitory neurotransmitter within the gastrointestinal tract (Boeckxstaens *et al.*, 1990; Bult *et al.*, 1990; Hata *et al.*, 1990; Toda *et al.*, 1990), I wanted to rapidly determine if this agent could be involved in neurogenic relaxation of internal anal sphincter smooth muscle. Although my primary intention was to investigate the behaviour of this muscle in man, I performed some preliminary studies using pig internal anal sphincter smooth muscle. I chose the pig because the research group in which I worked has extensive experience of studying pig urinary tract smooth muscles, and these have provided some good models of equivalent structures in the human (Sibley, 1985). In addition, the group has links with a local abattoir which regularly makes fresh tissue available for collection and experimentation. This ready supply of material allowed me to develop and perfect techniques for tissue acquisition, dissection and *in vitro* study without needlessly wasting valuable human tissue.

The aims of these preliminary experiments were;

> ***i.* to establish if the conditions used previously for isometric tension recording of pig urinary smooth muscles (Brading and Sibley, 1983) are suitable for studying neurogenic relaxation in isolated strips of internal anal sphincter smooth muscle and,**
>
> ***ii.* to determine if NO plays a role in neurogenic relaxation. I investigated this by determining if nerve mediated relaxation could be mimicked by application of NO from an exogenous source, and inhibited by L-nitroarginine, an antagonist of nitric oxide synthase.**

## METHODS

Internal anal sphincter smooth muscle was obtained from 9 female Landrace/ Large White cross pigs killed by exsanguination at the local abattoir. All the pigs were females and were slaughtered at 9-12 months of age. Anal canals were excised prior to the animals being immersed in scalding water. The tissue was then handled and dissected in the same way as described for human material.

Strips of smooth muscle were excised from the distal 2cm of the internal anal sphincter and these were then mounted for isometric tension recording in superfusion organ baths as described previously. Once the strips had reached a steady state of activity, their response to electrical field stimulation, using stimulation parameters similar to those described previously by Speakman *et al.*, (1990) was determined. The neurogenic nature of this response was assessed by incubating the strips with tetrodotoxin ($3x10^{-6}M$), a neurotoxin derived from the Japanese puffer fish *Spheroides rubripes*, which inhibits the conduction of nerve action potentials. The strips were superfused throughout with Krebs' solution which contained atropine ($10^{-6}M$) and guanethidine ($3x10^{-6}M$) to inhibit cholinergic and adrenergic neurotransmission (Maggi *et al.*, 1991). (Atropine inhibits the action of agonists at muscarinic cholinoceptors, whereas guanethidine reduces the release of neurotransmitters from adrenergic nerve endings.)

The dose dependent effect of sodium nitroprusside, an exogenous donor of NO, was then determined. Following this, the action of L-nitroarginine upon the response to electrical field stimulation was investigated.

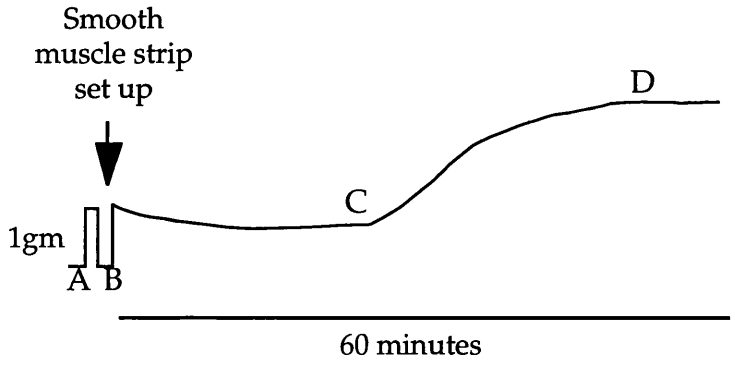
# RESULTS

## 1. Spontaneous tension and neurogenic relaxation

As described in the methods section, tension was initially established in the muscle strips by stretching them with a force equivalent to that provided by a 1gm weight. Following this, the strips contracted spontaneously and reached a plateau or *steady state* tension of 0.614±0.062 gms/mg tissue after approximately 1 hour (*n=24(4)*- the baseline was the tension present when in calcium free solution). The behaviour of the muscle strips during this time is illustrated in figure 1.

Figure 1.

The activity of pig internal anal sphincter smooth muscle strips when they are first set up for isometric tension recording is illustrated by this characteristic trace. A 1gm weight is attached to the tension transducer at point A. This produces an upward deflection. The weight is then removed and the muscle strip is attached- point B. The strip is stretched with a force equivalent to that provided by the 1gm weight. After a period of relative inactivity, the strip then contracts spontaneously (point C) producing further upward deflection of the trace. Eventually a steady state of tension is achieved (point D).




In this contracted state, isolated strips of pig internal anal sphincter smooth muscle relaxed in response to electrical field stimulation. Relaxant responses of

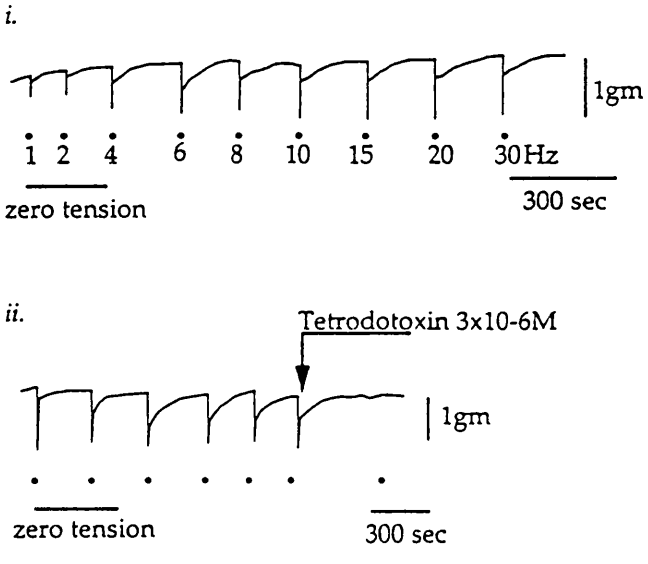
suitable size were obtained using a stimulus strength of 10V and a stimulus duration of 0.5ms. The degree of relaxation produced was determined by the frequency of stimulation and increased in size over a short range between 2 and 10Hz. Relaxations occurred in the presence of atropine ($10^{-6}$M) and guanethidine ($3x10^{-6}$M), but were abolished if tetrodotoxin ($3x10^{-6}$M) was added to the superfusate. This indicates that the relaxations are neurogenic in origin and are mediated by a NANC neurotransmitter(s). These points are illustrated in figure 2.

Figure 2.

The response of isolated strips of pig internal anal sphincter smooth muscle to electrical field stimulation are illustrated in the form of characteristic traces.

(*i.*) Each • represents an episode of electrical field stimulation using an impulse strength of 10V and a stimulus duration of 0.5ms. The frequency of stimulation increases from 0-30 Hz. Relaxant responses are produced (downward deflections of the trace). Atropine ($10^{-6}$M) and guanethidine ($3x10^{-6}$M) are present in the superfusate throughout.

(*ii.*) Repeatable relaxations are produced if electrical field stimulation (•= 10V, 0.5ms duration, 8Hz, for 1 sec) occurs every 180 seconds. These responses are abolished by tetrodotoxin ($3x10^{-6}$M). (Zero tension in this and subsequent figures is the level of tension remaining in the strip when it was superfused with calcium free solution at the end of the experiment. Superfusion with calcium free solution produces maximal relaxation in the smooth muscle strip.)



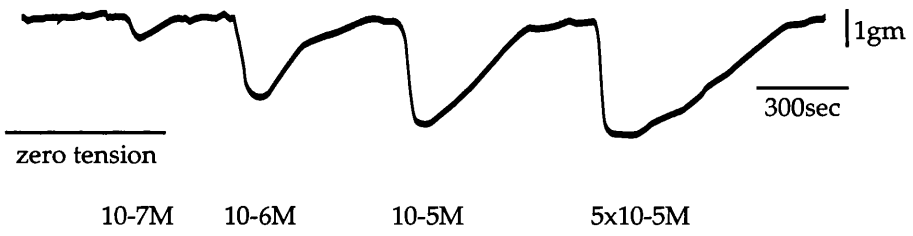

• 10V, 0.5ms duration, 8Hz .

## 2. Effect of exogenous nitric oxide

Sodium nitroprusside is an exogenous donor of nitric oxide (Feelisch and Noack, 1987). Its addition to the superfusate for 60 seconds at increasing concentrations caused dose dependent relaxations of the smooth muscle (*n=12(2)*). The effect of sodium nitroprusside is illustrated in figure 3.

Figure 3.

This characteristic trace illustrates the effect of sodium nitroprusside on isolated strips of pig internal anal sphincter smooth muscle. Sodium nitroprusside was added to the superfusate for periods of 60 seconds and its concentration was increased from $10^{-7}$ M to $5x10^{-5}$M. Downward deflections of the trace are produced which represent relaxation.



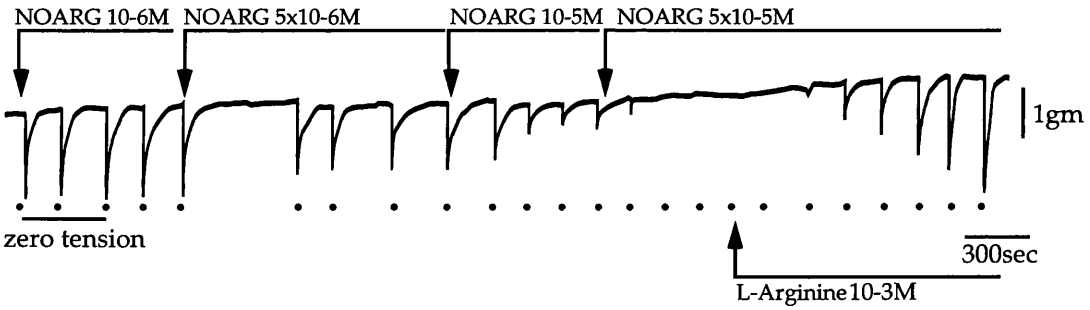

## 3. Effect of L-nitroarginine, an antagonist of nitric oxide synthase

L-nitroarginine is a synthetic analogue of L-arginine, the specific substrate of nitric oxide synthase. Its addition to the superfusate produced a dose dependent inhibition of nerve mediated relaxation and, at a concentration of $5x10^{-5}$M, L-nitroarginine completely abolished the neurogenic response observed in this tissue. Subsequent addition of an excess concentration of L-arginine ($10^{-3}$M)

completely reversed this inhibitory action (*n=15(3)*). These points are illustrated in figure 4.

Figure 4.

The concentration dependent effect of L-nitroarginine (NOARG) on nerve mediated relaxation of pig internal anal sphincter smooth muscle. Neurogenic relaxation occurs in response to electrical field stimulation (•= pulses of 10V, 0.5ms duration, 8Hz, for 1 sec) and the size of the relaxant response observed decreases as the concentration of NOARG in the superfusate increases. The action of NOARG is antagonised by the subsequent addition of L-arginine ($10^{-3}$ M) to the superfusate.



## DISCUSSION

The results from these preliminary investigations demonstrated that the conditions for isometric tension recording previously used for the study of urinary smooth muscles are also suitable for the study of relaxant responses in internal anal sphincter smooth muscle. In addition, the results imply the involvement of the L-arginine/NO pathway in neurogenic relaxation in this tissue.

The involvement of the L-arginine/NO pathway in neurogenic relaxation of pig internal anal sphincter smooth muscle is suggested by the fact that NANC nerve mediated responses are mimicked by the application of NO from an exogenous source and are inhibited by L-nitroarginine, an antagonist of nitric oxide synthase. Indeed, neurogenic relaxation can be completely abolished by this agent but its effect can be reversed by addition of an excess concentration of L-arginine, which is the specific substrate for NO production by nitric oxide synthase.

These preliminary experiments provided invaluable initial experience in handling tissue for isometric tension recording as well as important information about the role of NO in neurogenic relaxation of internal anal sphincter smooth muscle. Based on these findings I embarked upon a more detailed study of this tissue in humans.

# *Chapter 4*

## ASPECTS OF THE BEHAVIOUR OF HUMAN INTERNAL ANAL SPHINCTER SMOOTH MUSCLE *IN VITRO* AND COMPARISON WITH OTHER ANORECTAL SMOOTH MUSCLES.

## INTRODUCTION

The first step in my investigation of the involvement of NO in NANC innervation of human internal anal sphincter smooth muscle was to define the behaviour of this tissue in the isometric tension recording apparatus utilised here, and in particular to determine its response to adrenergic and cholinergic agonists. The latter studies were undertaken to aid subsequent confirmation of NANC relaxation and to ensure the integrity of intrinsic nerves. Inconsistent responses to cholinergic agents have been reported previously. Parks *et al.*, (1969) observed a contractile response in this tissue, whereas others have demonstrated relaxation in response to a similar stimulus (Burleigh *et al.*, 1979; Speakman *et al.*, 1992). Burleigh *et al.* (1979) found that the response to cholinoceptor stimulation was mediated partly by nerve stimulation and subsequently suggested (Burleigh 1992) that responses other than relaxation might reflect damage to intrinsic nerves sustained during tissue acquisition and dissection.

The response of internal anal sphincter smooth muscle to adrenergic and cholinergic agonists was also compared with that of the other anorectal smooth muscles. Up until now the activity of human internal anal sphincter smooth muscle *in vitro* has been considered in isolation. However, it is only one of four substantial smooth muscle structures in this region of the gut, the others being the conjoint longitudinal coat and the circular and longitudinal muscle layers of the rectum. It is uncertain if the behaviour of internal anal sphincter smooth muscle is unique within the anorectum or if it is shared by other smooth muscles, perhaps reflecting sphincter specialisation with in the region. As a general rule, sphincter specialised gastrointestinal smooth muscles generate spontaneous myogenic tension and contract in response to $\alpha$–adrenergic stimulation but relax in response to $\beta$– adrenergic stimulation. Smooth muscles from non-sphincter

specialised regions relax in response to stimulation by both α–and β-adrenergic agonists (Furness and Costa, 1987).

The aims of the experiments described in this chapter were thus two-fold;

***i.*** **to characterise the behaviour of human internal anal sphincter smooth muscle in the isometric tension apparatus used here and,**

***ii.*** **to determine if the behaviour of the internal anal sphincter is unique or if it is a manifestation of sphincter specialisation in this region of the human gut.**

## METHODS

Sphincter tissue was obtained from patients undergoing abdomino-perineal resection of the rectum and anal canal for low-lying rectal carcinoma (5 males, 6 females; median age 67 years, range 62-80). Rectal smooth muscle was derived from this source, and from patients with more proximal rectal carcinoma undergoing low anterior resection with colo-anal anastomosis (4 males, median age 63 years, range 60-65). Strips of smooth muscle were excised from the distal 2cm of the internal anal sphincter. Smooth muscle from the conjoint longitudinal coat was obtained from the proximal anal canal before the layer became obviously fibrous in nature. Rectal circular and longitudinal smooth muscle strips were acquired from the 'body' of the rectum, which was assumed to be mid-way along the cranio-caudal axis of the resection specimen. All strips contained parallel bundles of smooth muscle fibres.

Once dissected, smooth muscle strips were mounted for isometric tension recording in superfusion organ baths as described previously. The response of the strips to carbachol, a cholinergic receptor agonist, and noradrenaline was then determined. Carbachol was used as the cholinergic receptor agonist because of its resistance to breakdown by acetylcholinesterase. Noradrenaline was chosen as the adrenergic agonist because it is the principal neurotransmitter released by autonomic adrenergic nerves. Selective agonists and antagonists were then used to investigate the type of receptor stimulated by these agents.

# RESULTS

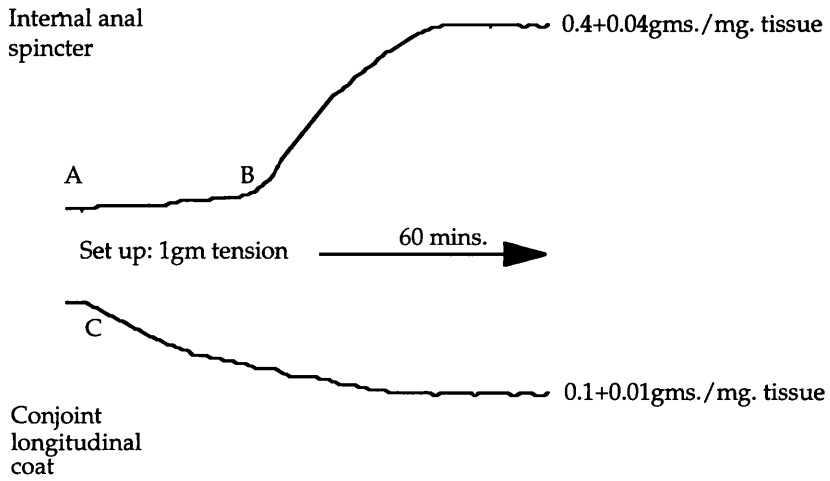
## 1. Spontaneous tension

During the period of equilibration, muscle strips taken from the internal anal sphincter contracted spontaneously and developed a steady state tension of 0.48 ± 0.04 gm/mg tissue (*n=30 (6)* - the baseline was the tension present when in calcium free solution). By comparison, those from the conjoint longitudinal coat relaxed spontaneously but maintained a steady state tension of 0.1±0.01 gm/mg tissue (*n=24(5)* ). This difference was significant ($p<0.001$) and is illustrated in figure 1.

Both layers of smooth muscle from the rectum (*n=24(4)* ) relaxed completely in the organ baths and had no residual tension once the steady state was achieved (equal to that present in calcium free solution).

Figure 1.

Two characteristic traces which illustrate the behaviour of smooth muscle strips from the internal anal sphincter and the conjoint longitudinal coat during the equilibration period at the start of experiments. At point A, both strips were stretched with a force equivalent to 1gm. Subsequently, the strip from the internal anal sphincter contracts (B- upward deflection) whereas that from the conjoint longitudinal coat relaxes (C- downward deflection)

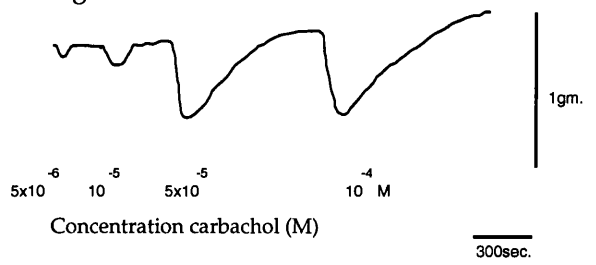


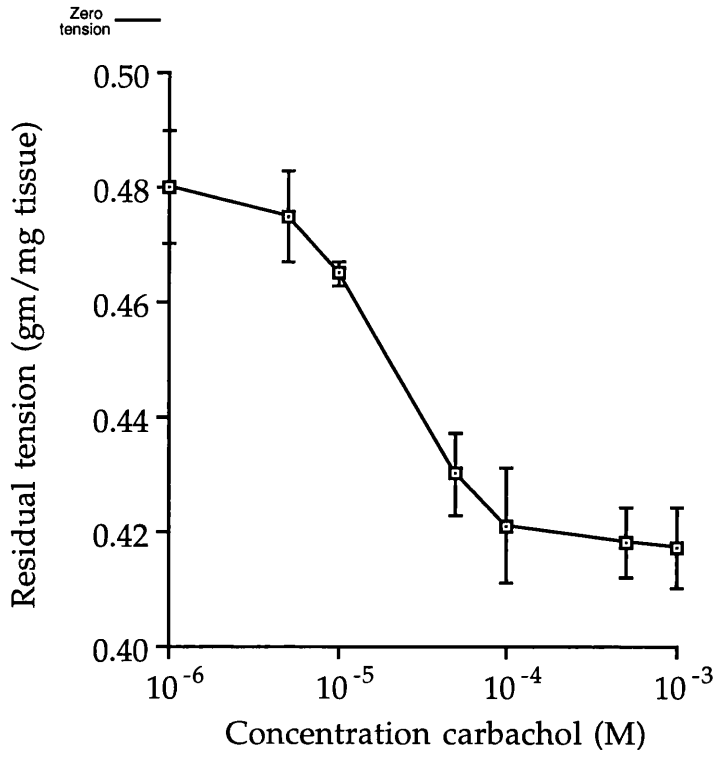
## 2. Response to cholinergic receptor stimulation

Smooth muscle strips from the internal anal sphincter (*n=30(6)* ) relaxed in a dose dependent manner on addition of carbachol for 10 seconds at increasing concentrations. Strips from the conjoint longitudinal coat (*n=18(3)* ) and both layers of the rectum (*n=18(4)* ) contracted in response to similar stimulation . These points are illustrated in Figure 2.

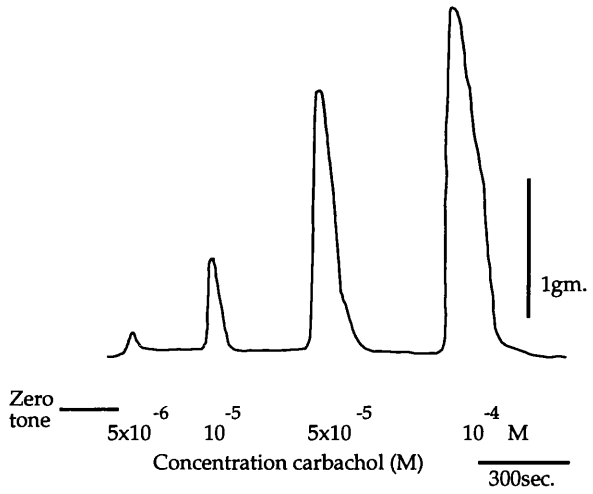
Figure 2.

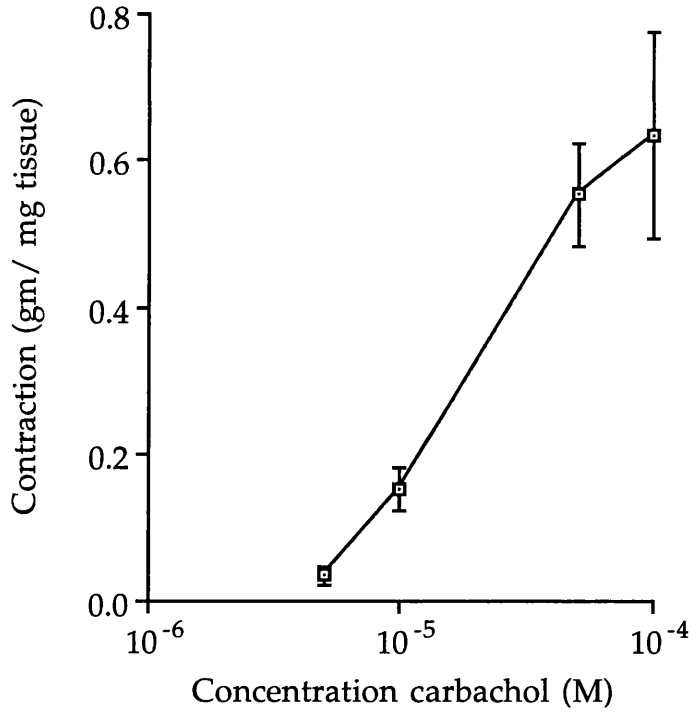
*i.* The response of internal anal sphincter smooth muscle to carbachol, illustrated in the form of a characteristic trace (above) and a cumulative dose response curve (below). Carbachol produced downward deflection of the trace indicating relaxation. In the cumulative dose response curve, the degree of relaxation produced is calculated in terms of the residual tension left in the strips after application of the drug.



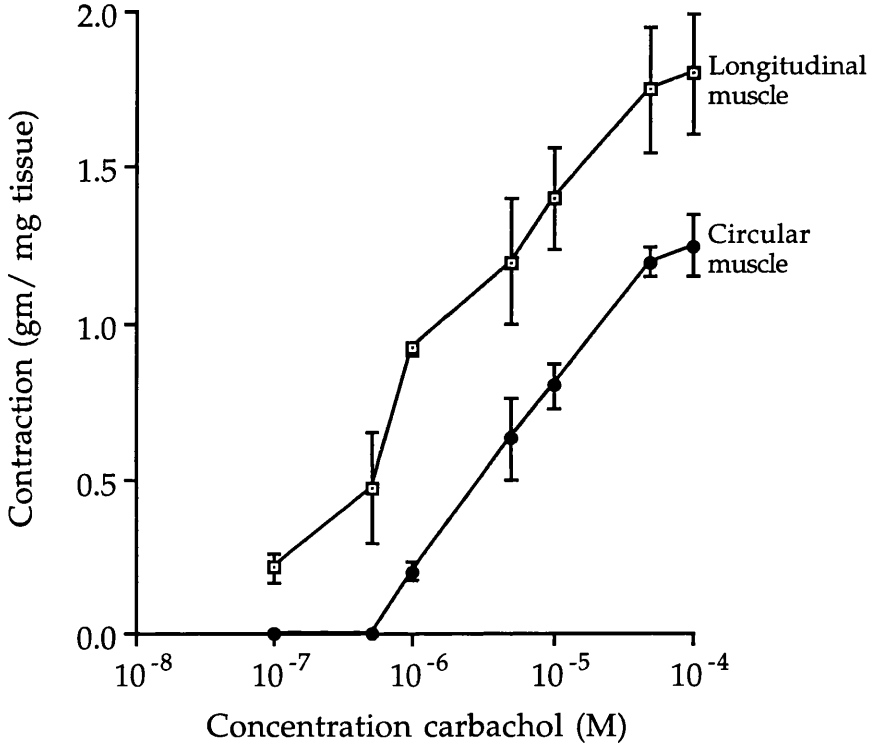



ii. The response of smooth muscle from the conjoint longitudinal coat to carbachol, illustrated in the form of a characteristic trace (above) and a cumulative dose response curve (below). Carbachol produced upward deflection of the trace indicating contraction.



iii. The response of rectal smooth muscle to carbachol. Both the circular and longitudinal muscle layers contracted in a dose dependent manner in response to this agent, as illustrated in this dose response curve.




## 3. Response to adrenergic receptor stimulation

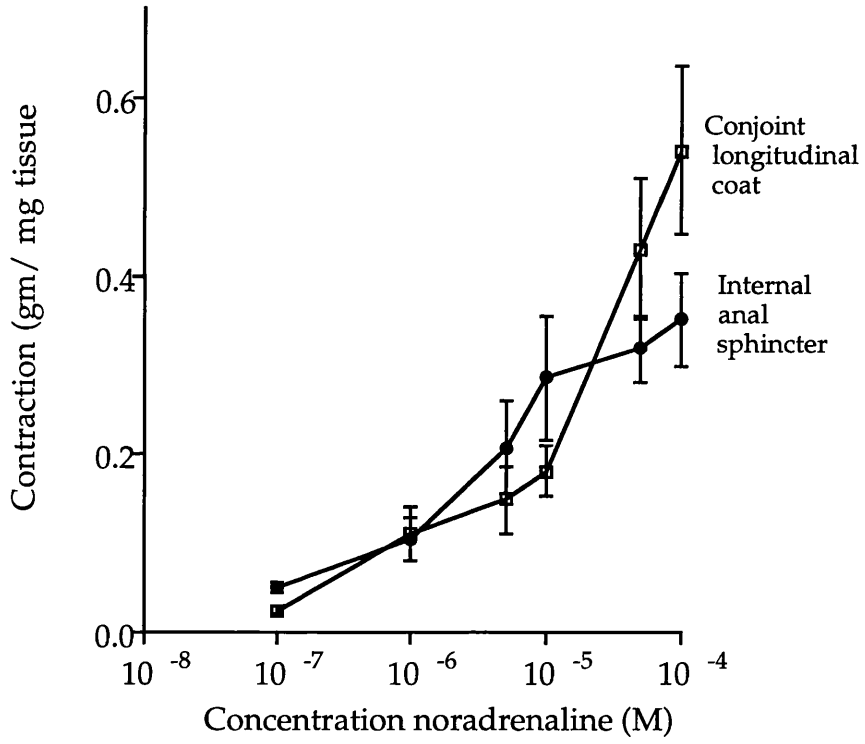
Noradrenaline caused dose dependent contractions in muscle strips from both the internal anal sphincter (*n=18(3)* ) and conjoint longitudinal coat (*n=18(5)* ). In each case, the drug was applied for 20 seconds. Isoprenaline, a β-adrenergic

agonist, produced dose-dependent relaxation of the internal anal sphincter when applied for the same time (*n=10(2)*). These effects are illustrated in figure 3.

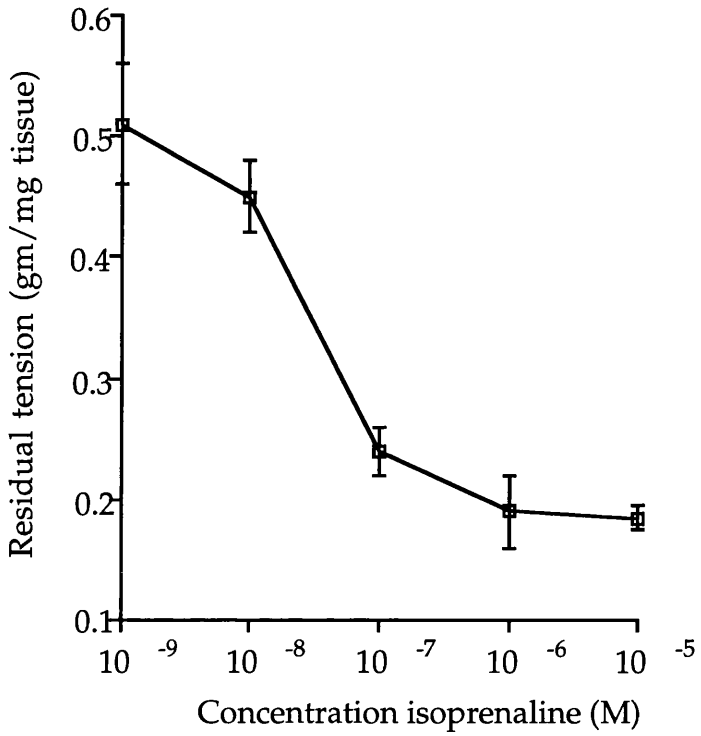
Figure 3.

The response of the internal anal sphincter and conjoint longitudinal coat smooth muscle to noradrenaline (i.), and the response of the internal anal sphincter to isoprenaline (ii.).

i.



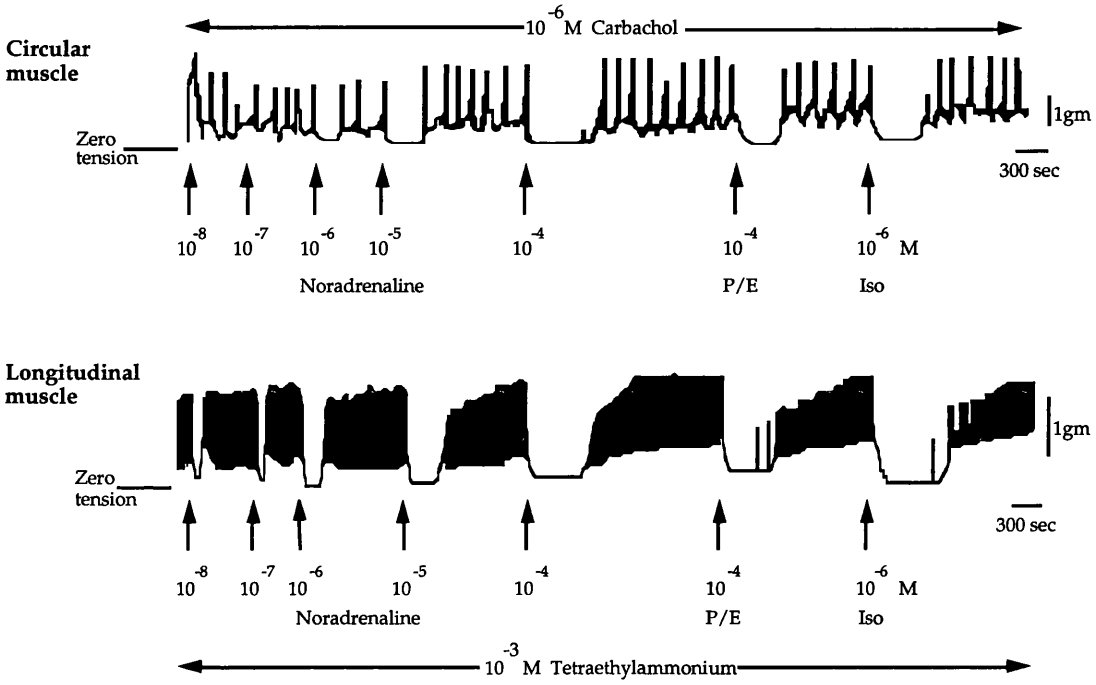
ii.




In their relaxed state, strips from neither layer of rectal smooth muscle responded to noradrenaline *(n=18(3)* ). If however, tension and activity were raised by the addition of carbachol (to circular strips-*n=12(2)*) and tetraethylammonium (to longitudinal strips-*n=12(2)* ), then noradrenaline was seen to have an inhibitory effect on both the frequency and amplitude of the resulting contractions. This inhibition could be reproduced by stimulation of both α– and β– adrenoceptors with phenylephrine or isoprenaline respectively (*n=12(2)* for each layer- Figure 4). (Tetraethylammonium is a potassium channel blocker which increases the tendency of smooth muscle membranes to depolarise. Incubation of rectal longitudinal muscle with this agent produced a 'stable' level of activity which did not decay over time. In contrast, carbachol (which was used first) did not produce such a stable response in this tissue whereas it did when it was applied to the circular muscle layer of the rectum.)

Figure 4.

The response of rectal smooth muscle to adrenergic stimulation. (P/E- phenylephrine; Iso- isoprenaline)




Analysis of the responses of the internal anal sphincter and the conjoint longitudinal coat reveals that in the presence of phentolamine, an α-adrenoceptor antagonist, the response of conjoint longitudinal coat strips to $5x10^{-5}$M noradrenaline was abolished *(n=12(2)* ) however, in the case of internal anal sphincter strips relaxation was revealed *(n=12(2)* ) (Figure 5). This modified internal anal sphincter response was abolished on addition of propranolol, a β–adrenoceptor antagonist *(n=6(2)* ). The responses to carbachol are discussed below.

Figure 5.

Analysis of the actions of carbachol and noradrenaline on isolated strips of human internal anal sphincter (*i.*), and conjoint longitudinal coat (*ii.*), illustrated in the form characteristic traces. Control responses to agonists are shown on the left, and the responses of the agonists in the presence of inhibitors is shown on the right.

Agonists: C- carbachol $10^{-4}$M NA- noradrenaline $5x10^{-5}$M

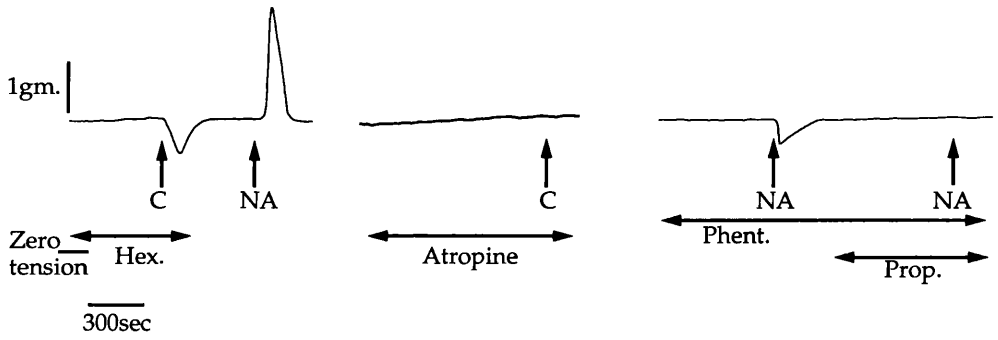
Antagonists: Atropine $10^{-6}$M (muscarinic cholinoceptors)

Hex- hexamethonium $5x10^{-6}$M (nicotinic cholinoceptors)

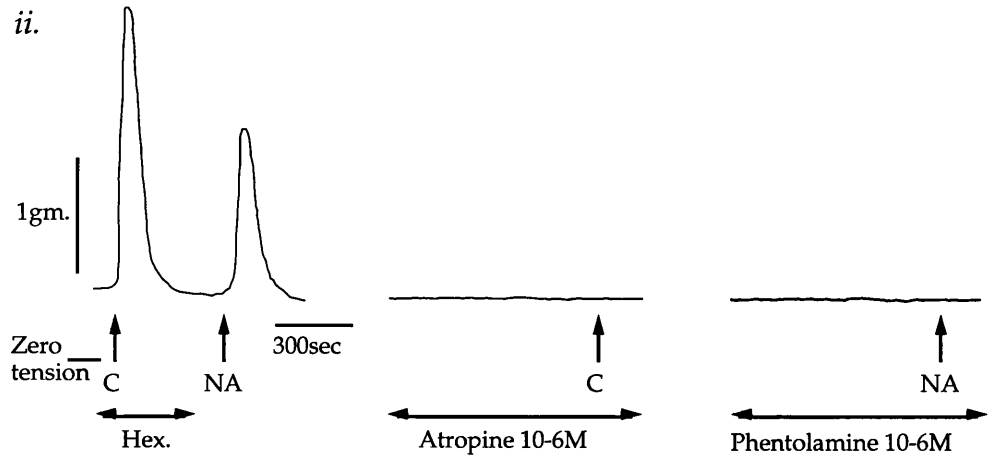
Phent- phentolamine $10^{-6}$M (α-adrenoceptors)

Prop- propranolol $5x10^{-6}$M (β-adrenoceptors)

*i.*



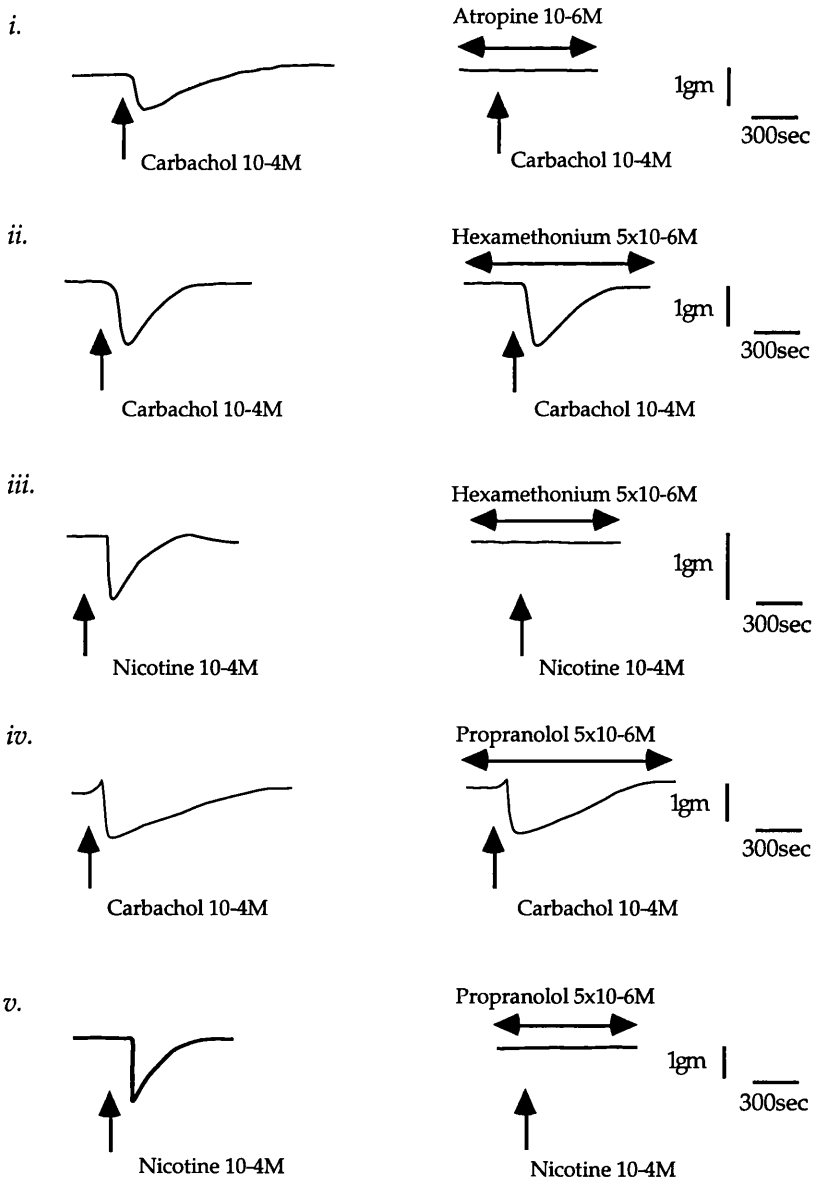

*ii.*



The response of internal anal sphincter smooth muscle to $10^{-4}$M carbachol was abolished in the presence of $10^{-6}$M atropine (a muscarinic cholinoceptor antagonist) (*n=11(2)*), but it was not reduced by $5x10^{-6}$M hexamethonium (a nicotinic, ganglionic cholinoceptor antagonist) (*n=8(4)*), which abolished the response of muscle strips to $10^{-4}$M nicotine (*n=7(2)*). In the presence of hexamethonium the relaxation produced by carbachol was 114.8±8.9% of control responses. In addition, the effect of carbachol ($10^{-4}$M) was unaltered by the addition of propranolol ($5x10^{-6}$M) (*n=6(2)*), a β-adrenoceptor antagonist. Relaxation in the presence of propranolol ($5x10^{-6}$M) was 94.6±5.19% of that seen in controls. Relaxation of muscle strips in response to nicotine ($10^{-4}$M) however, was abolished by propranolol ($5x10^{-6}$M) (*n=6(2)*). These points are illustrated in figures 5 and 6.

Figure 6

Characteristic responses of internal anal sphincter smooth muscle strips illustrating the effect of muscarinic and nicotinic cholinoceptor stimulation. *i.* Carbachol ($10^{-4}$) produces relaxation, a response which is abolished by atropine ($10^{-6}$M- a muscarinic cholinoceptor antagonist). *ii. and iv.* The response to carbachol is affected by neither hexamethonium ($5x10^{-6}$M- a nicotinic cholinoceptor antagonist) nor propranolol ($5x10^{-6}$M- a β-adrenoceptor antagonist). *iii. and v.* Relaxation produced by nicotine ($10^{-4}$), which stimulates nicotinic cholinoceptors, is abolished by both hexamethonium and propranolol at the same concentrations. Control responses to agonists are shown on the left and the corresponding response in the presence of an antagonist is shown on the right.



## DISCUSSION

Results obtained from the experiments described above indicate that *in vitro*, human internal anal sphincter smooth muscle generates and maintains spontaneous myogenic tension, contracts in response to α-adrenoceptor stimulation and relaxes in response to muscarinic, nicotinic and β-adrenoceptor stimulation. These finding agree with those previously reported by both Burleigh *et al.* (1979) and Speakman *et al.*, (1990; 1992) and as explained in the introduction to this chapter, relaxation observed in response to muscarinic cholinoceptor stimulation suggests that intrinsic nerves are not damaged by the tissue preparation methods used here.

The ability of muscle strips from the internal anal sphincter to generate and maintain spontaneous myogenic tension is shared to a lesser extent by the conjoint longitudinal coat and this behaviour is clearly different from that seen in the muscle layers of the rectum which had no spontaneous active tension in the organ baths. Smooth muscle strips from the internal anal sphincter and conjoint longitudinal coat are also alike in their contractile response to α-adrenergic stimulation again, clearly different from the relaxant responses of rectal smooth muscles. These aspects of internal anal sphincter and conjoint longitudinal coat behaviour suggests that they are both sphincter specialised smooth muscles in this region of the gut.

The mechanism responsible for the generation of spontaneous myogenic tension has been explored by Bouvier and Gonella (1981). Using the sucrose gap technique they examined tension and electrical activity in both layers of smooth

muscle taken from the anal sphincter of the cat. In those from the internal anal sphincter they noted slow variations in smooth muscle membrane potential called *electrical slow waves*. In strips from the longitudinal muscle layer, spike potentials were superimposed on these. Each slow wave (internal anal sphincter) or spike potential (conjoint longitudinal coat) was associated with an increase in basal tension. Both slow wave activity and tension disappeared in calcium free solution and in the presence of manganese ions, indicating that electro-mechanical coupling is calcium dependent. Similar studies have not been performed on these muscles in the human and research is required to confirm if the mechanisms are the same. This area may well be a fertile ground for the pharmacological manipulation of sphincter activity in the future.

The difference between the response of the circular smooth muscles of the anal canal and rectum to α- and β-adrenoceptor stimulation has been described previously in the cat (Kerremans and Penninckx, 1970) and the vervet monkey (Rayner, 1979). This has not previously been described in man nor has the difference in the behaviour of the rectal and anal longitudinal muscle layers. The mechanisms which underlie these differences in activity were not determined in this study.

These results show that there is a difference in the mechanism which is responsible for internal anal sphincter smooth muscle relaxation in response to nicotinic and muscarinic cholinoceptor stimulation. Nicotinic receptor stimulation produces relaxation through release of an adrenergic neurotransmitter that stimulates β-adrenoceptors. The mechanism by which muscarinic cholinoceptor stimulation produces relaxation is clearly different and it is an outstanding finding because most other smooth muscles contract in

response to this stimulus. Indeed, this phenomenon is shared only by endothelialised vascular smooth muscle preparations, where relaxation is dependent upon production of NO (Furchgott and Zawadzki, 1980). Clearly this aspect of the behaviour of internal anal sphincter smooth muscle requires further attention as it might have important implications with regard to the interaction of cholinergic nerves, including postganglionic parasympathetic nerves with the sphincter itself.

# *Chapter 5*

## INVOLVEMENT OF NITRIC OXIDE IN NEUROGENIC RELAXATION OF THE HUMAN INTERNAL ANAL SPHINCTER SMOOTH MUSCLE *IN VITRO*.

# INTRODUCTION

## 1. Non-adrenergic, non-cholinergic neurotransmission in the internal anal sphincter

Non-adrenergic non-cholinergic (NANC) nerves play an important role in the activity of the internal anal sphincter, particularly as they are responsible for mediating the rectoanal inhibitory reflex (Garrett *et al.*, 1974; Meunier and Mollard, 1977; Rayner, 1979; Bouvier and Gonella, 1981). However, little is known of the morphology, connections and distribution of these nerves and one of the main reasons for this is that the neurotransmitter they release has not been identified with any degree of certainty. This is especially true in man.

Adenosine triphosphate (ATP) has been implicated as a neurotransmitter in the NANC innervation of the guinea pig internal anal sphincter (Lim and Muir, 1986; Baird and Muir, 1990), whereas involvement of vasoactive intestinal peptide (VIP) has been suggested in the opossum (Nurko and Rattan, 1988) and rabbit (Biancani *et al.*, 1985). In man, the role of these agents seems unlikely. Adenosine triphosphate does relax human internal anal sphincter smooth muscle *in vitro.* In addition, ATP desensitisation and the ATP antagonist 2-2′ pyridylisatogen tosylate reduce the response of the tissue to both ATP and electrical field stimulation but, this effect was non-selective as the response to isoprenaline was also diminished (Burleigh, 1979). Vasoactive intestinal peptide also relaxes human internal anal sphincter smooth muscle *in vitro* but its involvement as an inhibitory neurotransmitter here seems unlikely because α-chymotrypsin reduces the effect of VIP but has no attenuating effect on neurogenic relaxation (Burleigh, 1983).

## 2. Is nitric oxide involved?

The discovery that in lesser mammals NO is involved in inhibitory neurotransmission in some gut smooth muscles (stomach-Boeckxstaens *et al.*, 1990; duodenum-Toda *et al.*, 1990; ileocolonic junction-Bult *et al.*, 1990; Hata *et al.*, 1990) suggested a potential role for NO in neurogenic relaxation of human internal anal sphincter smooth muscle. This proposition was supported by the results of preliminary experiments conducted using pig tissue. To determine if NO is involved in the human, the following was investigated:

**Is NO is involved in neurogenic relaxation of human internal anal sphincter smooth muscle *in vitro* ? If it is, then the effect of inhibitory nerve stimulation should be mimicked by exogenous application of NO and antagonised both by inhibitors of nitric oxide synthase and by agents which scavenge NO.**

## METHODS

Sphincter tissue was obtained from patients undergoing abdomino-perineal resection of the rectum and anal canal for low-lying rectal carcinoma (4 males, 7 females; median age 69 years, range 57-82). Strips of smooth muscle were excised from the distal 2cm of the internal anal sphincter and all strips contained parallel bundles of smooth muscle fibres.

Once dissected, muscle strips were mounted for isometric tension recording in superfusion organ baths as described previously. Once the strips had reached a steady state of tension, their response to electrical field stimulation was determined. The stimulation parameters used were similar to those described previously by Speakman *et al.*, (1990) and as described previously, these had been found to stimulate neurogenic relaxation in pig internal anal sphincter smooth muscle. The neurogenic nature of this response was assessed by incubating the strips with tetrodotoxin ($3x10^{-6}M$) and involvement of NANC neurotransmitter(s) was determined by studying the effects of cholinergic and adrenergic antagonists on the response of muscle strips to electrical field stimulation as well as cholinergic and adrenergic agonists. Following this, the dose dependent effect of sodium nitroprusside, an exogenous donor of NO, was determined. The action of inhibitors of nitric oxide synthase and oxyhaemoglobin on the response to electrical field stimulation was then investigated.

# RESULTS

## 1. Response to inhibitory nerve stimulation

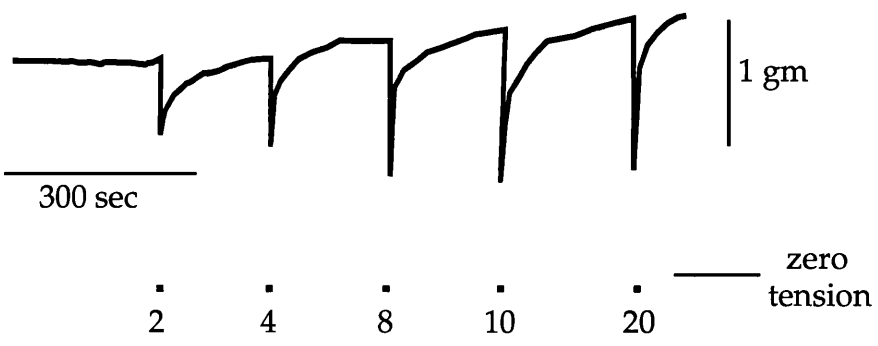
Smooth muscle strips from the human internal anal sphincter relax in response to electrical field stimulation using a pulse strength of 10 volts and a pulse duration of 0.5 ms. The relaxations are frequency dependent, reaching a maximum at 8-10 Hz. They can be repeated at 3 minute intervals. Responses occur in the presence of atropine ($10^{-6}$M), phentolamine ($10^{-6}$M) and propranolol ($5x10^{-6}$M) which abolish the response of the tissue to muscarinic cholinoceptor and $\alpha/\beta$ adrenoceptor stimulation. In addition, the frequency dependent relaxations are unaffected by pre-incubation with atropine ($10^{-6}$M) and guanethidine ($3x10^{-6}$M) for 20 minutes, but they are abolished by tetrodotoxin ($3x10^{-6}$ M).

These results, which are illustrated in figures 1, 2 and 3, confirm that the response of human internal anal sphincter smooth muscle to electrical field stimulation (as described here), are neurogenic and are mediated by a NANC neurotransmitter.

Figure 1.

The response of isolated strips of internal anal sphincter smooth muscle to electrical field stimulation is illustrated in the form of a characteristic trace *(i.)* and a frequency response curve *(ii.)*. At each •, impulses of 10V are applied for 0.5ms duration, over a range of frequencies.

*i.*



*ii.*

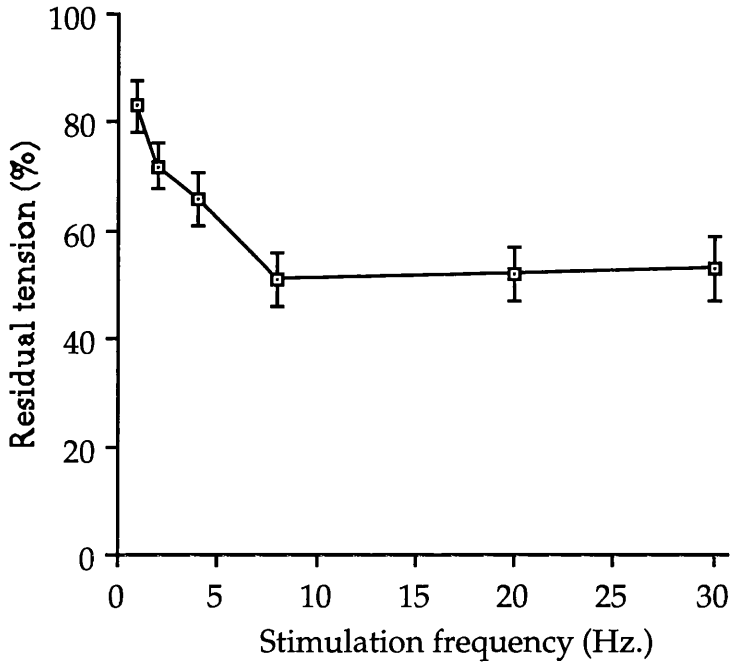
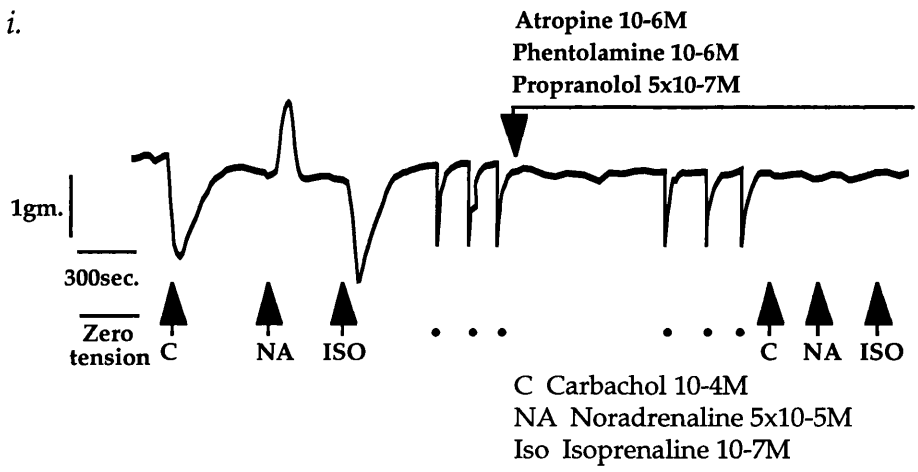



Figure 2.

*i.* A characteristic trace which illustrates the response of a muscle strip to cholinergic and adrenergic agonists, and to repetitive electrical field stimulation (•= 10V, 0.5ms duration, 10Hz, for 1 sec). In the presence of muscarinic (atropine), α-adrenergic (phentolamine) and β-adrenergic (propranolol) receptor antagonists, the response to electrical field stimulation is unchanged but, the action of the agonists is abolished. *ii.* The frequency response curve of internal anal sphincter smooth muscle strips in the presence and absence of atropine and guanethidine, which inhibit adrenergic and cholinergic neurotransmission.

*i.*



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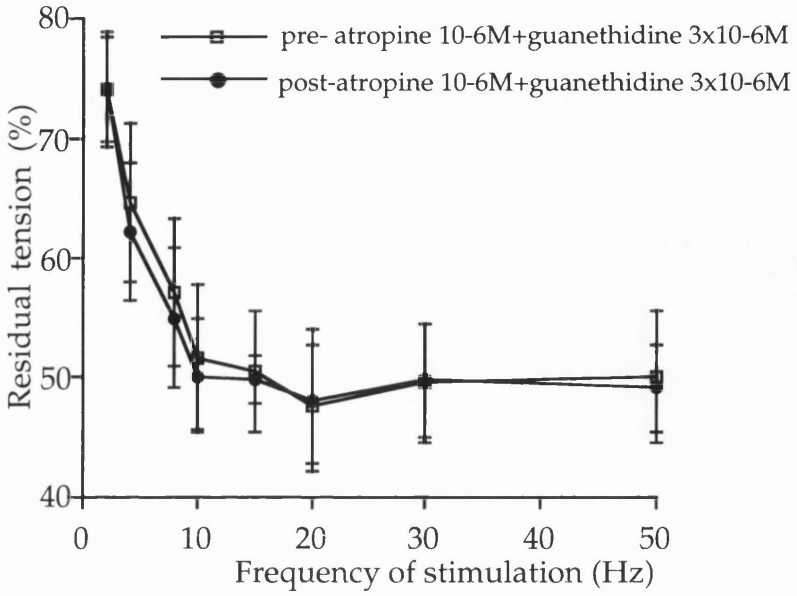
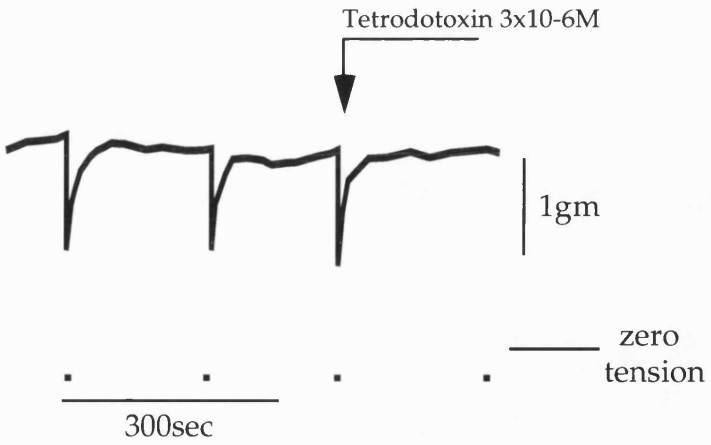



Figure 3.

A characteristic trace to illustrate the neurogenic nature of the response of internal anal sphincter smooth muscle to electrical field stimulation (•= 10V, 0.5ms duration, 10Hz, for 1 sec) . The response is abolished following addition of tetrodotoxin to the superfusate.

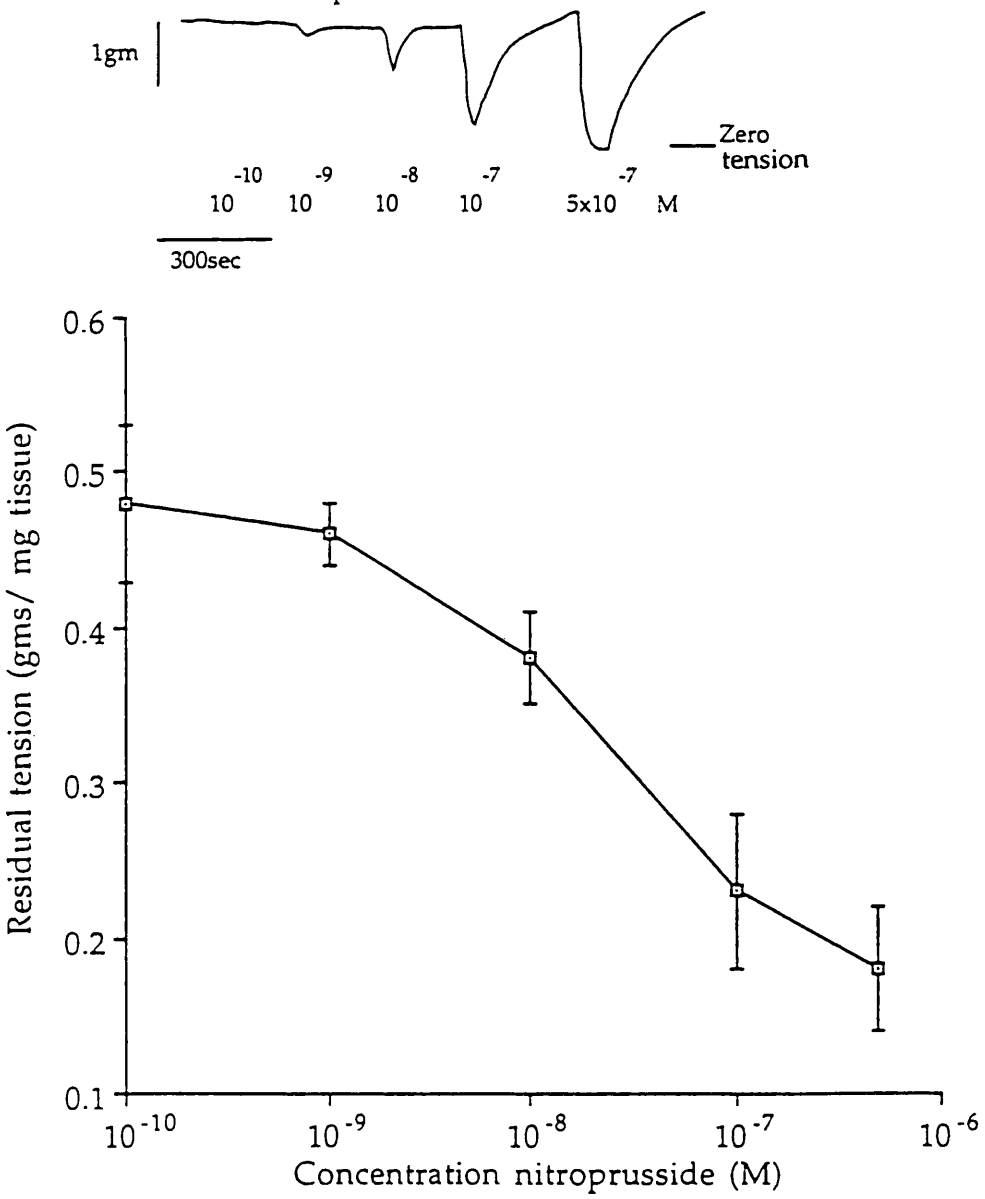


## 2. Response to sodium nitroprusside

Sodium nitroprusside is an exogenous donor of nitric oxide (Feelisch and Noack, 1987), and its addition to the superfusate caused dose dependent relaxation of the muscle strips (*n=18 (4)*) (Figure 4). Sodium nitroprusside was applied for 60 seconds and a maximal response was achieved at a concentration of $5x10^{-7}M$, when the residual tone in the strips was equivalent to that present in calcium free solution (zero tension).

Figure 4.

The effect of sodium nitroprusside shown in the form of a characteristic trace and a cumulative dose response curve.

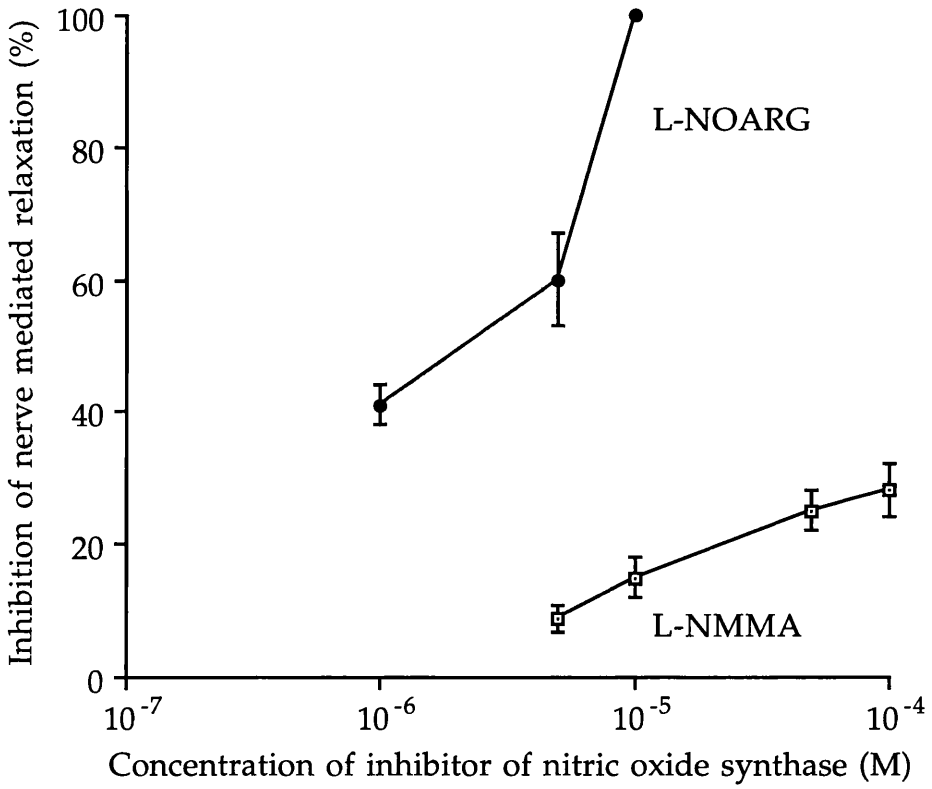


## 3. Response to inhibitors of nitric oxide synthase

N-monomethyl-L-arginine and N-nitro-L-arginine are synthetic analogues of L-arginine, and competitive antagonists of nitric oxide synthase. Their dose dependent effect upon electrically induced neurogenic relaxation of human internal anal sphincter smooth muscle is illustrated in figure 5 (*n=24(5)* ).

Figure 5.

The effect of N-monomethyl-L-arginine (L-NMMA) and N-nitro-L-arginine (L-NOARG, which are both inhibitors of nitric oxide synthase, on nerve mediated relaxation of internal anal sphincter smooth muscle induced by electrical field stimulation (10V, 0.5ms duration, 10Hz, for 1 sec).



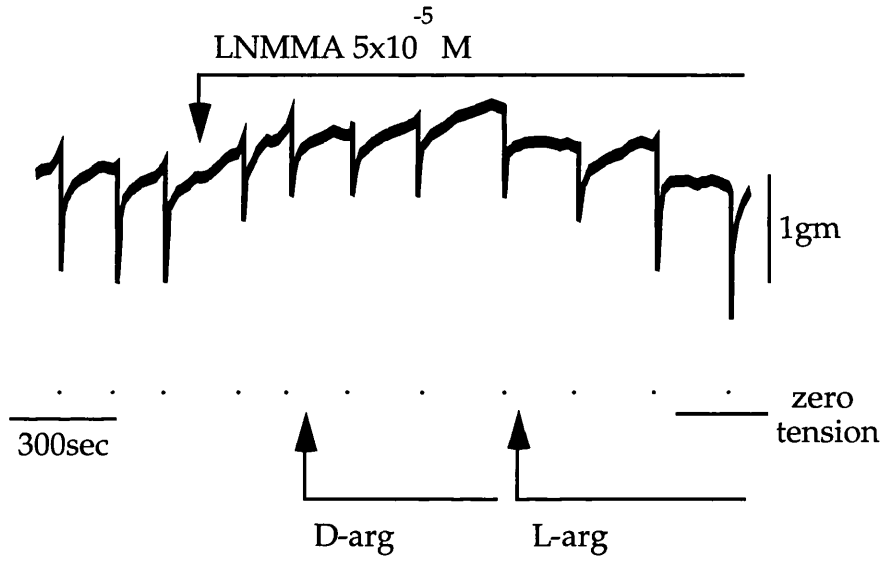

At a concentration of $5x10^{-5}$ M, N-monomethyl-L-arginine produced a partial but significant inhibition of the nerve mediated relaxation (10V, 0.5ms. duration, 10Hz, for 1 sec), reducing the relaxant response to 72.4± 3.5% of its original size ($p<0.05$). Addition an excess concentration of D-arginine ($5x10^{-4}$ M) had no

effect upon this inhibition, but it was reversed by L-arginine at the same concentration. N-monomethyl-D-arginine, the enantiomer (stereoisomer) of N-monomethyl-L-arginine, had no effect upon the tissue. These points are illustrated in figure 6.

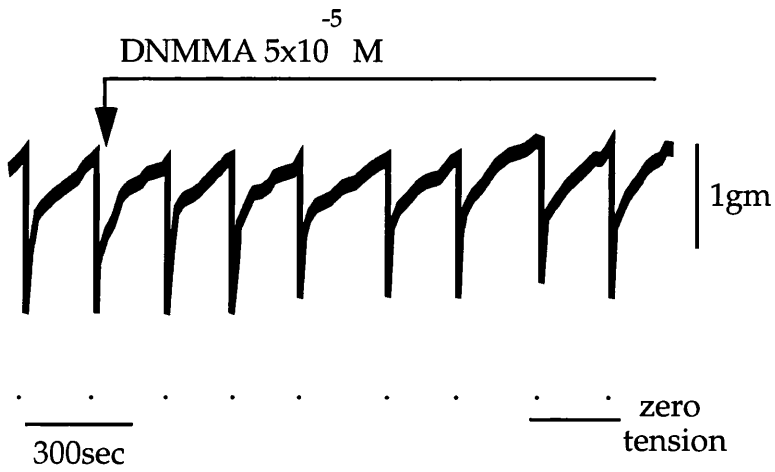
Figure 6.

Characteristic traces illustrating the effects of *(i.)* N-monomethyl-L-arginine (L-NMMA) and *(ii.)* N-monomethyl-D-arginine (D-NMMA) on nerve mediated relaxation of human internal anal sphincter smooth muscle (•= 10V, 0.5ms duration, 10Hz, for 1 sec). The reversing action of L-arginine (L-arg $5x10^{-4}$ M), but not D-arginine (D-arg $5x10^{-4}$ M) is evident.

*i.*



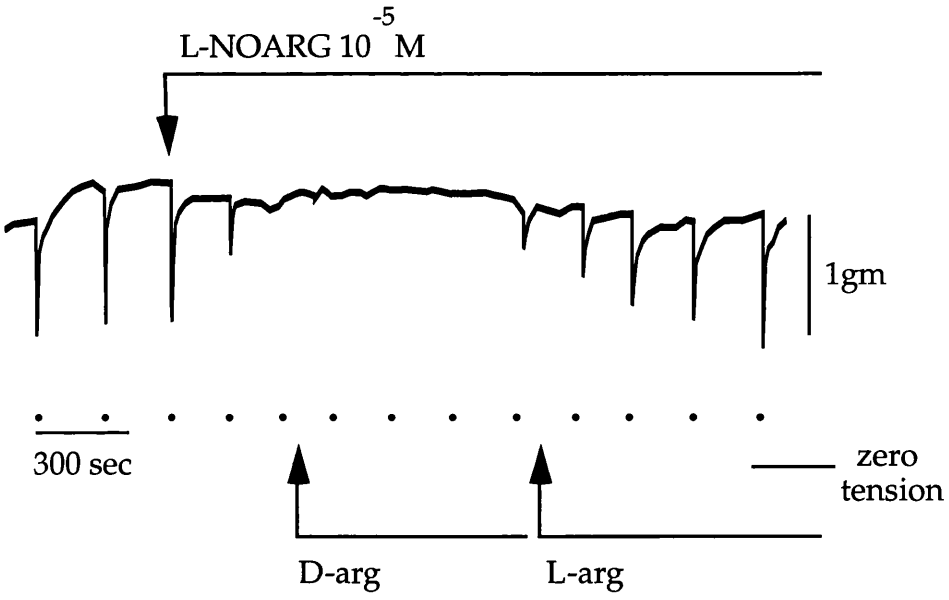

*ii.*



N-nitro-L-arginine is a more powerful antagonist of nitric oxide synthase. Its addition to the superfusate ($10^{-5}$M) abolished the response of the strips to electrical field stimulation (10V, 0.5ms. duration, 10Hz, for 1 sec;*n=30 (6)*). D-arginine ($5x10^{-4}$M) had no effect upon this inhibition but it was reversed by the addition of L-arginine at the same concentration. These points are illustrated in figure 7.

Figure 7.

A characteristic trace which illustrates the effect of $10^{-5}$M N-nitro-L-arginine (L-NOARG) on nerve mediated relaxation of internal anal sphincter smooth muscle (• = 10V, 0.5ms duration, 10Hz, for 1 sec). In the presence of L-NOARG, neurogenic relaxation disappears. Its action is antagonised by L-arginine (L-arg $5x10^{-4}$ M), but not D-arginine (D-arg $5x10^{-4}$ M).



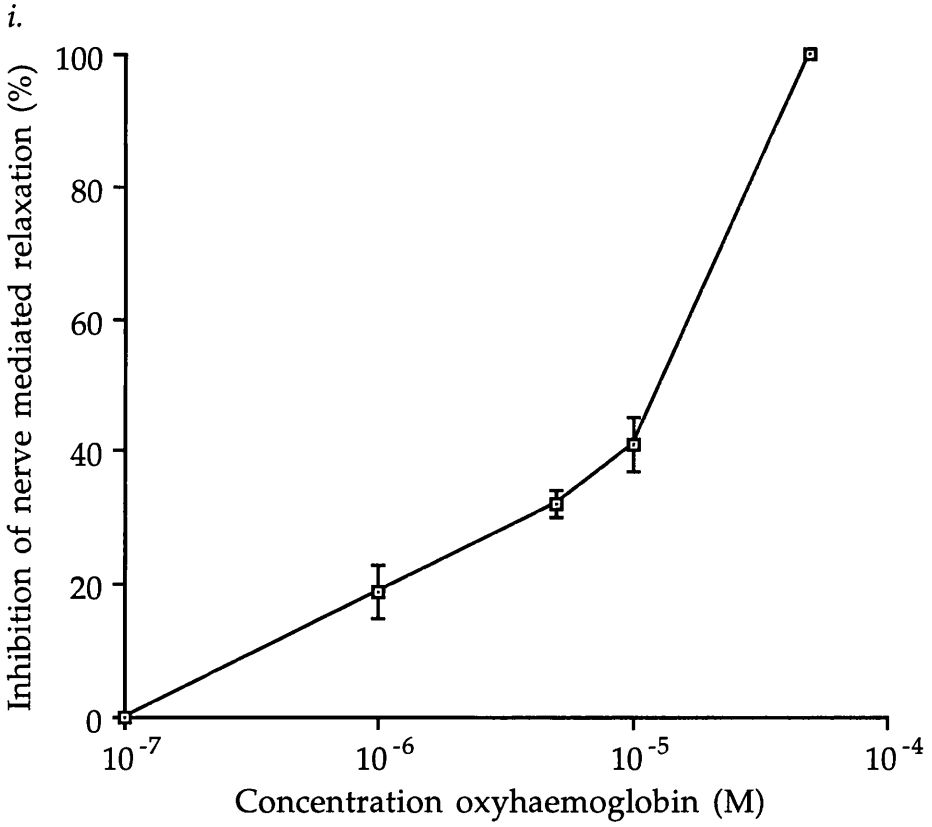

## 4. Effect of haemoglobin

Oxyhaemoglobin has a high affinity for nitric oxide and scavenges it from extracellular media; methaemoglobin has no such action (Martin *et al.*, 1986; Salvemini *et al.*, 1990). Addition of oxyhaemoglobin produced inhibition of nerve mediated relaxation in a dose dependent manner (*n=12(2)*), and the

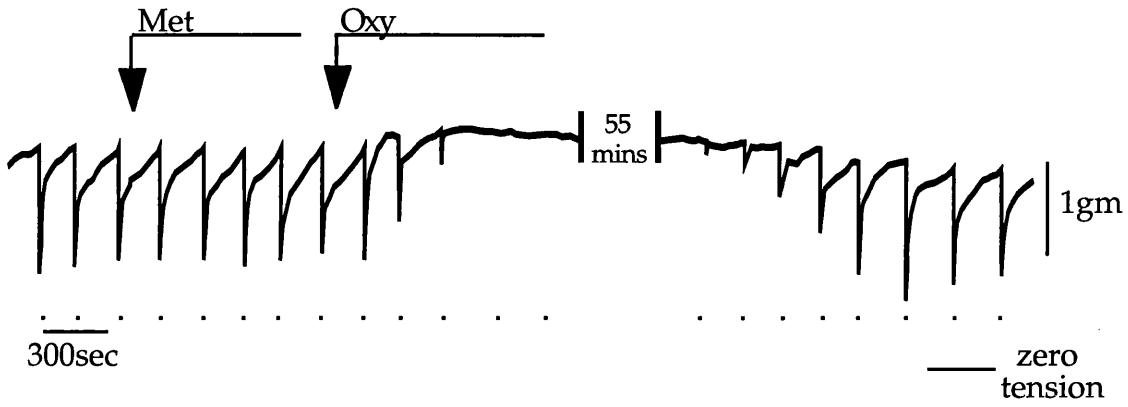
neurogenic response was abolished at a concentration of $5x10^{-5}M$ (*n=20(5)*). Relaxations returned after withdrawal of oxyhaemoglobin and a period of recovery. Methaemoglobin had no effect upon the relaxant response of the tissue. These points are illustrated in figure 8.

Figure 8.

*(i.)*. Oxyhaemoglobin inhibits nerve mediated relaxation of human internal anal smooth muscle in a concentration dependent manner. *(ii.)* A characteristic trace which illustrates that neurogenic relaxation (•= 10V, 0.5ms duration, 10Hz, for 1 sec) is reversibly abolished by oxyhaemoglobin (oxy) at a concentration of $5x10^{-5}M$ Methaemoglobin (met) has no such action at the same concentration.



*ii.*



## DISCUSSION

Using the parameters defined in these experiments, electrical field stimulation of isolated strips of human internal anal sphincter smooth muscle resulted in NANC nerve mediated relaxation. Such relaxations were inhibited and indeed abolished by antagonists of nitric oxide synthase as well as by oxyhaemoglobin which scavenges NO in extracellular media. The action of inhibitors of nitric oxide synthase was dose dependent and enantiomer specific. In addition, the effects of nerve stimulation were mimicked by sodium nitroprusside, an exogenous source of NO. These results therefore suggest that in this tissue NO is involved in neurogenic relaxation but, neither its source nor its precise role as a messenger molecule are revealed. Involvement of other putative neurotransmitters cannot be excluded but, if NO is released from nerves, then there appears little need to implicate their involvement especially because NO seems to play such a pivotal role in the relaxant response seen here.

The potential sources of NO within the internal anal sphincter include enteric neurones, macrophages, myocytes and endothelial cells. Two of these can probably be discounted on theoretical grounds. Production by macrophages is unlikely because the pattern of NO generation observed here is characteristic of that associated with the constitutive form of nitric oxide synthase rather than the inducible form of the enzyme. The constitutive form of nitric oxide synthase (present in nerves and endothelial cells) produces NO for short periods of time in response to receptor or physical stimulation, whereas the inducible form of the enzyme which is present in activated macrophages produces NO for long periods and continues once the stimulus which initiated its production is withdrawn.

Production of NO by myocytes is also unlikely as this source would not explain the effect of oxyhaemoglobin observed in these experiments. Oxyhaemoglobin remains outside cells and thus to inhibit the action of NO, oxyhaemoglobin must scavenge it as NO passes extracellularly. It is unlikely therefore, that NO is produced in the same cell in which it exerts its action.

Neurones and endothelial cells are thus the most plausible sources of NO within the internal anal sphincter. Strong evidence in favour of the former, and hence NO being a neurotransmitter in the internal anal sphincter, would be provided by the demonstration of nitric oxide synthase containing nerves within this tissue.

# *Chapter 6*

## STRUCTURE AND DISTRIBUTION OF NITRIC OXIDE SYNTHASE CONTAINING NEURONES IN THE HUMAN ANORECTUM

# INTRODUCTION

## 1. The rectoanal inhibitory reflex and the involvement of nitric oxide

The rectoanal inhibitory reflex is a fundamental component of normal internal anal sphincter behaviour which is mediated by NANC enteric inhibitory neurones (Denny-Brown and Robertson, 1935; Garrett *et al.*, 1974; Meunier and Mollard, 1977; Rayner, 1979; Bouvier and Gonella, 1981; Lubowski *et al.*, 1987; Kamm *et al.*, 1989). The neuronal circuitry involved is presumed to be analogous to that which subserves the intestinal descending inhibitory reflex (Furness and Costa, 1987). This implies that relaxation of the internal anal sphincter is mediated by inhibitory motor nerves whose cell bodies lie within rectal myenteric ganglia and whose processes traverse the rectal myenteric plexus to gain access to the internal anal sphincter.

The finding in the previous chapter that NO is possibly the neurotransmitter which mediates neurogenic relaxation of human internal anal sphincter smooth muscle, makes it also a plausible candidate for the neurotransmitter which mediates the rectoanal inhibitory reflex in man. This proposition is supported by studies by Hata *et al.*, (1990) who demonstrated involvement of NO in the intestinal descending inhibitory reflex in isolated segments of rat colon. These workers found that localised distension of the gut tube caused caudal circular smooth muscle relaxation, a response which was unaffected by desensitisation with ATP, neurotensin or vasoactive intestinal peptide. The reflex however, was inhibited by N-nitro-L-arginine and mimicked by exogenous application of NO. The effect of N-nitro-L-arginine was counteracted by L-arginine.

In order to establish further the involvement of NO as the neurotransmitter in the human internal anal sphincter and if it could mediate the rectoanal inhibitory reflex in man, it is necessary to demonstrate that there is a neuronal source for NO within the sphincter and that the morphological characteristics and distribution of such nerves are appropriate for them to transfer information between the rectum and the internal anal sphincter. I therefore sought to determine if this was the case.

## 2. Demonstration of nitric oxide synthase

Following the discovery that NO is an endogenous messenger molecule, several groups worked to isolate nitric oxide synthase in order that it could be characterised, and its distribution determined. Bredt *et al.* (1990) were the first to report success. They extracted the constitutive form of nitric oxide synthase from rat cerebellar homogenate and showed it to be a monomer with a molecular mass of 150kDa. This enzyme has an absolute dependence on calmodulin and calcium for its activity and requires several electron donors as co-factors; NADPH, FMN, FAD and tetrahydrobiopterin (Bredt *et al.*, 1990). Structurally, nitric oxide synthase has been shown to have recognition sites for these and its the C-terminal end resembles cytochrome P-450 (Bredt *et al.*, 1991). Transfection of cloned DNA (coded for nitric oxide synthase) into human kidney 293 cells in tissue culture resulted in expression of nitric oxide synthase protein and acquisition of nitric oxide synthase enzymatic activity (Bredt *et al,.* 1991). These were not present in untransfected cells.

Following its isolation and purification, Bredt *et al.* (1991) inoculated neuronal nitric oxide synthase in to rabbits and generated an anti-nitric oxide synthase antiserum. With this they investigated the distribution of nitric oxide synthase immunoreactivity in rat brain and peripheral tissues. They demonstrated that

nitric oxide synthase immunoreactivity occurs in discrete neuronal populations in the rat brain and is concentrated in the neural innervation of the posterior pituitary, in autonomic nerve fibres in the retina, in cell bodies and nerve fibres in the duodenal myenteric plexus, in the adrenal medulla, and in vascular endothelial cells. Neuronal nitric oxide synthase immunoreactivity was not found in the parenchymal cells in other organs (liver, kidney, spleen, thymus, lung, testis and salivary gland), nor was it exhibited in fat, fibroblast, epithelial, or muscle cells.

## 3. Association between nitric oxide synthase and NADPH diaphorase

The dependence of nitric oxide synthase on NADPH as a co-factor for its activity and the distribution of nitric oxide synthase immunoreactivity, suggested a possible association between nitric oxide synthase and a second enzyme, namely NADPH diaphorase (Hope *et al.*, 1991). Activity of the latter in paraformaldehyde-fixed tissue had been well defined previously in both the brain and the gut, where discrete populations of neurones are stained (Vincent, 1992). The histochemical technique used to reveal NADPH diaphorase activity is based on the presence in such cells of a NADPH-dependent enzyme which can catalyse the conversion of a soluble tetrazolium salt to an insoluble blue formazan dye. The function of the enzyme remained a mystery however until Hope *et al.* (1991) isolated and purified NADPH diaphorase and showed that its immunological and biochemical properties were indistinguishable from those previously described for nitric oxide synthase. Subsequently Dawson *et al.* (1991) demonstrated co-localisation of nitric oxide synthase immunoreactivity with NADPH diaphorase activity in rat brain and peripheral tissues, including the gut. In addition they showed that human kidney cells transfected with nitric

oxide synthase coded DNA, acquire NADPH diaphorase staining whereas, this is not present in untransfected cells.

The importance of this co-localisation is two-fold. Firstly, the staining pattern produced by NADPH diaphorase histochemistry is explained and secondly, a relatively simple staining technique can be used for the further investigation of nitric oxide synthase containing cells. The proviso of course is that co-localisation is consistent in the animal and tissue being studied.

The purpose of the experiments described in this chapter was therefore three-fold;

***i.* to demonstrate nitric oxide synthase immunoreactivity in the human anorectum,**

***ii.* to explore co-localisation of nitric oxide synthase immunoreactivity and NADPH diaphorase staining in this tissue and,**

***iii.* to determine the morphology and distribution of nitric oxide synthase containing neurones in the human anorectum and to assess if these are consistent with them mediating the rectoanal inhibitory reflex.**

Previous studies (Weinberg, 1970 and Baumgarten *et al.*, 1973) have demonstrated that intrinsic nerve processes are sparse within the internal anal sphincter, and few if any nerve cell bodies are found here. Instead, the cell bodies of intrinsic nerves are situated above in the rectal myenteric plexus and their axons pass caudally to innervate the smooth muscle of the sphincter (Baumgarten *et al.*, 1971 and Furness and Costa 1987). In view of this and the

limited availability of the antibody directed against nitric oxide synthase, rectal tissue was used for immuno-localisation studies.

## METHODS

Tissue was obtained from 9 patients undergoing abdomino-perineal resection for low rectal carcinoma (median age 65 years, range 40-80 years). In addition, specimens were also obtained from 3 patients undergoing anterior resection for more proximal rectal carcinoma (median age 62 years, range 43-78 years). Tissue from the specimens was processed for cryostat sections and for whole-mount histochemistry.

Sequential cryostat sections of the rectum were stained for the following;

*i.* Nitric oxide synthase immunoreactivity

*ii.* Nitric oxide synthase immunoreactivity followed by NADPH diaphorase activity

*iii.* NADPH diaphorase activity

*iv.* SMI 311 and 312 (pan-neuronal/pan-axonal) immunoreactivity.

Whole-mount preparations were stained for NADPH diaphorase histochemistry.

The following controls were included:

*a.* Immunohistochemistry

- primary antibody omitted and,
- primary and secondary antibodies omitted and,
- endogenous peroxidase activity.

*b.* NADPH diaphorase histochemistry;

- Nitro blue tetrazolium omitted and,
- NADPH omitted.

In order to demonstrate co-localisation of nitric oxide synthase immunoreactivity and NADPH diaphorase activity, sections were stained first for nitric oxide synthase immunoreactivity and then stained again for NADPH diaphorase activity as it was not possible to visualise nitric oxide synthase immunoreactivity after staining for NADPH diaphorase activity. In addition, it proved impossible to stain for NADPH diaphorase activity following demonstration of nitric oxide synthase immunoreactivity using diamino-benzine (DAB) as the chromagen. Either no staining occurred at all or there was non-specific darkening of the section which gave a false impression of positivity. The cause of this remains obscure. In view of these difficulties, the chromagen was changed to amino-ethyl carbazole (AEC), which revealed nitric oxide synthase immunoreactivity and permitted subsequent NADPH diaphorase staining.

# RESULTS

## 1. Controls

There was no staining following omission of the primary antibody, primary and secondary antibodies, nitro blue tetrazolium or NADPH. In co-localisation studies, the appearance of nitric oxide synthase immunoreactivity was unchanged if nitroblue tetrazolium or NADPH were omitted from the secondary reaction medium. No staining of whole mounts occurred if either NADPH or nitro blue tetrazolium was omitted from the incubation medium. Endogenous peroxidase activity was present principally within the epithelium where it was contained in neutrophils. Occasional inflammatory cells (lymphocytes and neutrophils) were also seen in the other tissue layers but no endogenous peroxidase activity was seen in nerve or muscle.

## 2. Nitric oxide synthase immunoreactivity

In the rectum, nitric oxide synthase immunoreactive nerve cell bodies are found predominantly within ganglia in the myenteric plexus. There are few nerve cell bodies containing nitric oxide synthase immunoreactivity in the submucosal plexus. The intensity of staining is variable but occurs throughout the cytoplasm (figure 1). Nuclei remain pale. Immunoreactive nerve fibres are present in the circular and longitudinal muscle layers, where they are aligned parallel (running around the gut) and perpendicular to muscle fibres (passing towards or away from the gut lumen). Some nerve fibre bundles run through the circular muscle layer to reach the submucosal plexus.

Nitric oxide synthase immunoreactivity was not seen in any other cell and in particular, smooth muscle cells showed no evidence of immunoreactivity.

## 3. Co-localisation of nitric oxide synthase immunoreactivity and NADPH diaphorase activity

Staining of individual ganglia is illustrated in figure 1. Out of 180 nerve cell bodies stained, 164 (91.1%) contained both nitric oxide synthase immunoreactivity and NADPH diaphorase activity. Of the remainder, 12 (6.7%) were nitric oxide synthase immunoreactive only and 4 (2.2%) contained NADPH diaphorase activity only. Thus the probability that NADPH diaphorase histochemistry will stain positive a cell body which also contains nitric oxide synthase immunoreactivity is 91.1%, whereas the chance of a NADPH diaphorase active cell not containing nitric oxide synthase is only 2.2%. It appears therefore that NADPH diaphorase histochemistry is an accurate marker for nitric oxide synthase immunoreactivity in myenteric neurones and it was used as such in subsequent experiments.

Too few submucosal neurones were stained for nitric oxide synthase immunoreactivity to allow meaningful quantitative analysis of co-localisation however, of those stained, all appeared to also contain NADPH diaphorase activity.

## 4. NADPH diaphorase staining and SMI 311 and 312 (pan-neuronal/ pan-axonal) immunoreactivity.

When sections were stained for both NADPH diaphorase activity and SMI 311 and 312 (pan-neuronal/ pan-axonal) immunoreactivity it was apparent that NADPH diaphorase stained cell bodies and their processes form a sub-population of the total neurones present because some nerve cell bodies contain SMI 311/312 immunoreactivity but not NADPH diaphorase activity. Figure 2

illustrates this. Nerve cell bodies which do not stain for NADPH diaphorase activity are also present in the submucosal plexus.

## 5. Morphology of nitric oxide synthase containing neurones

By performing NADPH histochemistry on whole-mount preparations of the rectum, the morphology of individual neurones becomes apparent. Although not all identical, positively stained neurones have similar morphological features which are characteristic of those ascribed to Dogiel Type 1 neurones within the enteric nervous system (Furness and Costa 1987). Nerve cell bodies are 18-26μm wide and 18-34μm long. They have several broad dendrites (called dendritic lamellae), an oval nucleus and a single axonal process which emerges from the apex of the cell (figure 3).

## 6. Distribution of nitric oxide synthase containing nerves in the rectal myenteric plexus.

In the proximal two-thirds of the rectum, most of the NADPH diaphorase containing neurones lie within or are in close proximity to ganglia. Ganglia in this region of the rectum contain 33±4 NADPH positive neurones and have a surface area of 0.2±0.06 $mm^2$ (*n=50*). A few neurones are seen outside these ganglia, in small nerves which pass into or run within the muscle layers (secondary and tertiary strands). The geometry of the myenteric plexus in this region varies (rectangular, pentagonal or hexagonal), but it is continuous and evenly distributed both around the circumference and along this segment of the gut. These points are illustrated in figure 4.

Axonal processes from positively stained nerve cell bodies are seen throughout the plexus in the primary, secondary and tertiary meshworks. The latter is seen in both the circular and longitudinal muscle coats, where nerve processes are seen lying parallel to smooth muscle fibres.

The appearance of the myenteric plexus in the distal 3-4cm of the rectum is markedly different from that in the majority of the rectum above. Here the primary meshwork has definite polarity, with ganglia and internodal strands aligned along the cranio-caudal axis of the gut. There is a paucity of processes passing circumferentially around the rectum in this region. Ganglia are smaller ($0.04\pm0.007mm^2$) and appear densely packed with positively stained neurones ($794\pm57$ neurones/$mm^2$ compared with $250\pm19$ in the proximal part of the rectum, $p<0.001$; $n=50$). Interestingly, the number of neurones in each ganglion in this region of the rectum is similar to that above ($29\pm3$ neurones per ganglia).

Some axons communicate with specialised nerve trunks which traverse the plexus along the cranio-caudal axis of the rectum. These nerves bypass ganglia and their appearance is similar to that previously described for structures called *shunt fascicles*. Shunt fascicles are a prominent feature in the distal rectal myenteric plexus and are seen to extend distally into the anal canal. They stain intensely and lie along the cranio-caudal axis of the rectum. These features are illustrated in figure 5.

## 7. Distribution of nitric oxide synthase containing nerves in the rectal submucosal plexus

NADPH diaphorase activity is present within the submucosal plexus in the rectum. However, as can be seen in figure 6 this is a much less substantial structure than the myenteric plexus in this region and contains comparatively

few positively stained neurones and their processes. The neurones which are present appear to have similar morphological characteristics to those described previously. No obvious cranio-caudal architectural differences were apparent but, due to its fragility and distribution across the submucosa, it proved very difficult to prepare large, intact sections of the plexus for detailed comparative analysis.

## 8. Distribution of nitric oxide synthase containing nerves in the myenteric plexus in the anal canal

The primary meshwork of the myenteric plexus can be followed distally into the anal canal as far as the junction of the proximal and middle thirds of the internal anal sphincter. In this region the plexus consists predominantly of linear nerve trunks which divide distally and disappear as they pass into the body of the internal anal sphincter. There are few ganglia here. Those that are present are densely packed with positively stained cells and are separate from, but connected with descending nerve trunks (figure 7).

## 9. Nitric oxide synthase containing nerves in the internal anal sphincter

Nerve processes which contain NADPH diaphorase activity are found throughout the internal anal sphincter. Both nerve trunks and single axons are seen. Particles of blue formazan dye are deposited homogeneously along the former, both here and in the rectal myenteric plexus above. However, in single axons traversing close to smooth muscle cells, dye is concentrated in discrete areas which gives them a beaded appearance. Some nerve cell bodies are seen at both the myenteric and submucosal borders in the proximal sphincter but none are present distally. These points are illustrated in figure 8.

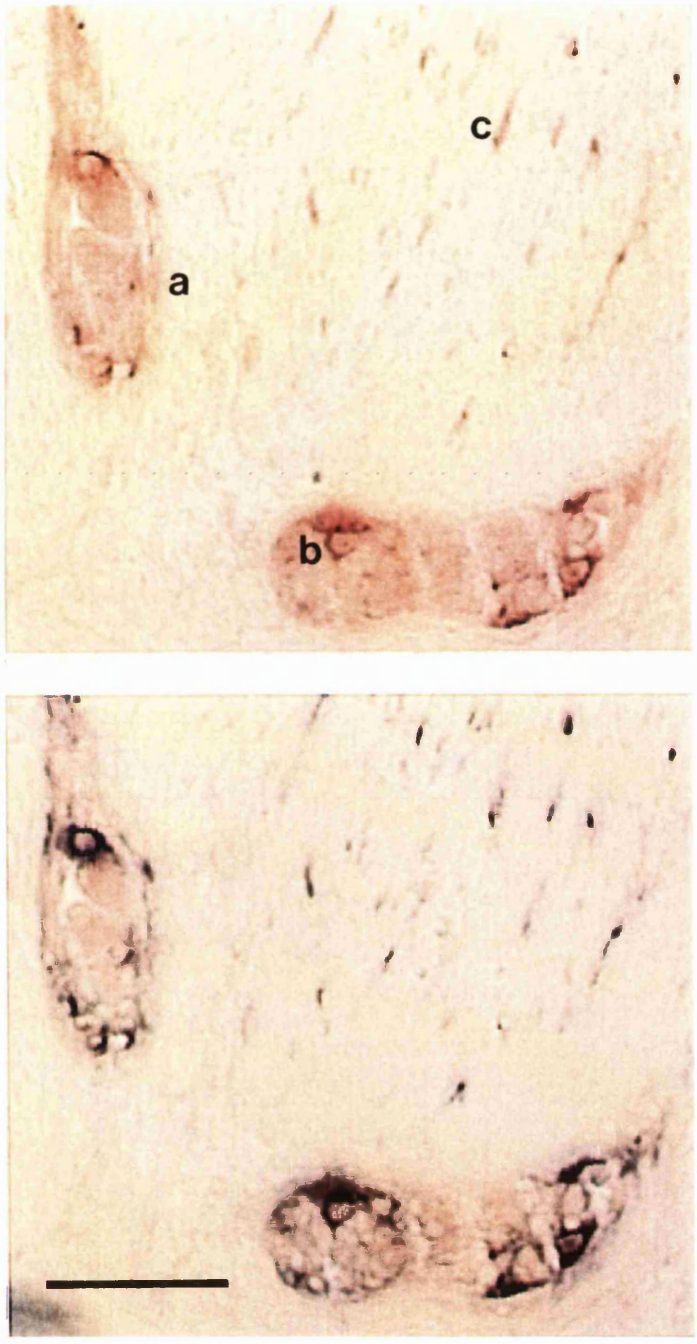



Figure 1.

A cryostat section through rectal myenteric ganglia (a) stained first for nitric oxide synthase immunoreactivity (top) and then for NADPH diaphorase activity (bottom). It is seen that the same population of enteric neurones (b) stains positive for the two enzyme activities. Positively stained nerve fibres are also seen in the circular smooth muscle layer here (c). (bar=100μm)

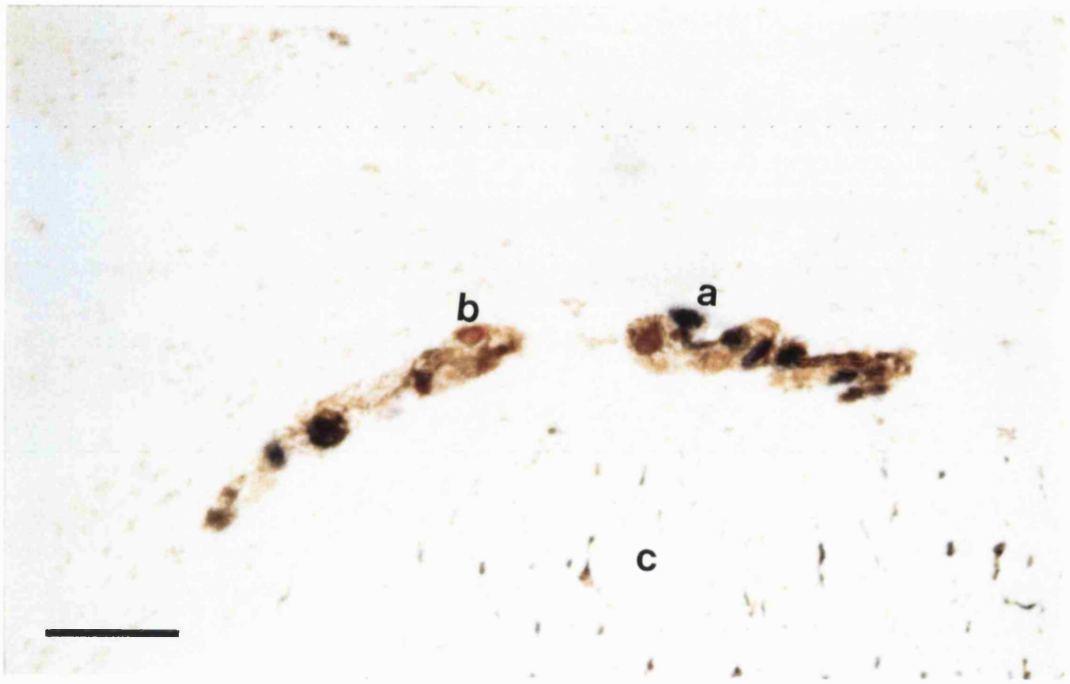



Figure 2.

A cryostat section of a rectal myenteric ganglion showing nerve cell bodies stained for NADPH diaphorase activity (blue; a) and SMI 311/312 (pan-axonal/pan-neuronal) immunoreactivity: brown; b). It is apparent that nitric oxide synthase containing neurones form a sub-population of the total number of enteric neurones present. (Circular muscle layer; c: bar=100μm)

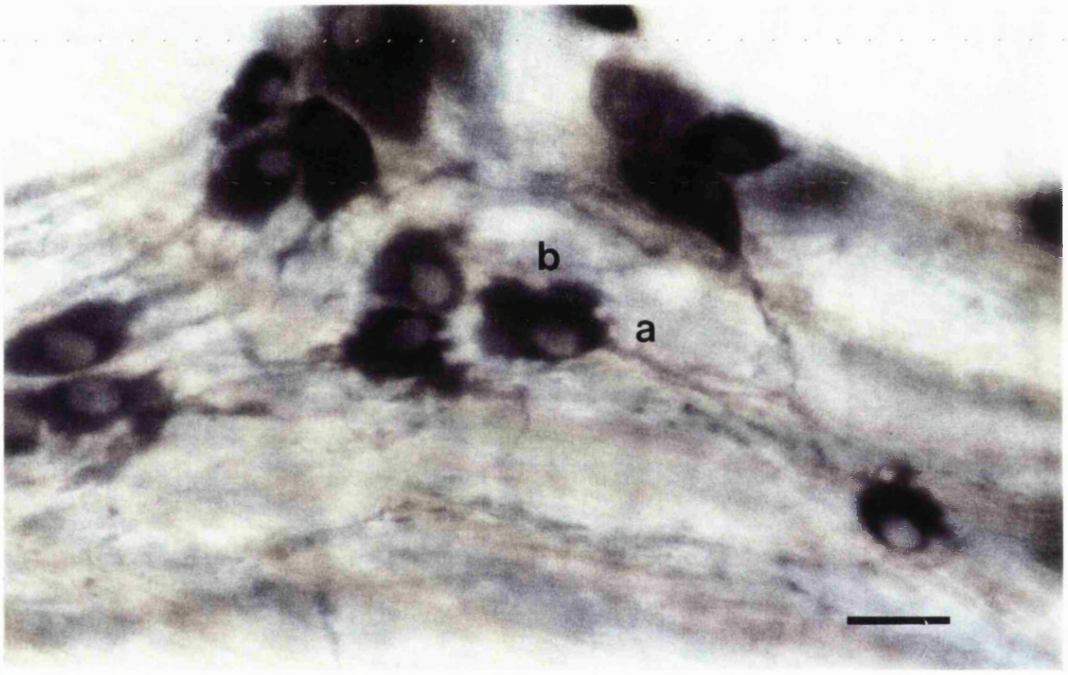



Figure 3.

A whole-mount of the rectal myenteric plexus stained using NADPH diaphorase histochemistry. This illustrates the appearance of individual nitric oxide synthase containing neurones. These have a single axonal process (a) and broad dendrites (b). This is typical of Dogiel type 1 enteric neurone morphology. (bar=25μm)

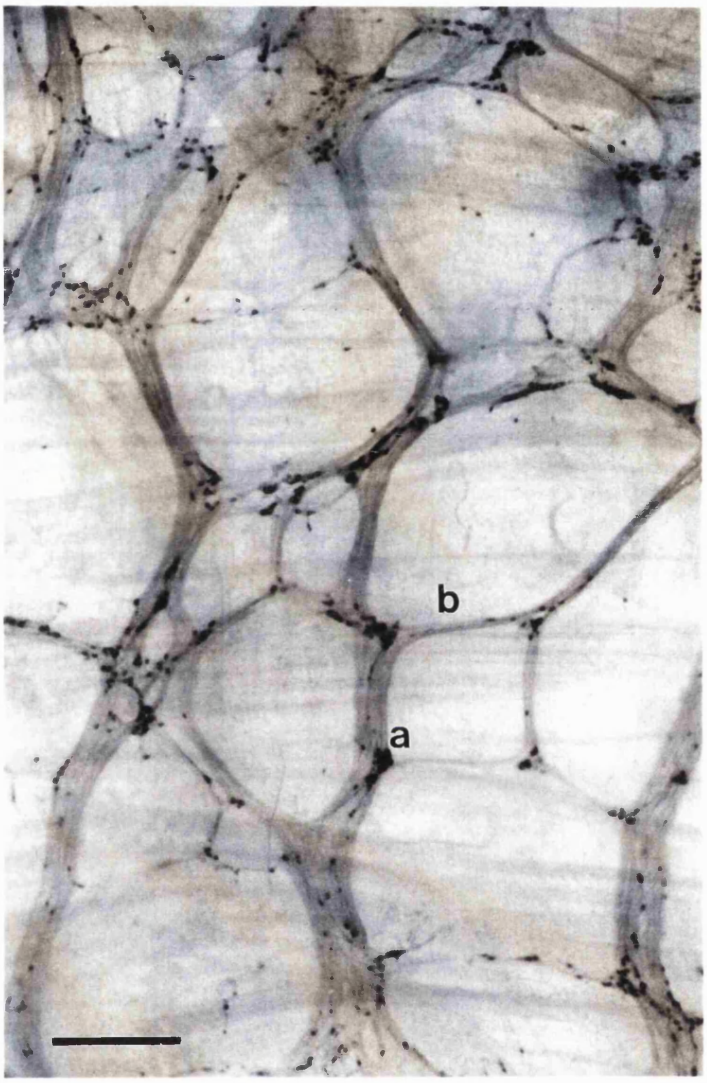



Figure 4.

i. The appearance of the myenteric plexus in the proximal two-thirds of the rectum as stained by NADPH diaphorase histochemistry. Nitric oxide synthase containing neurones aggregate together in ganglia (a) which are joined by internodal strands (b) to form a meshwork (primary plexus) which does not have apparent polarity. A few nerve cell bodies lie outside the confines of the primary plexus (c). (Cranial: top; bar=1mm)

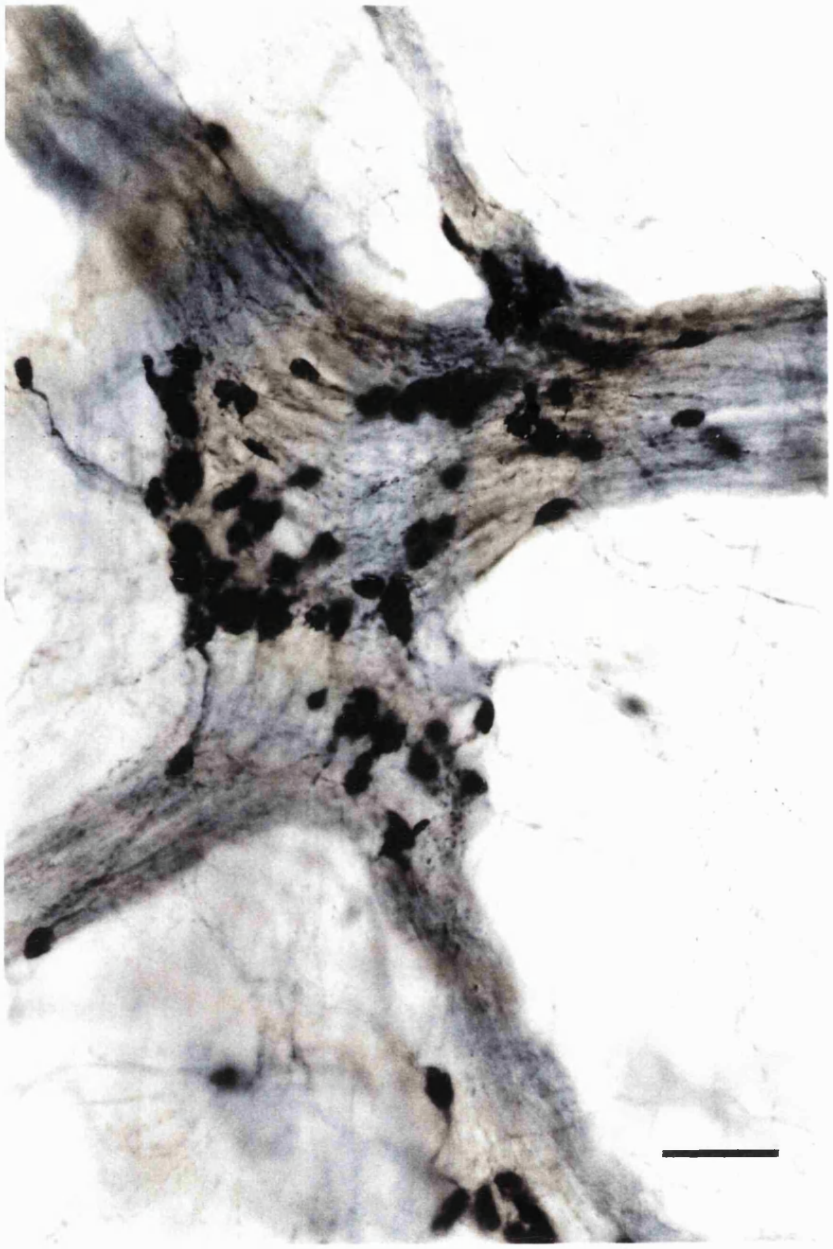


Figure 4.

ii. An individual ganglion from the proximal two-thirds of the rectum stained by NADPH diaphorase histochemistry. Note that individual nerve cell bodies are scattered throughout the structure. (bar= 100µm)

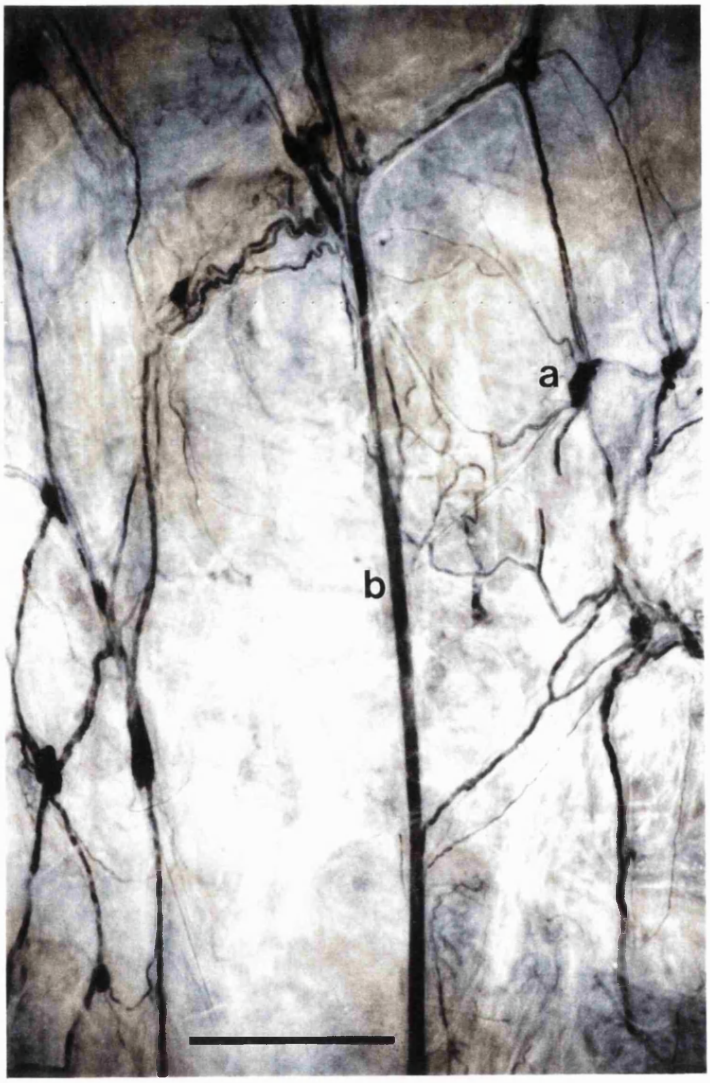



Figure 5.

i. The appearance of the distal rectal myenteric plexus stained by NADPH diaphorase histochemistry. Nitric oxide synthase containing neurones are again clustered in ganglia (a). Some of their axons traverse shunt fascicles (b) to descend to the anal canal. Note the cranio-caudal polarity here. (Cranial: top; bar=1mm)

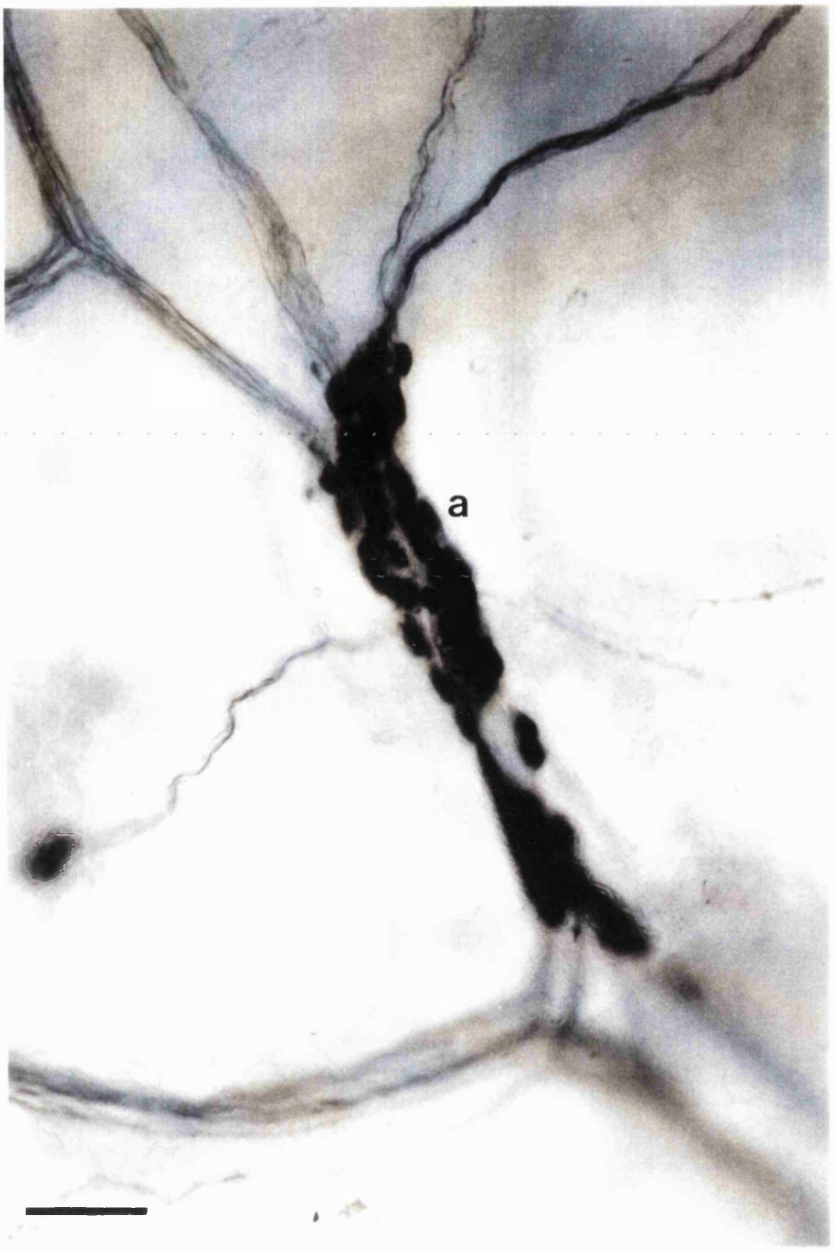



Figure 5.

ii. A ganglion in the distal rectal myenteric plexus stained by NADPH diaphorase histochemistry. Note that nitric oxide synthase containing neurones (a) are densely packed together with little room for other neurone types. (bar=100μm)

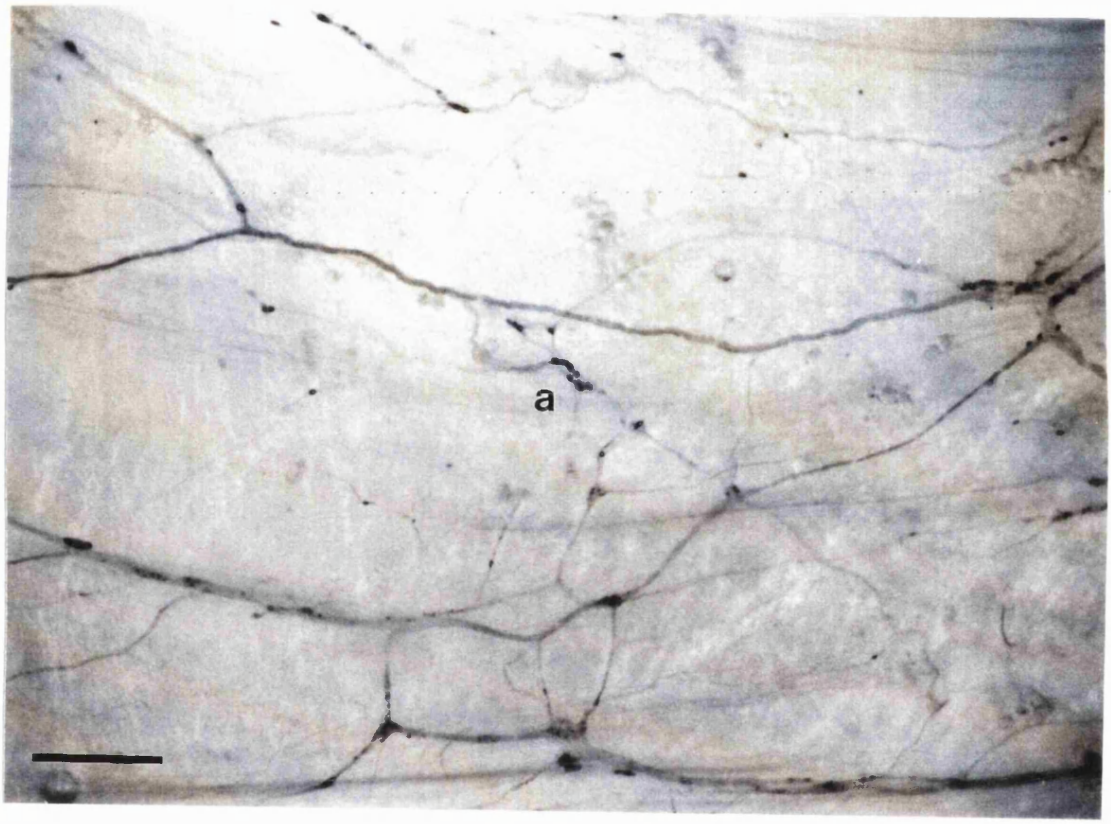



Figure 6.

The submucosal plexus in the proximal rectum stained by NADPH diaphorase histochemistry. Nitric oxide synthase containing neurones (a) are present but they are sparse. (Cranial: top; bar=1mm))

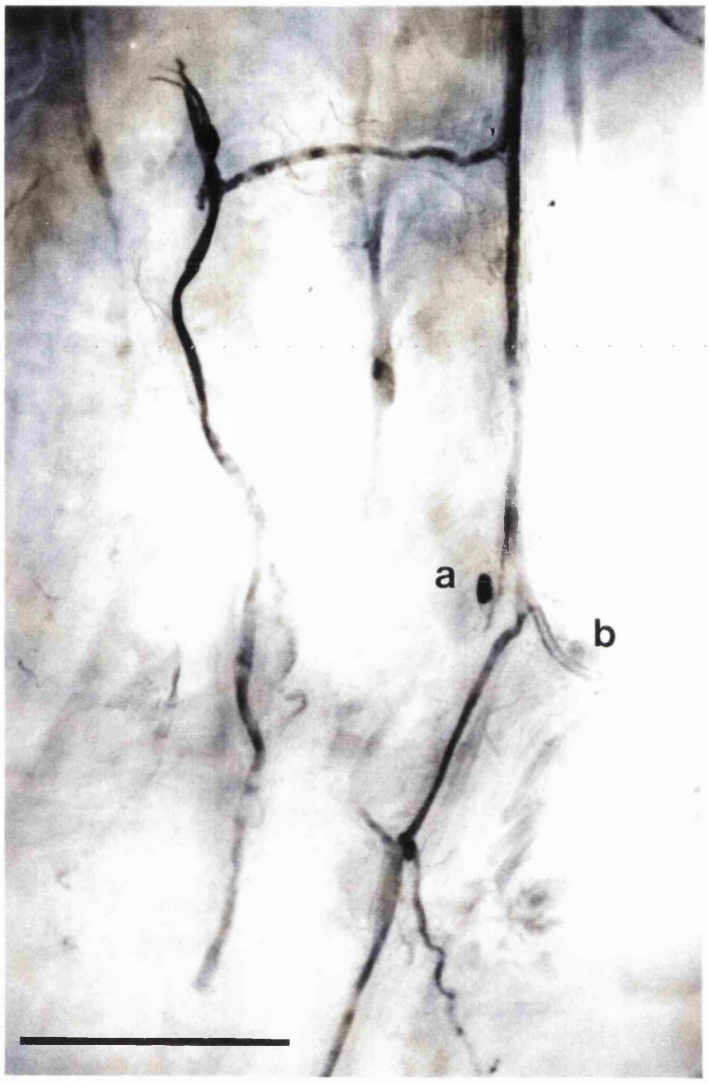



Figure 7.

The appearance of the myenteric plexus in the upper anal canal stained by NADPH diaphorase histochemistry. The plexus is seen here passing caudally just outside the internal anal sphincter at the junction of its proximal and middle thirds. There are few ganglia here and those that are present are densely packed with nitric oxide synthase containing nerve cell bodies (a). Stained nerves disappear as they branch and ramify into the internal anal sphincter itself (b). (Cranial: top; bar= 1mm)

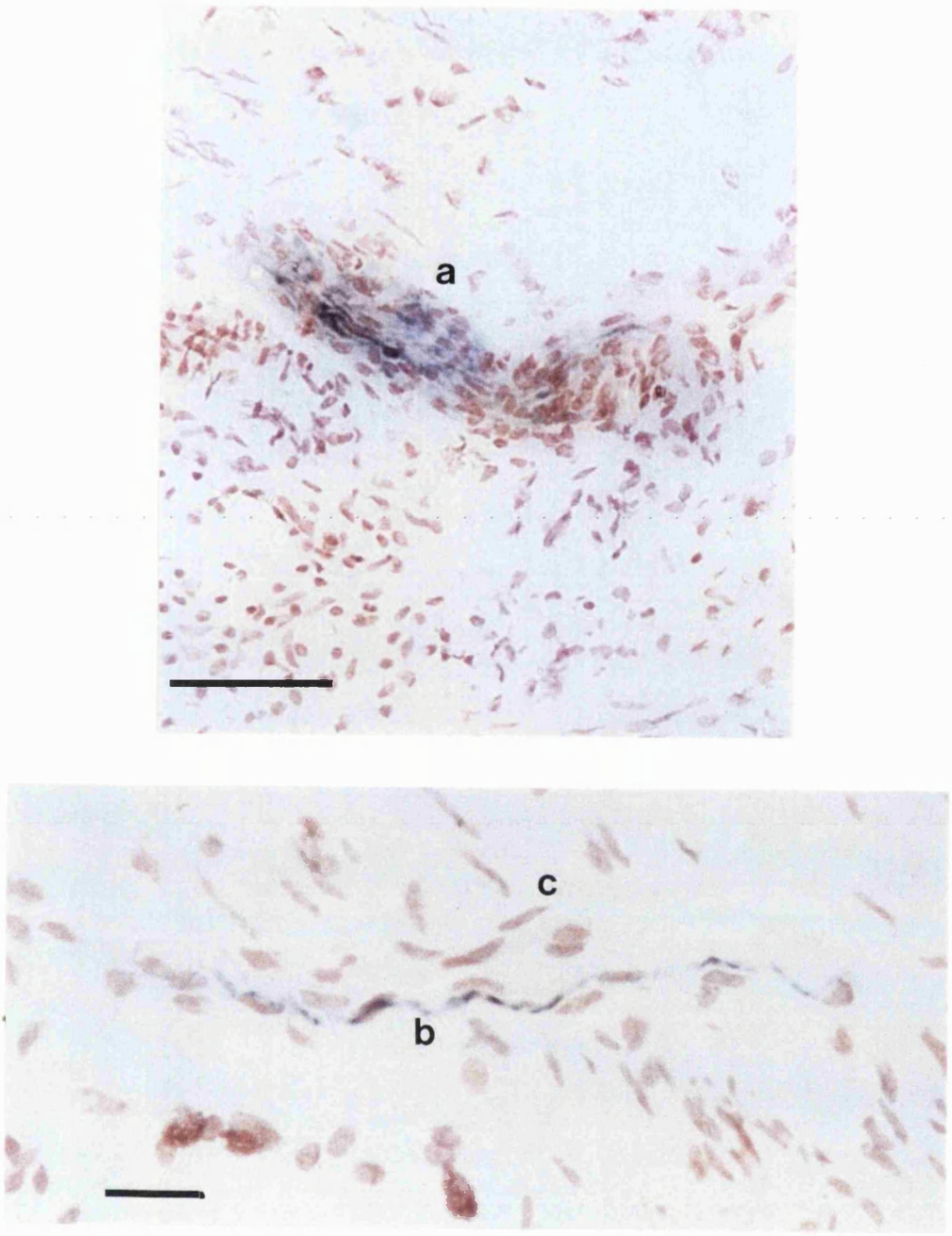



Figure 8.

Cryostat sections of the distal internal anal sphincter stained by NADPH diaphorase histochemistry, with a light neutral red counter stain;

(i) nitric oxide synthase containing nerve trunk (a; bar=100μm)

(ii) nitric oxide synthase containing axon (b). This is seen passing between smooth muscle cells whose nuclei are stained red (c). Note the distribution of stain here which gives the nerve a beaded appearance. (bar=10μm)

## DISCUSSION

On the basis of the morphological data presented here it is apparent that there is a neuronal source for NO in the human internal anal sphincter and that these nerves descend from cell bodies which lie above within rectal myenteric ganglia. The appearance of individual neurones is consistent with them being motor nerves within the gut. Thus, there is an anatomical basis for the suggestion that NO mediates NANC neurogenic relaxation of human internal anal sphincter smooth muscle and in addition, it seems reasonable to suggest that NO released upon stimulation nitric oxide synthase containing nerves, mediates the rectoanal inhibitory reflex in man.

The co-localisation nitric oxide synthase and NADPH diaphorase activity demonstrated here appears to be slightly less consistent than that described in lesser mammals (Dawson *et al.*, 1991). This is almost certainly attributable to the imperfect nature of the polyclonal anti-nitric oxide synthase antibody used in these experiments and subtle but unknown differences between the structure of human neuronal nitric oxide synthase and enzyme present in lesser mammals. In spite of this, NADPH diaphorase histochemistry is clearly an adequate marker for nitric oxide synthase immunoreactivity in the adult human anorectum, especially if one considers that the possibility of a NADPH diaphorase stained nerve cell body not containing nitric oxide synthase immunoreactivity is only 2%.

The advantage of using NADPH diaphorase histochemistry as a marker for nitric oxide synthase immunoreactivity is that it permitted the study of neuronal morphology and distribution in whole-mounts, which I found impossible using nitric oxide synthase immunocytochemistry. Tissue laminae in human gut are bound together by a great deal of connective tissue which not only hinders their

separation, but it also impedes neuronal penetration of large molecules used in immunocytochemical staining. NADPH diaphorase histochemistry however, relies on smaller molecules which easily penetrate connective tissue and this allowed the myenteric plexus to be visualised and dissected without untoward disruption. Even so, such preparations are extremely technically demanding, a point illustrated by my inability to dissect the submucosal plexus to the same extent as the myenteric, particularly in the distal rectum. Because of such technical difficulties, whole-mounts have seldom been utilised in anatomical studies of the human gut but it is clear that they provide invaluable information about neuronal morphology which can not be readily gleaned from sectioned tissue.

The individual neurones demonstrated here which contain nitric oxide synthase, have morphological characteristics previously ascribed to Dogiel type 1 enteric neurones. These are thought to be motor nerves within the gut (Furness and Costa 1987). In view of their distribution throughout the anorectal myenteric plexus, it is likely that nitric oxide synthase containing nerves influence smooth muscle activity in both the rectum and internal anal sphincter. In the former they might control 'reservoir' function in the same way that they appear to mediate receptive relaxation in the stomach (Desai *et al.*, 1991).

The gross architecture of the anorectal myenteric plexus as revealed by NADPH histochemistry, is broadly similar to that reported in previous studies which utilised different staining techniques. Christensen et al. (1984) compared the anatomy of the myenteric plexus in rodent and non-rodent species using silver-impregnation to stain whole-mounts. In non-rodents, including the cynomolgus monkey, the appearance of the most distal 5-20% of the rectal myenteric plexus was different to that seen in the rest of the rectum. The distal plexus was very irregular with few small ganglia present. Proximally, a stellate plexus was

apparent which contained large ganglia and in addition, shunt fascicles appeared. The hypoganglionic nature of the distal rectum in the human was commented upon by Aldridge and Cambell (1968) and Weinberg (1970) who studied this region using sectioned tissue from paediatric specimens. Finally, Kumar and Phillips (1989) used silver-impregnation of whole-mounts to survey the structure of the human myenteric plexus. In the rectum a dense plexus was noted, whose appearance corresponds with that found in the proximal portion of the rectum in this study. Kumar and Phillips (1989) also noted rectal shunt fascicles, but indicated that they were less common in the human than in other non-rodents (two or three, rather than five or six around the circumference of the rectum). They made no comment about the architectural change which occurs in the distal rectal myenteric plexus.

Shunt fascicles are a consistent finding in the myenteric plexus of both rodent and non-rodent mammals (Christensen *et al.*, 1984; Fukai and Fukuda, 1984; Kumar and Phillips, 1989). They are thought to act as pathways for rapid transfer of neuronal information between gut segments and can relay nerve impulses in both the cranio-caudal and caudo-cranial directions (Fukai and Fukuda, 1984). In the human, shunt fascicles have been described in the myenteric plexus from the oesophagus to the rectum and are particularly prominent in junctional regions such as the oesophago-gastric, gastro-duodenal and ileo-caecal junctions (Kumar and Phillips, 1989). They contain both myelinated and unmyelinated nerve fibres. In the colon and rectum, the myelinated component of shunt fascicles is thought to consist of extrinsic nerves which arise from the pelvic plexus, while the unmyelinated fibres can arise from intrinsic, enteric nerves (Furness and Costa, 1987). The results presented here suggest that nerves which contain nitric oxide synthase are an important constituent of rectal shunt fascicles. This is particularly true in the distal rectum where intensely stained, NADPH diaphorase positive nerves are present which

descend into the anal canal. Although thick nerve bundles have been described as encircling junctional regions elsewhere in the human gut (Kumar and Phillips, 1989), no similar positively stained neuronal structure is apparent at the anorectal junction.

Continuation of the myenteric plexus distal to the anorectal junction has been reported previously in studies using sectioned tissue (Holmes, 1961;Aldridge and Campbell, 1968; Weinberg, 1970; Baumgarten et al., 1971) but the topography of the plexus in this region, as described here, has not been reported before. Ramification of nitric oxide synthase containing nerves throughout the internal anal sphincter implies that there is a neuronal source for NO in this tissue. The precise localisation of nitric oxide synthase containing nerve cell bodies which innervate the internal anal canal was not determined in this study. Experiments using ante- or retrograde neuronal tracer/labelling techniques are required to define the distribution of individual neurones further.

Within the sphincter, the beaded appearance of individual stained axons is notable and contrasts with the staining pattern of larger nerve trunks, where formazan dye is deposited in a particulate but more or less homogeneous way. The consistency of this finding means that it is unlikely to be caused by the serpiginous distribution of axons here. Instead it might indicate that nitric oxide synthase is present in varicosities. Additional morphological studies utilising electron microscopy would be required to investigate this possibility.

# *Chapter 7*

## NITRIC OXIDE SYNTHASE CONTAINING NEURONES IN HIRSCHSPRUNG'S DISEASE, A CONDITION IN WHICH THE RECTOANAL INHIBITORY REFLEX IS ABSENT

# INTRODUCTION

## 1. Definition

Hirschsprung's disease takes its name from the Danish paediatrician who first described the condition in 1886. It is a congenital abnormality of the gastrointestinal tract which is characterised by the presence of a non-propulsive, non-relaxing, aganglionic segment of gut which extends proximally for a variable distance from the distal rectum. This results in functional intestinal obstruction. Although adult presentation does occur, Hirschsprung's disease is most commonly diagnosed in childhood and symptoms can usually be traced back to the immediate post natal period.

## 2. Aetiology

Enteric neurones originate from the neural crest and migrate to the gut prior to differentiation and maturation. For some reason, as yet unknown, this process is abnormal in Hirschsprung's disease. It has been suggested that the vagus is the sole conduit for passage of enteric neurones to the gut (Okamoto and Ueda, 1967) and that Hirschsprung's disease arises because distal migration of these neurones and longitudinal growth of the gut are out of phase (Webster, 1973). This is a somewhat simplistic view however, which does not take account of evidence which suggests that enteric neurones enter the gut at vagal and trunk levels, and that there is a dual gradient of differentiation and maturation of these cells towards the middle of the gut (Gershon *et al.*, 1980). A second hypothesis is that the micro-environment in which the cells develop is defective and certainly abnormalities in the extra-cellular matrix of aganglionic bowel have been demonstrated (Kumagoto and Donahoe, 1985; Payette *et al.*, 1987; Parikh *et al.*, 1992). The cause of these changes is unclear but they help to explain why other

enteric neuronal abnormalities, such as intestinal neuronal dysplasia, can occur in association with Hirschsprung's disease (Pistor *et al.*, 1987). Vascular abnormalities (Earlam, 1985) and infection with cytomegalovirus are also possible causative factors (Hershlag *et al.*, 1984). A genetic component in the aetiology of Hirschsprung's disease is suggested by a definite familial incidence (Bodian and Carter, 1963) and by an association with other congenital conditions such as Down's syndrome (Garver *et al.*, 1985)

## 3. Pathophysiology

In the aganglionic segment in Hirschsprung's disease, intramural ganglia and nerve cell bodies are absent from both the myenteric and submucosal plexuses (Whitehouse and Kernohan, 1948; Zeulzer and Wilson, 1948). In their place are large nerve bundles and trunks which may originate outside the gut (Tam and Boyd, 1990). Proximal to this is a hypoganglionic transition zone and more proximal still is bowel which appears to be normally ganglionated. Hirschsprung's disease most commonly affects the rectum and sigmoid colon but the condition can be limited to the distal rectum only (short/ ultra- short segment disease- Meier-Ruge, 1985) or the entire colon can be involved (Cass and Myers, 1987). Uncommonly, aganglionosis can also affect the small bowel or rarely, the entire gut (Di Lorenzo *et al.*, 1985: Rudin *et al.*, 1986).

Apart from the absence of ganglia, several other neuronal abnormalities are recognised in the aganglionic bowel in Hirschsprung's disease. The large, hypertrophied nerve trunks which replace the normal enteric nerve plexuses have intense acetylcholinesterase activity and there is an abnormal abundance of similarly reactive fibres in the muscularis mucosa and lamina propria (Garrett *et al.*, 1969). There is a loss of the normal peri-cellular network of adrenergic fibres around ganglia. Cholinergic and adrenergic fibres are present in both the

circular and longitudinal muscle layers and ultrastructural studies suggest that these nerves form synaptic communications with myocytes (Howard and Garrett, 1970; Baumgarten *et al.*, 1973).

Innervation of the aganglionic bowel by NANC nerves is also abnormal, and both excitatory and inhibitory fibres are affected. Substance P is a putative excitatory neurotransmitter in the gut and the number of substance P immunoreactive nerve fibres is reduced in the circular and longitudinal muscle layers in the aganglionic segment in Hirschsprung's disease, but their presence in the mucosa and along-side blood vessels is similar to that seen in normally ganglionated gut (Tam and Boyd, 1991). Of the putative inhibitory NANC nerves, those containing vasoactive intestinal peptide immunoreactivity are also reduced in number in the muscle layers in the aganglionic segment (Bishop *et al*, 1981) but, in a study using whole-mount immunocytochemistry they were found to be more abundant in this region than was previously thought (Tam and Boyd, 1990). In addition, neuronal immunoreactivity for met-encephalin, galinin, somatostatin, calcitonin gene related peptide and serotonin is also reduced in the aganglionic region in Hirschsprung's disease ( Larsson *et al.*, 1988; Tam and Boyd, 1991).

Abnormalities of NANC nerves also manifests in the response of aganglionic smooth muscle to electrical field stimulation. In ganglionated bowel stimulation of intrinsic nerves (by electrical field stimulation) produces a biphasic response; relaxation followed by contraction. Here, relaxation is mediated by NANC inhibitory nerves and in the aganglionic segment the relaxant response is absent (Larsson *et al.*, 1987). The neuronal abnormality responsible for this has not been identified.

The explanation for the contraction of the aganglionic gut in Hirschsprung's disease is also uncertain. Hypersensitivity of denervated smooth muscle has been suggested (Wright and Shepherd, 1965; Okamoto *et al.*, 1967) but this has not been substantiated by others (Larsson *et al.*, 1987). Abnormal activity of cholinergic (Hanani *et al.*, 1986; Garrett *et al.*, 1969) and adrenergic nerves (Nirasawa *et al.*, 1986) has also been demonstrated which could result in contraction (cholinergic nerves) or failure of relaxation (adrenergic nerves).

## 4. The rectoanal inhibitory reflex in Hirschsprung's disease

Callaghan and Nixon (1964) were the first to report that the rectoanal inhibitory reflex is absent in Hirschsprung's disease, a finding subsequently confirmed by the same group and by other investigators (Lawson and Nixon HH, 1967; Schnaufer *et al.*, 1967; Tamate *et al.*, 1984). Testing for the reflex has been used as diagnostic tool in some centres and accuracy rates (diagnosis confirmed by subsequent biopsy) of 100% have been claimed (Tamate *et al.*, 1984). Others have found the investigation to have less diagnostic reliability but suggested that this reflected deficiencies in equipment and technique rather than variation in the physiological response of the anorectum in this condition (Aaronson and Nixon, 1972, Meunier *et al.*, 1978). In addition, it has been suggested that in normal children the reflex can be absent in the immediate post-natal period due to immaturity of enteric inhibitory neurones (Howard and Nixon, 1968; Holschneider *et al.*, 1976; Ito *et al.*, 1977). More recent studies however, using microtransducers, suggest that this is in fact not the case and the reflex can be demonstrated throughout the neonatal period, even in premature infants (Tamate *et al.*, 1984).

## 5. Are nitric oxide synthase containing nerves present in Hirschsprung's disease?

If NO mediates the rectoanal inhibitory reflex, then one explanation for the absence of the reflex in Hirschsprung's disease is that the nerves which produce NO, namely those which contain nitric oxide synthase, are missing from the myenteric plexus of the aganglionic gut in this condition. The purpose of the experiments described in this chapter was to test this hypothesis. The aims were;

**i. to determine if nitric oxide synthase containing neurones are present in the colon in infants,**

**ii. to investigate if nitric oxide synthase containing nerves are present in aganglionic bowel in Hirschsprung's disease and,**

**iii. to determine the distribution of nitric oxide synthase containing neurones across the transition zone.**

## METHODS

Tissue was obtained from the surgical specimen excised from seven infants with proven Hirschsprung's disease, who underwent definitive resection of aganglionic bowel at the John Radcliffe Hospital in Oxford. (Hirschsprung's disease was diagnosed histologically on the basis of enteric aganglionosis by Dr. S. Gould, Consultant Paediatric Histopathologist at the same hospital). All patients had recto-sigmoid aganglionosis and underwent anterior resection of the affected segment followed by colo-anal anastomosis. The median age at the time of definitive surgery was 9 months (range 5-12 months). In addition, normal control colon specimens were obtained from three age-matched infants with anorectal agenesis undergoing closure of colostomy. Tissue from the specimens was processed for cryostat sections and for whole-mount histochemistry. For the former, specimens were taken from the normally ganglionated colon as well as from the aganglionic segment. For whole mounts, a continuous strip of bowel was obtained from the resection specimen which included the ganglionated, transitional and aganglionic zones.

In view of possible differences between enteric neurones in young children and in the older patients studied in the previous investigations, the degree of co-localisation between nitric oxide synthase and NADPH diaphorase was established first, prior to using NADPH diaphorase histochemistry to determine the distribution of nitric oxide synthase containing neurones.

Adjacent cryostat sections from ganglionated and aganglionic bowel were stained as follows:

*i.* Nitric oxide synthase immunoreactivity

*ii.* Nitric oxide synthase immunoreactivity followed by NADPH diaphorase activity.

*iii.* NADPH diaphorase activity

*iv.* SMI 311/312 (pan-neuronal and pan-axonal) immunoreactivity.

*v.* Appropriate controls, as described previously.

To investigate the distribution of nitric oxide synthase containing neurones in whole-mounts, strips of tissue, extending from the ganglionated to the aganglionic zone, were fixed and then dissected 'in continuity'. This technique was utilised so that any change in the architecture of the enteric nerve plexuses could be detected as accurately as possible.

# RESULTS

## 1. Controls

There was no staining following omission of; primary antibody, primary and secondary antibodies, nitro blue tetrazolium and NADPH. In co-localisation studies, the appearance of nitric oxide synthase immunoreactivity was unchanged if nitro blue tetrazolium or NADPH were omitted from the secondary reaction medium. Very little endogenous peroxidase activity was present. When it occurred, it was contained within inflammatory cells.

## 2. Co-localisation of nitric oxide synthase immunoreactivity and NADPH diaphorase activity

Staining of individual myenteric ganglia for nitric oxide synthase immunoreactivity, followed by NADPH diaphorase activity was carried out as described for adult rectal tissue. Assessment of 176 nerve cell bodies from both the myenteric and submucosal plexuses revealed that 174 (98.8%) contained both nitric oxide synthase immunoreactivity and NADPH diaphorase activity. One nerve cell body was immunoreactive for nitric oxide synthase only (0.6%) and one cell body contained NADPH diaphorase activity only (0.6%). The probability therefore, that NADPH diaphorase histochemistry will demonstrate a nerve cell body which contains nitric oxide synthase is 98.8%, whilst the probability that it will stain positive a cell body which does not contain nitric oxide synthase is only 0.6%. Thus, in the infant colon, as in the adult rectum, NADPH diaphorase histochemistry is an accurate marker for nitric oxide synthase. This relationship was utilised in subsequent experiments.

## 3. Distribution of nitric oxide synthase activity across the bowel wall from the serosa to mucosa in normally ganglionated and aganglionic bowel.

In ganglionated bowel in patients and controls, NADPH diaphorase activity is seen in ganglia both in the myenteric plexus and at the submucosal border of the circular smooth muscle layer (submucous plexus). In addition, NADPH diaphorase activity is present within neuronal processes in both the circular and longitudinal muscle layers, where fibres run parallel and perpendicular to smooth muscle cells. Very fine nerves containing NADPH diaphorase activity are also seen passing from the submucous plexus into the mucosa. No other structures stained positive for NADPH diaphorase activity. These points are illustrated in figure 1.

In the aganglionic segment, no NADPH diaphorase activity is apparent. Hypertrophied nerve trunks are present in this region but they do not contain NADPH diaphorase activity and therefore, they do not contain nitric oxide synthase. These points are illustrated in figure 2 and 3.

## 4. Distribution of nitric oxide synthase containing neurones in the myenteric plexus of normally ganglionated colon, aganglionic colon and across the transition zone

In the myenteric plexus of normally ganglionated colon, positively stained neurones are found principally within ganglia. These are joined by internodal strands to form a meshwork with varied geometry. This meshwork is devoid of polarity in that it is evenly distributed both longitudinally in the cranio-caudal axis of the gut and circumferentially around it (figure 4i.). NADPH diaphorase activity is also seen in nerve fibres running parallel with muscle bundles in both

the circular and longitudinal muscle layers. In addition, positively stained neurones are noted in the submucosal plexus but they are relatively scarce here in comparison with the number present at the myenteric border. Individual nerve cell bodies have the morphological characteristics typical of Dogiel Type 1 enteric neurones. They have several short dendrites, an oval nucleus and a single axonal process (figure 4ii.).

Passing caudally from normally ganglionated colon into the transition zone, distinct changes in the architecture and orientation of the myenteric plexus are apparent. Ganglia and internodal strands are aligned progressively in a linear fashion along the cranio-caudal axis of the gut. There are few internodal strands passing circumferentially around the gut. More caudal still, ganglia disappear and individual nerve cell bodies lie in series along stained nerves. Distally, nerve cell bodies become increasingly sparse and then disappear completely. Their axonal processes pass caudally in nerve bundles for a further 1.5-2.0cm but these then disappear too, leaving the aganglionic gut devoid of NADPH diaphorase activity. These points are illustrated in figure 5.

Due to the fragility and filamentous nature of the submucosal plexus in the infant colon, it proved technically impossible to dissect it in a similar way to the myenteric plexus.

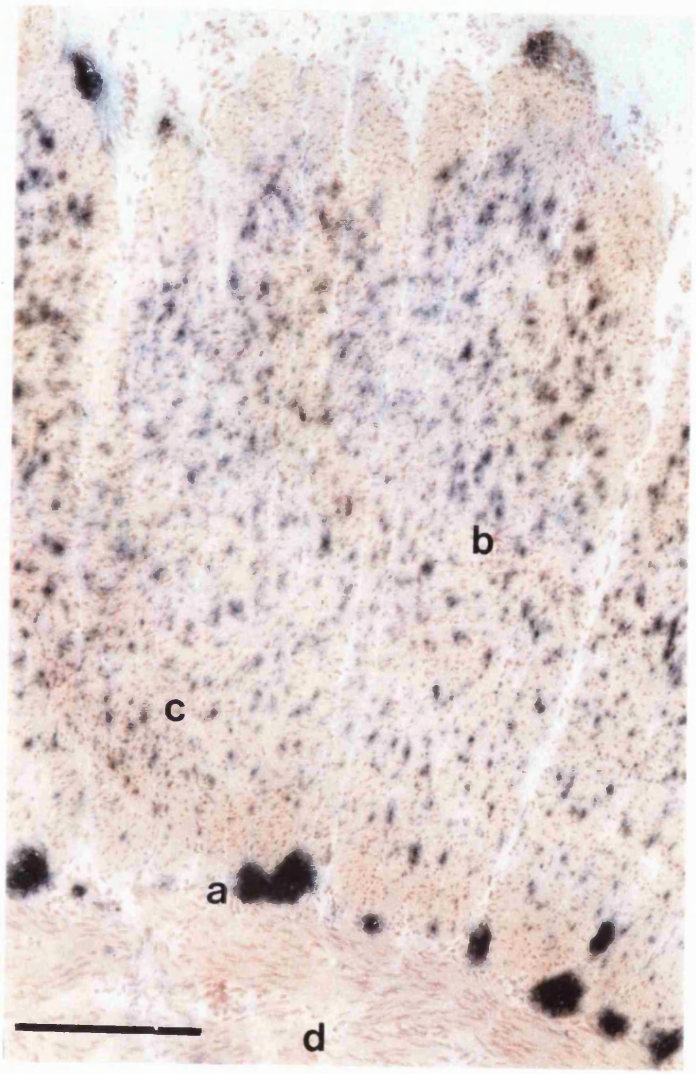



Figure 1

(i) A cryostat section of normally ganglionated sigmoid colon in Hirschsprung's disease stained to reveal NADPH diaphorase activity and hence the presence of nitric oxide synthase. Positively stained nerve cell bodies (blue; a) are seen in the myenteric plexus and their processes (b) traverse the muscularis externa (circular muscle-c; longitudinal muscle-d). (Counter stain: haematoxylin; bar=100μm)

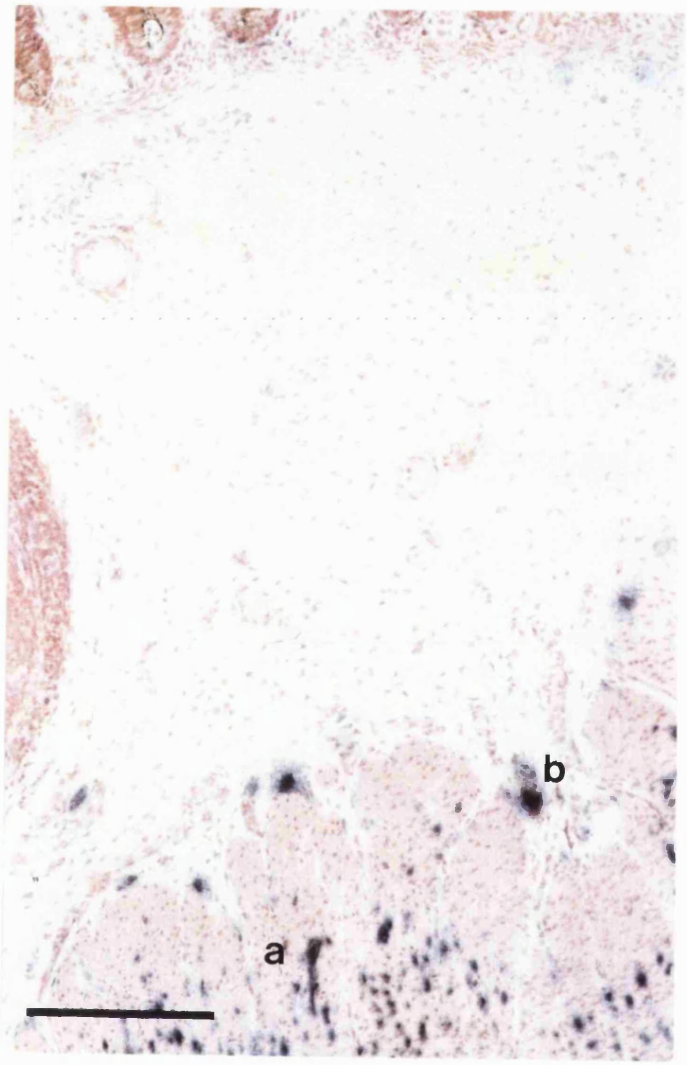



Figure 1

(ii) A cryostat section of normally ganglionated sigmoid colon in Hirschsprung's disease stained to reveal NADPH diaphorase activity and hence the presence of nitric oxide synthase. Positively stained nerve processes are seen in the circular muscle layer (blue; a) while nerve cell bodies (b) are present along its submucosal border. Some very fine nerves extend from the submucous plexus into the mucosa but no other structures are stained positive. (Counter stain: haematoxylin; bar=100μm)

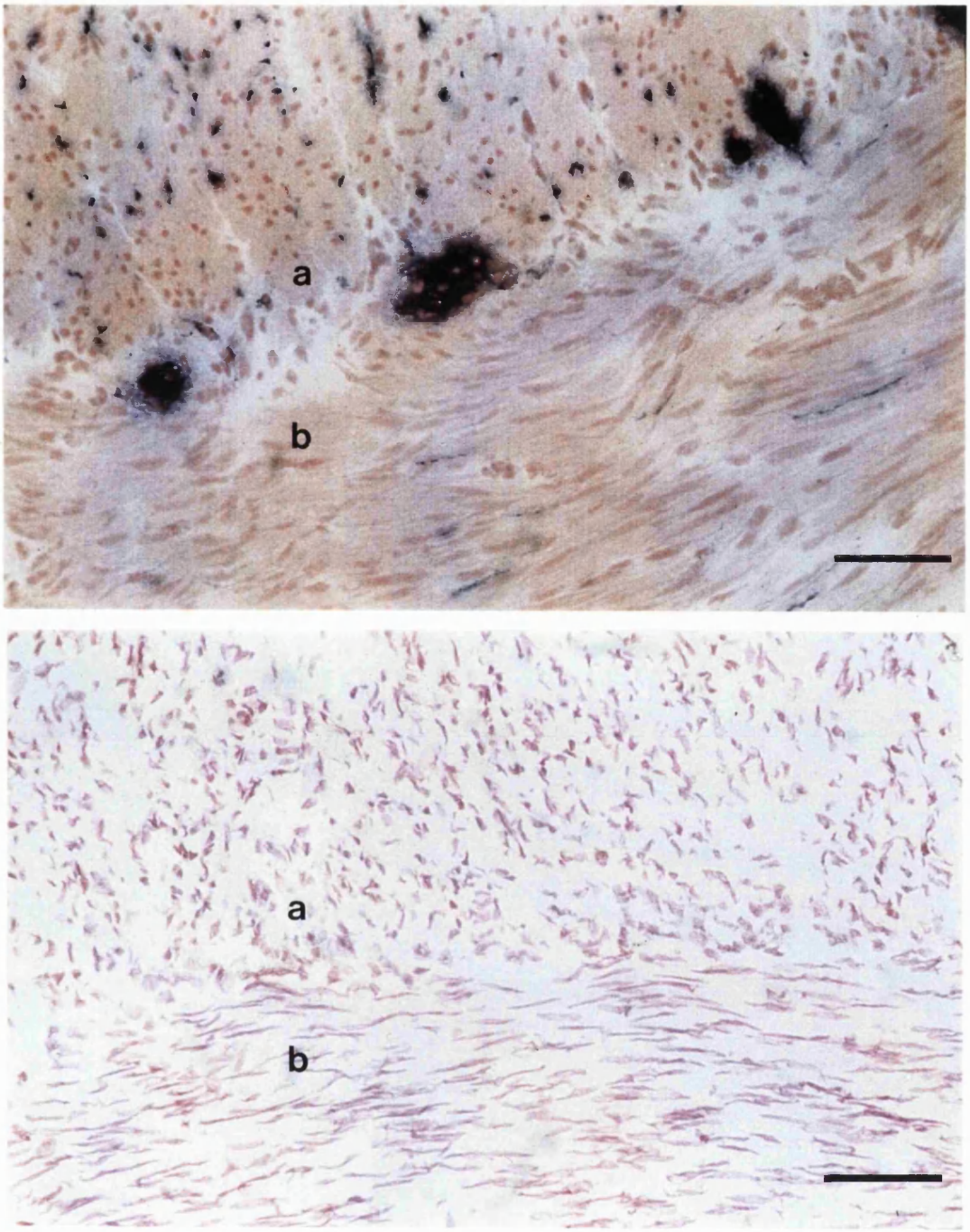



Figure 2.

Cryostat sections of the junction between the circular (a) and longitudinal muscle layers (b) of the ganglionated and aganglionic segments in Hirschsprung's disease stained to reveal NADPH diaphorase activity and hence the presence of nitric oxide synthase. Note the presence of the positive staining (blue) in the former and its absence in the latter. (Counter stain: haematoxylin; bar=100μm)

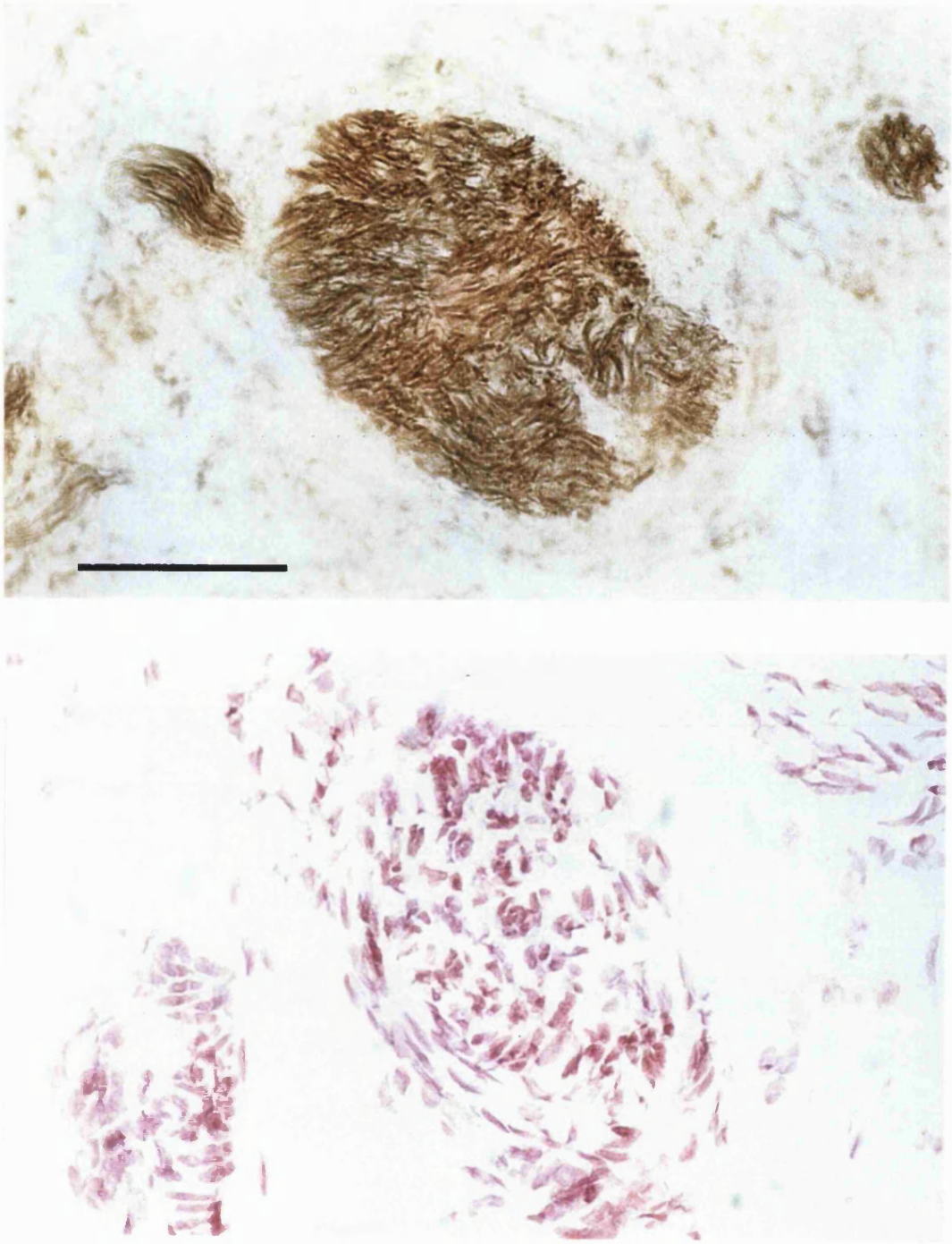


Figure 3.

Adjacent cryostat sections of a nerve trunk in the aganglionic segment of gut in Hirschsprung's disease, stained for;
(i) SMI 311/312 (pan-axonal/pan-neuronal) immunoreactivity (brown) and,
(ii) NADPH diaphorase activity. Note the absence of positive staining (blue). (Counter stain: neutral red; bar=100μm)

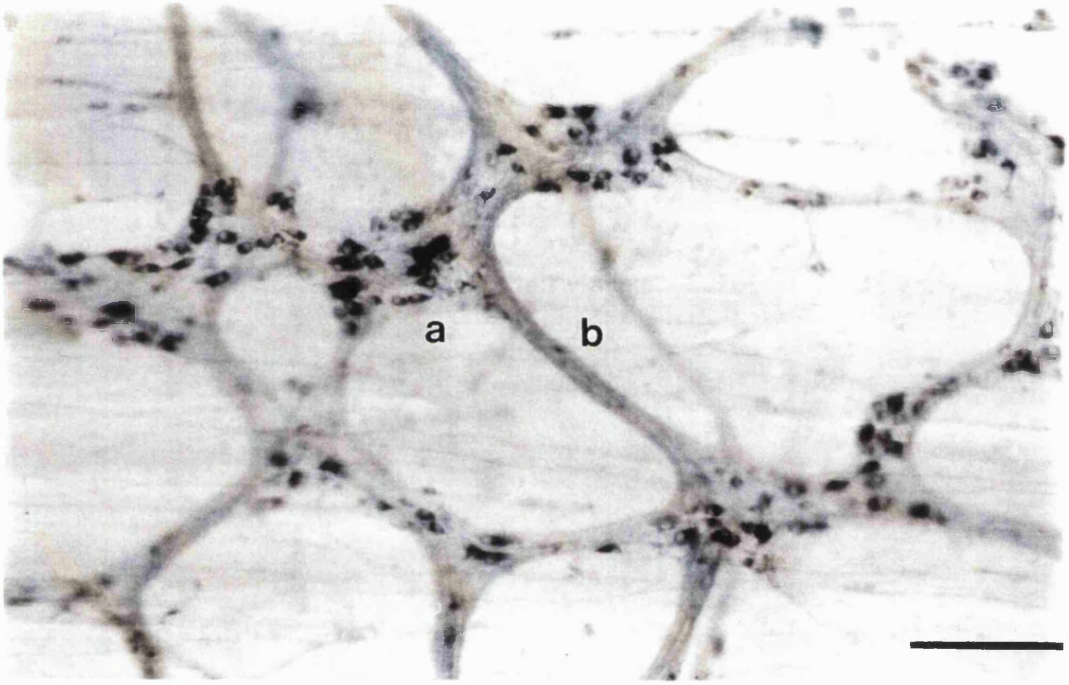



Figure 4.

(i) A whole mount of the myenteric plexus in the normally ganglionated colon of a child suffering from Hirschsprung's disease, stained to reveal NADPH diaphorase activity and hence the presence of nitric oxide synthase. The plexus here lacks polarity and consists of ganglia (a) joined by internodal strands (b). (Cranial: top; bar=100μm)

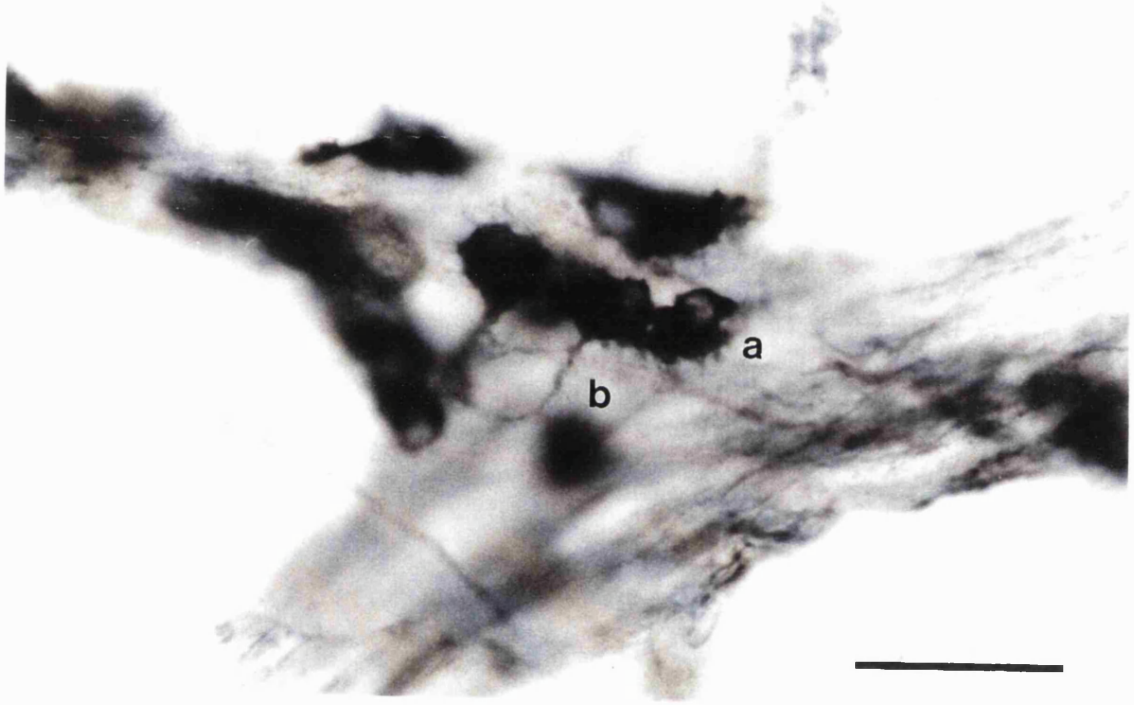



Figure 4.

(ii) A whole mount of the myenteric plexus in the normally ganglionated segment of gut in Hirschsprung's disease stained to reveal NADPH diaphorase activity and hence the presence of nitric oxide synthase. The appearance of individual positively stained neurones is shown. These have several dendrites (a) and a single axon (b). This is typical of Dogiel type 1 enteric neurones. (bar= 50μm)

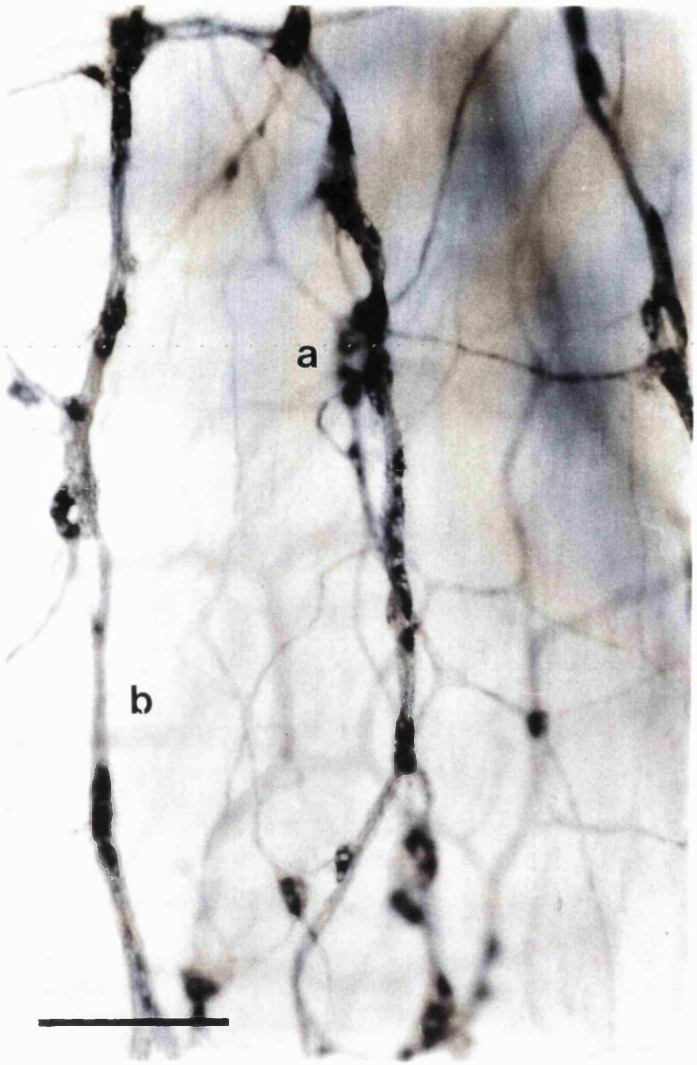



Figure 5.

A sequence of whole mounts of the transition zone in Hirschsprung's disease, stained to reveal NADPH diaphorase activity and hence the presence of nitric oxide synthase.

(i) Initially, ganglia (a) and internodal strands (b) become aligned linearly along the cranio-caudal axis of the colon. (Cranial: top; bar= 100μm)

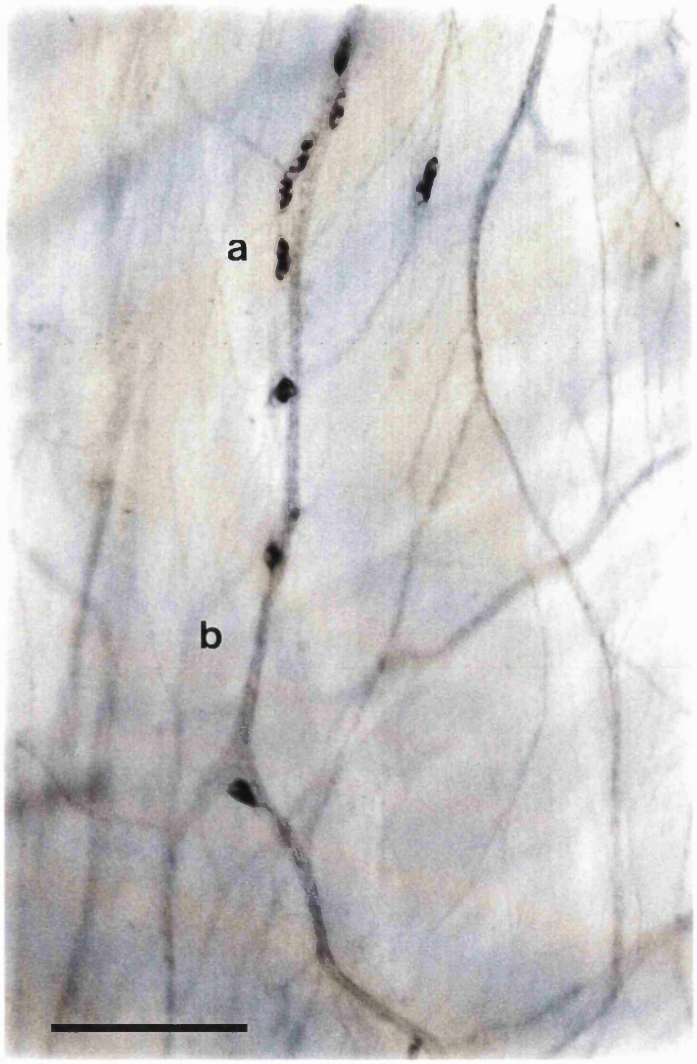



Figure 5.

(ii) Further distally in the transition zone, discrete ganglia disappear and positively stained neurones (a) lie in series along nerve trunks (b). (Cranial: top; bar= 100μm)

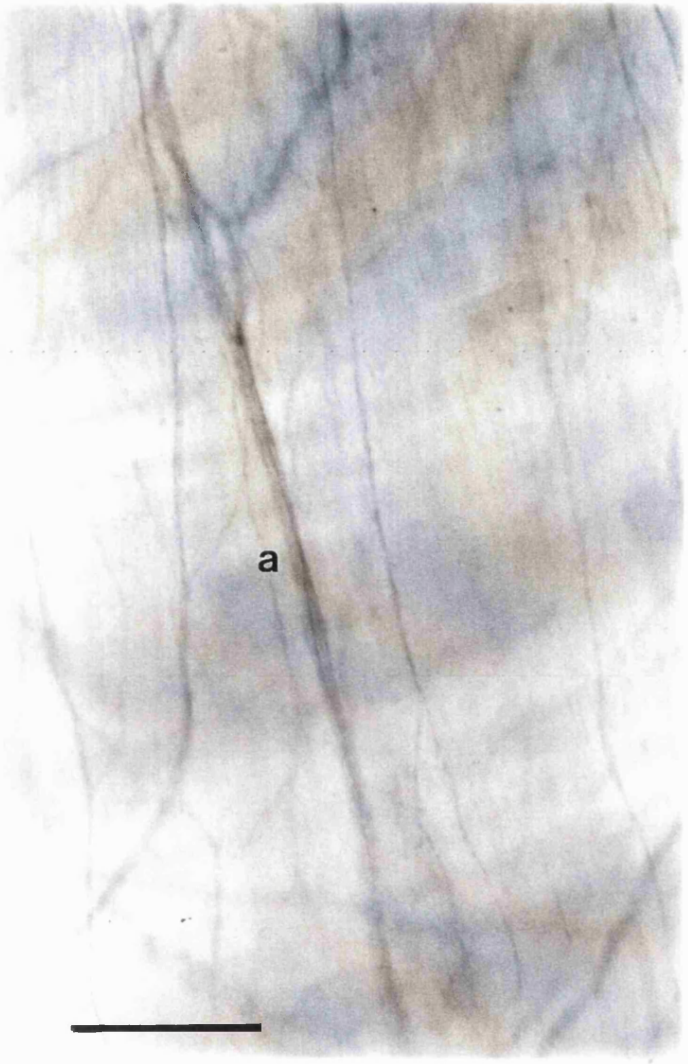



Figure 5.

(iii) Further distal still, positively stained nerve cell bodies are absent. Stained axons (a) can be traced distally for a short distance but these too disappear. (Cranial: top; bar= 100μm)

## DISCUSSION

The major conclusion that can be drawn from these experiments is that in Hirschsprung's disease, nitric oxide synthase containing neurones are present in normally ganglionated colon but are completely absent from the aganglionic segment. This finding is therefore consistent the hypothesis that nitric oxide synthase containing nerves mediate the rectoanal inhibitory reflex because both the nerves and the reflex are absent in Hirschsprung's disease.

The absence of nitric oxide synthase containing neurones in aganglionic gut in Hirschsprung's disease helps to explain why this segment acts as a functional obstruction to the passage of intestinal contents. Physiological evidence suggests that NO plays an important role in nerve mediated relaxation of the human colon (Boeckxstaens *et al.*, 1993; Burleigh 1992; Keef *et al.*, 1992). This is likely to manifest in two ways, based on results from animal experiments. Firstly, nitric oxide synthase immunoreactive neurones which have identical morphological characteristics to those described here, have been demonstrated to have the correct projections and neurochemistry to be the inhibitory neurones which mediate the descending relaxation phase of the peristaltic reflex (Costa *et al.*, 1992). Their absence in the aganglionic segment in Hirschsprung's disease might therefore account for the failure of circular muscle relaxation in this region during peristalsis. Secondly, spontaneous, oscillating contractile activity in colonic circular muscle (mechanical slow waves) is controlled in part by electrical slow waves generated in a pacemaker region along the submucosal border (Szurszewski 1981; Smith *et al.*, 1987). This area is densely innervated by NANC inhibitory nerves which appear to utilise NO as a neurotransmitter (Smith *et al.*, 1989; Ward et al., 1992). Release of NO reduces the amplitude and duration of electrical slow waves which in turn diminishes the force of muscle contraction (Smith *et al.*, 1992; Ward *et al.*, 1992). If slow wave activity in human

colonic circular muscle is controlled in a similar way to this, then one can speculate that in the aganglionic segment in Hirschsprung's disease, absence of NO producing nerves removes an important inhibitory factor in the control of spontaneous circular smooth muscle contraction.

The changes in the arrangement of the myenteric plexus in the transition zone in Hirschsprung's disease have not been reported before. This re-emphasises the value of using whole-mounts to assess neuronal architecture. Quite why ganglia and nerves change their alignment in the transition zone is unclear, but this might result from a change in the extracellular matrix during development. Abnormalities of neurotrophic factors have been described (Parikh *et al.*, 1992). The effect the abnormal neuronal architecture in the transition zone has on motility in this region is also uncertain but clearly, normal innervation cannot be assumed on the basis of the presence of nerve cell bodies (ganglion cells) alone.

Finally, in view of its simplicity and accuracy as a marker for an important group of enteric neurones, NADPH diaphorase histochemistry might be a useful addition to the diagnostic armoury in Hirschsprung's disease. Its value is currently being evaluated and it seems likely that it will have a role as an adjunct to cholinesterase staining in equivocal cases.

# *Chapter 8*

## THE ROLE OF NITRIC OXIDE IN CHOLINERGIC INDUCED RELAXATION OF HUMAN INTERNAL ANAL SPHINCTER SMOOTH MUSCLE *IN VITRO*.

# INTRODUCTION

## 1. The link between muscarinic cholinergic receptors and nitric oxide

The discovery that nitric oxide is an endogenous bioactive substance arose from the investigation of the mechanism which is responsible for relaxation of vascular smooth muscle in response to stimulation of muscarinic, cholinergic receptors (Furchgott and Zawadzki, 1980). As demonstrated in chapter 4, human internal anal sphincter smooth muscle also relaxes in response to muscarinic cholinoceptor stimulation, as evidenced by the following results;

*i.* relaxation of human internal anal sphincter smooth muscle in response to $10^{-4}$M carbachol is abolished by atropine,

*ii.* relaxation of internal anal sphincter smooth muscle in response to $10^{-4}$M carbachol is not affected by hexamethonium ($5x10^{-6}$M), but this agent does abolish the response to nicotine $10^{-4}$M,

The mechanism which subserves this response has not been established. It is possible that NO is involved here as it is in the response of vascular smooth muscle. Clearly this warrants further attention as it will increase our understanding of the interaction between cholinergic nerves and the internal anal sphincter.

## 2. Relaxation of internal anal sphincter smooth muscle following muscarinic cholinergic receptor stimulation involves release of a non-adrenergic, non-cholinergic neurotransmitter. Could this be nitric oxide?

The relaxant effect of muscarinic cholinoceptor stimulation on the anal canal has been demonstrated *in vivo* by Gutierrez and Shah (1975) who infused bethanechol (a muscarinic cholinoceptor agonist) in to 7 normal healthy human volunteers. They recorded a significant fall in resting anal canal pressure, indicating internal anal sphincter relaxation. This response was abolished by atropine confirming that bethanechol acted through stimulation of muscarinic cholinoceptors. The location of these receptors was not determined further.

Bethanechol has also been shown to relax isolated strips of internal anal sphincter smooth muscle *in vitro,* as has acetylcholine (Burleigh *et al.,* 1979). In their detailed account, Burleigh *et al.* ( 1979) demonstrated that the relaxant effect of both these agents could be antagonised by tetrodotoxin, an agent which inhibits the propagation of nerve impulses. Tetrodotoxin significantly inhibited the action of acetylcholine on strips of smooth muscle from the proximal part of the internal anal sphincter however, it did not affect the response of those from the distal part of sphincter to the same degree. In addition, the action of acetylcholine was unaffected by both hexamethonium and propranolol but it was blocked by hyoscine, a muscarinic cholinoceptor agonist. Burleigh *et al.* (1979) concluded that acetylcholine stimulated nerves to release an unknown neurotransmitter which is most probably NANC in nature.

Similar aspects of the behaviour of feline internal anal sphincter smooth muscle have been examined *in vitro* by Todorov and Papasova (1984). As in the human, application of acetylcholine resulted in relaxation which was abolished in the

presence of both atropine and hexamethonium. The response was not affected to the same extent when either of these agents was applied in isolation. In addition, the effect of acetylcholine was abolished by tetrodotoxin but it was not inhibited by either reserpine or guanethidine. Based on these findings, Todorov and Papasova (1984) concluded that acetylcholine induces relaxation in this tissue by stimulating release of a NANC inhibitory neurotransmitter from nerves which possess both nicotinic and muscarinic cholinoceptors.

Acetylcholine can also relax other sphincter-specialised smooth muscle within the gastrointestinal tract. In the lower oesophageal sphincter (Velkova *et al.*, 1979), relaxation in response to acetylcholine is mediated through stimulation of nicotinic cholinoceptors situated on postganglionic adrenergic nerves and in the canine ileo-caecal sphincter relaxation is mediated through stimulation of nicotinic receptors situated on NANC nerves (Pelkmans *et al.*, 1989). In this respect, relaxation of internal anal sphincter smooth muscle is unusual as it appears to be mediated through stimulation of muscarinic rather than nicotinic cholinoceptors and it is a response shared only by endothelialised vascular smooth muscle preparations.

The aims of the experiments described in this chapter were to determine;

***i.* if relaxation of human internal anal sphincter smooth muscle which occurs in response to stimulation of muscarinic cholinoceptors is mediated through production of nitric oxide and,**

***ii.* the site of nitric oxide production.**

## METHODS

Sphincter tissue was obtained from patients undergoing abdomino-perineal resection of the rectum and anal canal for low-lying rectal carcinoma (4 males and 3 females; median age 66 years, range 40-86). Strips of smooth muscle were excised from the distal 2cm of the internal anal sphincter and all strips contained parallel bundles of smooth muscle fibres.

Once dissected, muscle strips were mounted for isometric tension recording in superfusion organ baths as described previously. After the strips had reached a steady state of tension, their response to electrical field stimulation was determined. The stimulation parameters used were similar to those described previously. The role that nitric oxide plays in carbachol induced relaxation of internal anal sphincter smooth muscle was determined by examining the effect of L-nitroarginine and oxyhaemoglobin on this response. Both agents were applied at concentrations which had previously been shown to abolish nerve mediated relaxation. Each strip acted as its own control i.e.. carbachol was administered before and after incubation with the antagonist, and to ensure responses did not change over time, test strips were run parallel with strips which did not receive antagonists.

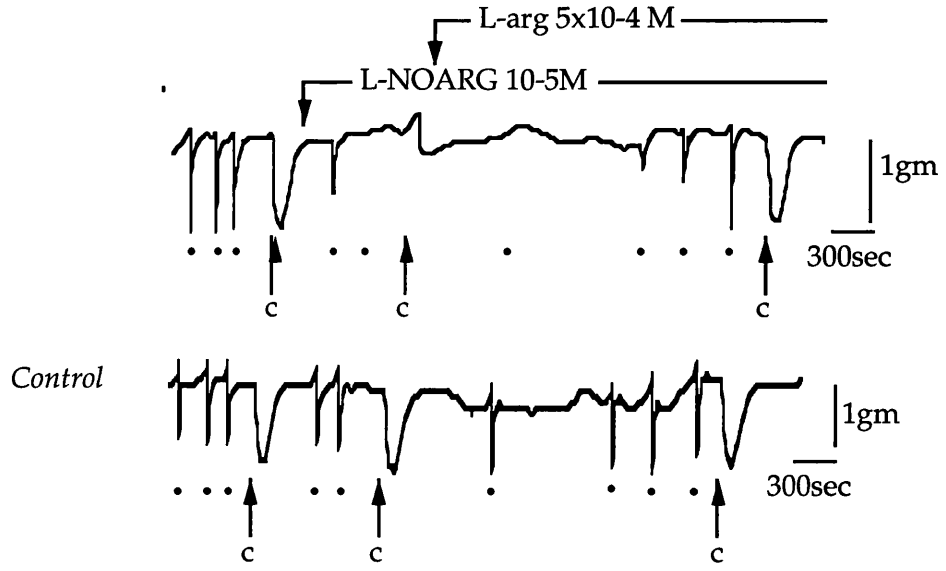
# RESULTS

## 1. Involvement of nitric oxide in carbachol induced relaxation of internal anal sphincter smooth muscle

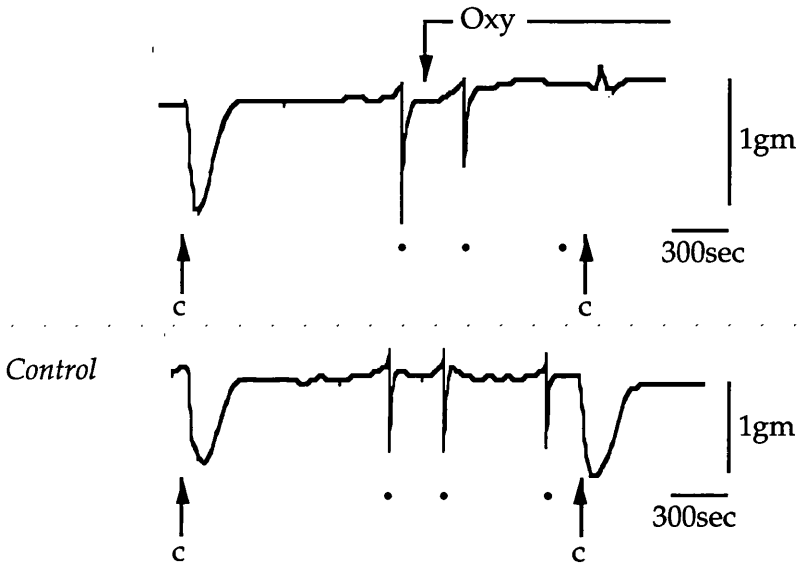
As can be seen in figure 1, at the concentrations applied both N-nitro-L-arginine and oxyhaemoglobin abolish neurogenic relaxation and produce a marked reduction in the response to carbachol. N-nitro-L-arginine ($10^{-5}$M) reduced the response to carbachol ($10^{-4}$M) by 54.5±9%, $p<0.0001$ (*n=24(7)*). The effect of L-nitroarginine is reversed by L-arginine, added at a sufficient concentration to restore nerve mediated relaxations. Carbachol induced relaxation of strips incubated with N-nitro-L-arginine ($10^{-5}$M) and L-arginine ($5x10^{-4}$M) is 97±4.7% of that seen in controls (*n=15(4)*). Oxyhaemoglobin ($5x10^{-5}$M) reduced the response to carbachol ($10^{-4}$M) by 81.5±9%, $p<0.0001$ (*n=8(2)*).

Figure 1

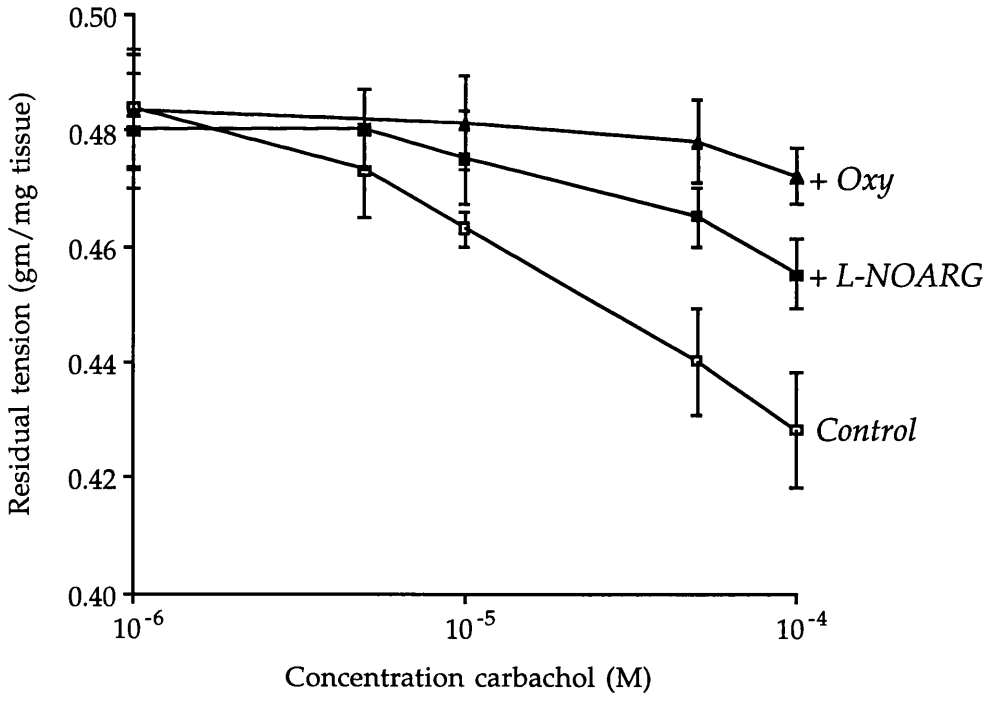
*i.* The effect of N-nitro-L-arginine (NOARG) and L-arginine on neurogenic relaxation (• = 10V, 0.5ms. duration, 10Hz, for 1 sec) and the response to carbachol (c: $10^{-4}$M). The effect of N-nitro-L-arginine is antagonised by L-arginine (L-arg). Test strips were run in parallel with control strips which were not incubated with the antagonist.



*ii.* The effect of oxyhaemoglobin (Oxy : $5x10^{-5}M$) on the response to carbachol (c: $10^{-4}M$) and electrical field stimulation (•= 10V, 0.5ms.duration, 10Hz, for 1 sec).




*iii.* The effect of oxyhaemoglobin (Oxy: $5x10^{-5}M$) and N-nitro-L-arginine (L-NOARG: $10^{-5}M$) on the dose dependent response of internal anal sphincter smooth muscle to carbachol.



## 2. The site of carbachol induced nitric oxide synthesis

Tetrodotoxin (3x10-6M) did not inhibit the effect of carbachol ($10^{-4}$M) observed here. In the presence of tetrodotoxin $3x10^{-6}$M, the response to carbachol was 105±8.3% of that seen in controls (*n=11(3)*). Venom from the scorpion *Leiurus quinquestriatus* blocks sodium channels (Hille, 1992) and has been shown to inhibit NANC inhibitory neurotransmission in the lower oesophageal sphincter (Daniel and Posey-Daniel, 1984). It did abolish neurogenic relaxation of internal anal sphincter smooth muscle but it did not affect the response to carbachol which, in the presence of 20μm/ml scorpion venom (Daniel and Posey-Daniel, 1984), was 102±9.5% of that seen in controls (*n=6(2)*). ω-Conotoxin, derived from the cone shell *Conus geographus*, blocks neuronal N type calcium channels (Hille, 1992) but has no effect on neurogenic relaxation of internal anal sphincter smooth muscle and has no action on the response to carbachol in this tissue (*n=3(1)*). The effect of potential antagonists of the response to carbachol is illustrated in figures 2 and 3.

Figure 2.

The effect of *i.* tetrodotoxin ($3x10^{-6}$M) and *ii.* scorpion venom (20μm/ml) on the response of internal anal sphincter smooth muscle to carbachol $10^{-4}$M (c). At this concentration, both neurotoxins abolish electrical field stimulated relaxation (•= 10V, 0.5ms.duration, 10Hz, for 1 sec), but the effect of carbachol is unchanged.

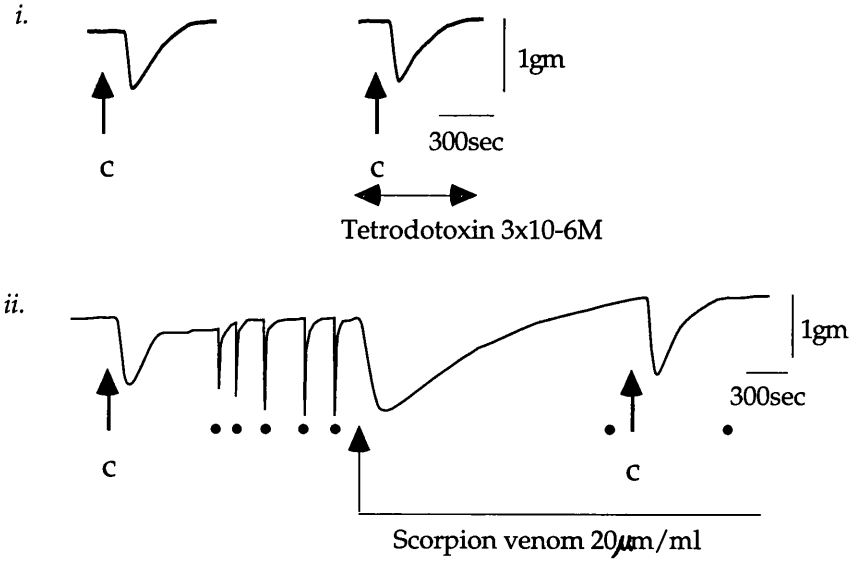


Figure 3.

The effect of putative antagonists on the response of internal anal sphincter smooth muscle to carbachol ($10^{-4}$M). This figure includes data from chapter 4.

L-NOARG- N-nitro-L-arginine $10^{-5}$M

L-ARG- L-arginine $5x10^{-4}$M

Oxyhaemoglobin $5x10^{-5}$M

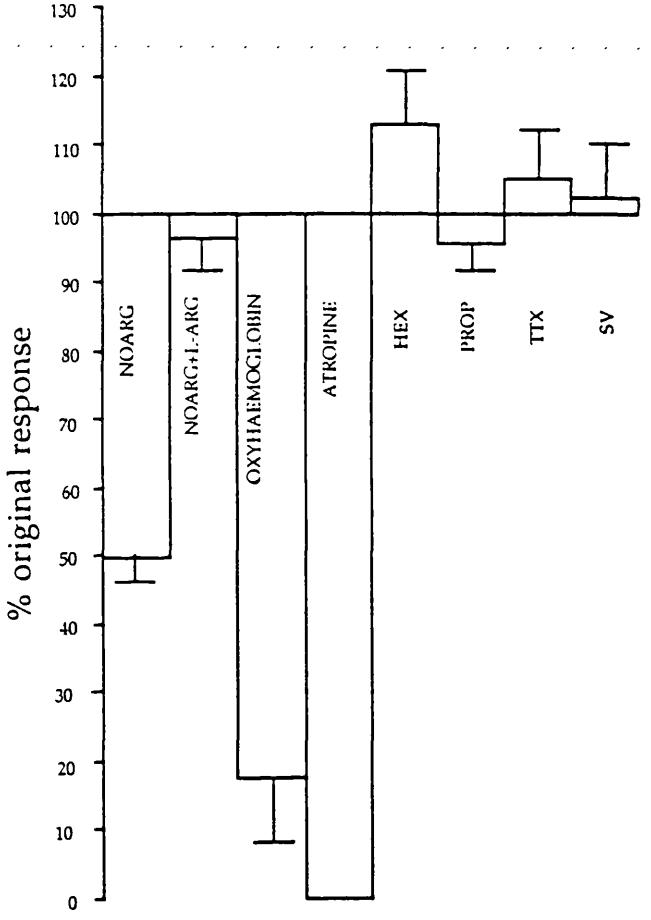
Atropine $10^{-6}$M

HEX-Hexamethonium $5x10^{-6}$M

PROP- Propranolol $5x10^{-6}$M

TTX- Tetrodotoxin $3x10^{-6}$M

SV- Scorpion venom 20µm/ml



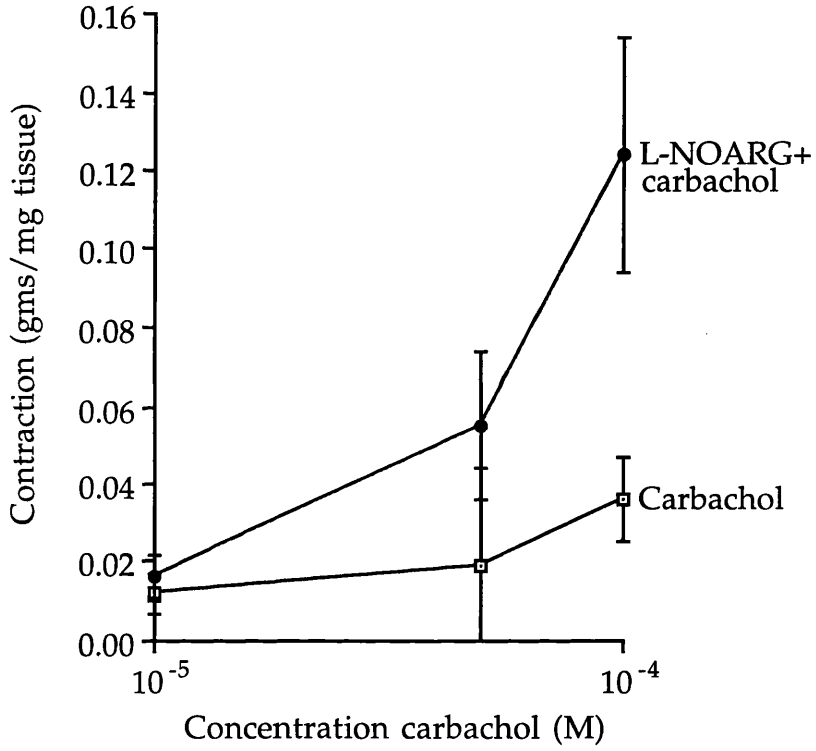

## 4. Contraction in response to carbachol

A small initial contraction was seen in some strips in response to carbachol, prior to relaxation. This was noted in 8 of the 24 strips which were subsequently incubated with N-nitro-L-arginine. Following administration of L-nitroarginine,

all strips showed a dose dependent contractile response which preceded the attenuated relaxation (*n=24(4)*). This is illustrated in figure 4.

Figure 4.

The initial contraction seen in muscle strips in response to carbachol, before and after administration of N-nitro-L-arginine (L-NOARG 10-5M).



## DISCUSSION

The results presented here indicate that relaxation of isolated strips of internal anal sphincter smooth muscle in response to muscarinic cholinoceptor stimulation is mediated by NO. This is evidenced by the inhibitory effect of both N-nitro-L-arginine and oxyhaemoglobin. Both agents were applied at a concentration which had previously been demonstrated to abolish neurogenic relaxation in this preparation. The action of N-nitro-L-arginine was reversed by L-arginine, further implicating involvement of the L-arginine/nitric oxide pathway here. Neither N-nitro-L-arginine nor oxyhaemoglobin completely abolished the dose dependent action of carbachol. Possible explanations for this resistance are; *i.* the concentrations of antagonists used were sufficient to abolish neurogenic relaxation in response to electrical field stimulation but they were not high enough to inhibit all nitric oxide synthase and nitric oxide activity, *ii.* muscarinic receptor stimulation releases a second (or more) inhibitory neurotransmitter whose synthesis and action is not blocked by N-nitro-L-arginine or oxyhaemoglobin, iii. muscarinic receptors on smooth muscle cells induce relaxation.

Based on findings presented earlier in this thesis, it seems that a neuronal source for nitric oxide is most likely here. If this is the case then one must conclude that the muscarinic receptors involved are situated at nerve terminals (as evidenced by the lack of ganglia in this tissue and the inactivity of tetrodotoxin), and that their activation is not coupled to tetrodotoxin or scorpion $\alpha$-toxin sensitive sodium channels. In addition, one must conclude that activation of nitric oxide synthase within these nerves is independent of neuronal *N* type calcium channels in view of the lack of activity of $\omega$- conotoxin.

In spite of the morphological evidence presented in this thesis, it is conceivable that a muscarinic receptor-linked, extra-neuronal source for NO exists within the internal anal sphincter. Endothelial cells, macrophages, and smooth muscle cells are possible synthetic sites. Production of NO by endothelial cells in response to muscarinic cholinoceptor stimulation cannot be excluded on the basis of data presented here but, synthesis within macrophages and myocytes is very unlikely.

The pattern of nitric oxide production studied here (synthesis in short bursts rather than for prolonged periods), is not that expected from the inducible form of nitric oxide synthase, and is instead characteristic of the pattern one would expect emanating from the constitutive form of the enzyme. On this criterion therefore, production of NO within macrophages is unlikely to account for the responses seen here.

Smooth muscle cells themselves represent another possible source of NO, but this is difficult to reconcile in view of morphological data which has failed to demonstrate the presence nitric oxide synthase within myocytes in the internal anal sphincter and experiments which examined the effect of oxyhaemoglobin on the response to carbachol. Oxyhaemoglobin remains out-side smooth muscle cells and exerts its inhibitory action by sequestering NO in the extra-cellular space. One must conclude therefore, that after synthesis in response to muscarinic cholinoceptor stimulation, NO also traverses the extracellular space and this implies that synthesis and action of NO within the same effector cell is most unlikely, but it cannot be ruled out completely. In fact, such a mechanism has been proposed by Grider *et al.* (1992). These authors studied the effects of electrical field stimulation and vasoactive intestinal peptide upon relaxation of

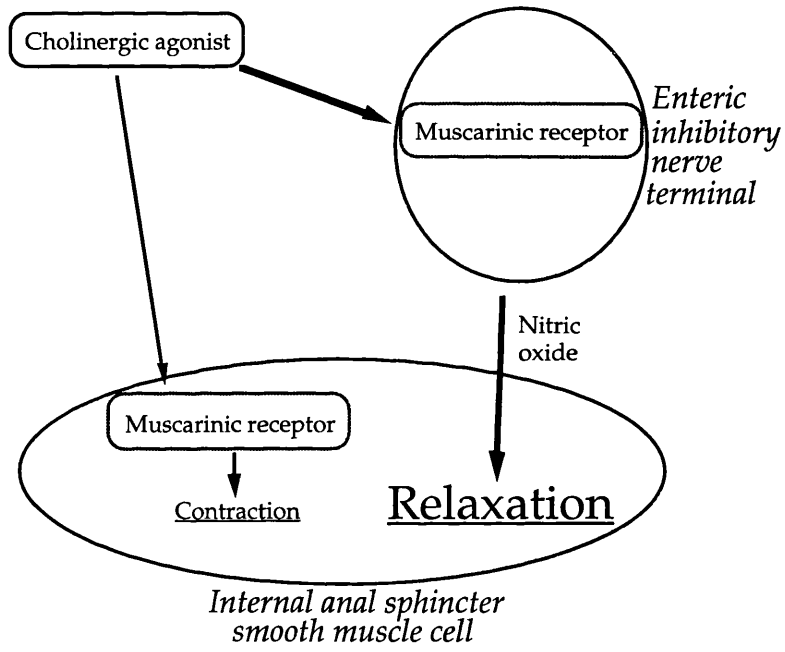
smooth muscle from the guinea pig gastric fundus. In muscle strip experiments they demonstrated that electrical field stimulation induced relaxation was associated with the release of both vasoactive intestinal peptide and NO. Addition of N-nitro-L-arginine abolished NO production and partially inhibited relaxation as well as the release of vasoactive intestinal peptide. This effect was reversed by L-arginine but not D-arginine. In subsequent studies using isolated myocytes, the authors reported that vasoactive intestinal peptide stimulated the release of NO. Again, this was inhibited by N-nitro-L-arginine. Grider *et al.* (1992) concluded that vasoactive intestinal peptide is the primary relaxant neurotransmitter in guinea pig gastric smooth muscle and that its release stimulates the production of NO which in-turn amplifies the effect of vasoactive intestinal peptide and enhances its release. Needless to say, these results are controversial and are at variance with other available data. Importantly, Grider *et al.* (1992) did not provide evidence of the purity of their myocyte preparation and its contamination by nerve cells or fragments of nerve fibres and they did not examine the effects of tetrodotoxin or oxyhaemoglobin. In addition, a constitutive form of nitric oxide synthase has never been demonstrated within smooth muscle cells. Thus, although interesting, this work from Grider *et al.* (1992) work must be viewed with caution until it can be verified by other investigators.

The dose dependent contractile component of the response to muscarinic receptor stimulation seen after application of N-nitro-L-arginine is interesting and suggests that, irrespective of the site of those mediating relaxation, muscarinic cholinoceptors which mediate contraction are almost certainly present on smooth muscle cells within the internal anal sphincter. Their influence on the activity of internal anal sphincter smooth muscle is only revealed when the inhibitory effects of nitric oxide have been removed. In figure

5, I propose a scheme which might explain the responses of internal anal sphincter smooth muscle strips which occur in response to muscarinic receptor stimulation.

Figure 5.

A possible arrangement of muscarinic receptors which would explain the responses of internal anal sphincter smooth muscle to carbachol.



# *Chapter 9*

# CONCLUSIONS

*Thesis* can be defined as a proposition to be maintained or proved. The thesis examined in the experiments described here is that NO mediates neurogenic relaxation of human internal anal sphincter smooth muscle, and that it is the hitherto undiscovered agent which mediates the rectoanal inhibitory reflex in man.

## 1. Nitric oxide is a neurotransmitter within the human internal anal sphincter

A neurotransmitter can be regarded as a substance which is released from a neurone that has an effect on that neurone or on a cell near its site of release. If a substance is to be considered as a neurotransmitter then at least two criteria should be fulfilled, firstly its release from neurones should be detectable and secondly there should be a uniformity of action on a post-junctional site when the substance is applied from an exogenous source and when it is released by nerve stimulation (Furness and Costa, 1987).

Based on these criteria and the evidence presented in this thesis, NO does appear to be an inhibitory neurotransmitter within the human internal anal sphincter. Although release of NO has not been demonstrated directly, its synthesis within, and release from nerves is strongly inferred. Firstly, there is a neuronal source for NO within the internal anal sphincter and in fact nerves were the only structures demonstrated to contain nitric oxide synthase in this tissue. Secondly, the effect of inhibitory nerve stimulation is antagonised and can be abolished by inhibitors of nitric oxide synthase. These agents are analogues of L-arginine (the specific substrate required for NO production), their action is enantiomerically specific and it can be reversed by addition of L-arginine itself. Thirdly, the effect of inhibitory nerve stimulation can be inhibited by an agent, oxyhaemoglobin, which scavenges NO as it traverses extracellularly. Finally, NO applied from an

exogenous source reproduces the effect of inhibitory nerve stimulation on internal anal sphincter smooth muscle.

My assertion that NO is an inhibitory neurotransmitter in the human internal anal sphincter is consistent with findings published by other researchers. As highlighted in the introduction to this thesis, my investigations were conducted at a time of exploding interest in NO and since initial reports appeared in 1990, it has been implicated as an enteric neurotransmitter in most regions of the gut, both *in vitro* and *in vivo*, and in a variety of mammals including man. Available evidence suggests that NO is involved in inhibitory neurotransmission in the oesophagus (Du *et al.*, 1991; Murray *et al.*, 1991; Yamato *et al.*, 1992), lower oesophageal sphincter (De Man *et al.*, 1991;Tottrup *et al.*, 1991), stomach (Desai et al., 1991; Boeckxstaens et al., 1990), pylorus (Bayguinov and Sanders, 1992), duodenum (Toda et al., 1990), jejunum (Stark *et al..*, 1991), ileum and ileocolonic junction (Boeckxstaens *et al.*, 1990; Bult *et al.*, 1990, Maggi *et al.*, 1991; Ward *et al.*, 1992), colon (Boeckxstaens *et al.*, 1993; Dalziel *et al.*, 1991; Huizinga *et al* 1992; Middleton *et al.*, 1993; Thornbury *et al.*, 1991) and the internal anal sphincter (Craig and Muir, 1991; Chakder and Rattan, 1992; Rattan *et al.*, 1992; Rattan and Chakder, 1992; Tottrup *et al.*, 1992). On the basis of immunocytochemical studies, there is morphological evidence for a neuronal source of NO in the canine proximal colon (Ward *et al.* 1992), the guinea pig small intestine and colon (Costa *et al..*, 1992; Young *et al.*, 1992) and throughout the rat gastrointestinal tract (Bredt et al., 1991; Schmidt *et al.*, 1992).

In addition to man (and the pig), involvement of NO in neurogenic relaxation of internal anal sphincter smooth muscle has now also been demonstrated *in vitro* in the guinea pig (Craig and Muir, 1991) and both *in vitro* and *in vivo* in the opossum (-Chakder and Rattan, 1992; Rattan and Chakder, 1992; Rattan *et al.*, 1992; Tottrup *et al.*, 1992).

In the guinea pig Craig and Muir (1991) demonstrated that internal anal sphincter smooth muscle relaxed in a dose dependent manner on application of exogenous NO. In addition, nerve mediated relaxation was significantly inhibited by oxyhaemoglobin and by L-nitroarginine methylester (54-66% of control responses), an effect which was partially reversed by L-arginine. The authors also demonstrated that apamin abolishes the relaxant action of ATP in this tissue and showed that this agent has no effect on neurogenic relaxation when applied alone. However, it did inhibit residual neurogenic relaxation when applied in the presence of oxyhaemoglobin and L-nitroarginine methylester, suggesting the possible release of a second neurotransmitter, although this was not identified.

In the opossum, N-nitro-L-arginine inhibits neurogenic relaxation of internal anal sphincter smooth muscle *in vitro* in a dose dependent manner, and at higher concentrations relaxations can be completely abolished (Tottrup *et al.*, 1992). The effect of N-nitro-L-arginine can be reversed by an excess of L-arginine but not by D-arginine at a similar concentration. Neurogenic relaxation is mimicked by application of sodium nitroprusside, an exogenous source of NO.

Results presented in this thesis also concur with those contained in a report from Burleigh (1992) which was published whilst the experiments described here were being completed. Burleigh also demonstrated the actions of N-nitro-L-arginine and L-arginine on neurogenic relaxation of the human internal anal sphincter smooth muscle *in vitro*. However, the dose dependent effect of sodium nitroprusside, the enantiomeric specificity of inhibitors of nitric oxide synthase (and its substrate L-arginine) and the action of oxyhaemoglobin were not described by Burleigh.

Conventional neurotransmitters such as acetylcholine and noradrenaline are stored in membrane bound vesicles prior to their release from presynaptic nerves, and they influence postsynaptic cells by interacting with membrane bound receptors on the cell surface. Nitric oxide does not appear to conform to this pattern of behaviour and indeed challenges classical concepts of neurotransmission. First, its receptor, guanylate cyclase, is cytosolic rather than membrane bound. Second, NO is both labile and very lipid soluble. It is thus unlikely to be packaged in membrane bound vesicles prior to release. In an effort to address these conceptual problems, it has been suggested that NO is stored in a more stable form, bound to another molecule such as cysteine, and there is some evidence supporting this (Thornbury *et al.*, 1991). Alternatively, NO could be produced on demand only, and it is possible that depolarisation makes calcium available for nitric oxide synthase activation within presynaptic neurones. Another possibility is that depolarisation releases nitric oxide synthase into the synaptic cleft and it is this free enzyme which produces NO. This theory is improbable however, as the proteolytic enzyme α-chymotrypsin enhances rather than inhibits nerve mediated relaxation of human internal anal sphincter smooth muscle and this militates against release of a peptide or protein (Burleigh, 1983).

The mechanism by which NO causes smooth muscle relaxation is also unclear. It is recognised that, by activating soluble guanylate cyclase, NO increases synthesis of cyclic guanosine monophosphate (cGMP), and this has been shown to produce membrane hyperpolarisation in colonic smooth muscle (Thornbury *et al.*, 1991). Such membrane changes are the hallmark of NANC nerve mediated relaxation and are thought to result from an increase in potassium conductance (Thornbury *et al.*, 1991). The processes responsible for electro-mechanical coupling have not been determined, but possible mechanisms include enhanced

$Ca^{2+}$ sequestration and reduced sensitivity of the contractile apparatus to $Ca^{2+}$. Involvement of additional second messengers is also possible.

## 2. Nitric oxide mediates the human rectoanal inhibitory reflex.

The rectoanal inhibitory reflex is thought to be a modified descending inhibitory peristaltic reflex which is mediated by NANC inhibitory nerves that arise from cell bodies within rectal myenteric ganglia and descend in the rectal myenteric plexus to innervate the internal anal sphincter. Results from experiments described here therefore provide good evidence that NO mediates this reflex in man.

Firstly, as described in the previous section, NO is intimately involved in nerve mediated relaxation of human internal anal sphincter smooth muscle and there are nitric oxide synthase containing nerves within this tissue. Secondly, these nerves arise from cell bodies which lie within rectal myenteric ganglia and they traverse the rectal myenteric plexus as they descend to the internal anal sphincter. Hence their distribution is appropriate for them to mediate the reflex. In addition, the morphological characteristics of these nerves is similar to that of equally reactive ones in the guinea pig myenteric plexus which have been extensively studied by Costa *et al.* (1992) and have been demonstrated to have the correct projections and neurochemistry to be the inhibitory motor neurones which mediate the descending inhibitory reflex in intestinal peristalsis. Finally, in Hirschsprung's disease, a condition in which the rectoanal inhibitory reflex is absent, nitric oxide synthase containing nerves are absent from the aganglionic, non-relaxing segment of gut but are present in the normally ganglionated segment which peristalses.

Unfortunately, due to the potentially dangerous vasoactive side-effects of nitric oxide and agents which affect its synthesis and function, it was not possible during the course of my experiments to establish the involvement of nitric oxide in the human rectoanal inhibitory reflex *in vivo*. However, such experiments have been conducted in the opossum (Rattan *et al.*, 1992). Rattan et al. (1992) demonstrated that relaxation of the internal anal sphincter occurs in response to rectal distension, local intramural nerve stimulation, sacral nerve stimulation and 1,1-dimethyl-4-phenylpiperazinium. Relaxant responses were inhibited in a dose dependent manner by N-nitro-L-arginine but not its stereoisomer N-nitro-D-arginine. The action of N-nitro-L-arginine was reversed by excess L-arginine. D-arginine had no such effect. In addition, exogenous NO, applied as sodium nitroprusside, caused dose dependent relaxation of the internal anal sphincter. These data strongly support the assertion that NO mediates the rectoanal inhibitory reflex.

## 3. Future considerations and therapeutic implications

My attention has focused on the motor component of the rectoanal inhibitory reflex and further research is required to determine sensory and inter-neurone inputs here. In addition, the mechanisms by which nitric oxide initiates smooth muscle relaxation must also be established. These investigations are possible now that the role of NO is established. It is conceivable that such investigations will suggest ways in which the rectoanal inhibitory reflex can be modulated to therapeutic advantage. Inhibition of the reflex in incontinent patients might reduce the amount of faecal leakage experienced and thus result in effective relief of symptoms. Also, a better understanding of the mechanism of internal anal smooth muscle relaxation will be important in determining the intrinsic properties of this muscle and this might in turn suggest ways in which spontaneous myogenic activity could be enhanced pharmacologically. It is

possible that high tone smooth muscles, such as the internal anal sphincter, have unique properties which could be utilised to therapeutic advantage.

The importance of NO in the function of the internal anal sphincter is further emphasised by its role in the response of internal anal sphincter smooth muscle to muscarinic cholinoceptor stimulation. This might indicate that NO is a mediator of inhibitory influences on the internal anal sphincter which emanate from both enteric and parasympathetic (post ganglionic) cholinergic nerves. It is important to establish if this is indeed the case as this is likely to cast new light on the mechanism of defaecation.

Recognition of the apparently important role that NO plays in internal anal sphincter relaxation has already stimulated research in the manipulation of the L-arginine/nitric oxide pathway in patients with high sphincter tone. Use of a nitrate paste is being investigated by a group at St. Mark's Hospital in London (UK) for the treatment of fissure-in-ano. High resting anal canal pressure is characteristic in this condition and indeed it has been implicated in its causation. Current management is directed at reducing pressure within the distal anal canal by either dividing or stretching the internal anal sphincter. In normal volunteers, nitrate paste (applied topically) results in a significant fall in internal anal sphincter pressure and thus produces what is essentially a chemical sphincterotomy (Loder *et al.*, 1993). Reports of the use of this paste in those with fissures is awaited.

# *Publications arising from this thesis*

## Papers

**O'Kelly TJ**, Mortensen NJMcC.
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**O'Kelly TJ**, Mortensen NJMcC and Brading AFB.
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*Gut* 1993;34:689-693.

**O'Kelly TJ**, Mortensen NJMcC and Brading AFB.
In vitro response of the longitudinal muscle layer of the human anal canal to cholinergic and adrenergic stimulation, compared with the internal anal sphincter and rectal smooth muscles.
*Br J Surg* (accepted for publication January 1993)

**O'Kelly TJ**, Davies J, Mortensen NJMcC and Brading AFB.
The distribution of nitric oxide synthase containing neurones in the human rectal myenteric plexus and anal canal; morphological evidence that nitric oxide mediates the rectoanal inhibitory reflex.
*Dis Colon Rectum* (accepted for publication January 1993)

**O'Kelly TJ**, Davies J, Tam P, Mortensen NJMcC and Brading AFB.
Abnormalities of nitric oxide producing neurones in Hirschsprung's Disease; morphology and implications.
*J Paed Surg* (accepted for publication February 1993)

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Inhibitory neurotransmission in the pig internal anal sphincter: The role of nitric oxide.
The Physiological Society of Great Britain, *J Physiol* 1992;446:523P.

**O'KellyTJ**, Brading AF, Mortensen N.
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The sphincter properties of the internal anal sphincter and the conjoint longitudinal coat of the anal canal.
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# *Statement by candidate*

All the investigations and results described here were undertaken solely by the candidate in the University Department of Pharmacology, Oxford with the exception of morphological experiments performed on cryostat sectioned material. These were performed by Dr. Jan Davies in the same department but the studies were all instigated, designed and analysed by the candidate.

This is the first morphological and physiological study which has investigated the role of nitric oxide in the innervation of the human internal anal sphincter and in particular the first to consider whether nitric oxide producing nerves could mediate the rectoanal inhibitory reflex in man. Throughout the text the candidate has indicated which findings are new and original, and which support those described by others either prior to the start of this research or whilst it was being conducted. In addition, where appropriate, the candidate has explained the importance of his findings with relevance to the advancement of medical science.

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