AETIOLOGY OF ~~ENTEROCOLITIS ASSOCIATED WITH~~ HIRSCHSPRUNG'S DISEASE

ASSOCIATED ENTEROCOLITIS.

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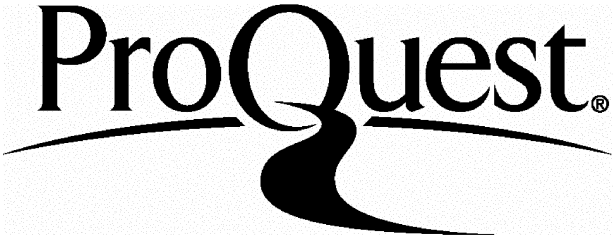

ABSTRACT

The aetiology of Hirschsprung's disease-associated enterocolitis was investigated. Similar isolation rates and toxigenicity of *C.difficile* were found in children with Hirschsprung's disease with or without diarrhoea and from children without Hirschsprung's disease. No evidence of cholera toxin-like enterotoxins was found in *Esch coli* or *Bacteroides spp* isolated from children with Hirschsprung's disease. Neither was secretory activity demonstrable in culture filtrates tested on piglet colon in Ussing chambers or cultured cell lines.

The efficacy of creating colonic aganglionosis using benzalkonium chloride was evaluated in neonatal piglets. Insufficient ablation of ganglia occurred with either BAC or the 14-carbon homologue for the model to be of use in assessing neurally-mediated colonic secretion.

The effect of the neural abnormalities in children with Hirschsprung's disease on the response to secretory stimuli was investigated using Ussing chambers. Basal electrogenic ion transport in aganglionic and ganglionic rectosigmoid and transverse colon from children with Hirschsprung's disease and in normally-innervated colon from children with anorectal anomalies were similar. Impaired neurally mediated secretion to Iloprost (prostacyclin PGI_2 analogue) and acetylcholine was demonstrated in aganglionic colon. The response to carbachol varied between children but the mean response was not significantly reduced.

Ganglionic colon proximal to the aganglionic colon also had a reduced response to acetylcholine despite a normal acetylcholinesterase staining pattern. Direct colonocyte secretion with *Esch coli* STa enterotoxin was unimpaired and resistant to neural blockade with tetrodotoxin. The responses to theophylline but not IBMX were reduced in aganglionic colon. Hypertrophic acetylcholinesterase-positive nerve fibres are likely to contribute to the reduced secretion to acetylcholine.

Conclusions :

i) No evidence was found for *C difficile*, or of other toxigenic bacterial products in the aetiology of idiopathic diarrhoea in children with Hirschsprung's disease.

ii) In contrast, human aganglionic colon had an overall reduced net secretory potential, particularly to neurally-mediated secretagogues.

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To my mother and father

"Truth lies within a little and certain compass,

but error is immense"

-Henry St.John, Viscount Bolingbroke,

1678-1751.

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1.1 **Congenital colonic aganglionosis - Hirschsprung's disease**

Hirschsprung's disease (HD) is a disorder of the distal colon which occurs in approximately 1 in 5000 live births and is the commonest cause of intestinal obstruction in the newborn. The main features, as listed by Schärli (1982), are:

1. A spastic, constricted segment of colon extending proximally from the anorectum for a variable length, but usually up to the sigmoid colon. The affected segment fails to peristalse due to a disorganised migrating motor complex.
2. The total absence of ganglion cells in both the myenteric and submucosal plexus. The aganglionosis extends proximally from the anorectum along a variable length of colon. Most commonly the affected area extends to the sigmoid colon (around 80% of cases) but with reducing frequency it can extend to affect the splenic flexure, the proximal transverse colon or the entire colon (total colonic aganglionosis). Between the normally innervated colon and the aganglionic segment is a transitional oligoganglionic zone. The level of aganglionosis does not necessarily correspond with the length of constriction.
3. Failure of the internal anal sphincter to relax following distension.

The most common presentation of HD is neonatal intestinal obstruction with bilious vomiting and a delayed passage of meconium. A proportion of patients will present later in childhood with chronic constipation.

Histochemistry

The abnormal control of the aganglionic muscle is thought to result from a disorder of the non-adrenergic, non-cholinergic inhibitory innervation (NANC) although abnormalities were first established in the cholinergic and adrenergic system. Considerable proliferation of acetylcholinesterase-positive nerve fibres (AchE) is seen in the aganglionic segment of colon (Meier-Ruge, 1974) and physiological studies have shown increased acetylcholine release along with raised tissue

concentrations of AchE (Garrett, Howard & Nixon, 1969; Ikawa *et al.*, 1980; Bonham *et al.*, 1985). In cases of long segment and total colonic aganglionosis the density hypertrophied acetylcholinesterase-positive nerve fibres falls and they never extend beyond the splenic flexure (Meier-Ruge, 1974). This distribution corresponds to their origin from extrinsic sacral S2-S4 parasympathetic innervation. Increased adrenergic innervation to the muscle layers and the submucosa with increased tissue concentrations of catecholamines have been repeatedly described although appears to vary with individual patients and is not a consistent finding (see Schärli, 1982).

The actual NANC neurotransmitter has yet to be identified but a number of candidates have been suggested: adenosine triphosphate (Burnstock, 1971), various neuropeptides including vasoactive intestinal peptide (VIP), somatostatin, substance P and, most recently, nitric oxide (Bult *et. al.*, 1990).

Several groups have investigated the neuropeptide profile in the colon of HD. Many studies report considerable variation between individual tissues in their neuropeptide staining patterns but the overall pattern includes the following:

Reduced concentrations of VIP and SP in both the aganglionic and transitional sections of colon compared with control tissue have been reported by several groups when measured by both immunohistochemistry and radioimmunoassay (Bishop, 1981; Dupont, 1980; Hamada *et al.*, 1987; Larsson *et al.*, 1988a; Taguchi *et al.*, 1983; Tsuto *et al.*, 1982, 1985; Wattchow *et al.*, 1991). Other peptides have also been shown to be reduced in the aganglionic section of colon: met-enkephalin, galanin, somatostatin, calcitonin gene-related peptide, gastrin-releasing peptide, neurokinin A (Hamada *et al.*, 1987; Hirose *et al.*, 1989; Larsson *et al.*, 1988a; Tsuto *et al.*, 1985; Wattchow *et al.*, 1991). Rogawski *et al.*, (1987) demonstrated a reduction in the serotoninergic (5HT) nerves.

In addition, increased frequency of fibres staining for neuropeptide Y have been demonstrated in aganglionic colon (Hamada *et al.*, 1987; Larsson *et al.*, 1988).

The immunohistochemical analysis of the ENS is becoming more complex with the

recognition of coexistence of different peptides within a single neuron but clearly alterations in the innervation to the colon exist and aganglionic zone cannot, therefore, be considered as totally denervated. While functional studies on the effect of purified peptides on intestinal function are accumulating, conclusions as to the possible effects of the different peptide patterns in Hirschsprung's disease remain highly speculative.

Physiological studies

In an attempt to unravel the underlying pathophysiology, *in vitro* physiological studies of HD have concentrated almost exclusively on the abnormal innervation and function of the smooth muscle innervation. Altered or absent muscle responses (principally contraction without any subsequent relaxation) have been demonstrated following electrical field stimulation (EFS) which activates all nerves (Hanini *et al.*, 1986; Larsson *et al.*, 1987; Nirasawa *et al.*, 1986; Okasora & Okamato, 1986) and the addition of the cholinergic agonists acetylcholine and carbachol (Hiramoto & Kieswetter, 1974); serotoninergic agonists (Beleslin, 1980); and adrenergic agonists (nicotine and dimethylphenylpiperazinium) (Beleslin, 1980).

In contrast, the consequences of the abnormal innervation on intestinal ion transport has received little attention (discussed further in chapter 4).

Embryology

The studies on avian neural crest development by Le Douarin (1982) and Gershon (1987) in particular have made significant advances in the understanding of the development of the intestinal nervous system. The ENS is derived *in-utero* from neural crest precursor cells which migrate both proximally and distally along the developing intestine. During migration, precursor cells are continually deposited where they develop and mature. Earlier studies led to the view that aganglionosis resulted from a failure of the migrating neural crest cells to reach and colonise a part of the colon (Webster, 1975). However, by explanting neural crest precursors into the ls/ls mouse intestine, a murine model of Hirschsprung's disease, it can be shown that migration into the developing colon itself is not defective but that

colonisation and maturation of the ganglion cell precursors is inhibited by the local microenvironment of the affected colon (Gershon, 1987), indicating that the defect lies in an inhibitory effect of the aganglionic colon and not in the ganglion cell precursors. The inhibitory factor(s) are not known. The aganglionic segment of colon in the ls/ls mouse contains an overabundance of laminin and type IV collagen and shows a hypertrophy of the muscularis mucosae (Payette *et al.*, 1988; Tennyson *et al.*, 1986). These substances can act as cell adhesion molecules and may regulate migration of the neural crest precursor cells. The inhibitory effect appears to affect the ganglion cells and not the axonal development: the neuronal axonal outgrowths are not inhibited in the affected segment and continue to proliferate resulting in the excessive cholinergic fibre production.

Immunopathology

Increased expression of MHC class II antigens throughout the colonic wall (including neural tissue) has been demonstrated (Hirobe *et al.*, 1992) and the authors suggest that aganglionosis may result from an autoimmune destruction of incoming ganglion cell precursors by host lymphocytes. Such findings add an extra degree of complexity to the possible embryological events resulting in Hirschsprung's disease.

Related disorders of the enteric nervous system

It seems likely that HD represents only one specific disease in a spectrum of disorders of the ENS, ranging from complete aganglionosis (HD), through hypoganglionosis, to hyperganglionosis (Howard & Garrett, 1984). The differences in the histopathology of total colonic aganglionosis to that of Hirschsprung's disease has led some authors to suggest that they are two separate diseases. Similarly, neuronal intestinal dysplasia (NID) represents a further variant intestinal neuropathy. NID is characterised by hyperganglionosis, with ganglion cells appearing in the lamina propria, together with an increase in the number of cholinesterase-positive nerve fibres and can occur in the proximal bowel in children with HD. Coexistence with HD has been reported but its true incidence is uncertain

(Meier-Ruge, 1974; Athow *et al.*, 1991; Schärli, 1992). Other intestinal disorders have been described in which children suffering from chronic constipation have intestinal neuropathological changes (Krishnamurthy & Schuffler, 1987).

Surgical Management of HD

HD is treated by surgical removal of the affected length of bowel. At the Hospitals for Sick Children, Great Ormond St, London this is performed in three stages:

i) The intestinal obstruction is relieved by forming a stoma in ganglionic bowel (usually the proximal transverse or sigmoid colon).

ii) The pullthrough procedure is performed when the infant is six to nine months of age. The aganglionic bowel is resected and the normal bowel is mobilised and anastomosed to the recto-anal margin. The surgical technique varies with surgical preference and the extent of the aganglionosis. The pulled-through colon usually adapts into a functional rectum.

iii) Once it has been established that there is no anastomotic leak or stricture the stoma is closed, usually 3-4 weeks after pullthrough.

1.2 Complications:Enterocolitis

Having undergone corrective surgery, a high proportion of children suffer from further complications. The most common are constipation, faecal incontinence and diarrhoea (Holschneider, 1982; Joosten *et al.*, 1988; Kleinhaus *et al.*, 1979; Lister & Tam 1990; Soave, 1977; Tariq, Brereton & Wright, 1991). The aetiology remains unclear in the majority of these cases. The different types of pullthrough procedure each have particular risks of complications.

Although an important component in the morbidity undoubtedly involves surgical damage to the internal anal sphincter (Cass, 1986), the problem of the diarrhoea and constipation can present both preoperatively and post operatively, seemingly irrespective of the time of operation.

The incidence of the complications is also considered by many paediatric surgeons to be greater in children with HD than those who have undergone similar surgery

for anorectal anomalies.

The enterocolitis associated with HD (HDE) remains the foremost cause of mortality and an important cause of morbidity in children with HD (Holschneider, 1982; Ikeda & Goto, 1984; Kleinhaus *et al.*, 1979; Nixon, 1982; 1985; Lister & Tam, 1989; Sherman *et al.*, 1989).

The early report by Bill & Chapman (1962) described the cardinal features of HDE: fever, abdominal distension and explosive watery stools. The histopathology of the inflamed colon was nonspecific and resembled that of colitis seen in intestinal obstruction due to carcinoma of the colon.

The incidence of HDE varies according to centre, most probably due to different diagnostic criteria and different operative procedures. Large patient number reviews give the incidence of enterocolitis between 18-29% with a mortality rate of up to 29% (Kleinhaus, 1979; Ikeda & Goto, 1984; Nixon, 1982; Sherman *et al.*, 1989).

A reduction in the mortality rate towards later years was noted in two of the reports which was ascribed to improved supportive clinical management. It was again noted that HDE occurs both before and after the surgical correction. Late postoperative enterocolitis occurred in 22% of cases in the review by Sherman *et al.*, 1989).

The aetiology of enterocolitis in HD (HDE) remains unknown. Several hypotheses have been suggested.

i.) *Intestinal distension.*

Bill & Chapman (1962) hypothesised that the colon proximal to the obstruction secretes water and electrolytes, creating excess faecal fluid despite intestinal faecal impaction. Intestinal distension is a long recognised potent stimulus of secretion (Florey, Wright & Jennings, 1941; Greenwood, 1989) supported by experimental work in animals (Shields, 1965). However, two principal problems remain with this explanation of HDE. Firstly, the enterocolitis appears to be specific to children with HD and is not reported in cases of neonatal intestinal obstruction due to other causes such as imperforate anus, intestinal malrotation and atresia; and secondly,

HDE can occur after corrective surgery in children with no intestinal obstruction.

ii). *Hypersensitivity reaction.*

Berry & Fraser (1968) artificially created colonic obstruction in rabbits by creating a ligature. The animals were then "sensitised" by an intraluminal injection of E.coli endotoxin and finally given an i.v injection of endotoxin twelve hours later. In contrast to the control animals which were only obstructed, 6/9 of the sensitised animals developed colitic changes and mucosal infarction. The authors proposed that a hypersensitivity reaction involving the intestinal vasculature could occur in the distended proximal colon in HD as bacteria invade the partially ischaemic mucosa. The interpretation that immune response to luminal bacterial endotoxin is acause of intestinal colitis has had little support in the ensuing years of immunulogical research in immunology and the explanation remains unconvincing. Perhaps because of this its relevance remains undetermined.

iii). *Immunological defects.*

Impaired neutrophil chemotaxis (reduced by 36%) was reported in nine cases of HD by Wilson-Storey *et al.*, (1988) when compared to age matched controls. Other broad immunological parameters, (total serum IgG, IgM and IgA, total WBC counts and lymphocyte subset ratios) were no different between HD, HDE and controls. The same workers also reported the complete absence of salivary IgA in children with HDE (Wilson-Storey & Scobie, 1989). It seems unlikely that local defects of the colonic immune system will be picked up with such tests and children with HDE do not suffer from the repeated local and systemic infections characteristic of immunodeficiency. Armstrong and Raafat (1988) found no difference in the immunoglobulin classes produced by local (gut) lymphocytes in children with HD and children with ulcerative colitis but did detect an increase in IgA-positive cells in both groups. In a similar study, Imamura *et al* (1992) demonstrated increased IgA- and IgM-containing plasma cells in the colon of children with HDE. Such studies of immunological cell populations in inflammed colon give no indication as to causal or secondary roles for immunopathology but

merely confirm that inflammation is present. Significant immunodeficiency is not confirmed in the clinical observations of children with HDE, but the increasing awareness of neural-immune cooperation might generate a more convincing argument for such a defect. The possibility of autoimmune destruction of ganglion cells (Hirobe *et al.*, 1992) turns the picture on its head by suggesting that the inflammatory cells are present in aganglionic colon for an altogether different reason.

iv). *Microbial infection.*

Recognised enteric pathogens in HDE were not isolated by early authors (Bill & Chapman, 1962; Stockdale & Miler, 1957) or Nixon (1985) but other studies have demonstrated the presence of certain microbial enteropathogens. Teitelbaum *et al.*, (1988) described enterocyte-adherent organisms in 7 out of 18 patients with HDE. The organisms reported were toxigenic *Esch.coli*, toxigenic *Clostridium difficile*, and *Cryptosporidium sp*. Rotavirus were reported from 7/9 cases of HDE by Wilson-Storey *et al.*, (1990). *C difficile* has also been isolated from children with HDE (Thomas *et al.*, 1986). Strains of *C difficile* producing the cell culture cytotoxin (toxin B) were isolated from a greater number of children with HDE than children with HD without HDE or control children. This association was not confirmed in a study by Wilson-Storey *et al.*, (1990) in children with Hirschsprung's disease up to 2 years of age.

Such studies have failed to implicate *C difficile* in a pathogenic role and its significance in paediatric colonic disease and its relevance to HDE remains unresolved. These findings have been investigated and are considered in more detail in chapter 2.

v). *Other findings*

Changes in the pattern of colonic mucin production have been described (Akkary *et al.*, 1981, Fujimoto & Puri, 1988) in human aganglionic colon. It is impossible to tell from these studies whether the changes in mucin type are secondary responses to inflammation or primary defects specific to HD. The authors suggest that

alterations in the mucin layer of the colon might lead to an increased susceptibility to the initiating events that lead to enterocolitis.

1.3 The enteric nervous system and its involvement in intestinal ion transport

The enteric nervous system (ENS) has a central regulatory role in the coordination of intestinal motility and intestinal ion transport as well as acting on intestinal vasculature, enteroendocrine cells and immunological tissues (Cooke, 1987; Hubel, 1989; Keast, 1987; Tapper, 1983). The disturbances in muscle contraction and relaxation seen in HD, Chagas' disease and achalasia illustrate the importance of a normal ENS in maintaining correct intestinal function. The generally accepted view that the ENS regulates the intestinal ion transport has important implications in the consideration of the aetiology of the diarrhoea associated with HD. As will be discussed below abnormalities of the ENS could result in a defective intestinal ion transport. The possible outcome could result in 1) an altered basal ion transport ("basal tone"), either through increased basal secretion or absorption with the net effect being a result of the flow of two opposite forces: active and passive absorption against active and passive secretion; 2) altered response to secretory or absorptive stimuli. The aganglionic colon would then respond either by secreting excessively or having a reduced ability to secrete. The neural influence on colonic ion transport is given below. Although much of the work in this area has been carried out on the small intestine the data quoted relate to the colon unless otherwise stated.

Colonic Neuroanatomy

The entire intestine is well served by neural tissues. The total number of enteric neurons is estimated to exceed that found in the central nervous system (Furness & Costa, 1987). The nerve fibres extend into the lamina propria and have been shown to originate from both the myenteric and submucosal plexus but a mucosal plexus has been also been described. At first it was thought that the myenteric plexus was only involved in the muscle control and the submucosal plexus was involved in the

submucosal mechanisms, in particular regulation of electrolyte transport, but interconnections have been shown running between the plexus layers in a number of species and such a separation of function is probably artificial.

The majority of the intestinal nerve fibres are of intrinsic origin and this is felt to represent the relative importance of the enteric nervous system in intestinal ion transport over the extrinsic autonomic input.

In addition to acetylcholine and noradrenaline, a large list of putative neurotransmitters (mostly peptides) have been shown to exert secretory or absorptive effects on the colon.

Adrenergic effects

Noradrenaline and other adrenergic agonists have been shown to increase the absorption of Na^+ and Cl^- in the rat colon *in vitro* (Racusen & Binder, 1979; Pamucku & Chang 1990) and in adult human distal colon (Sellin & DeSoigne 1987) acting via α_2 receptors on the enterocyte basolateral surface. The adrenergic fibres are of extrinsic sympathetic origin, consequently, *in vitro* studies are considered to lack a complete adrenergic input.

Cholinergic effects

In contrast to the absorptive influence of the adrenergic nerves the cholinergic nerves are secretory. Acetylcholine and carbachol have been shown to exert a secretory effect in the colon of rat (Zimmerman & Binder, 1983; Diener *et al.*, 1989), rabbit (Kuwahara *et al.*, 1987) and pig (Traynor *et al.*, 1991) *in vitro*. Muscarinic receptors (possibly M3) have been identified on rat enterocyte basolateral membranes.

Neuropeptidergic effects

A growing list of putative neuropeptides is emerging of different neurohormonal substances which have been shown to alter intestinal ion transport (reviewed in Brown & Miller, 1991). Mostly, the studies have been carried out in the small intestine of various animals but effects on the colon have been described with vasoactive intestinal peptide (VIP) which has a secretory effect on rat distal colon *in*

vitro (Racusen & Binder, 1977).

Neural control of basal ion transport

A "tonic" neural tone ie continual release of neurotransmitter which exert net secretory action, has been described in the small and large intestine of some but not all animals. The addition of the neural Na^+-channel blocker tetrodotoxin (TTX) to the serosal side of the mucosa will abolish all nerve fibre conduction of action potentials. The loss of any electrogenic tonic neural tone will immediately be shown by a fall in the transepithelial potential difference (Vt) and short-circuit in a preparation of mucosa in an Ussing chamber.

This has been reported in rat and rabbit distal colon (Andres *et al.*, 1985; Biagi *et al.*, 1990) where the addition of TTX significantly reduced the basal current through enhancing Na^+ and Cl^- uptake. The authors suggest that a continual secretory nervous influence maintains a balance between absorption and secretion and if the nervous activity is blocked with TTX then the epithelium is intrinsically set to net absorption rather than secretion. Kuwahara *et al.*, (1987) did not find a basal neural tone in guinea pig colon as TTX did not alter basal short-circuit current. Only one report exists that studied the effect of TTX on the human colon. Hubel *et al.*, (1987) found that TTX caused an accelerated fall in the basal short-circuit current over 20 minutes following addition to the serosa and suggested that this represented an inhibition of tonic neural activity. This finding is difficult to interpret. The slow decline is in marked contrast to the immediate drop shown in the rat (Andres *et al.*, 1986; Bridges *et al.*, 1986) and might represent a reduced ability to permeate the submucosa. Slow falls in the electrical properties of an *in vitro* preparation of mucosa could well mean that the tissue was dying. The presence of a neural tone in human colon is, therefore, unconfirmed and needs further investigation.

Response to secretagogues

The secretory response of the colon to certain secretagogues has been shown to be mediated, at least in part, by the submucosal nerves. Direct stimulation of the

submucosal (and mucosal) nerve fibres by electrical field stimulation in human colon (Kuwahara *et al.*, 1989) or by anemone toxin in the rat distal colon (Bridges *et al.*, 1986) results in electrogenic secretion and inhibition of Na^+ and Cl^- uptake. Certain prostaglandins cause secretion in the rat distal colon when applied to the serosal surface. Diener *et al.*, (1988) found that Iloprost (a synthetic prostacyclin analogue) induced Cl^- secretion which was mediated by the submucosal neurons as it was completely inhibited by serosal atropine and markedly reduced in an epithelial preparation with the submucosa (and, therefore, the submucosal plexus) removed. Prostaglandin E2 (PGE2) was also able to cause Cl^- secretion and its efficacy was reduced by about 50% in a submucosal preparation but not in a mucosal preparation. Bradykinin also has been shown to cause secretion by stimulating prostaglandin synthesis which in turn activates the ENS (Diener *et al.*, 1988). Such findings support the regulatory role of the ENS in the normal colonic function.

The involvement of the ENS in the response to "foreign" secretagogues acting from the luminal side is more problematic. Considerable evidence has been documented by Lundgren *et al.*, (1989) working *in vivo* on cat small intestine. The addition of TTX, hexamethonium, a cholinergic nicotinic receptor antagonist or lignocaine, a local anaesthetic agent, either abolished or reduced the fluid secretion into the lumen by approximately 50-70% after exposure to cholera toxin, *E.coli* STa enterotoxin, prostaglandin E2 and sodium deoxycholate. They postulate that certain epithelial cells (the enteroendocrine cells) are sensory receptor cells which transmit a signal via intermediary nerves to effector nerves which release secretory neurotransmitters. To support this they have also documented the release of 5-HT from EC cells on exposure to cholera toxin (Nilssen *et al.*, 1983). The reflex is also suggested to involve VIP-ergic neurons as they found increased levels of VIP in the jejunal venous return of cats during exposure to cholera toxin (Cassuto *et al.*, 1981).

In contrast, *in vitro* experiments have not been able to reproduced these findings.

Using rabbit and guinea pig ileum, Moriarty *et al.*, (1989) and Carey & Cooke (1986) found the response to cholera toxin unaffected to the presence of TTX when examined in Ussing chambers. Clearly, the tissue preparations used in Ussing chamber experiments do not have a completely intact neural network as the outer muscle layers are removed but the interneurons within the mucosal and submucosal plexus would still be intact and therefore susceptible to TTX inhibition. Changes in intestinal submucosal muscle tone can affect net hydraulic conductance in rabbit ileum (Ashan, Naftalin & Smith, 1988; Smith 1984). Consequently, in the studies of Lundgren *et al* it is unclear whether TTX attenuates secretion *in vivo* by an indirect action on the innervation to the outer muscle rather than on the nerves supplying the mucosa.

In vivo studies using the suckling mouse by Guerrant *et al.*, (1980) also found no inhibition of STa-induced secretion with propanolol or atropine. These data leave the question of neural regulation of luminal secretagogues open to discussion.

Specific disease states

Clinical evidence of altered intestinal ion transport resulting from abnormalities in the ENS is limited due to the difficulty of studying such changes in humans.

The best example for which there is clinical and experimental evidence of an underlying neuronal abnormality is diabetes mellitus. Diarrhoea is a well recognised complication in elderly patients with uncontrolled diabetes mellitus. It also develops in rats with streptozocin-induced diabetes. Evidence suggests that the diarrhoea results from a reduced adrenergic input to the small intestine as the absorptagogues epinephrine and tyramine added to the serosa failed to cause a reduction in the short circuit current and fluid loss in the intestine *in vitro* and *in vivo* (Chang *et al.*, 1985; 1986).

Other conditions exist in which the ENS is known to be altered such as Chagas' disease and oesophageal achalasia the effects have only been studied in relation to the motility disturbances and not the possible effects on ion transport.

From these data it can be seen that the ENS exerts an important influence on the

normal functioning of the gastrointestinal tract, particularly the motor activity and the ion transport functions. The understanding of the ion transport regulation has been derived almost exclusively from animal experiments, and has concentrated on the small intestine. The species differences that exist in animal work highlight the uncertainty in extrapolating the results to humans.

1.4 Hypothesis

HD is an important example of how abnormalities in the ENS can result in disturbances in the normal functioning of the intestine. It is hypothesised that abnormalities in the colonic mucosal innervation are responsible for episodes of enterocolitis in these children as a result of dysfunctioning neural control of water and electrolyte absorption and secretion. These will be manifest in the basal electrogenic ion transport across the colonic mucosa as well as in the electrogenic secretory events in response to secretory stimuli present in the colonic flora.

To examine this hypothesis, the following investigations were carried out:

i) The role of *C.difficile* as an aetiological agent of diarrhoea in children with Hirschsprung's disease (Chapter 2).

ii) The creation of a piglet model of colonic aganglionosis using benzalkonium chloride (Chapter 3).

iii) An examination of the effect of aganglionosis on the basal electrogenic colonic ion transport and the response to secretory stimuli in human paediatric colon *in vitro* using Ussing chambers (Chapter 4).

iv) The identification of putative microbial secretagogues in children with Hirschsprung's disease (Chapter 2 & 5).

Chapter 2.

Diarrhoea in children with Hirschsprung's disease: the association with toxigenic *Clostridium difficile*.

2.i Introduction

2.ii Methods
-EDTA-soluble antigen production
-HEp2 calcium mobilisation
-HEp2 adhesion

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-Review of clinical histories of children with Hirschsprung's disease and diarrhoea: aetiology of diarrhoea
-Incidence of *C.difficile* isolation and production of toxin B
-EDTA-soluble antigen profiles
-HEp2 calcium mobilisation
-HEp2 adhesion

2.iv Discussion

2.i Introduction

Several hypotheses have been proposed concerning the aetiology of Hirschsprung's disease-associated enterocolitis (HDE) (as discussed in Chapter 1).

Thomas *et al* (1982; 1986) reported an association between the presence of *Clostridium difficile* in children with Hirschsprung's disease-associated enterocolitis (as defined as onset of fluid stools) which was not found in those children without diarrhoea. Similarly, high titres of toxin B could be detected in the stool filtrates. These findings were supported by the prompt improvement seen in those treated with oral vancomycin. Pseudomembranous colitis was described in some of the children (Thomas *et al.*, 1982) and fatal cases have been described in Hirschsprung's disease by others (Brearly *et al.*, 1987; Price *et al.*, 1990).

In contrast, a more recent study by Wilson-Storey *et al* (1990) found no difference in the incidence of *C difficile* in newly diagnosed cases of Hirschsprung's disease up to 2yrs of age with or without diarrhoea. As a number of letters to the Lancet duly pointed out following the first report by Thomas *et al*, *C difficile* can be isolated from the faeces of children without any intestinal pathology or symptoms. The incidence in newborn infants is high, ranging from 15 to 70% of neonates but this falls quickly after 12-18 months to adult rates (0 to 3%) by 3 years of age (George, 1986). This high neonatal incidence creates difficulties in establishing a primary pathogenic role for *C difficile* in children despite the symptoms of diarrhoea.

To determine the incidence, aetiology and pattern of presentation of diarrhoea in children with Hirschsprung's disease the clinical notes of children seen at the Hospitals for Sick Children, London were reviewed. In addition, the relationship between *C difficile* and the aetiology of diarrhoea in Hirschsprung's disease was investigated in a prospective longitudinal study on the isolation rate and toxigenicity of *C difficile* from children with and without diarrhoea.

The possibility of differentiating pathogenic strains of *C difficile* was also

investigated by testing for adhesion to HEp2 cell lines and ability to mobilise intracellular calcium in these cells. Adhesion to the mucosa is an essential step in the pathogenesis of intestinal infection. Differentiation of virulent strains of *C difficile* was noticed in challenge studies using hamsters (Borriello *et al.*, 1988) in which highly virulent strains were found adherent to the mucosa in high numbers in contrast to the avirulent strains.

The concentration of free intracellular calcium is a central component in the regulation of most eukaryotic cells and is therefore tightly controlled. Small fluctuations occur in the cytoplasmic calcium concentration through either the release of calcium from intracellular stores or uptake from the external solution. Various hormones and secretagogues raise intracellular calcium concentrations as a precursor event to secretion in enterocytes. Studies on the enteropathogenic *Esch coli* by Baldwin *et al* (1991) have shown that adhesion to cultured HEp2 cells resulted in release of calcium from intracellular stores. A similar event has been shown in neutrophils exposed to *C difficile* toxin A (Pothoulakis *et al.*, 1988).

2.ii Methods

Clinical histories

The records of 92 patients undergoing treatment for Hirschsprung's disease over a period of three years (1989-1991) were reviewed. The details of operations and the occurrence and, where stated, the causes of diarrhoea were noted in 44 children who had one or more episode of diarrhoea. The aetiology of the diarrhoeal episodes were considered independent to the isolation of *C difficile* as this is examined separately and stool samples were not obtained from all of the children whose notes were reviewed.

Distinction between enterocolitis, diarrhoea, gastroenteritis and loose stools

is frought with difficulty and although separate definitions can be made, the criteria necessary for such distinctions are not usually available. Therefore, the diagnosis given in the notes were used and the terms diarrhoea and enterocolitis were not substantiated with additional tests. For the purpose of this study, the term diarrhoea was used unless the evidence was available to substantiate an alternative description. This meant that diarrhoea with proven colonic inflammation and or systemic signs such as fever and requirement of intravenous fluid replacement was termed enterocolitis and milder forms of diarrhoea (e.g. was not fluid) was called loose stools.

Incidence of *C difficile* in children with Hirschsprung's disease

Samples Faecal samples (stools, rectal swabs, soiled nappies) were obtained from children with HD who were admitted to the Hospital or seen as outpatients. The samples from non-HD children were obtained either from surgical or medical wards and outpatients without any prior selection other than they did not have HD. If more than one specimen was obtained from a child during the stay in hospital it was considered as a separate episode only if obtained after 4 weeks from the previous specimen. Each visit was considered as a separate episode.

Bacteriological culture

Stool specimens were emulsified where necessary in PBS at approximately a 1 in 10 (w/v) proportion. For the selective isolation of clostridia, the broth suspension was treated with a volume of chloroform to a ratio of 1:3 chloroform:suspension. The suspension was then mixed on a vortex shaker for 10 seconds and left at room temperature for 5 minutes before culturing on cycloserine-cefoxitin-fructose agar (Oxoid) and blood agar. Cultures were incubated anaerobically at 37^0C for 48 hours.

Identification

Gram positive bacilli growing on CCFA were identified as *C.difficile* by fluorescent yellow colony colour under UV illumination, together with the production in cooked meat broth cultures of major peaks of iso-caproic and iso-butyric acid along with iso-valeric and butyric acid detected by gas liquid chromatography, and by biochemical identification using the API ATB 32A kit.

C.difficile toxin B testing

Production of toxin B was carried out using a human embryonic lung (HEL) cell line. The isolates were incubated in 20ml reduced BHI broth (rBHI) with 0.05% w/v cysteine HCl for 48 hours. 2ml of the culture suspension was centrifuged at 10000rpm (minifuge) for 10 mins and filtered through a sterile 0.2μm membrane filter. 200μL of a 1 in 10 dilution in Dulbecco's minimal essential medium (DMEM; Difco) was added to a tube of HEL fibroblasts containing 2ml growth medium and incubated on a roller at 37°C for 24-48hrs (final dilution: 1 in 100). Cytotoxicity was characterised by rounding up and detachment from the tube of the fibroblasts usually after overnight incubation. The cytopathic effect was completely neutralised by 200μl of *C.sordelli* antitoxin (Wellcome Ltd) from a stock solution diluted 1 in 100. Broth control and cell controls were also included.

Adhesion to cultured HEp2 cell monolayers

The *C difficile* strains were incubated in 20ml rBHI at 37°C for 48 hrs (without shaking) and centrifuged at 2000rpm at 15°C for 10 mins and the cell deposit was

gently resuspended in warmed DMEM to A_{620} 0.08-0.13 optical density, corresponding to $10^{-6}$to 10^{-8} cfu/ml. 100μl of the cell suspension was added to 7-10 day old cell monolayers of HEp2 human laryngeal epithelial cell line grown on glass coverslips (shellvials) and incubated at 37°C for 60 & 180 minutes anaerobically. The cell layer was then gently washed x3 with warmed PBS before fixing with 70% methanol for 10 minutes. The cell layers were stained with 10% Giemsa (BDH) in pH 6.8 buffer for 5 minutes and then destained with buffer for 2-5 minutes. Coverslips were dried at 37°C and examined under oil immersion. Adhesion to the cells was determined qualitatively by visual estimation of the number of bacilli to each cell, where positive adhesion was considered to be >5 bacilli per cell. The number of cells with adherent bacilli was then estimated over the entire coverslip. Adherent and non-adherent strains of *Esch coli* (characterised previously, kindly supplied by Dr P Everest, University of Leicester) were used as positive and negative controls for the method employed and tested identical to the method for the *C difficile* isolates except cultures were used after 24hours incubation. All isolates were tested on three separate occasions using up to 4 coverslips per strain.

Mobilisation of intracellular calcium in cultured HEp2 cell monolayers

The method described by Baldwin *et al* (1991) was employed using the methylester derivative 9-(4-bis-4(carboxymethyl)amino-3-(2-(2-bis(carboxymethylamino-5-methylphenoxy)ethoxy)phenyl)-2-7-cichloro-6-hydoxy-3H-xanthin-3-one (Fluo-3, Sigma). Strains of *C difficile* were inoculated into 20ml rBHI for 48 hours at 37°C. 1.5ml of the culture supernatant was centrifuged using a minifuge to obtain a clear fluid and then diluted by a ratio of 1 in 200 in DMEM to neutralise the acidity. Approximately 150μl was added to the 5-10 day old HEp2 cells cultured on glass coverslips (23mm x 23mm) after washing three times in warmed PBS. The supernatant-treated cells were incubated for 2, 4 and 6 hours at 37°C in a moist atmosphere with 10% CO_2. Cells were then washed three times in PBS and then incubated for 60 minutes with 50μM Fluo-3, prepared in DMEM without

phenol red (phenol red autofluoresces). After washing three times with PBS the cells were examined under a Zeiss Axiophot microscope with exitation and emission wavelengths at 490 and 530nm respectively. To minimise fluorescence quenching, all manipulations were carried out in low light conditions.

This work was carried out at the Department of Genetics, University of Leicester with Dr P. Everest.

EDTA-soluble antigen typing

Isolation of ethylenediamine tetraacetic acid (EDTA)-soluble antigens was carried out using the method described by Poxton & Byrne (1981). Isolates were inoculated into 100ml rBHI and incubated for 24hrs at 37°C (static). Cultures were centrifuged for 20mins at 8000g @ 15°C and the deposit washed in cold PBS and respun at 8000g for 15 mins. 500μl of the cell deposit was mixed with 1ml of EDTA-chelation buffer (50M EDTA in PBS) and incubated in a 45°C waterbath with regular mixing for 30 minutes. The cell suspension was spun at 10000rpm (minifuge) and the supernatant was removed and dialysed against tap water overnight. The antigens were then stored at -26°C.

Sodium dodecyl -Polyacrylamide gel electrophoresis (SDS-PAGE)

Gel electrophoresis was used to separate the EDTA-soluble antigens extracted from *C difficile*. Antigen preparations were diluted 1 in 2 with sample buffer to give a final concentration of 2.5% SDS, 5% mercaptoethanol & 1mM EDTA in 10mM Tris buffer and boiled for 5mins before loading on 12.5% polyacrylamide SDS-gels. Bands were visualised using a silver stain method as given by the manufacturer.

Statistical analysis

Differences between groups were tested for using the Chi squared test with Yates' correction. Significance was taken at 95%

2.iii Results

Review of causes of diarrhoea in children with Hirschsprung's disease

Of the 44 children with HD who had one or more episodes of diarrhoea 12 (27%) had total colonic aganglionosis or subtotal colectomy for long segment HD.

Certain common features in the patterns of presentation of diarrhoea were noted. In 9 cases (21%) the diagnosis of HDE was made in neonates presenting in the first week of life with intestinal obstruction, fever and the explosive expulsion of fluid faeces on rectal examination. The symptoms were relieved in all but one case on the formation of defunctioning colostomy.

Twenty episodes of gastroenteritis were diagnosed (46.5%) in which a recognised enteropathogen was isolated in 18: rotavirus (2), adenovirus (8), small round structured viruses (1), enteropathogenic *E.coli* (2), enterotoxigenic *Esch coli* (1), *Campylobacter sp* (1) and *Aeromonas hydrophila* (2). Cysts of *Cryptosporidium sp.* were seen in one case.

Four children (9%) suffered from chronic loose stools related to faecal incontinence, two of whom were successfully treated with either rectal myectomy (one case) or repeat pullthrough procedure (one case).

Three children (7%) were diagnosed as having colonic neuronal dysplasia (Schärli, 1991) after repeated occurences of diarrhoea following an original diagnosis of HD treated by pullthrough procedures. Subsequent treatment by a further ileorectal pullthrough procedures resulted in no further episodes of diarrhoea.

Four cases of chronic diarrhoea (9%) were in children with ileostomies for either total or long segment HD which had been treated by extensive or complete colectomy. A definite diagnosis of small intestinal bacterial overgrowth was made in one child.

One child with glucose 6-phosphate deficiency had episodes of haemorrhagic colitis associated with haemolytic crises.

No cases of food intolerance or inflammatory bowel disease resembling Crohn's

disease or ulcerative colitis were diagnosed in these patients.

The number of children remaining in whom episodes of diarrhoea occurred of unknown aetiology was 13 (30%).

Stool culture for *C.difficile*

The incidence of *C difficile* in relation to the occurrence of diarrhoea was investigated. A total of 122 stools were cultured, 75 from children with HD and 47 from non-HD controls (children without HD or other intestinal neuropathies). The samples were categorised into four groups on the basis of whether the child had Hirschsprung's disease and/or diarrhoea.

The isolation rates of *C.difficile* from the four groups of children are given Table 2.1. No statistically significant differences were found between the isolation rate of *C difficile* from episodes of diarrhoea from children with Hirschsprung's disease (16 out of 44: 36%), and those without diarrhoea (7 out of 31 episodes: 31%). These values were not significantly different from those obtained in the children without Hirschsprung's disease, with diarrhoea (16%) and without diarrhoea (27%).

A number of children with Hirschsprung's disease presented on more than one occasion, one child (SM) presenting 4 times with diarrhoea, and twice without diarrhoea (*C.difficile* was isolated on three occasions, two from episodes of diarrhoea). To correct the undue weighting of repeated isolation of *C.difficile* from the same children, the data were also analysed for the incidence of *C difficile* in children rather than by episode (data shown in Table 1). Again, no significant differences were found between the isolation rate from all four groups.

The relationship between isolation of *C difficile* and the severity of the diarrhoea was examined. 17 children with Hirschsprung's disease had a single recorded episode of diarrhoea of short duration (less than 2 weeks) of whom 6 grew *C difficile* (35%). Chronic or recurrent diarrhoea occurred in 16 children with Hirschsprung's disease and *C difficile* was isolated from 6 children (37.5%). No

significant difference was found between the severity of diarrhoea and incidence of *C difficile*.

The relationship between age and *C.difficile* carriage is shown in Fig 1. A prolonged carriage of *C.difficile* was found in the children with HD and diarrhoea (Group II mean age 40mths, range < 1-180mths) with one child carrying *C.difficile* at 15 years of age, whereas colonisation was not found in the other groups beyond 12 months of age (data given in Table 1).

Toxin B production

The proportion of strains of *C.difficile* that produced enterotoxin B was similar in the strains from children with HD and diarrhoea (8/16: 50%) and those without diarrhoea (4/7: 57%). Similarly the production of enterotoxin B was comparable in the children without HD that did (2/6:33%) and did not have diarrhoea (2/4: 50%).

Table 2.1. Incidence of *C.difficile* (CD) in children with Hirschsprung's disease

	Hirschsprung's disease				Control			
	No diarrhoea		With diarrhoea		No diarrhoea		With diarrhoea	
	CD+	CD-	CD+	CD-	CD+	CD-	CD+	CD-
No of episodes	7	24	16	28	4	21	6	16
No of patients	7	20	12	25	4	21	6	16
Mean age (mths)	3.7	29.3	40.5	50.4	3.25	27.3	7.7	26.2
Range	≤8	<1-132	<1-180	<1-162	1-7	<1-156	4-12	2-156
No. >18mths[1]	0(0%)		7(46%)		0(0%)		0(0%)	
Toxin B[2]	4(57%)		8(50%)		2(50%)		2(33%)	

Abbreviations: CD+: *C.difficile* isolated; CD-: *C.difficile* NOT isolated.

1: Number of episodes in children over 18mths old.
2: Production of toxin B by strains of *C.difficile*.

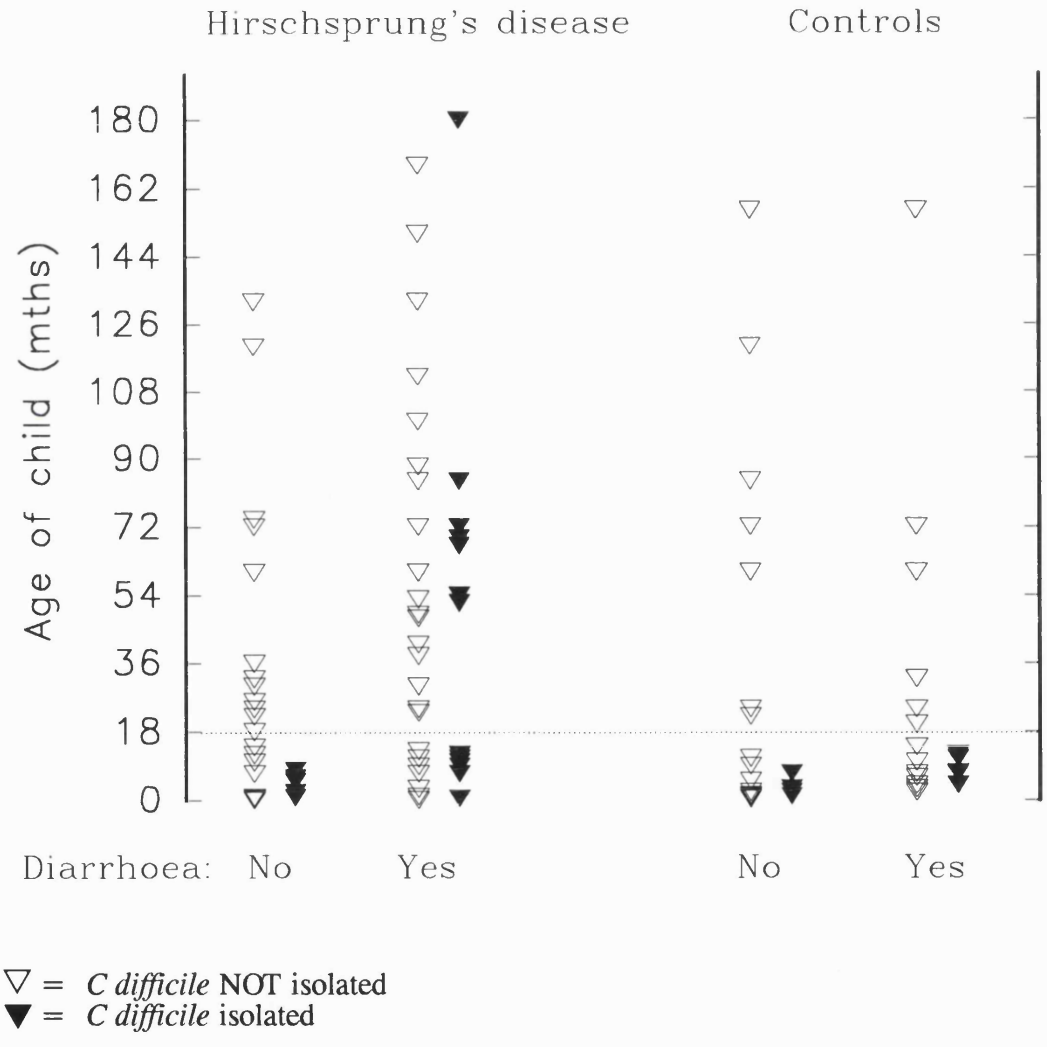


Fig. 2.1. Distribution of *C difficile* and age in children with Hirschsprung's disease and control children with and without diarrhoea.

EDTA-soluble antigen profiles

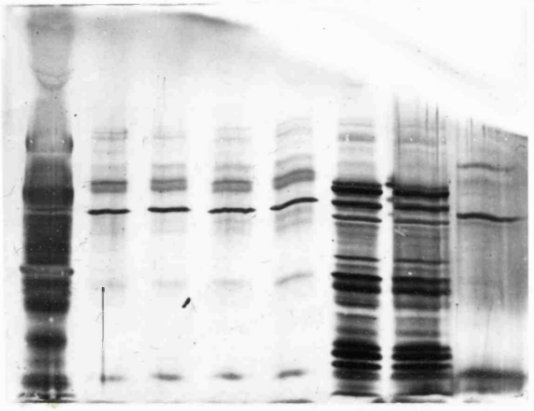
To determine whether the repeated isolation of *C.difficile* from children with HD and diarrhoea represented chronic carriage or different infections from exogenous sources the strains were typed by EDTA-soluble antigen profiles on SDS-PAGE gels.

Repeated isolation of *C.difficile* was encountered in two children with HD who had recurrent episodes of diarrhoea.

One child (P2) had 4 episodes of diarrhoea in which stool samples were collected. *C.difficile* was isolated in two of the episodes of diarrhoea. Both strains were toxigenic and had identical EDTA antigen profiles (Fig 2.3). In the other two samples adenovirus was seen on electron microscopic analysis in one and no enteric pathogen (excluding *C difficile*) was identified in the other. In both children treatment with oral vancomycin eliminated *C.difficile* from the faeces. *C difficile* was also isolated during a period in which the child did not have diarrhoea (P1c).

The other child (P1) had undergone colonic resection for long segment Hirschsprung's disease. Stool samples were obtained from four episodes of diarrhoea from which *C.difficile* was isolated in each case. The child was between the age of 4 to 5 years during the time the samples were collected. The strains were all non-toxigenic and had identical EDTA-soluble antigen profiles on SDS-PAGE analysis (P1a-c; Fig2). No other enteric pathogens were isolated.

FIG.2.2 EDTA-soluble antigen profiles of *C.difficile*



Strain No	P1a	P1b	P1c	P1d	P2a	P2b	P2c
Age of child (mths)	52	54	68	69	8	9	11
Diarrhoea	Y	Y	Y	Y	Y	Y	N

Fig2.2. EDTA-soluble antigen profiles of *C difficile* obtained after silver staining. Molecular weight markers are in the first column from the left (Mr=29K,45K,66K & 116K).

Adhesion of *C difficile* to HEp2 cells

Nine strains (5 from children with Hirschsprung's disease and diarrhoea, 4 from children with Hirschsprung's disease without diarrhoea) were tested for their ability to adhere to HEp2 epithelial cell line. None of the 9 strains demonstrated any cell-specific adhesion to the HEp2 cell monolayers after 60 or 180 minutes' incubation in aerobic or anaerobic conditions. Occasional bacilli could be seen but these were randomly distributed and not found overlying over the cells. In contrast, significant adhesion was observed with the enteropathogenic *Esch coli* O127; H6 (E2348/69) after 180 minutes (but not 60 minutes) in aerobic and anaerobic conditions in which localised clumps of greater than 20 bacilli could be seen in approximately 1 in every 10 HEp2 cells). No localised patterns of bacilli could be seen in the non-adhesive strain *Esch coli* XL-1 with only sparse cells distributed randomly, comparable to that seen with the clostridia.

Effect of *C difficile* on intracellular calcium in HEp2 cells

Six strains of *C difficile* isolated from children with Hirschsprung's disease were examined for their ability to mobilise intracellular calcium in HEp2 cells. Three strains were isolated from children with diarrhoea (F482 tox B-; F80 toxB+; F1166 toxB+) and three strains from children without diarrhoea (F99 tox B+; F649 tox B-; F759 tox B-). None of the six strains was able to induce any increases in cellular fluoresence within the the cytoplasm or nucleus of the cells after 2, 4 and 6 hours incubation. After 6 hours' incubation, rounding up and detachment of the cells was noted in the cells that had been challenged with toxigenic strains of *C difficile*. The rBHI alone did not induce any fluorescence at all three time points. Culture supernatants of strains of *Campylobacter jejuni* were used as positive controls. The ability of these strains to induce cytoplasmic fluoresence with fluo-3 had been identified previously by Dr P Everest.

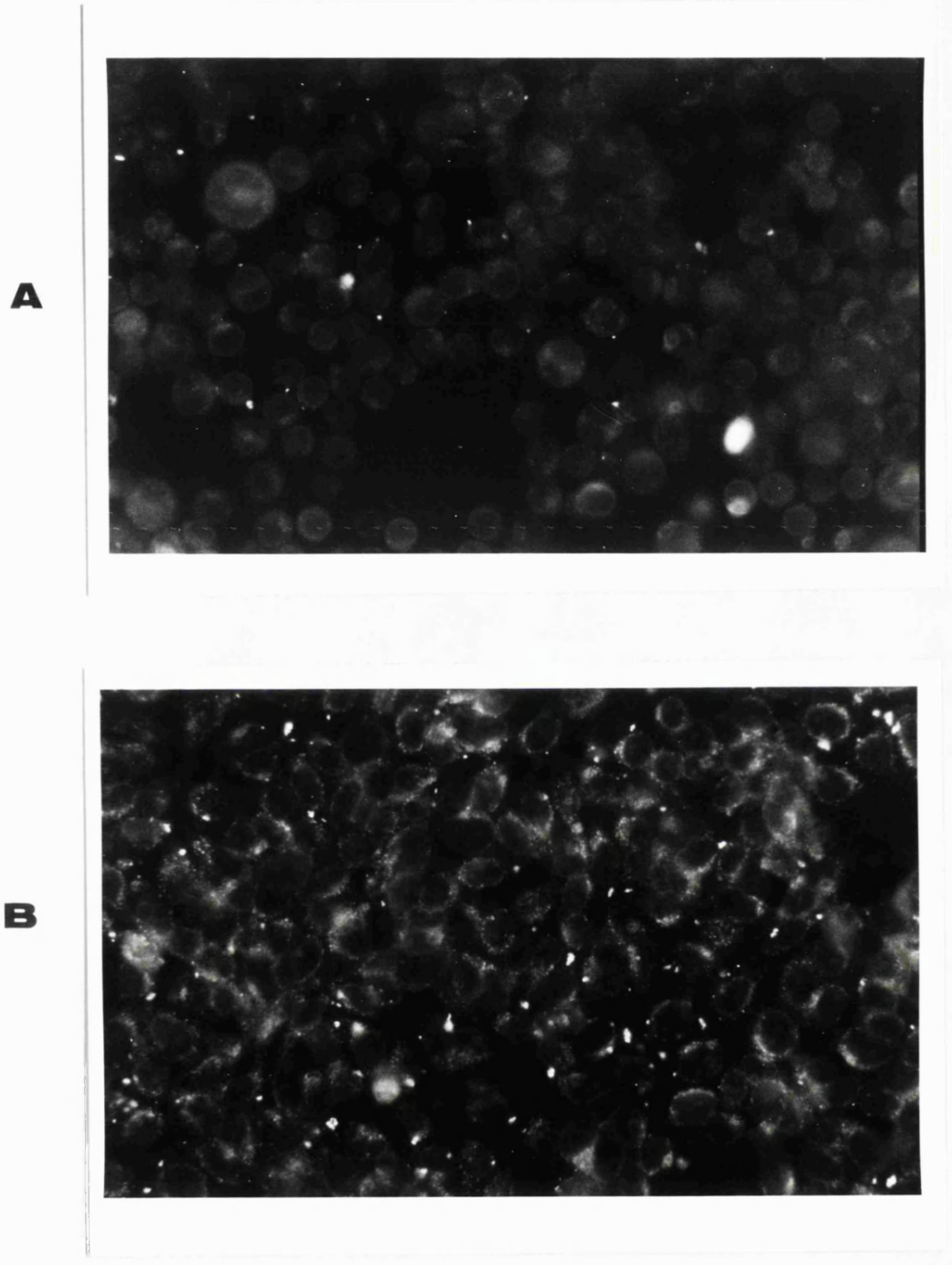


Fig.2.3. Intracellular fluorescence of Fluo-3 in HEp2 cells after 4 hours exposure to (**A**)*C difficile* and (**B**) *Campylobacter jejuni* culture supernatants.

2.iii Discussion

A high prevalence of enterocolitis both before and after pullthrough surgery has been reported in several large series (Kleinhaus *et al.*, 1979; Ikeda & Goto, 1984, Nixon, 1985) and this is supported by the findings of this study. However, the extent to which sporadic diarrhoea, particularly gastroenteritis in these children is distinguished from chronic/recurrent diarrhoea remains unclear. This will be due largely to difficulty in making useful definitions between diarrhoea, gastroenterits and enterocolitis. The term enterocolitis should strictly apply to cases of diarrhoea with colonic involvement and in particular inflammation but this information requires histological analysis of biopsies obtained at endoscopy. Such investigations are not practical in all cases of diarrhoea and are even contraindicated in suspected colitis. The clinical evidence to suggest this will be obtained either at the laparotomy during the formation of the defunctioning colostomy or by sigmoidoscopy. Deciding on whether colonic involvement occurs requires analysis of stool Na^+ and K^+ concentrations which are time consuming and cumbersome. It is clearly understandable why a blanket term "enterocolitis" is used, based on the clinical picture alone, rather than carrying out extensive analyses to obtain a more correct diagnosis. The description "Hirschsprung's disease-associated enterocolitis" will serve to emphasise the potential severity of the condition rather than describe accurately the clinical findings.

In the present study, severe enterocolitis occurred in the neonates seen before defunctioning colostomy but not in the children who had either had defunctioning surgery or pullthrough procedure. In the latter group gastroenteritis without systemic signs was the most common diagnosis. Although microbial enteropathogens have not been found in most studies on HDE, enteropathogenic *Esch. coli* (Teitelbaum *et al.*, 1989) and rotavirus (Wilson-Storey *et al.*, 1990) have been described in more recent studies. These studies also indicate that microbial enteropathogens, particularly viruses, are an important cause of diarrhoea in these

children.

A high incidence of colonisation with *C difficile* in children with Hirschsprung's disease was found but no significant difference was found in the isolation rate or toxigenicity of isolates of *C difficile* from those children with or without diarrhoea.

The isolation of *C difficile* in children over 18mths of age could explain the apparently contradictory previous findings of the association of *C difficile* with diarrhoea in children with Hirschsprung's disease. The data presented in this study suggests that a specific association of *C difficile* with episodes of diarrhoea will not be demonstrable in children under 18 months of age (as found by Wilson-Storey *et al*) but may be seen in children over this age limit (as seen by Thomas *et al*).
The prolonged carriage of *C difficile* seen in the older children with Hirschsprung's disease and diarrhoea might result from repeated exposure to the organism through hospital admission. In addition, previous studies have indicated that repeated exposure to antibiotics leads to colonisation with *C difficile* in adults with inflammatory bowel disease (George, 1986) and in children with cystic fibrosis (Peach *et al.*, 1986). The children with HD, both with and without diarrhoea, underwent staged surgical correction which involved 2 or 3 operations and appropriate antibiotic prophylaxis (a penicillin, aminoglycoside and metronidazole) and were not maintained on antibiotics except in the case of several children with who were given oral vancomycin or metronidazole for repeated isolation of *C difficile* (eg P2).
Other studies have found no evidence for this phenomenon (Ellis *et al.*, 1984; Tullus *et al.*, 1989) and in the study by Tullus of children up to 18mths old, they also noted a correlation with *C difficile* present at 11 months and the development of diarrhoea. This finding is comparable to the data obtained in this study but no explanation is offerred to account for this association. One possible interpretation which incorporates these findings is that children with HD who develop problems resulting in repeated episodes of diarrhoea will be admitted to hospital and treated

with antibiotics as a precaution against the development of enterocolitis. This initiates a vicious cycle by increasing the risk of (cross-) infection with *C difficile* and permitting colonisation through antibiotic treatment, even with vancomycin and metronidazole which will not eradicate spores but will reduce the colonisation resistance offered by the normal colonic flora.

The two children from whom *C difficile* was repeatedly isolated are difficult to interprete and illustrate the difficulty in establishing a pathogenic role for *C difficile* in children over 18mths old. In case P2, all the *C difficile* isolates were toxigenic and disappeared after vancomycin treatment suggesting a causal role in diarrhoea. However, toxigenic *C difficile* was also isolated when the child was asymptomatic (Fig.2: SM3) and further episodes of diarrhoea from which *C difficile* was not isolated, responded to treatment with oral vancomycin (similar to the findings of Thomas *et al* (1982) and others (Ellis *et al.*, 1984). The other child P1 had repeated isolation of nontoxigenic *C difficile* with identical EDTA-soluble antigen profiles. As the isolates were non-toxigenic, it seems likely that intestine was colonised with a single strain rather than suffering from repeated episodes of diarrhoea due to *C difficile*. The abnormal anatomy of the intestine in this child due to the extensive colonic resection might have predisposed to prolonged colonisation.

Whether these children had pseudomembranous colitis is also unclear. Sigmoidoscopy to detect colonic pseudomembranes was not routinely undertaken in the hospital for cases of sporadic diarrhoea. Uncertainty has existed on previous studies in which detection of toxin B has been used as an indication of the production of toxin A. However, as production of toxin B and toxin A are genetically linked (Wren, 1992) and assuming that toxin A is the most important virulence determinant, it can be reasonably assumed that the strains are able to cause diarrhoea and pseudomembranous colitis.

Attempts to differentiate between virulent and avirulent strains of *C difficile* were

made using adhesion to HEp2 cells and ability to mobilise intracellular calcium. Adhesion to HEp2 cells is a characteristic of the enteropathogenic serotypes of *Esch coli* (Cravioto *et al.*, 1979) and this was used as a control for the methodology. The similarity to *C difficile* is remote but adhesion of *C difficile* has not been described. The clostridia were incubated with the cell lines for 60 and 180 minutes. Longer periods of incubation were not tested because of the problems of maintaining viability in both the anaerobic bacteria and aerobic eukaryotic cells simultaneously. The failure to detect any significant binding to HEp2 cells does not eliminate possible adhesive mechanisms in *C difficile* disease. The adhesion to intestinal mucous and a more appropriate intestinal cell line might yield differences between strains and indicate virulence determinants. Such classification could be seen in the ability of strains of *C difficile* to adhere to the intestine of hamsters in challenge studies (Borriello *et al.*, 1988).

The failure of *C difficile* to induce any changes in intracellular calcium concentrations particularly in the toxigenic strains was surprising. The use of intestinal cells would be of greater relevance but attempts to load fluo-3 into Caco-2 intestinal epithelial cell line were unsuccessful. The use of cell culture supernatant rather than purified toxin A might also explain the present results. Cell rounding and detachment from the bottle indicates that toxin B at least was produced in the toxigenic strains.

Eukaryotic intercell tight junctions are maintained by the intracellular microfilaments which are can be altered by cAMP and Ca^{++} (Madara, 1990). Hecht *et al* demonstrated a fall in the tissue resistance of cultured intestinal epithelial cells after 6-8 hours with purified toxin A (Hecht *et al*, 1988). The increased permeability was not due to plasma membrane damage but linked to alterations in the cytoskeletal F-actin microfilaments. Other studies have found similar permeabilty changes (Mitchell *et al.*, 1986; Moore *et al.*, 1990). Theoretically, the distended distal colon in children with Hirschsprung's disease might permit greater access to the colonocyte tight junctions than normal, placing

children with Hirschsprung's disease at greater risk of developing colitis by *C difficile*. However, with the morphological arrangement of the colonic epithelia such that the ratio of exposed intercrypt apical colonocytes to the crypt depth makes it difficult to imagine that distension of the colon will occur to such an extent that the reserve capacity to stretch will result in colonocyte intercell junctions forced apart.

The adhesive properties and ability to mobilise intracellular calcium do not permit differentiation between virulent and avirulent strains. Many additional factors and events are likely to be necessary for the full pathogenic events that lead to intestinal disease with *C difficile*.

In summary, although the overall incidence and toxigenicity of *C difficile* was similar in all of the four groups of children, an association could be seen between children with Hirschsprung's disease and diarrhoea over 18mths of age. These findings reconcile previous contradictory studies on the association of toxigenic *C difficile* with children with Hirschsprung's disease and diarrhoea but leaves the question of aetiology unresolved.

Chapter 3.

The use of benzalkonium chloride in the ablation of enteric ganglia in the piglet

3.i Introduction

3.ii Methods

3.iii Results
- -clinical findings
- -morphology:H&E
- -enzyme histochemistry :NADH diaphorase
- -immunohistochemistry: S-100,PGP.
- -complications

3.iv Discussion

3.i Introduction

The enteric nervous system plays a central role in the regulation of normal colonic function (Furness & Costa, 1987) and its importance in the coordination of intestinal motility is amply illustrated by the profound disturbances seen in Hirschsprung's disease (Holschneider, 1982). The effect of the abnormal innervation in Hirschsprung's disease on colonic ion transport, however, remains unknown. As discussed in chapter 1 the aetiology of the enterocolitis remains unknown. To investigate the possible interaction between any microbial enterotoxic activity and the abnormal innervation of the distal colon which might result in abnormal secretory or absorptive responses a suitable animal model is needed.

Such a model ideally would i) possess similar responses to secretory stimuli, ii) have similar colonic neuropathology and iii) permit analysis of the contribution of the different neuropathological processes *ie* nerve fibres or ganglia.

Existing animal models of Hirschsprung's disease

Spontaneous aganglionosis has been described in a number of different animal species: white foals (Hultgren, 1982; Vonderfecht, Bowling & Cohen, 1983) and the pig (Osborne, Davis & Farley, 1968) but clearly they present difficulties as a laboratory model. The conventional animal model of human HD is the piebald lethal (sl/sl)/ lethal spotting mouse (ls/ls) (Webster, 1974; Wood, 1985).

The trait is recessive and obtained by breeding the heterozygous piebald NYZ strain. Both strains develop megacolon early in life and die of diarrhoea and enterocolitis before breeding age. The piebald lethal strain has an 25mm aganglionic zone extending from the rectum and a further transitional zone of 20mm in which the number of ganglia gradually increases to normal. The mice live for up to 2 weeks before dying of megacolon. The lethal spotting strain (ls/ls) survive for a slightly longer period, sufficient to breed for several months before succumbing to the megacolon. Only the terminal 4mm of the colon is aganglionic although the

presence of occasional ganglia has been shown in the aganglionic colon which means that the mouse should strictly be termed hypoganglionic (Bolande & Towler, 1972). The histochemical changes are not identical to that seen in human HD and they also differ between the two strains of mice. Cholinergic and adrenergic fibres are increased in human aganglionic colon whereas both cholinergic and adrenergic fibres are either reduced or unchanged in density in the mice strains (Webster, 1974; Wood, 1985). The reduction in the number and distribution of peptidergic nerves is a common feature in both the mouse model and human Hirschsprung's disease.

A rat model of congenital colonic aganglionosis has been described (Nagahama *et al.*, 1985) in which the aganglionic region extends to the distal ileum and so resembles total colonic HD rather than the more usual pattern of aganglionosis. Similar to the piebald mouse mutants, the constricted colon has a reduced density of AchE-positive nerve fibres. This is in contrast to the abundant AchE-positive fibres infiltrating human aganglionic colon. As yet experience of the model appears to be confined to Japan.

The use of benzalkonium chloride in enteric neuronal ablation

The ablation of the ganglia in both the myenteric and submucosal plexus in the descending colon of the rat has been described using benzalkonium chloride (BAC) as well as other surfactants (membrane damaging detergents). Sato *et al.* (1978, Imamura *et al.*, 1975; Sakata *et al.*, 1979) treated the serosal surface of adult Wistar rat descending colon for 30 minutes and followed the changes over 7 weeks. At 3-6 weeks all nervous elements were absent though no other degenerative or inflammatory changes were reported. The advantage of the method is the control over the selective ablation of ganglia within a defined length of colon whilst retaining the nerve fibres.

The differences in the pattern of neuropathology in the rodent models and human Hirschsprung's disease are compounded both by the extent of neural regulation of intestinal ion transport (Cooke, 1987; Cooke & Carey, 1990) and the basic

mechanisms of colonocyte ion transport (Sandle & McGlone, 1987). To overcome these difficulties in interpretation that would arise through using these animal models, experimental ablation of colonic myenteric and submucosal plexus was examined in the neonatal piglet. The piglet gastrointestinal tract is considered to have a closer resemblance to that of man in anatomy, innervation and physiology than the usual laboratory animals, rat, mouse or rabbit (Argenzio, 1985; Stevens, Argenzio & Roberts, 1986).

All previous work using BAC has been carried out on adult rats. The method described by Sato *et al*, using the rat distal colon could be adapted for use in the neonatal piglet descending colon as a means of selectively ablating the ganglion cells in the colon. In this way the toxic effect of BAC would occur at an age closer to the proposed embryological events occurring in human Hirschsprung's disease.

BAC is a mixture of differing carbon-chain-length compounds of similar overall structure. The most active component, as determined in studies in rat jejunum is the 14-carbon length homologue benzyldimethyltetradecylammonium chloride (Herman & Bass, 1990). The efficacy of C14 was examined in comparison with BAC.

3.ii Methods

ANIMALS

Pregnant Large White/Landrace crossed pigs were obtained from the National Pig Development Corporation (Drillfield, Yorkshire) and farrowed in-house. The piglets were kept with the sow for up to 4 weeks before weaning onto pellet food. Piglets were weaned onto pellets at 4-5 weeks of age.

APPLICATION OF BAC

The piglets were operated on at 48-80 hours of age, when they weighed between 1200-2200g. BAC was applied to the colon using three surgical procedures:

i) BAC-soaked gauze

The animals were anaesthetised using an open mask delivering halothane, oxygen and nitrous oxide. A lower midline abdominal incision was made from below the costal margin. The descending colon was identified and mobilised without disruption of the vascular supply. A 5-7 cm length of the descending colon was exteriorised by the use of a pair of widened forceps placed under the colon and the bowel was mobilised to allow access to the distal descending colon. A length of descending colon was mobilised from the posterior abdominal wall and a piece of BAC-soaked gauze was wrapped around a 4cm length of colon, taking care to avoid damage to the mesenteric vessels. The gauze was kept moist by the application of the BAC solution every 2-5 minutes. After BAC treament the gauze and the proximal and distal ends of the treated segment of bowel were marked with serosal silk sutures. After treatment, the bowel was replaced into the abdominal cavity and the laparotomy wound was closed in two layers, using 4/0 Dexon (Ethibond) for the muscle layers and 2/0 Prolene (Ethibond) for the fat and skin layer. Control animals underwent a similar laparotomy, but physiological saline was substituted for BAC. After surgery, piglets were returned to the sow and littermates.

ii) BAC gauze on colon stripped of the serosal membrane

The possible interference with the permeation of BAC across the serosal membrane was also examined. The serosal membrane was removed from the colon at the site

of gauze treatment in the distal descending colon using microdissecting scissors at the operation immediately prior to applying the BAC. This procedure caused the bleeding of a number of the small blood vessels on the bowel surface but retained the main arterial/vascular supply.

iii) Serosal and mucosal exposure to BAC

Two 48hr old piglets were treated with serosal BAC in gauze as described in method (i) but were simultaneously exposed to 40ml 0.1% BAC held in the colonic lumen which was administered using a urinary bulb catheter inserted from the anus and held in position with adhesive tape.

iv) Infusion of BAC via arterial supply

A method for destroying the enteric nervous tissue in adult dog small intestine has been described (Frantzides *et al*, 1990) using cobalt chloride infused into an isolated segment of jejunum. The principle of intra-arterial injection of the neurotoxin was modified to inject BAC into the distal colon of the piglet via the arterial supply.

All solutions of BAC, C14 and saline were maintained at 37°C during the operative procedures by the use of waterbath. All animals received analgesic immediately after operation (Temgesic).

MONITORING

Animals were monitored for signs of abdominal obstruction. If present, characterised by abdominal distension, profound diarrhoea with dehydration or lethargy, the animals had barium enemas performed for abdominal x-ray and were then examined under anaesthetic with a second laparotomy. After removal of the treated colon, the animals were killed by anaesthetic overdose with halothane.

BARIUM ENEMA

Micropaque (Nichols) was diluted 1 in 2 in tap water (warmed to hand temperature) and delivered via tubing under gravity. The volume given varied depending on the age and size of the animal (25ml in small animals, $\geq$50 ml in larger animals. The animals were placed over Kodak TMG X-ray film cartridges

whilst the X-rays were taken (Mobilux; 40mA, 0.08secs, 55kV) from a distance of 55 cm.

HISTOLOGY

Haematoxylin and Eosin

Tissue was placed into formal calcium (20g calcium acetate in 1000ml 4% formalin) and processed after wax embedding using standard methods.

NADH-diaphorase

Slides were stained for NADH-diaphorase activity using the method of Hoyle & Burnstock (1989). Tissues for NADH-diaphorase activity were cut into 1-2cm squares and pinned out on silicone rubber and fixed in 4% (w/v) paraformaldehyde in PBS (pH7.3) at 4°C for 90 minutes. Tissues were washed at RT for 15-30 minutes in PBS and then stored at 4°C in 7% sucrose-0.1% sodium azide-PBS until processed. Tissue sections were prepared in O.C.T. (BDH) and snap frozen in liquid nitrogen-chilled iso-pentane. 5-10 serial sections of 10μm thickness were cut from each block every 80-100μm using a Reichert cryostat.

Slides were incubated at 37°C in 0.1M PBS containing 1mM NADH (Fluka) and nitroblue tetrazolium (0.6mM) (Sigma) for 30-60 minutes. The staining intensity was controlled visually. The tissues were then further fixed in formol calcium for 10 minutes before dehydration through graded alcohols, cleared in graded xylene and mounted in D.P.X. (BDH).

S-100 antigen

The S-100 protein is expressed on certain neuronal cells such as the glial cells and in Schwann cells of both myelinated and non-myelinated nerve fibres. The ganglion cells do not express this antigen (Taguchi *et al.*, 1985). Immunocytochemical staining of the S-100 antigen was carried out using an avidin-biotin complex/alkaline phosphatase method. Wax-embedded tissue sections were de-waxed, trypsinised and rehydrated through graded alcohols. Nonspecific antibody binding was blocked with 25% normal pig serum for 10 minutes at room temperature. Slides were incubated for 30 minutes with rabbit anti S-100 (1 in 2000 dilution, Dako) and

washed in PBS before incubation with the anti-rabbit biotinylated IgG for 30 minutes. After washing slides were incubated for 30minutes with the Streptavidin-Biotin complex-horseradish peroxidase (prepared according to manufacturer's instructions). Visualisation of the ABC complex was achieved by placing slides into the diaminobenzidine solution for 10 minutes and then counterstained lightly with Mayer's haematoxylin for 2-5 minutes and then rinsed in distilled water. Slides were dehydrated through graded alcohols and mounted in DPX. Control slides were treated similarly except for the replacing of the primary antiserum with non-immune pig serum.

PGP 9.5 antigen

The pan-neuronal marker protein PGP 9.5 was used to visualise all neuronal tissues (Thompson *et al.*, 1983). Cryostat cut sections were incubated with PGP9.5 antiserum (1 in 2000 dilution in PBS-0.1% Triton-X, antisera raised in rabbit; Ultraclone Ltd) for 18 hours at 4°C. After 3 washes in PBS the slides were then incubated with biotin labelled anti-PGP 9.5 IgG antisera for 90 minutes. After 3 washes the reaction was visualised with fluoroscein labelled streptavidin for 90 minutes. Slides were mounted in Citifluor and examined using a Zeiss Axoplan microscope.

ANALYSIS

Ganglion counts

The number of ganglia per mm were counted in each of the serial sections in which all layers of the colon, ie outer muscle, submucosa, muscularis mucosae and mucosa were present. The total length of tissue was measured using a calibrated eyepiece graticule (Agar Ltd) using a Nikon fluophot microscope with x10 and x40 magnification eyepieces. 6-10 serial sections were counted per tissue. The total number of ganglia was then calculated to give the mean count of ganglia per mm of tissue.

3.iii Results

Method i: BAC soaked gauze

Initial pilot experiments testing a range of concentrations of BAC (0.05%-0.25%w/v) indicated that 0.1% exerted a destructive effect on the ganglion cells in the treated section of piglet colon.

A constricted segment of colon, corresponding to the treated area was found in 6/7 animals animals treated with 0.1% for 15 minutes (n=3) or 30 minutes (n=3). The constriction was present at laparotomy in the animals killed at week 1 (n=1), 2 (n=1), 4 (n=2), 6 (n=1) and 10 (n=1). Marked dilatation of the colon immediately proximal to the treated area was noted in 3 animals. An example is shown in Fig.3.1.

Diarrhoea developed in three animals and was of sufficient severity that all three animals had to be killed. No virus particles were seen in the stools and *Salmonella spp* were not isolated. Gene probe analysis for the presence of heat labile and heat stable enterotoxins as well as verotoxins on the predominant coliforms (*Esch coli* 2 and *Serratia sp* 1) from these three piglets (2 *Esch coli* and 1 *Serratia* species) were negative for all three toxins in each of the isolates. (Gene probe analysis performed by Dr M Woodward, MAFF, Weybridge. Methods detailed in Woodward *et al.*, 1990). The presence of the constricted segment of distal colon with proximal distension corresponded with this diarrhoea and was thought to be responsible.

The histological examination of the treated segment using H&E showed reduced numbers of normal ganglia in both the myenteric and submucosal plexus (Fig 3.1). Oval gaps could often be seen between the circular and longtitudinal muscle layers corresponding to the myenteric plexus. Those neurons that were present in the ganglia had abnormal cell architecture such as condensed nuclei and vacuolated cytoplasm (Fig 3.2). Two animals had complete transmural necrosis in the treated section of colon. One animal had been treated with 0.025% BAC and did not have a constriction at the operative site, the other animal had been treated with 0.1% BAC.

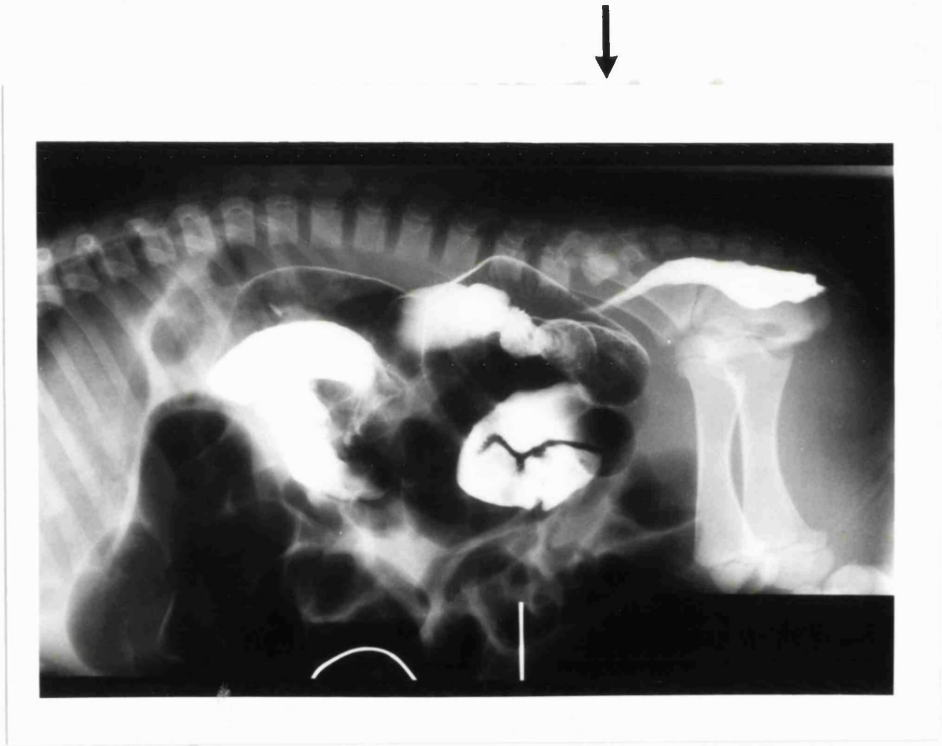


Fig.3.1. Radiological picture of barium enema taken from a piglet 2 weeks after treatment with 0.1% BAC. Note the constriction at the colon corresponding to the operation site together with the distended loops of bowel.

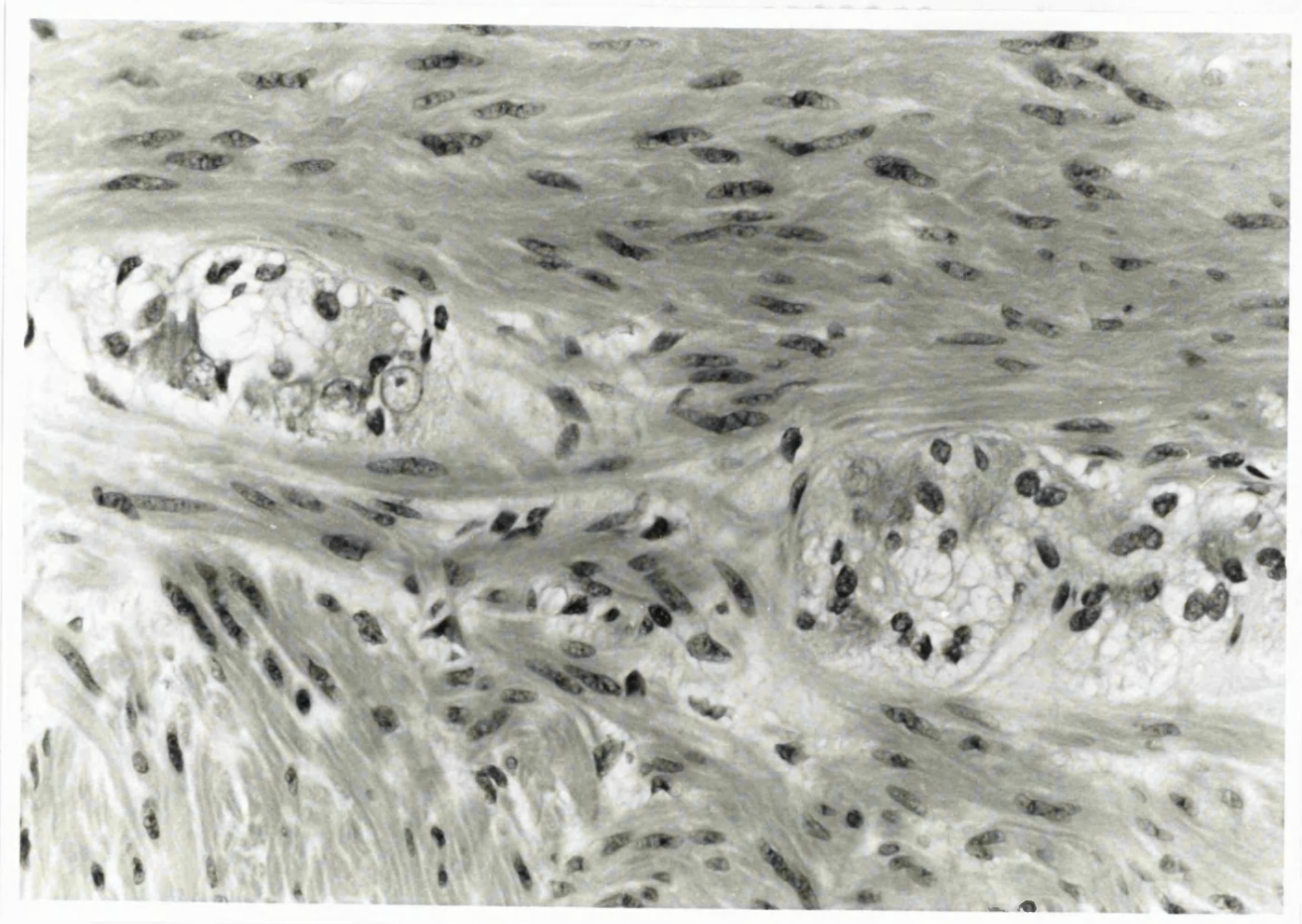


Fig.3.2. Photomicrograph of a ganglion in the myenteric plexus of the distal colon of a piglet treated with 0.1% BAC. The ganglion cells show marked cytoplasmic vacuolation (H&E x400).

Immunohistochemistry using antisera against the S-100 antigen, however, gave a normal pattern of staining.

These experiments indicated that 0.1% BAC was able to ablate the ganglia in the colon of piglets and lead to a constriction at the treated site after 1 week and could persist for up to 10 weeks after operation. The intact S-100 immunoreactivity was interpreted as a selective destruction of the ganglion cells with the neuronal support cells (eg glial cells) remaining unaffected.

Subsequent experiments on groups of piglets treated in the same way as the pilot experiments were carried out using BAC at a concentration of 0.1% (w/v).

Three different periods of exposure to BAC were tested, 15 mins (n=6), 20 mins (n=2) and 30 mins (n= 7). 4 saline controls were also treated for 15mins (n=1) and 30 mins (n=3). Animals were killed 3 weeks after operation.

Clinical features and anatomy

All the animals from all three groups failed to demonstrate any clinical signs of intestinal obstruction resulting from the BAC treatment. No constriction was seen at laparotomy in the operative site. Idiopathic diarrhoea and abdominal distension associated with megacolon similarly failed to develop in any of the animals.

Histology

On examination of the operative site stained with H&E ganglia were present in both the myenteric and submucosal plexus of animals treated with saline and 0.1% BAC for periods ranging from 2-9 weeks. The ganglion cells were of normal size and had normal staining patterns without signs of degeneration (Fig3.3) in both groups. No evidence of muscle thickening was seen.

Immunohistochemistry

S-100: Immunoreactivity with S-100 antisera was present in both the myenteric and the submucosal plexus of the saline (n=3) and BAC treated animals (n=3). No difference in the extent or staining intensity of immunoreactive ganglia was seen within the myenteric plexus. Figure 3.4 shows the pattern of staining seen in the

myenteric plexus of a piglet treated with BAC.

PGP9.5: Tissues from four piglets were also examined using antisera against the pan-neuronal marker protein PGP9.5. Extensive immunoreactivity of both ganglion cells and nerve fibres were seen in the myenteric and submucosal plexus of the BAC-treated colon (n=3 animals). The pattern of staining was indistinguishable from that seen in the saline-treated control animal (n=1) (data not shown).

Enzyme histochemistry

NADH-diaphorase staining is a marker of NAD-dependent dehydrogenases and can be considered as an indicator of ganglion cell metabolic activity. Furthermore, the NADH-diaphorase is a more selective stain for nervous tissue and would be easier to compare between normal and hypoganglionic tissue.

The results of the ganglion counts within the myenteric and submucosal plexus of BAC- and saline-treated animals is summarised in Table 3.1. No significant differences were found in the number of ganglia/mm between the two groups of animals after 2-3 or 6-9 weeks after operation. The NADH-diaphorase activity was confined to the cytoplasm of cells and confined to a proportion of the number of ganglion cells within a ganglion. Examples of the staining pattern are shown in Fig.3.5.

Method ii:Removal of serosal membrane

To facilitate penetration of the BAC across the serosal muscle layers experiemtns were carried in which the thin outer serosal membrane was removed from the colonic wall at the operative site prior to application of the BAC.

Three piglets were operated on three days after birth. One animal became ill two days after the operation and was found to have a necrotic colon at the site of operation due to impaired vascular supply, caused by a bleed at the original operation. The other two animals were killed after 2 weeks.

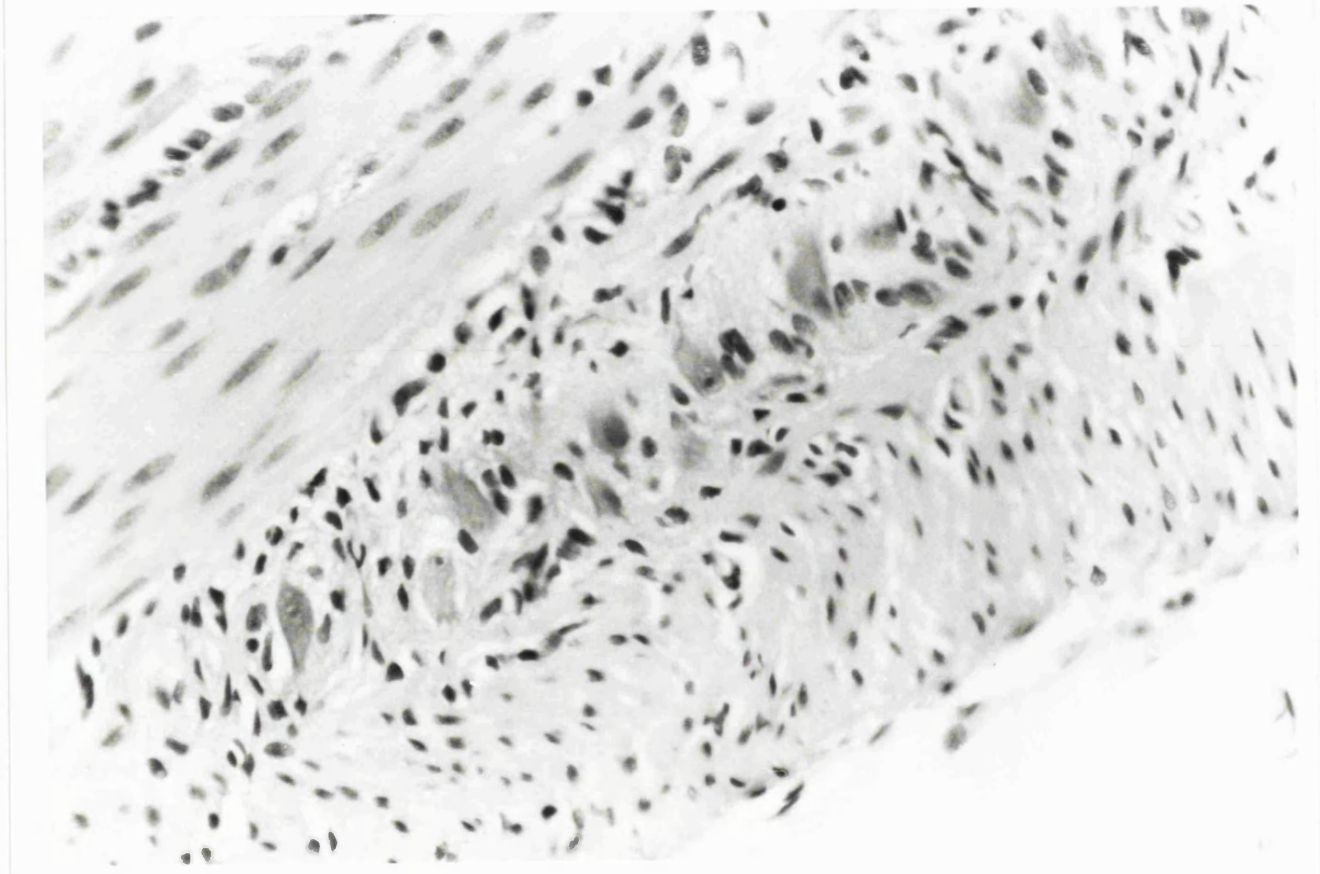


Fig.3.3. Photomicrograph of a ganglion from the distal colonic myenteric plexus of a piglet 3 weeks after treatment with 0.1% BAC. The ganglion cells are present in normal number and of normal staining pattern. (H&E x200)

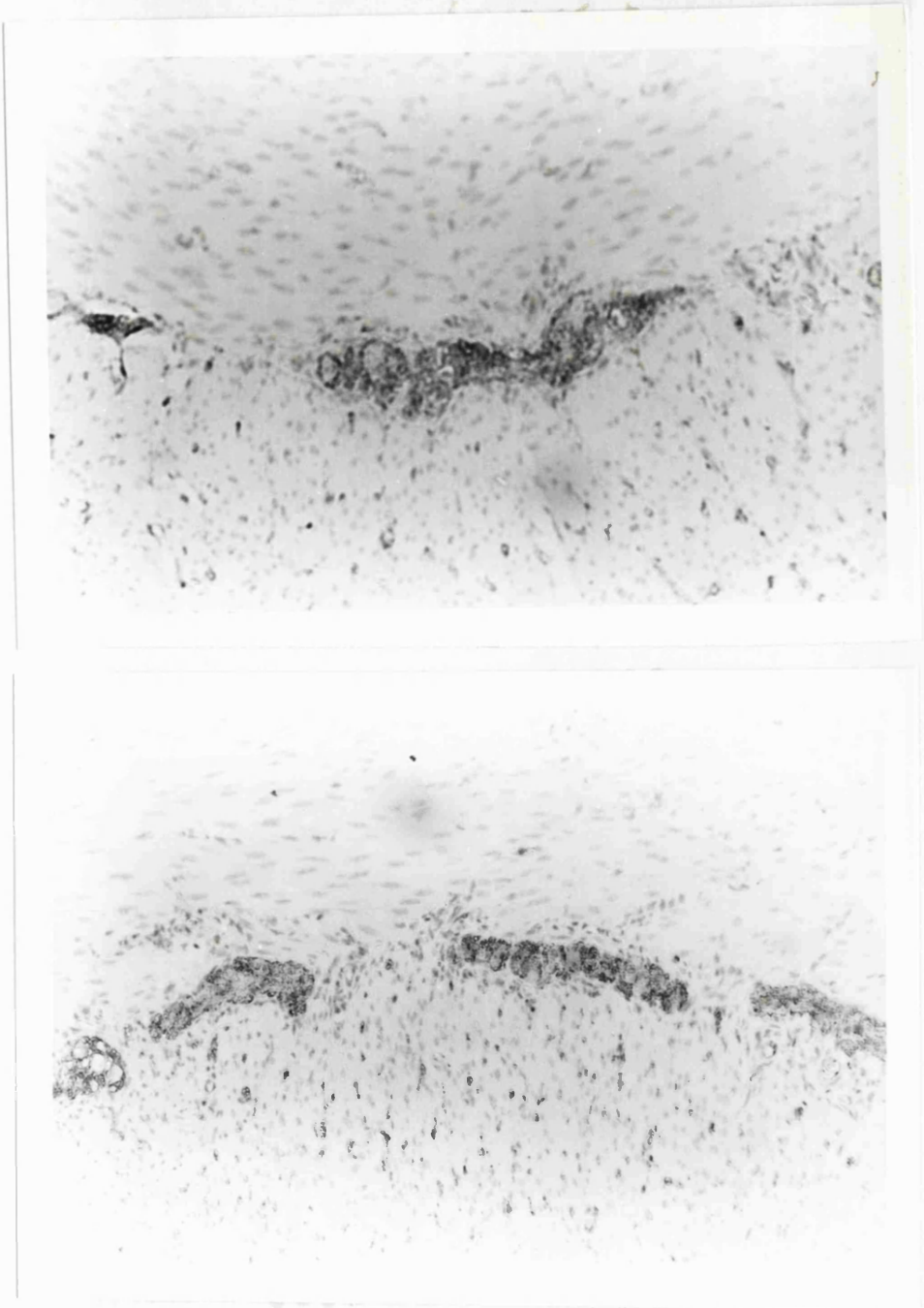


Fig.3.4. S-100 immunoreactivity in the myenteric plexus in the distal colon of a piglet 6 weeks after treatment with (**A**) 0.1% BAC and (**B**) 0.9% saline (x200).

Histology

The tissue had a pattern of NADH-diaphorase activity that appeared normal with ganglia present in both the myenteric and submucosal plexus.

On the basis of only two animals it was nevertheless felt that the removal of the serosal membrane did not result in ablation of either myenteric or submucosal plexus.

Method iii:serosal and mucosal exposure

Two animals were treated with simultaneous exposure to serosal and mucosal BAC solutions for 30 minutes. The animals were killed 7 weeks after the operation. No clinical signs of colonic obstruction were seen in the two animals. NADH-diaphorase activity was present in both plexi of the treated site of the colon. The mucosae of these animals also appeared normal.

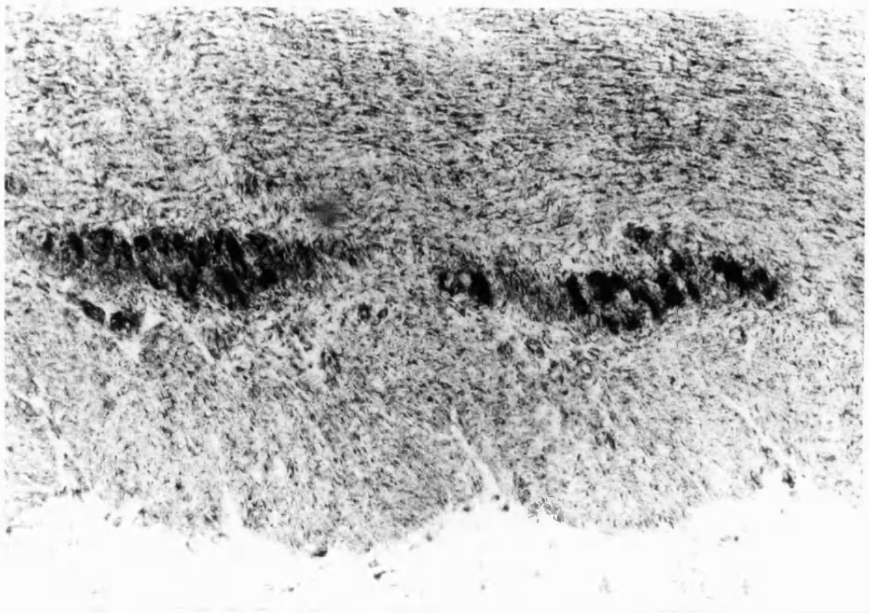
Method iv:BAC infusion

The method was initially attempted on 3 48hr old piglets. The limited access of the descending colon meant that intra-arterial and -vascular catheters were difficult to keep in place and the small size of the vessels meant that on removal of the catheters the vessels were impossible difficult to repair without the use of diathermy. Closure of the laparotomy was not possible due to bleeding from the colonic vasculature and all animals were killed immediately after the operation.

Intramuscular injection of BAC

The effect of intramuscular injections of 0.1%BAC into the colonic wall was studied in a 48hour old piglet. Multiple intramuscular injections of 0.1-0.2 ml 0.1% BAC were given around the surface of a 4cm length of distal colon. The animal remained well for 7 weeks and had no gross changes at laparotomy. NADH-diaphorase staining demonstrated ganglia in both the myenteric and submucosal plexus in the treated section of colon.

B



C

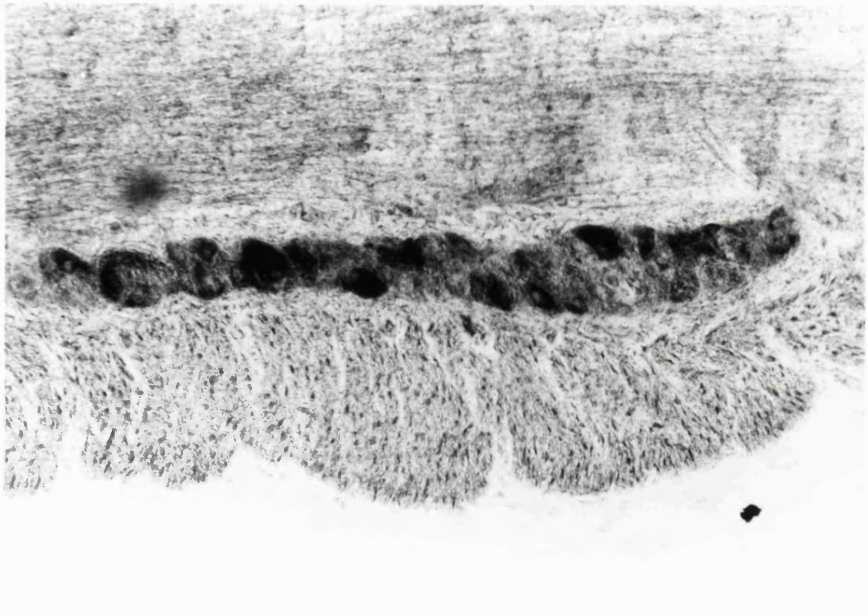


Fig.3.5b.

Benzyldimethyltetradecylammonium chloride

To examine the efficacy of the 14-carbon BAC homologue benzyldimethyltetradecylammonium chloride (C14) in ablating the colonic ganglia, a further group of animals were treated with 0.1% C14 for 30 minutes.

Three animals were killed after 2 weeks and 4 animals were killed at 7 weeks. No clinical features of intestinal obstruction developed in any of the piglets during the 7 weeks following the operation.

Histology

Normal numbers and staining patterns of ganglia using NADH-diaphorase staining were seen in the treated tissue sections. The data are shown in Table 3.1 and compared with the results of the BAC-treated animals and saline controls in Fig.3.6.

Complications related to surgery

A number of animals became ill due to complications of the surgical procedure. These included i) urethral obstruction due to accidental suturing of the urethral stalk in male piglets (n=2); ii) intestinal obstruction from adhesions developing around the colon at the operative site; iii) necrosis of the treated segment due to damage to the vasculature supplying the distal colon; iv) brief ($\leq$ 2-3days) episodes of diarrhoea developed in a number of animals during the first week of life. This was considered to be scours and resolved without any intervention. The other cause of diarrhoea in older animals was due to overfeeding which was easily remedied by reducing the diet volume. In one case of diarrhoea in a newborn piglet virus particles which resembled rotavirus were seen in the faeces using transmission electron microscopy. The diarrhoea resolved without intervention.

Table 3.1. Number of ganglia* in the distal colon of piglets treated with BAC or C14

Age post-op	Myenteric plexus			Submucosal plexus		
	BAC	C14	saline	BAC	C14	saline
2-4	0.6±0.1 (5)	0.8±0.1 (3)	0.95±0.3 (5)	1.7±0.2 (5)	1.3±0.3 (3)	1.8±0.3 (5)
6-8	0.8±0.2 (5)	0.8±0.2 (4)	0.9±0.1 (12)	1.4±0.1 (5)	1.8±0.2 (4)	1.3±0.2 (12)

* Results given as mean±SEM per mm colon, (n) = number of animals
BAC: benzalkonium chloride, C14: benzyldimethyltetradecylammonium chloride.

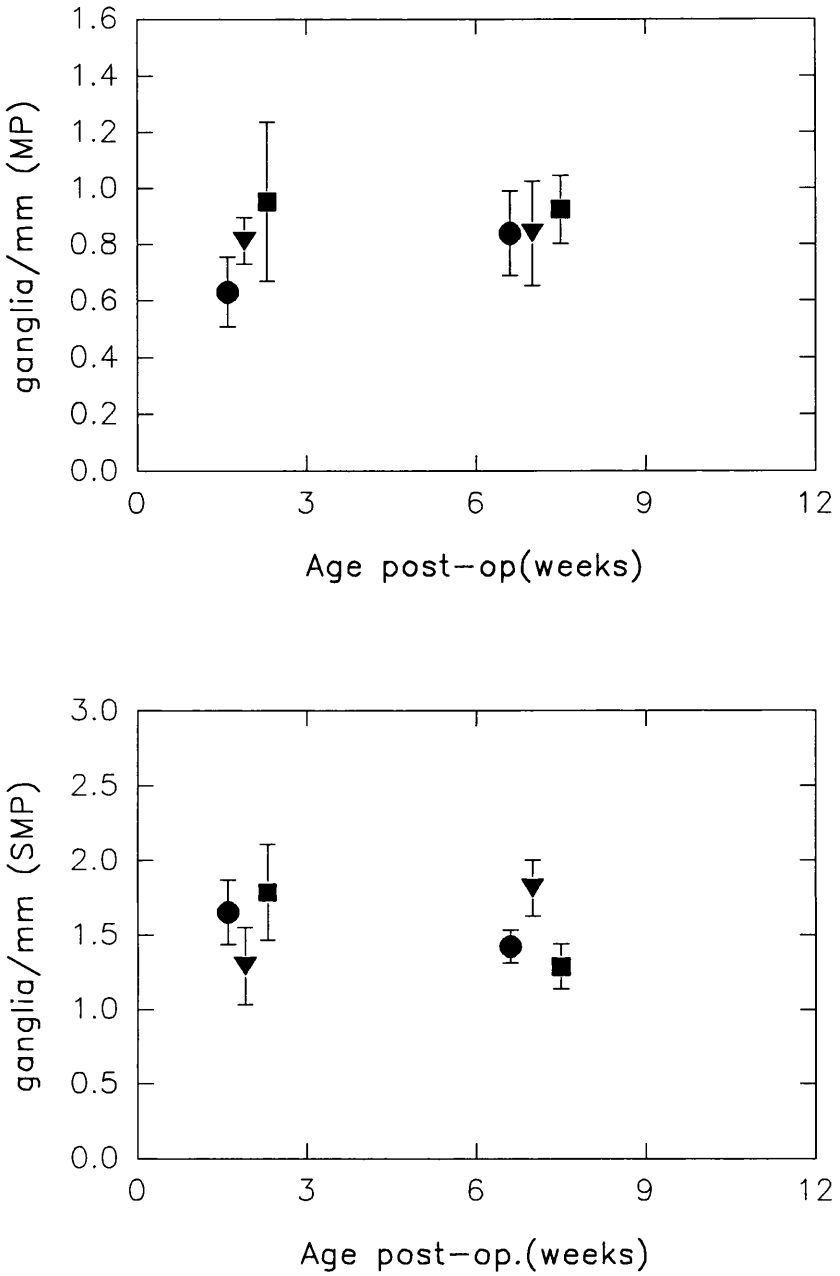


Fig.3.6. Mean number of ganglia in the myenteric plexus (MP) and submucosal plexus (SMP) of piglets treated with 0.1% BAC (●), 0.1% C14 (▼) and saline (■).

3.iii Discussion

Although a degenerative effect of BAC on nervous tissue appears to be a property common to surfactants, differences exist in the extent of the neuronal ablation when tested in different parts of the intestine. Fox *et. al.*, (1983) found that BAC ablated the ganglia in the myenteric plexus of the rat jejunum but did not alter the submucosal ganglia. Zucoloto *et al*, (1988) treated adult rat colon with 0.2% BAC and found that the neuronal count in the myenteric plexus was reduced by 78% to control tissues after 5 months. The submucosal plexus was not affected. The failure of the BAC to consistently reduce the numbers of myenteric ganglia in the neonatal piglets may be related to the thickness of the outer muscle layers in different parts of the intestine as well as between animal species impeding the penetration of surfactant.

The numeration of intestinal tract ganglia and neurons is difficult and time consuming if representative values are required including corrections for shrinkage of the tissue during fixation. In order to determine whether total aganglionosis was achieved, it was considered that a qualitiative evaluation of ganglion distribution coupled with the a comparative rather than absolute ganglion counting method (described above) were sufficient.

The inconsistent effect of BAC could result from variations in the composistion of the source chemical(s). BAC is a mixture of alkyldimethylbenzylammonium chlorides. The BAC supplied by Sigma varied in consistency even between samples with the same batch number. The consistency varied between a dry crystalline powder, a waxy substance and a viscous fluid. The BAC supplied from Fluka was supplied sealed under nitrogen and always remained as waxy flaky substance. However, no changes in efficacy were found after using the Fluka.

Herman & Bass (1989) have shown that the efficacy of destroying the ganglion cells corresponds with the carbon length of the different component compounds. Treating

rat jejunum with a range of carbon length compounds, applied to the serosa at 1-2mM concentrations, all chain lengths except C18 were able to reduce ganglion cell numbers with the C14 compound (benzyldimethyltetradecylammonium chloride) being the most effective. C14 was able to reduce the myenteric plexus ganglion cell counts by 80-95%. Similar to BAC, none of the homologues had significant effect on the submucosal plexus.

A significant effect of BAC might have been due to a compensatory ganglion cell replacement by new proliferating neurons. The ganglion cell number in the myenteric plexus appears to vary with age. Gabella (1971) found that the absolute number of ganglion cells in the myenteric plexus of the rat small intestine increased dramatically during the postnatal period. The number is assumed to remain stable during "middle age" and then start to fall in older (aged) animals (Santer & Baker 1988).

All previous studies with BAC have been carried out in adult animals rather than in the immediate postnatal period. In the present study, tissues were examined at 2 time points: 2-3 weeks and 6-9 weeks after BAC treatment. No significant changes were seen in the ganglion cell numbers between the two time points in the both the treated and untreated animals. This suggests that compensatory increases in ganglion cells were unlikely to have occurred within 2-3 weeks of exposure to BAC.

Recent studies have investigated the effect of BAC treatment on small intestinal enterocyte turnover rates in animals in which the myenteric plexus is significantly ablated (Holle, 1991). Significant increases in epithelial mitosis coupled with lenghtening of the villi were seen in BAC-treated animals. Ion transport effects were not studied. The author suggests that the myenteric plexus exert an inhibitory effect on mucosal turnover and its removal permitted unregulated epithelial growth. However, the influence of the regeneration of the muscle layers and other undefined events could be responsible rather than a specific effect of neuronal damage.

Other experimentally induced neuropathies using agents such 6-hydroxydopamine or capsaicin have varying degrees of neurotoxicity, acting on adrenergic and sensory fibres respectively. The absence of ganglia coupled with a proliferation of extrinsic nerve fibres in Hirschsprung's disease do not readily permit such a strategy in reproducing the neural changes. Equally, blockade of all nerve fibre transmission with tetrodotoxin is unsuitable as Hirschsprung's disease contains a proliferation of hypertrophied infiltrating nerve fibres.

These studies indicate that BAC is not sufficiently efficacious or reliable in the ablation of colonic ganglia in the piglet for the creation of a suitable animal model of Hirschsprung's disease in which to study the neural control of secretion. For this reason a study was carried out using human tissues. The results are given in the following chapter.

Chapter 4.

Electrogenic colonic ion transport in children with Hirschsprung's disease and anorectal anomalies

4.i Introduction
-Ion transport mechanisms in the mammalian colon

4.ii Methods
-Ussing chamber methods

4.iii Results: human paediatric rectosigmoid colon
-Basal electrical properties
-Neurally mediated secretagogues: ATXII, Iloprost
-Direct colonocyte secretagogues: theophylline, IBMX & STa enterotoxin
-effect of tetrodotoxin
Transverse colon
-basal properties
-neurally mediated secretagogues
-direct colonocyte secretagogues

4.iv Discussion

4.1Introduction

Colonic ion transport

The colon plays an important role in the maintenance of the total body fluid homeostasis through absorption of fluid passing from the ileum. Although the small intestine absorbs a large proportion of the intestinal fluid, the colon removes what is left with considerably greater efficiency. Regulation of electrolyte uptake can occur through endocrine signalling. Aldosterone has a powerful stimulatory effect on Na^+ uptake by increasing the amiloride-sensitive channel in the colonocyte apical membrane (Sandle & Binder, 1987; Sandle, 1989). Sodium depletion results in increased colonic uptake (Finkel *et al.*, 1991). Such adaptive mechanisms are relatively long-term events. The more immediate regulation of electrolyte transport across the intestinal mucosa is mediated by the ENS (as discussed in chapter 1).

Electrolyte transport across the mucosa consists of simultaneous absorptive and secretory activity with the net flow determined by the sum of electrolyte flow along the whole intestine. Diarrhoea will occur after certain conditions which lead to to the absorptive capacity of the colon being overwhelmed by the volume of fluid passing from the distal ileum (Read, 1982) or where the colon fails to absorb or is actively secreting.

To study the mechanisms of diarrhoea it is necessary to understand the cellular basis by which this occurs: that is the balance between absorption and secretion of electrolytes and water.

The exact mechanisms responsible for the transport of ions in the human colon are not completely established. Most mammalian epithelia have the recognised ion transporting pumps and ion channels in common but the exact coupling and net ionic balance of these mechanisms differs between species. Furthermore it is clear that segmental heterogeneity exists not just between small and large bowel but along the length of the colon (Binder & Sandle, 1987; Bleakman & Naftalin, 1988; Bridges & Rummel, 1986; Turnberg, 1991).

The colon normally absorbs Na^+ and Cl^- while secreting K^+ and HCO_3^-. Water passively follows solute due to osmotic pressure and hydraulic conductance gradients. The transport of a solute across the cell membranes creates an osmotic pressure difference across the membrane. This osmotic pressure gradient draws water in the same direction. the volume of water flowing across the membranes will depend on the conductance of the membrane to water (hydraulic conductance) and the magnitude and maintenance of the osmotic pressure gradient.

The passage of water and ions across the intestinal epithelium occurs by two routes: 1) the transcellular pathway, consisting of the apical and basolateral membranes of the enterocyte; 2) the paracellular pathway via the intercellular tight junctions. The movement of water and ions is also influenced by the composition of the submucosal tissues and the musculature (Bleakman & Naftalin, 1988).

Both active and passive transport mechanisms are involved in the transcellular transport of ions and water across the colonic epithelium. In the following paragraphs the current models of distribution of ion-transporting mechanisms (ion pumps, channels and transporters) in the colon will be summarised. The passage of electrolytes and water is a dynamic event coordinated by different factors. Therefore, the impression of a single driving force is probably oversimplistic.

Colonic Absorption

In the distal colon the principal mechanism of Na^+ uptake is the result of the ouabain sensitive Na^+/K^+/ATPase pump situated on the basolateral membrane. It pumps 3 moles of Na^+ out of the cell and 2 moles of K^+ into the cell for each mole of ATP hydrolysed. This maintains a low intracellular Na^+ concentration, a high intracellular K^+ concentration and an electrical potential difference across the the cell membrane (approximately -50mV). This electrochemical gradient permits the entry of Na^+ into the cell across an apical Na^+ specific ion channel.

In humans apical Na^+ uptake is sensitive to amiloride and aldosterone in the distal colon but not in the proximal colon (Sandle, 1989).

The other mechanism by which Na^+ is transported into the enterocyte involves

electroneutral Na^+-Cl^- entry into the enterocyte via the apical membrane. This could occur by either a single Na^+-Cl^- cotransporter or a parallel ion exchange mechanism where sodium entry is coupled with hydrogen exit and chloride entry is exchanged for bicarbonate exit. Experimental evidence does not permit a complete breakdown of the presence and contribution of these and other pumps and exchange mechanisms. It is clear however that differences exist between rat, rabbit and human colonic ion transport mechanisms (Binder & Sandle, 1987; Sandle, 1989). In the rat the electroneutral NaCl uptake predominates and the Na^+ uptake is amiloride resistant, whereas in humans the electrogenic Na^+ uptake accounts for almost all of the current and potential difference.

Water flow is also influenced by the leakiness of the epithelial paracellular pathway. This is determined by the number of tight junctions connecting the enterocytes. The electrical resistance of an epithelium gives an indication of the tightness with which the epithelium is held together. The small intestine is a leaky epithelium with resistance readings around 20-45Ωcm^2. Tight epithelia such as the gallbladder and oesophageal epithelia have resistances in the order of 500-1000$\Omega \cdot cm^2$. The colonic epithelium falls between these and is considered to be moderately tight with reported values around 100$\Omega \cdot cm^2$. The tightness of the colon helps to maintain a higher osmotic pressure gradient and transepithelial potential difference and to keep the absorbed fluid hypertonic.

Colonic Secretion

Na^+ is the principal ion determining absorption in the colon but in colonic secretion it appears to be Cl^-. Under basal conditions the colon secretes HCO_3 and K^+. In the presence of a secretagogue several events can occur which result in Cl^- loss across the apical membrane: 1) coupled Na^+-Cl^- absorption is inhibited, and may even be reversed; 2) Cl^- is secreted across the apical membrane. The current hypothesis is that Cl^- enters the enterocyte with Na^+ and K^+ via the basolateral $Na^+/K^+/2Cl^-$ cotransporter and accumulates above its electrochemical equilibrium. The Cl^- leaves via an apical channels down its electrochemical gradient. The K^+ leaves the cell via

a basolateral K^+ channel.

Certain circumstantial evidence indicates that electrolyte secretion and absorption occur at different sites ie secretion occurs from the crypt cells and absorption from the apical cells but the evidence is circumstantial and some studies of both small (Walters & Sepulveda, 1991) and large intestine (Bleakman & Naftalin, 1988) contradict this view. Further patch clamp analysis of the enterocytes in relation to their site along the crypt-villus axis will help resolve this issue.

Under normal conditions the absorptive processes appear to dominate the secretory events. Limited secretion of fluid is likely to facilitate the passage of the faecal bulk along the colon but a number of different agents can induce secretion in the colon. Different secretagogues can either merely inhibit absorption via the coupled Na^+-Cl^- absorption or additionally induce anion (HCO_3^-, Cl^-) secretion. Not surprisingly bacterial enterotoxins act via the apical membranes whereas host physiological agents such as prostaglandins, neurotransmitters and neuropeptides usually act via the basolateral membranes and have no effect from the luminal side.

Secondary messengers

There are three recognised key intracellular messenger systems that activate and regulate the activity of the ion transport systems. Changes in the concentration of available intracellular calcium ($[Ca^{++}]_i$), cyclic nucleotides (cAMP and cGMP) and phosphoinositides exert differing effects on the ion transport of the cell by activating phosphorylation of membrane proteins by protein kinases. The messenger compounds are in turn regulated by stimulatory or inhibitory proteins (G_s and G_i).

i) Agents that raise cAMP levels include cholera toxin (CT), *E.coli* heat labile toxin (LT), forskolin, prostaglandin E, VIP and theophylline. The individual mechanism by which cAMP is raised varies between the agonists eg. theophylline prevents cAMP breakdown by inhibiting phosphodiesterase action whereas cholera toxin activates the adenyl cyclase and G_s proteins.

ii) Very few agents have been described which act on intestinal epithelia via cGMP, the best characterised being *E.coli* heat-stable enterotoxin (STa) which has been

shown to act by stimulating guanylate cyclase.

iii) The mobilisation of free $[Ca^{++}]_i$ and the inositol phosphate pathway occurs after exposure to acetylcholine, carbachol, 5-HT, substance P.

By using various secretagogues it is possible to examine the effect of direct luminal secretion acting on the colonocytes or indirectly by stimulation of the neural fibres running through the submucosa and mucosal plexuses as well as different intracellular secretory pathways.

Studies on the effect of various neuropeptides and other neurotransmitters in different animal species have established the sensitivity of the colonic mucosa to a large number of neurotransmitters when applied to the serosal but not mucosal surfaces (Brown & Miller, 1991). *In vivo* evidence indicating a role of the ENS in mediating secretion in response to luminal secretagogues can be found in studies from two different groups of workers. As discussed in chapter 1, a significant body of evidence has been produced by Lundgren and his colleagues implicating the enteric nervous system in the pathophysiology of secretion in the small intestine. Additionally, studies on streptozocin-induced diabetic rat small intestine (Chang *et al*, 1985; 1986) have demonstrated a defect in the inhibitory adrenergic innervation which could contribute to the diarrhoea seen in these animals.

In contrast to the substantial body of research on the smooth muscle changes in Hirschsprung's disease, almost nothing is known of the role of the enteric nervous system in human colonic ion transport either under normal conditions or in various disease states. Defective neural regulation of colonic absorption and secretion could account for the episodes of diarrhoea and constipation seen in children with Hirschsprung's disease. Previously, studies of the absorptive function of the rectum *in vivo* in children with Hirschsprung's disease found greater absorption of sodium and chloride ions and higher rectal potential difference in the rectum of children with Hirschsprung's disease compared with control children (Heath, Milla & Spitz, 1985).

To determine the effect of the neural abnormalities in Hirschsprung's disease on

colonic ion transport we have examined the electrogenic colonic ion transport in children with Hirschsprung's disease in comparison with normally innervated colon from children with anorectal anomalies. The basal electrical properties and response to different secretagogues were examined in i) aganglionic and ganglionic rectosigmoid colon and ii) ganglionic transverse colon. The effect of neurally mediated secretagogues (ATXII, Iloprost), neurotransmitters (acetylcholine & carbachol) and luminally-active secretagogues which act from the apical membrane (*Esch.coli* STa enterotoxin, theophylline, 3-isobutyl-1-methylxanthine [IBMX]). The agonists chosen also would also yield information on any possible defects in activation of the three recognised secondary messenger pathways: calcium mobilisation by acetylcholine and carbachol, increases in cGMP concentrations by STa enterotoxin and increases in cAMP concentrations by IBMX and theophylline.

Methods

TISSUE SPECIMENS

Rectosigmoid tissues were obtained from children undergoing corrective pullthrough surgery for Hirschsprung's disease. Mostly the operations were Duhamel type procedures. The normally innervated "control" tissues were obtained mainly from children with anorectal anomalies. Several tissues were obtained from children undergoing colonic interposition.

The age range of the three groups (given as mean and range) are shown below and were broadly comparable :

	Age (mths)	Range
Aganglionic colon (HD)	8.0	3-36
Ganglionic colon (HD)	24.4	2-168
Ganglionic colon (ARA)	24.3	1-192

Samples of proximal transverse colon were obtained from right sided colostomy closure procedures from children with Hirschsprung's disease and compared with right sided colostomy closures from children undergoing staged operations principally for anorectal anomalies.

The ages of the children are shown below:

	Age (mths)	Range
Hirschsprung's disease	16.0	3-45
ARA controls	17.8	3-72

All specimens were obtained from scheduled operations in children with normal electrolyte status. The majority of the patients received 0.2mg atropine premedication. All children were anaesthetised with endotracheal administration of halothane and nitrous oxide with analgesia controlled using narcotic drugs: either Fentanyl or morphine and bipivucaine (given either as an epidural or peripherally).

Tissues were collected in theatre in ice-chilled NaCl-Ringer's solution which had been previously oxygenated for 30 minutes. Once removed from the patient the tissues were transported immediately to the laboratory. The tissue segments were opened longitudinally and the luminal surface rinsed in NaCl-Ringer's. The tissues were pinned out on wax, mucosal surface down and stripped free of the outer layers of muscle using microdissection scissors at x50 magnification (Nikon 102 dissecting microscope). Care was taken to remove as little submucosal tissue as possible. The tissues were bathed continously in chilled oxygenated NaCl-Ringer's during dissection, which took 10-15 minutes. The dissected outer muscle layers together with pieces of mucosa/submucosa adjacent to the exposed tissue surface in the chambers were fixed for histological analysis.

An adjacent piece of tissue was also fixed for histological analysis.

The mucosa/submucosa preparations were mounted in perspex open topped Ussing-type chambers against rubber "O-rings" to minimise edge damage. Two chamber designs, exposing differing areas of tissue, were used throughout the study. The design was modified from chambers used by Dr R. Naftalin, King's College, London. Exposed tissue area was either a rectangle of 1x2.0 cm^2 ($2.0cm^2$) or $0.17x0.06cm^2$ ($0.9cm^2$). Tissue surfaces were bathed with bicarbonate-Ringer's solution containing (in mmol/l) NaCl 113; KCl 4.5; $MgCl_2$ 1.0; $Na^+{}_2HPO_4$ 0.2; $NaHCO_3$ 25; $CaCl_2$ 1.25 and glucose 10.0. Each chamber was oxygenated and stirred directly by bubbling with 5%CO_2 in oxygen (delivered through syringe needles). The chamber was kept at 37°C by a heated water circulating jacket (Grant).

ELECTRICAL MEASUREMENTS

Experiments were carried out under open-circuit conditions. The electrical parameters were recorded using standard procedures via a computer-based voltage clamp modified from that described by Naftalin and Smith (1984).

The transepithelial voltage (Vt) was monitored continously via agar bridges (3M KCl in 3.5% agar in 1.57mm ID polythene tubing, Portex) positioned close

(<3mm) to the tissue faces connected to matched calomel electrodes (Russell pH Ltd). Agar bridges were stored at 4°C with opposite ends in 3M KCl and NaCl-Ringer's solution without glucose to reduce the high concentration of KCl at the tissue end.

Vt readings were automatically corrected for offset potential which were usually less than 1mV. Resistance (Rt) measurements were made using external current pulses (CED 2106) of 180μA delivered via Ag/AgCl electrodes (Clark Electromedical Ltd) through agar bridges positioned at opposite ends of the chamber (24mm from the tissue face). The Rt measurements were averaged over 200msecs to allow for capacitance transients. The external current pulse was delivered once every 180 secs during equilibration periods and increased during exposure to agonists to 10-30 second pulses. The Rt readings were corrected for solution resistance and recorded as ohms·cm^2. The short-circuit current values were calculated from the Vt and Resistance using Ohm's Law and recorded as μA/cm^2. The Vt, Isc and Rt were stored to a Dell computer hard disc for analysis via an analogue to digital interface (CED 1401, Cambridge).

The Clamp programme was calibrated using an artificial epithelium designed by Dr PM Smith, Dept of Physiology, University of Liverpool and built by the Department of Biomedical Electronics, Great Ormond St. Hospital, London. The artificial epithelium allows preset values for offset and open circuit readings to be set. The circuit diagram is shown in Fig 4.

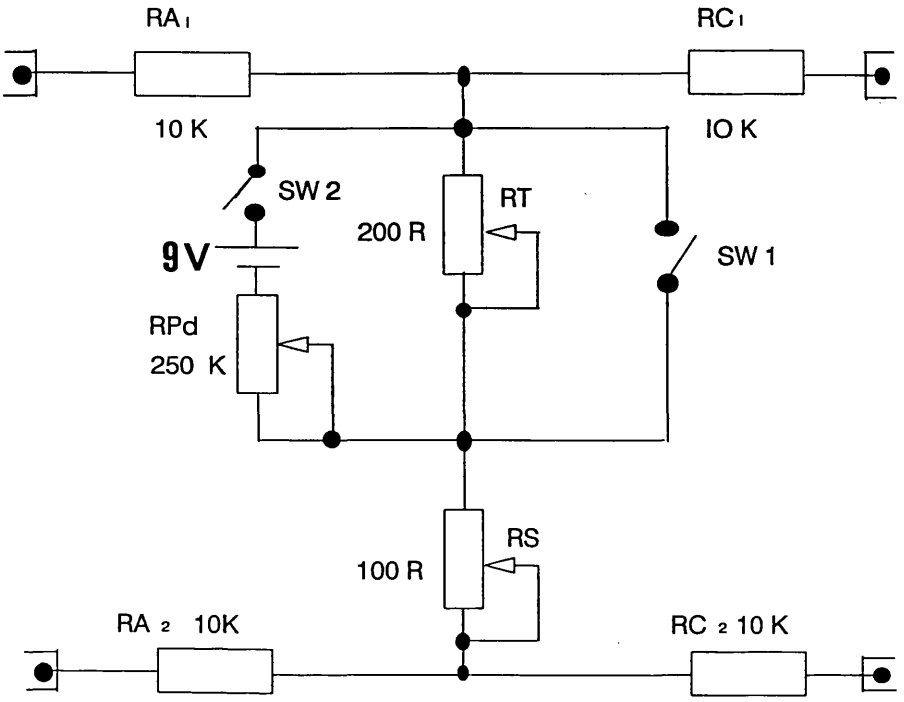


Fig.4.1. Test circuit for calibrating "Clamp" voltage clamp.

RC1, RC2: Vt measurement, calomel resistance
RA1, RA2: Current pulse delivery, AgCl resistance
RS: Solution resistance (variable 0-100Ω)
RT: Tissue resistance (vaiable 0-200Ω)
SW1: removes Rt (for offset readings)
SW2: creates Vt (varied by Rpd)

Resistors represented by rectangles along with their ohmic resistance values.

DRUGS

Acetylcholine, carbachol, theophylline, vancomycin ("Vancocin" Lilly Industries), atropine & IBMX were prepared in NaCl-Ringer's solution. Theophylline and IBMX were dissolved by vigorous shaking at 37°C for 30 minutes. ATXII was dissolved in 150mM saline. *Esch.coli* STa enterotoxin was dissolved in 0.1M phosphate-buffered saline (PBS) and stored as a stock of 1000MU/ml at -27°C in 0.5ml volumes. Tetrodotoxin was dissolved in 5mM disodium citrate and stored at 10^{-4}M concentration at 4°C, protected from the light. All agonists which were added in 100μl volumes were kept on ice during the experiment. IBMX, theophylline and *Esch.coli* STa enterotoxin were warmed to 37°C prior to use.

The efficacy and stability of the TTX was confirmed periodically by studies carried out on unstripped rat distal colon in Ussing chamber preparations. TTX ($9x10^{-7}$M) applied to the serosal (but not the mucosal) bathing solution caused an immediate drop of $\approx$88% of the Vt and Isc without any significant alteration in Rt and abolished the rises in Vt and Isc seen after neural stimulation by electrical field stimulation (Neurolog System, Digitimer Ltd) at 10Hz, 5 mSec pulse width, 1-5V for 30 seconds using gold foil electrodes (Electrode Supplies Ltd).

EXPERIMENTAL PROTOCOL

The offset potentials and Ringer's solution resistance were recorded in the Ussing chambers for a period of no shorter than 60 minutes to allow the Ringer's solution and chambers to reach 37°C.

After mounting, tissues were allowed to equilibrate to a stable baseline (25 to 90 minutes). Five minutes before addition of agonist the electrical sampling frequency was increased to 10-30 seconds. After exposure to agonists both mucosal and serosal bathing solutions were changed with at least three washes of oxygenated pre-warmed Ringer's solution and left for a period of 20-45 minutes in which to re-equilibrate before addition of further agonists. Changes in the Vt, Isc and Rt after agonist were compared with the mean of the baseline taken over the 5 mins prior to addition of the agonist. Tissues maintained stable electrical parameters for up to 5

hours after which tissues would usually demonstrate a steady decline in Rt and Vt.

HISTOLOGY

Tissues removed from children with Hirschsprung's disease were separated into the different groups after histological examination for the presence or absence of ganglion cells, which was performed after the Ussing chamber experiments. Tissues were fixed as described in Chapter 3.

Neural PGP-9.5 antibody staining

Cryostat-cut sections were incubated with PGP9.5 antiserum (1 in 2000 dilution in PBS-0.1% Triton-X, antisera raised in rabbit; Ultraclone Ltd) for 18 hours at 4°C. After 3 washes in PBS the slides were then incubated with biotin labelled anti-PGP-9.5 IgG for 90minutes. After 3 washes the reaction was visualised with fluoroscein labelled streptavidin for 90minutes.

Slides were mounted in Citifluor and examined using a Zeiss Axoplan microscope. Photomicrographs were taken using Kodak TMAX-400 film (monochrome).

NADH-diaphorase activity

The presence or absence of neurons within the submucosal and myenteric plexus of the tissues was confirmed by staining for NADH-diaphorase activity using the method of Hoyle and Burnstock (1989) as described in chapter 3.

Acetylcholinesterase activity

Acetylcholinesterase (AchE) activity was detected using a method based on that of Karnovsky and Roots (1964) in the presence of the nonspecific acetylcholinesterase inhibitor tetraisopropylpyrophosphoramide and stain intensified with 0.1% silver nitrate (60secs). The slides were lightly counterstained with Carazzi's Haematoxylin (10 secs). Slides were then dehydrated through graded alcohols and xylene and mounted in DPX (BDH).

CHEMICALS

Iloprost was generously donated by Schering (W.Sussex, England). Most other chemicals, unless otherwise stated, were obtained from Sigma (Poole UK).

STATISTICS

Results are given as the mean $\pm$ one standard error of the mean (SEM). Statistical significance of differences between the responses of the three tissue groups were first tested using the non-parametric Kruskal-Wallis ANOVA (K-W ANOVA) and then differences between the test and controls was tested by Kolgomorov-Smirnoff 2-sample test (K-S 2-sample). The K-W ANOVA value for the difference between the three groups of tissue are given in the text and in the comparative bar chart figures, and the K-W ANOVA values are given in the data tables. Non-parametric analysis was chosen in order to compensate for unknown variables in tissues obtained from children at theatre. Furthermore, the limited number of tissues available for study with any one particular agonist meant that the sample size was small.

4.iii Results

BASAL ELECTRICAL PROPERTIES: RECTOSIGMOID COLON

Ganglionic colon (ARA). Table 4.1 summarises the basal electrical measurements in bicarbonate-Ringer's solution after equilibration (usually 60 minutes, range 25-90 mins). The Vt readings normally fell quickly during the first 15 minutes after mounting and then either stabilised or then subsequently rose to their basal plateau readings. The Rt values gradually increased over the first 30-60 minutes.

Aganglionic colon (HD). The values obtained from the aganglionic rectosigmoid colon were not significantly different from the normally innervated control colon.

Ganglionic colon (HD). Similarly, the basal electrical properties of the ganglionic rectosigmoid colon from children with HD were comparable to the ganglionic control tissues. Data are given in Table 4.1.

The basal Vt, Isc and Rt values were similar for all three tissue groups. This is shown in Fig.4.2. No correlation was found between the Vt, Isc and Rt and age of the child for the values from all three groups of tissue.

The addition of 10^{-4}M ouabain (BDH) to the serosal bathing solution resulted in almost complete abolition of the Vt and Isc (Vt = <0.5mV) in ganglionic colon (5 tissues from 4 children: 3 transverse, 2 rectosigmoid colon).

Table 4.1. Basal electrical properties of human paediatric aganglionic and ganglionic rectosigmoid colon.

	N	Vt(mV) (n)	Isc(μA/cm^2) (n)	Rt($\Omega\cdot$cm^2) (n)
Aganglionic (HD)	28	+13.1±1.4 (37)	-147.3±13.3 (36)	93.1±6.0 (34)
Ganglionic (HD)	25	+12.1±1.5 (26)	-126.7±13.8 (26)	94.0±7.2 (25)
Ganglionic (Control)	26	+9.9±1.0 (37)	-114.0±11.9 (36)	92.2±5.6 (37)

Results are given as mean ± SEM. HD = Hirschsprung's disease; Vt = transepithelial potential difference (serosal side positive); Isc = calculated short-circuit current; Rt = transepithelial resistance; N= number of patients; n = number of tissues. No significant differences were found between the three groups of tissues.

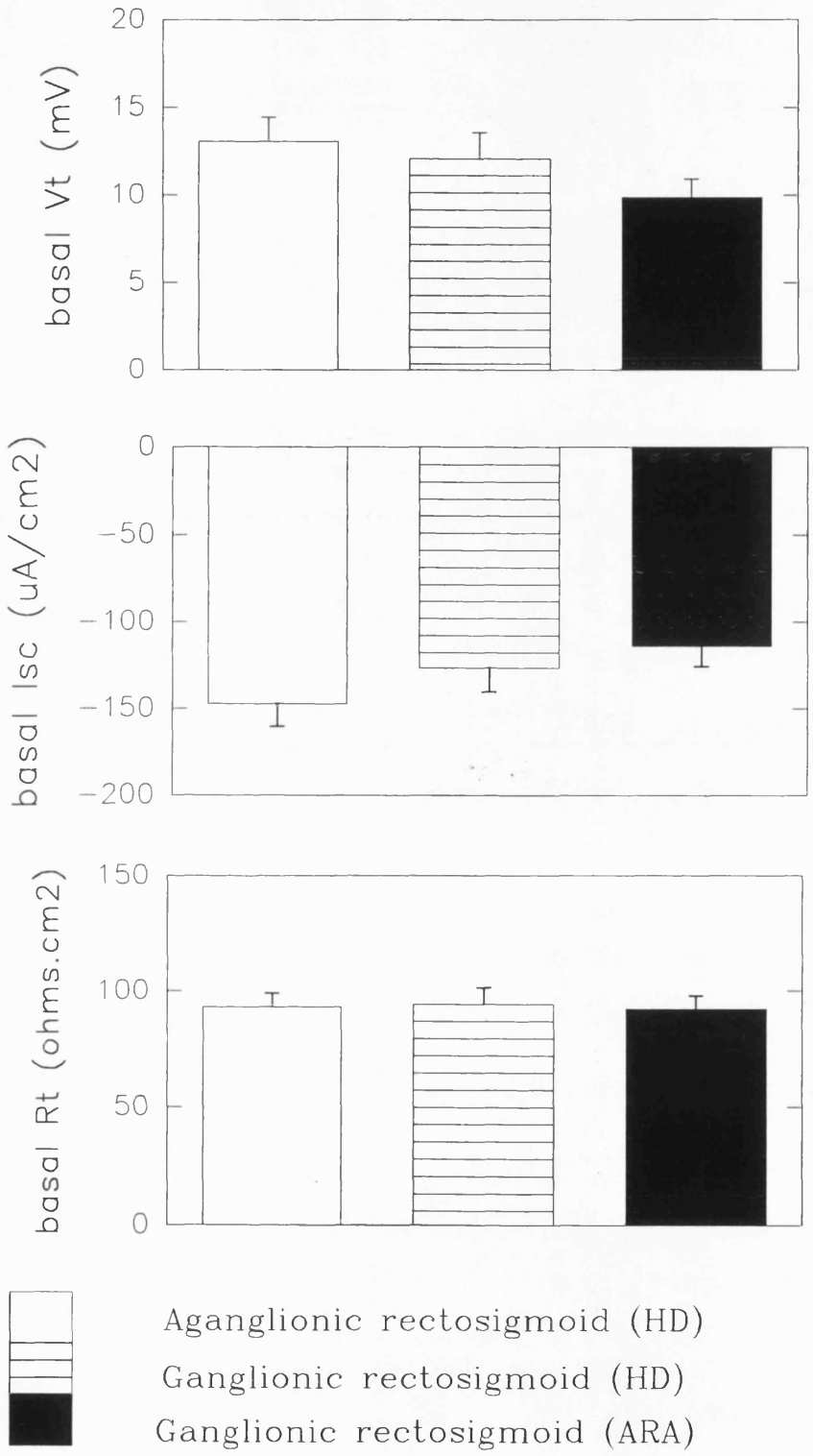


Fig.4.2. Basal electrical properties of human aganglionic and ganglionic rectosigmoid colon. Results given as mean ± SEM.

NEURALLY MEDIATED SECRETION: RECTOSIGMOID COLON

Immunohistochemical examination of the neural innervation to the colonic mucosa

Tissues from 3 normally-innervated rectosigmoid and one transverse colon were examined for the extent of the innervation supplying the colonic lamina propria and epithelium. Two aganglionic tissues were also examined for comparison. Fig 4.3A demonstrates the pattern of innervation in the rectosigmoid colon of a 6mth old female child with imperforate anus stained with PGP9.5. Dense innervation of the lamina propria was seen in all four ganglionic tissues. The neural fibres and varicosities could be seen extending along the length of the crypt from the base to the apical surface (Fig.4.3 C,D) and occasional lateral projections running into the mucosal crypt colonocyte layer were noticed.

The aganglionic rectosigmoid colon from 2 children with Hirschsprung's disease also demonstrated dense mucosal staining patterns but also had characteristic hypertrophied nerve fibres in the submucosa and myenteric plexus along with an increased density of staining of fibres in the muscularis mucosae and circular and longitudinal muscle layers (Fig 4.3 B).

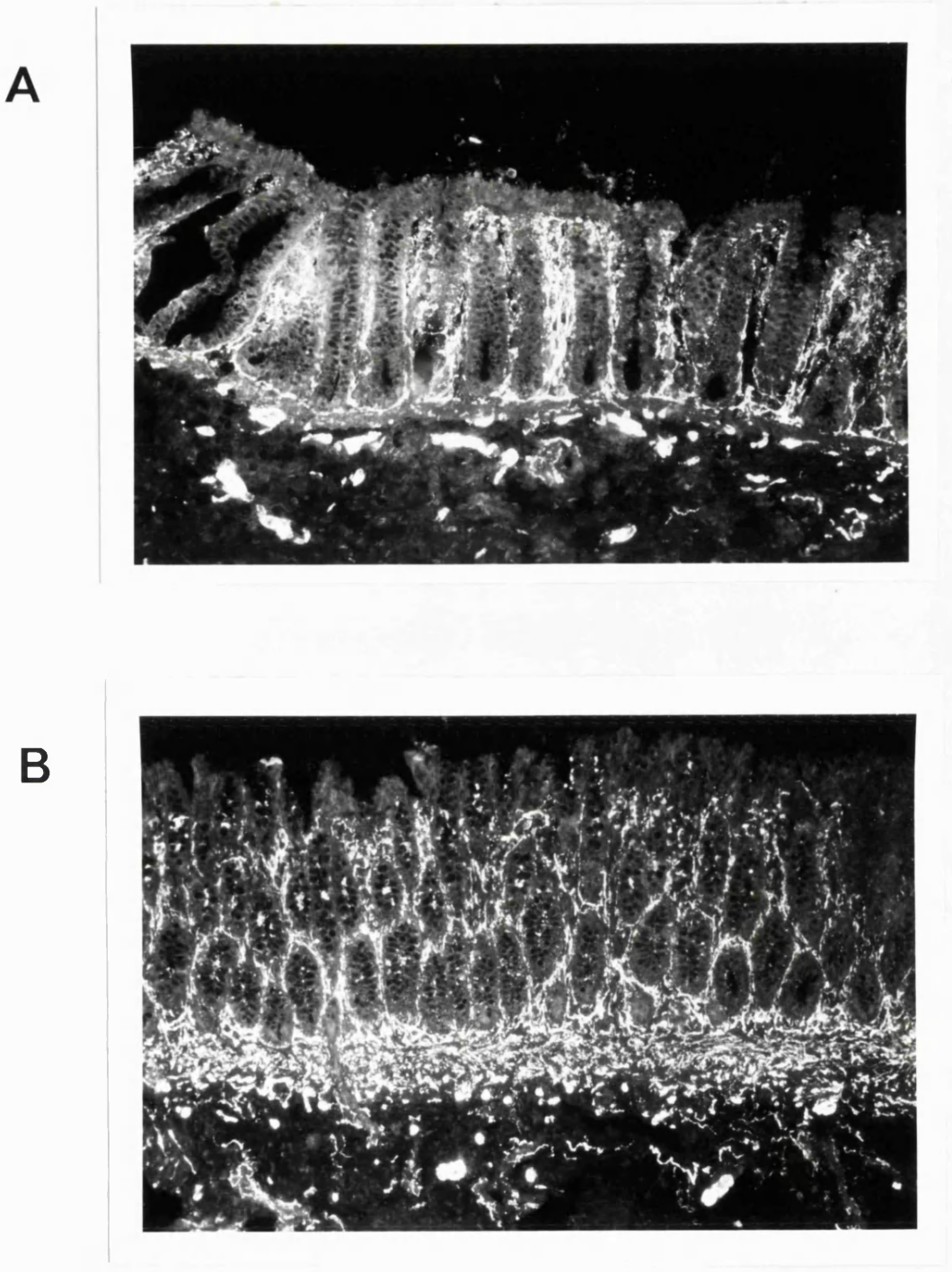


Fig.4.3a. Photomicrograph of **A**, **C**: human ganglionic rectosigmoid colon (3 mth ♂ child with imperforate anus) and **B**: aganglionic rectosigmoid colon (6 mth ♂ with Hirschsprung's disease). Stained with antisera to PGP9.5. Neural fibres can be seen extending to the apical surface the mucosa (**A** & **B** x200).

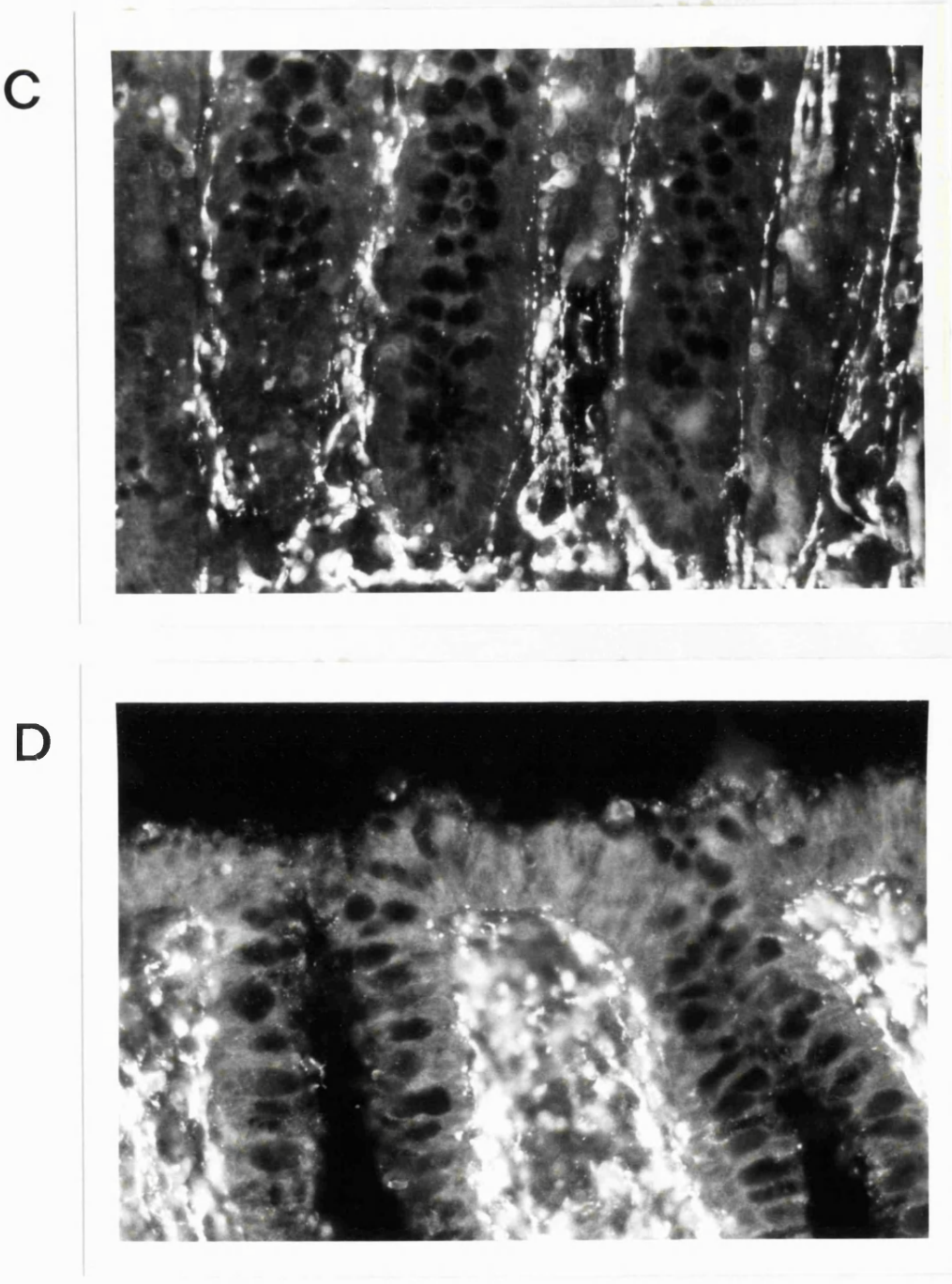


Fig.4.3b. Higher magnification picture of human ganglionic rectosigmoid colon (tissue **A**). A line of varicosities can be seen running along the length of the crypt (**C** x400) as well as across the apical surface epithelia (**D** x400).

To examine the effect of neurally mediated secretion of the aganglionic colon two different agonists were chosen which act exclusively via neural tissues: the neural sodium channel activator ATXII isolated from the sea anemone *Anemonia sulcata* and Iloprost, a stable prostacyclin (PGI_2) analogue. Both ATXII and Iloprost have been shown to act via the submucosal neural tissues, and not directly on the colonocyte, to cause chloride secretion in rat distal colon (Andres *et al*, 1985; Diener *et al*, 1988).

Effect of ATXII

Control ganglionic colon (ARA): ATXII (10^{-6}M) added to the serosal bathing solution resulted in an increase in Vt and Isc in 2/4 tissues from ganglionic rectosigmoid colon from children with anorectal anomalies (data given in Table 4.2). ATXII had no effect when added to the mucosal bathing solution (n=2) in these two children. No response to serosal ATXII was seen in the other 2 children. The children were not taking frusemide or diagnosed as having spina bifida.

Aganglionic colon (HD): ATXII caused an increase in the Vt, Isc and fall in Rt in 1 out of 6 children with Hirschsprung's disease. The other tissues exhibited no change in electrical values after challenge with ATXII. (Data summarised in Table 4.2)

Ganglionic colon (HD). Gradual increases in Vt and Isc were seen in the ganglionic rectosigmoid colon from children with HD. The data are given in Fig 4.4.

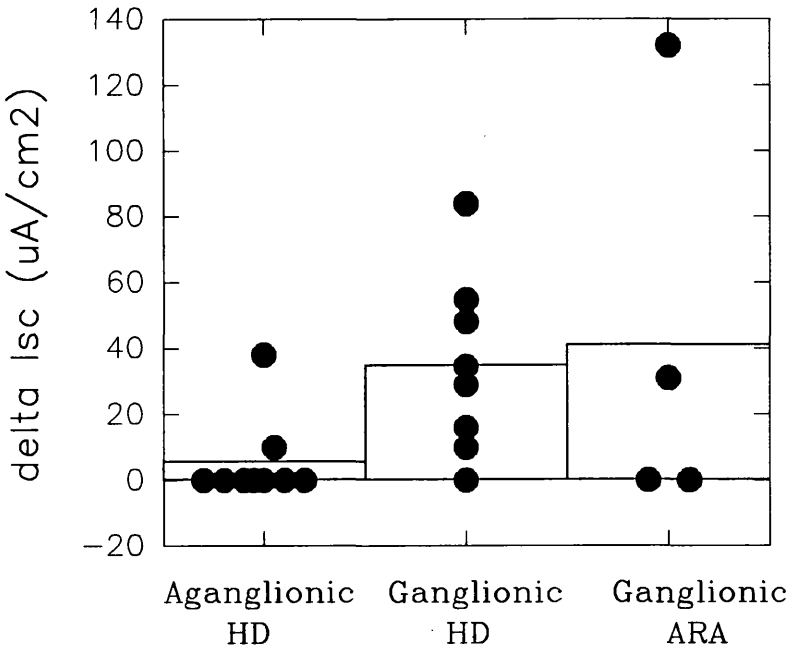


Fig.4.4 Response of aganglionic and ganglionic rectosigmoid colon to serosal ATXII (10^{-6}M). The mean increase in short-circuit current is shown (bar chart) as well as individual data points.

A different neurally-mediated secretagogue was sought because of the inconsistent response to ATXII seen in the first four control ganglionic tissues.

Effect of Iloprost

Control ganglionic colon (ARA): Neurally mediated secretion was also examined using Iloprost. 10^{-6}M Iloprost added to the serosal bathing solution of the normally-innervated control rectosigmoid colon (7 tissues from 6 children, mean age: 32mths) resulted in a marked rise in tissue Vt and Isc together with a fall in tissue resistance (data in Table 4. 3)

The time course of action is shown in Fig.4.5A. Prior addition of 10^{-6}M tetrodotoxin to the serosal bathing solution completely prevented any increase in Vt or Isc (n=3; data not shown). The addition of tetrodotoxin once the secretion had stabilised at maximum values after the addition of Iloprost reversed the increase in Vt, Isc and Rt (Fig4.5). Iloprost failed to result in any significant change in electrical properties when added to the mucosal bathing solution. Serosal atropine (10^{-6}M) also had no significant effect on the response to Iloprost (n=3).

*Aganglionic colon (HD):*In contrast addition of Iloprost to the serosal bathing solution of aganglionic rectosigmoid colon failed to induce any changes in the electrical parameters (increase in Vt: $+0.23\pm0.2$mV; Isc: $+5.2\pm2.8\mu A/cm^2$ and Rt: $-6.0\pm2.6\Omega\cdot cm^2$; n=7 from 5 children) which was statistically highly significant ($p<0.0001$ *vs* control ganglionic colon, unpaired t-test). A typical trace is compared to ganglionic colon Fig.4.5B.

Ganglionic colon (HD): The response of the ganglionic rectosigmoid colon to neural activation by 10^{-6}M Iloprost (Table 4.3) was not significantly different from the control ganglionic colon described above.

The comparison of the three groups of tissue are shown in Fig.4.6.

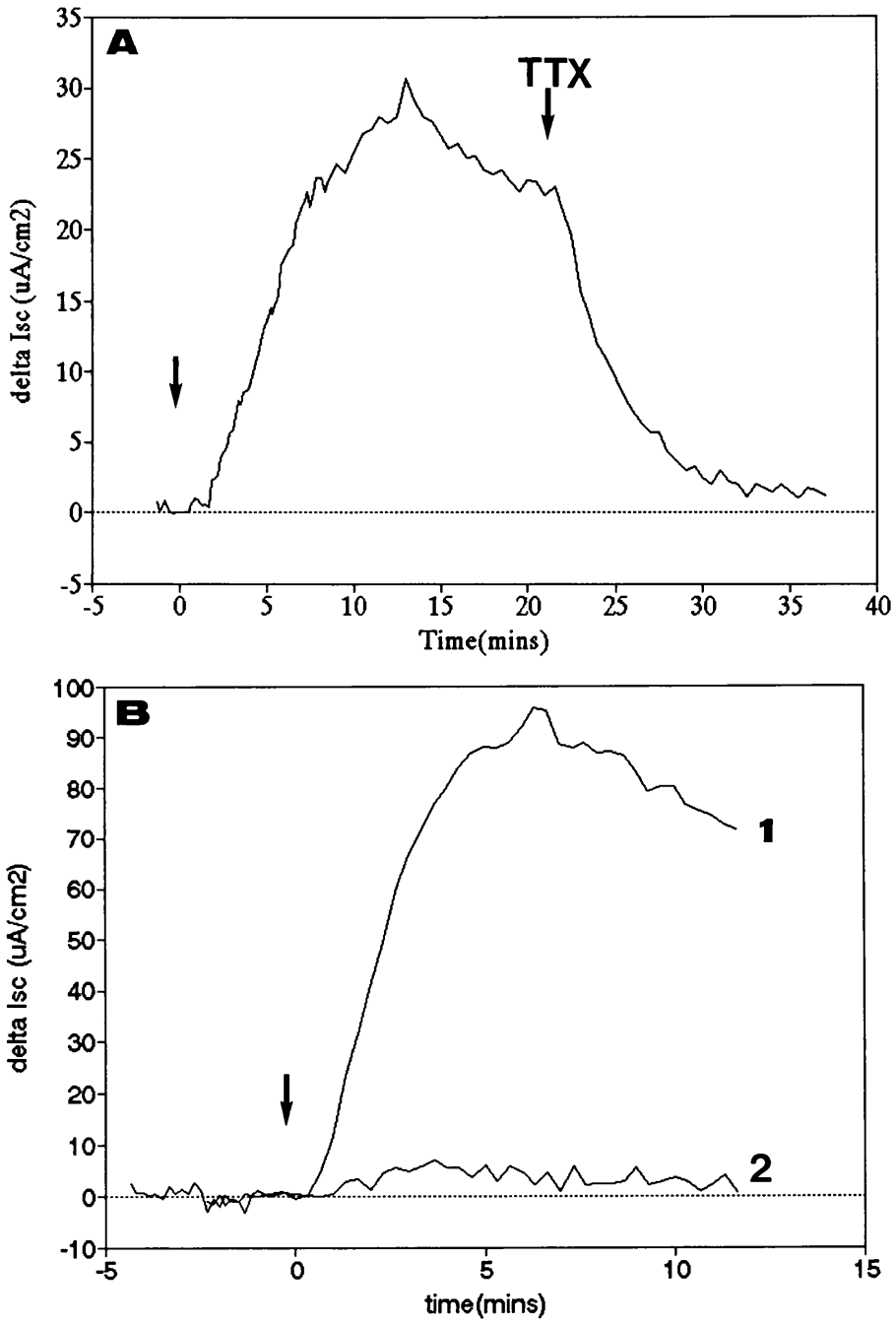


Fig.4.5. **A**: Effect of serosal Iloprost (10^{-6}M) added at Time 0 (arrow) on short-circuit of ganglionic rectosigmoid colon (3mth♂ anorectal anomaly) and the subsequent addition of serosal TTX (10^{-6}M) 23 minutes after addition of Iloprost. **B**: Comparison of response of ganglionic (**1**) and aganglionic rectosigmoid colon (**2**) to serosal Iloprost added at time O (arrow).

Table 4.3 Response of ganglionic and aganglionic colon to Iloprost (10^{-6}M)

	N	Vt(mV) (n)	Isc(μA/cm^2) (n)	Rt($\Omega\cdot$cm^2) (n)	Time (mins)
Aganglionic (HD)	10	+0.5±0.2* (14)	+12.0±5.6¶ (14)	-4.0±1.4§ (14)	NA
Ganglionic (HD)	8	+5.5±1.4 (10)	+74.8±11.8 (10)	-10.2±5.3 (8)	7.1
Ganglionic (ARA)	8	+6.9±1.1 (11)	+108.8±15.7 (11)	-15.7±4.1 (11)	6.3

Results are given as change in Vt, Isc and Rt and expressed as mean ± SEM.
NA: not applicable, other abbreviations are as given in footnote to Table 4.1.
* P<0.0001; ¶ P=0.002; § P=0.01 versus Ganglionic (ARA)(K-S 2-sample test).

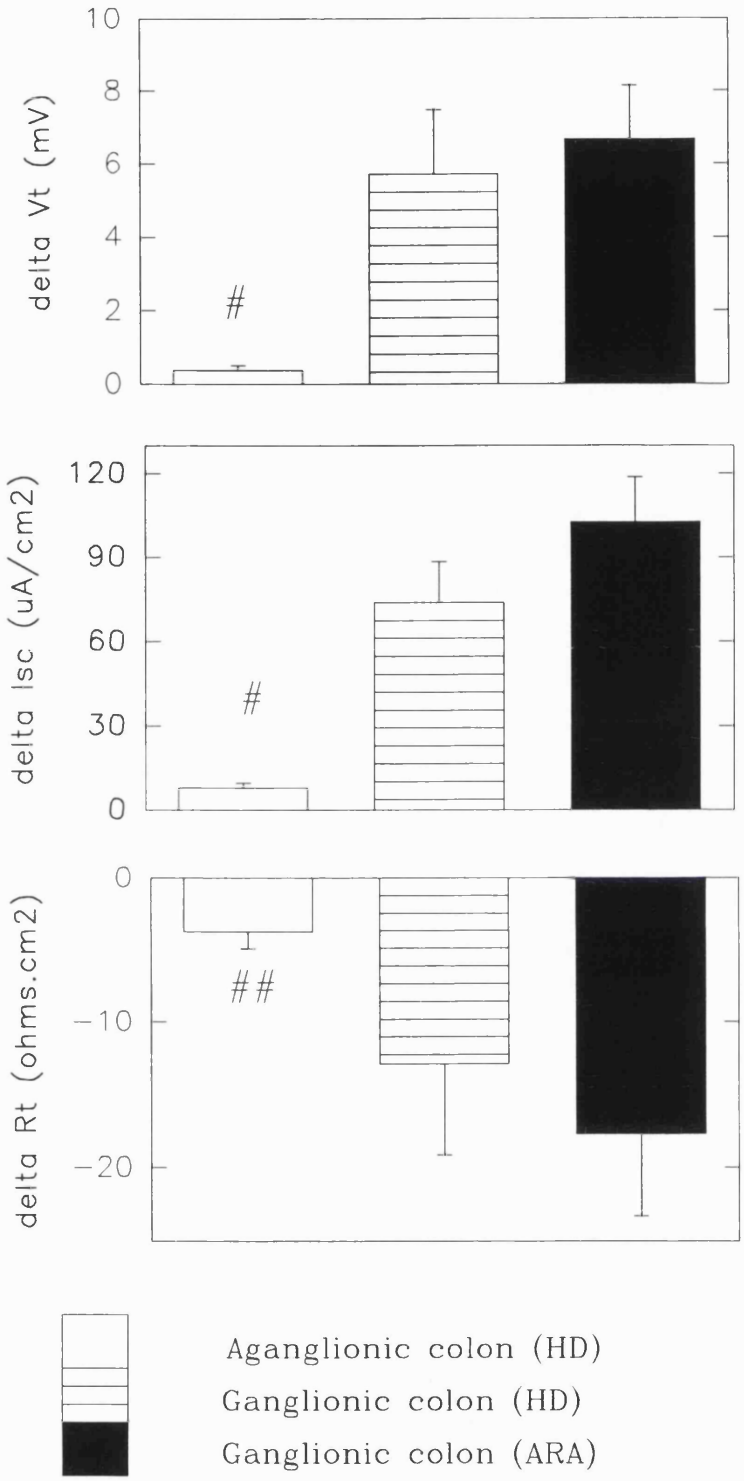


Fig.4.6 Comparison of response to Iloprost in human paediatric colon. Results given as mean±SEM.

# $P<0.0001$ (K-W ANOVA).
$P<0.05$ (K-W ANOVA).

CHOLINERGIC AGONISTS: RECTOSIGMOID COLON

Effect of acetylcholine

Control ganglionic colon (ARA): The effect of direct cholinergic neurotransmitter action on the colonocyte was examined. Addition of acetylcholine (900 & 9μM) to the serosal bathing solution of control ganglionic rectosigmoid colon gave transient increases in Vt and Isc coupled with a fall in Rt (Table 4.4 & 4.5). The time course of action of 900μM acetylcholine is shown in Fig.4.7. Maximum responses were obtained $\approx$2 (900μM) and $\approx$5 minutes (9μM) after addition to the serosal bathing solution. Prior addition of serosal atropine (10^{-4}M) completely abolished the response in 4 tissues (3 rectosigmoid, 1 transverse, data not shown).

*Aganglionic colon (HD):*In contrast to ganglionic colon, aganglionic colon gave either a small or an absent response to serosal acetylcholine (data summarised in Fig. 4.7). Fig.4.7 shows a typical time course of action of aganglionic rectosigmoid colon to high concentration of acetylcholine (900μM). Second challenge with 9μM acetylcholine after a lag of 30-50 minutes failed to alter the Vt, Isc and Rt (n=3).

*Ganglionic colon (HD):*The response of the proximal ganglionic rectosigmoid colon from children with HD to 9μM acetylcholine was also significantly reduced compared with the control ganglionic colon (Table 4.4 & 4.5). The response to the higher concentration was not statistically different from the ganglionic control group.

A comparison of the response to acetylcholine is shown in Fig.4.8.

Table 4.4 Response of aganglionic and ganglionic colon to acetylcholine ($9x10^{-4}M$).

	N	Vt(mV) (n)	Isc($\mu A/cm^2$) (n)	Rt($\Omega \cdot cm^2$) (n)	Time (mins)
Aganglionic (HD)	9	+1.5±0.3* (11)	+27.2±8.7¶ (10)	-2.2±1.3§ (10)	2.0
Ganglionic (HD)	2	+5.2±1.7 (3)	+77.6±15.4 (3)	-2.3±1.2 (3)	2.0
Ganglionic (ARA)	5	+7.3±1.3 (6)	+100.7±18.6 (6)	-15.8±4.3 (6)	2.3

Results are given as change in Vt, Isc and Rt and expressed as mean ± SEM.
Abbreviations are as given in footnote to Table 4.2.
* $P<0.001$; ¶ $P=0.004$; § $P=0.004$ versus Ganglionic colon (ARA) (K-S 2-sample test).

Table 4.5 Response of ganglionic and aganglionic colon to acetylcholine (9μM)

	N	Vt(mV) (n)	Isc(μA/cm²) (n)	Rt(Ω·cm²) (n)
Aganglionic (HD)	13	+1.7±0.6* (21)	+22.6±8.1¶ (21)	-6.3±2.4 (18)
Ganglionic (HD)	11	+2.0±0.7** (18)	+32.2±12.2¶¶ (18)	-9.1±2.8 (17)
Ganglionic (ARA)	10	+4.1±0.6 (13)	+70.9±13.1 (13)	-11.3±5.3 (13)

Results are given as change in Vt, Isc and Rt and expressed as mean ± SEM.
Abbreviations are as given in footnote to Table 4.2.
* P<0.0002; ¶ P<0.01 versus Ganglionic (ARA) (K-S 2-sample test).
** P=0.0002; ¶¶ P<0.01 versus Ganglionic (ARA) (K-S 2-sample test).

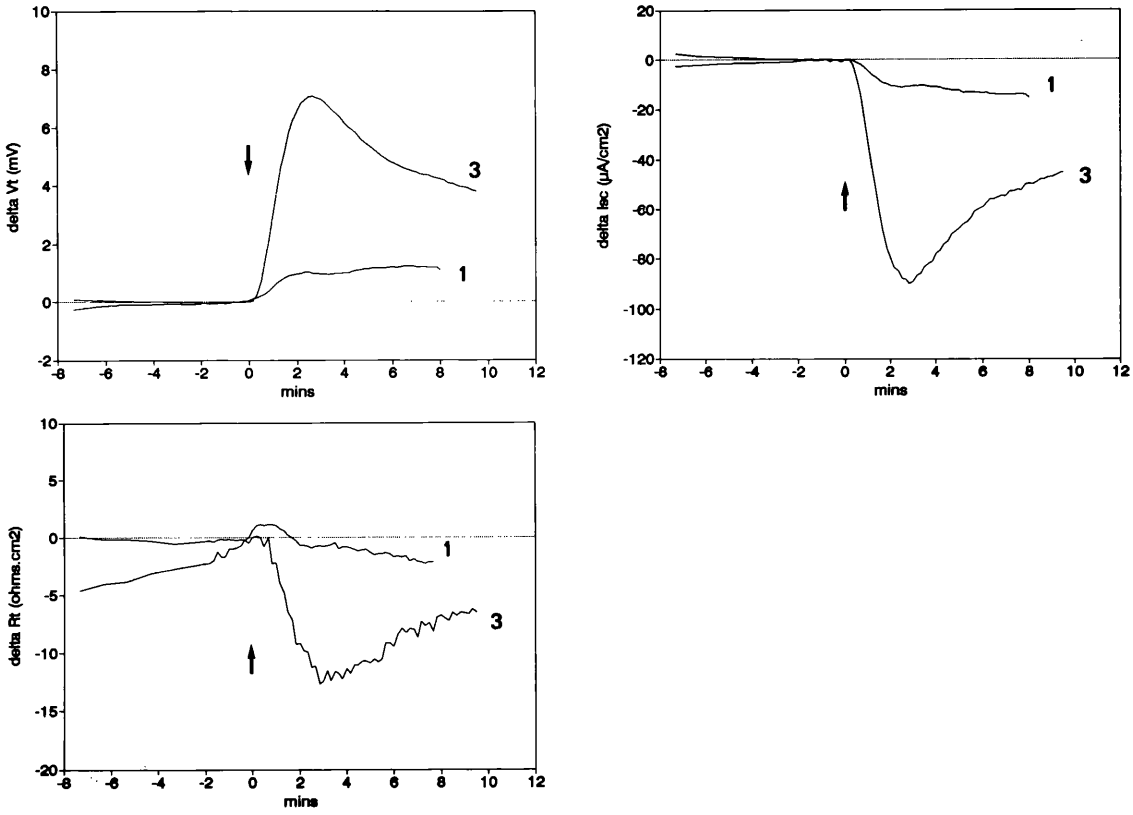


Fig.4.7 Effect of serosal acetylcholine (900μM) on **1**: aganglionic (6mth♂ child with Hirschsprung's disease) and **3**: ganglionic (2mth♂ with imperforate anus rectosigmoid colon).

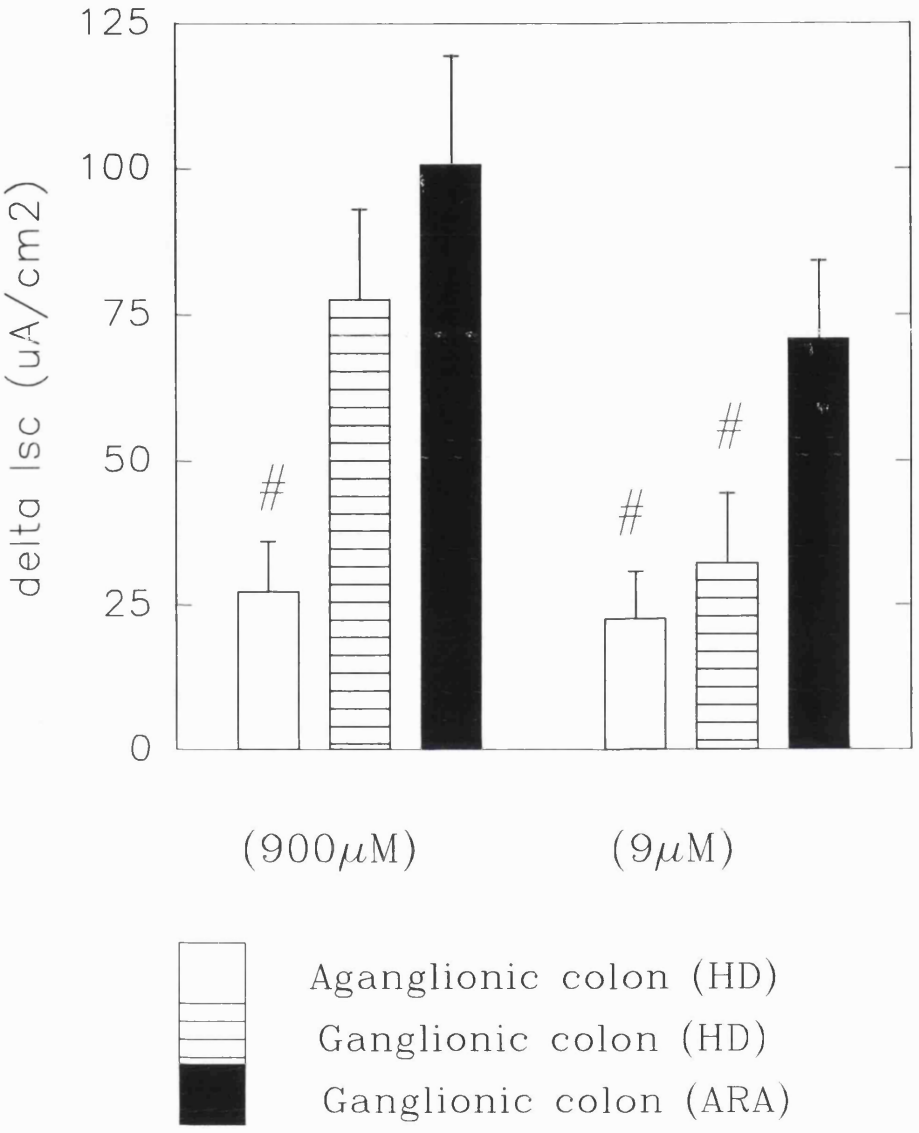


Fig.4.8 Comparison of increase in short-circuit current to acetylcholine in human rectosigmoid colon. Results given as mean±SEM.
# $P < 0.05$ (K-W ANOVA).

Effect of carbachol

Control ganglionic colon (ARA): Subsequent challenge with the acetylcholinesterase resistant acetylcholine analogue carbachol (9μM) to the serosal bathing solution of control ganglionic rectosigmoid colon resulted in a transient rise in Vt and Isc similar to that seen after acetylcholine (Table 4.6).

Aganglionic colon (HD): When added to the serosal bathing solution of aganglionic rectosigmoid colon, carbachol also gave variable responses with 10 out of 19 tissues giving increases in Isc of $< 10\mu A/cm^2$. Other tissues gave increases in Isc ranged from 10 to $137\mu A/cm^2$. The median response was not significantly different from that of the control ganglionic tissues ($p > 0.05$ KW-ANOVA, data summarised in Table 4.6).

To minimise this variability, the results were corrected so that where more than one tissue was tested from the same child, the responses were averaged and considered as a single data point so as to correct for bias that might arise in the statistical analysis. Two broad patterns of response to carbachol were seen with 7 out of the total 14 aganglionic children gave increases of less than $10\mu A/cm^2$ (mean increase $3.1 \pm 1.4\mu A/cm^2$; $p=0.001$ vs control tissues). The other 7 tissues responded to carbachol with a mean increase of $66.4 \pm 15.9\mu A/cm^2$; ($p > 0.05$ vs control tissues).

The median increases in Vt and Rt were +0.82mV (range: 0 - 8.05) and $-2.0\Omega \cdot cm^2$ (range: -20 - 0) respectively.

Ganglionic colon (HD): Prompt increases in Vt, Isc along with a fall in Rt were seen in 11 tissues from proximal ganglionic descending colon of 8 children with HD. The mean values were comparable with those of the control ganglionic tissues. The data are given in Table 4.6.

Differences between Hirschsprung's disease and total colonic aganglionosis

In analysing the variation of the response to cholinergic agonists differences were found between rectosigmoid colon from Hirschsprung's disease and total colonic aganglionosis. The 6 tissues from children with TCA responded to 9μM

acetylcholine with increases in Vt ($+4.4\pm1.3$mV n=6) and Isc ($+53.7\pm17.4\mu A/cm^2$ n=7) which were significantly greater than the aganglionic colon from Hirschsprung's disease (Vt: $+0.7\pm0.5$mV n=13; Isc: $+7.4\pm4.9\mu A/cm^2$ n=15; p=0.004 KS 2-sample t test). A similar pattern was seen in the responses to carbachol with the increases in Vt ($+3.2\pm1.5$mV n=3) and Isc ($+40.4\pm17.8\mu A/cm^2$ n=3) of colon from TCA significantly greater than the response of aganglionic colon from Hirschsprung's disease (Vt: $+1.7\pm0.6$mV n=16; Isc: $+29.1\pm10.9\mu A/cm^2$ n=15; p=0.02 KS 2-sample t test).

ACETYLCHOLINESTERASE STAINING PATTERN

The extent of the acetylcholinesterase activity within the submucosa and mucosal tissues was examined. The control ganglionic tissues exhibited areas of acetylcholinesterase activity in the ganglia of the myenteric and submucosal plexus. Nerve fibres could be seen within the submucosa and very occasional fibres were seen within the lamina propria. An example is shown in Fig.4.9B.

Aganglionic tissues showed increased density of large AchE-positive nerve fibres in the submucosa, muscularis mucosae and lamina propria when compared to the ganglionic control colon (Fig.4.9A).

Ganglionic colon proximal to the aganglionic area gave comparable acetylcholinesterase staining patterns to that of the control tissues, with no increased acetylcholinesterase activity within the muscularis mucosae.

Table 4.6 Response of ganglionic and aganglionic colon to carbachol ($9x10^{-6}M$)

	N	Vt(mV) (n)	Isc($\mu A/cm^2$) (n)	Rt($\Omega \cdot cm^2$) (n)	Time (mins)
Aganglionic (HD)	17	+1.9±0.5* (19)	+29.7±9.0¶ (19)	-3.9±1.2 (18)	NA
Ganglionic (HD)	8	+5.6±1.8 (11)	+91.4±25.7 (11)	-14.3±3.5 (11)	3.9
Ganglionic (ARA)	8	+4.6±0.7 (11)	+64.2±11.3 (11)	-8.9±2.3 (11)	4.2

Results are given as change in Vt, Isc and Rt and expressed as mean ± SEM.
Abbreviations are as given in footnote to Table 4.2.
* P=0.01; ¶ P<0.01; versus Ganglionic (ARA) (K-S 2-sample test).

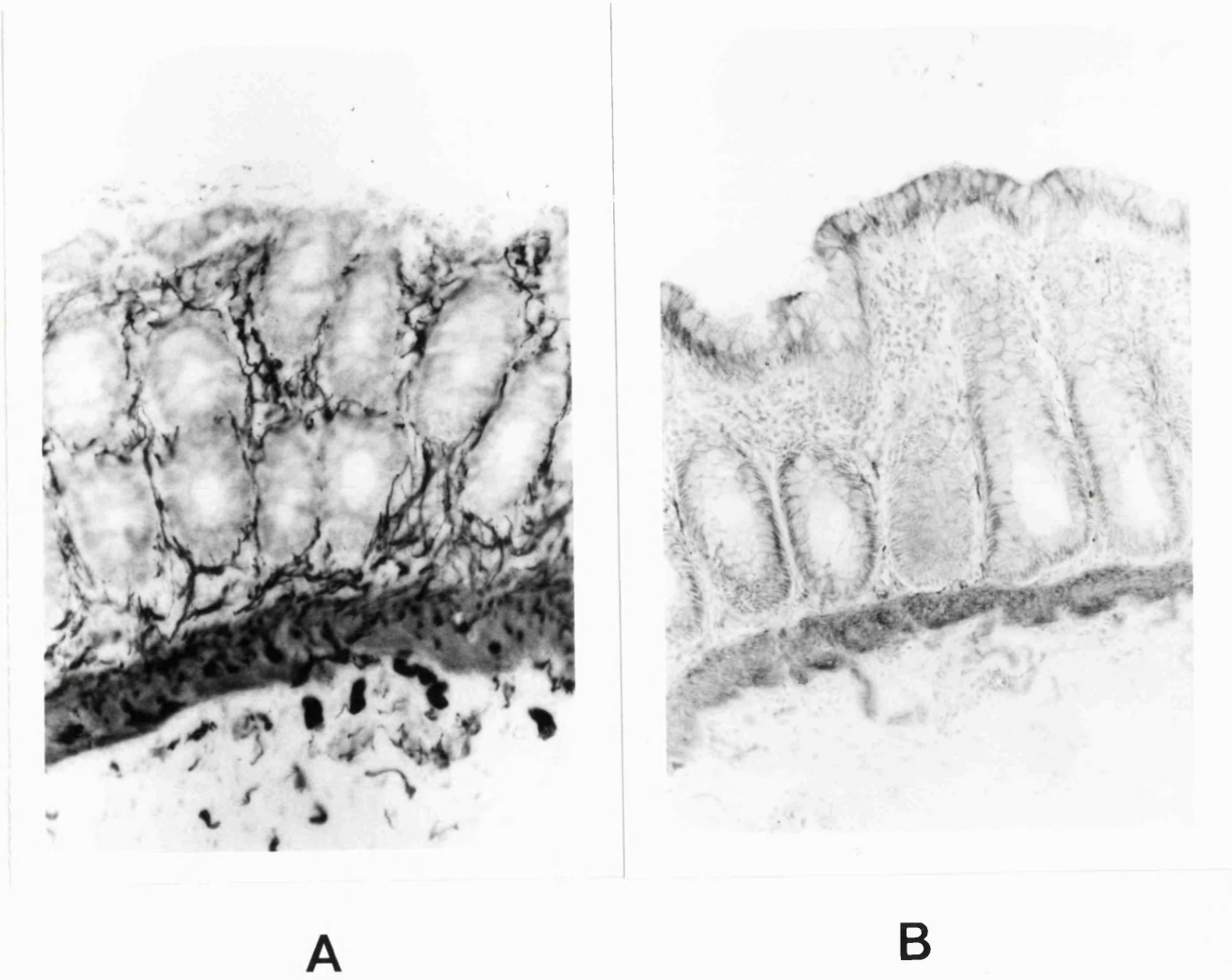


Fig.4.9 Photomicrograph of acetylcholinesterase staining pattern in the lamina propria of human aganglionic (**A**: 3mth♀ with Hirschsprung's disease, x200) and ganglionic colon (**B**: 8mth ♂ with imperforate anus, x200). Extensive staining can be seen in the lamina propria of aganglionic colon in contrast to the normally innervated ganglionic colon.

The secretory response of the mucosa to luminally-active secretagogues was examined using 50MU/ml *Esch.coli* STa enterotoxin, theophylline and IBMX.

ESCH. COLI STa ENTEROTOXIN: RECTOSIGMOID COLON

Control ganglionic colon (ARA): Addition of 50MU/ml STa enterotoxin to the mucosal bathing solution of ganglionic colon resulted in a prompt increase in Vt and Isc together with a distinct pattern of Rt readings which gave an immediate increase of $6.5\pm2.6\Omega\cdot cm^2$ before a subsequent fall of $-9.5\pm4.6\Omega\cdot cm^2$ from the peak value. A typical trace is shown in Fig. 4.10. The response to a second challenge of STa enterotoxin after prior equilibration with TTX (10^{-6}M) was not significantly different from the response to primary challenge (Table 4.7).

*Aganglionic colon (HD):*Aganglionic colon gave similar responses to STa enterotoxin in Vt, Isc and Rt (an initial increase of $+4.5\pm1.1\Omega\cdot cm^2$ followed by a fall of $-6.3\pm1.1\Omega\cdot cm^2$). No significant difference was found between the increase in Vt and Isc in the aganglionic and ganglionic colon to STa enterotoxin (data in Table 4.7. The increases in Vt and Isc were reversible on washing out the bathing solutions. Repeat challenge with STa enterotoxin in the presence of serosal tetrodotoxin (10^{-6}M) gave similar increases to those without TTX. The increase in Isc to STa enterotoxin in the presence of TTX was not significantly different between the aganglionic and ganglionic colon (increase in Isc$\pm$SEM: $+25.85\pm4.3\mu A/cm^2$ (aganglionic colon, n=4) *vs* $37.0\pm8.7\mu A/cm^2$ (ganglionic colon, n=4). The initial rise in Rt was unaffected by the presence of TTX. The data are summarised in Fig.4.11.

*Ganglionic colon (HD):*The ganglionic rectosigmoid colon from children with HD gave increases in Vt and Isc to STa enterotoxin that were not significantly different from the values obtained from the control ganglionic colon described above (n=7 tissues from 5 children, Table 4.8). The responses to second challenge with STa enterotoxin were unaffected by TTX (Table 4.8; Fig.4.11).

No correlation was found between age of the children from all three groups and the response to STa.

Table 4.7 Response of aganglionic and ganglionic colon to Esch.coli STa enterotoxin

	N	Vt(mV) (n)	Isc(μA/cm^2) (n)	+Rt (n)	-Rt($\Omega\cdot$cm^2) (n)	Time (mins)
Aganglionic (HD)	4	+4.1±0.9 (6)	+41.7±10.1 (6)	+4.5±1.1 (6)	-6.3±1.1 (6)	15.2
Ganglionic (HD)	5	+2.2±0.8 (7)	+43.3±7.6 (7)	+3.7±2.0 (7)	-16.6±4.2 (7)	11.7
Ganglionic (ARA)	9	+4.2±1.3 (11)	+39.4±5.3 (11)	+6.5±2.6 (11)	-9.5±4.6 (11)	12.9

Results are given as change in Vt, Isc and Rt and expressed as mean ± SEM.
Abbreviations are as given in Table 4.2. +Rt, -Rt = results given as the immediate increase seen in Rt and the subsequent fall in Rt.
No significant differences exist between the three groups of tissue.

Table 4.8 Response of aganglionic and ganglionic colon to Esch.coli STa enterotoxin in the presence of 10^{-6}M TTX

	N	Vt(mV) (n)	Isc(μA/cm^2) (n)	+Rt (n)	-Rt($\Omega\cdot$cm^2) (n)	Time (mins)
Aganglionic (HD)	3	+2.9±0.4 (4)	+25.9±4.3 (4)	+4.5±0.5 (4)	-8.5±1.2 (4)	13.5
Ganglionic (HD)	4	+1.1±0.3 (5)	+17.8±3.5 (5)	+0.8±0.4 (5)	-8.4±2.5 (5)	11.8
Ganglionic (ARA)	4	+5.2±1.9 (4)	+37.0±8.7 (4)	+11.5±6.7 (4)	-5.6±2.1 (4)	13.0

Results are given as change in Vt, Isc and Rt and expressed as mean ± SEM. Abbreviations are as given in Table 4.2. +Rt, -Rt = results given as the immediate increase seen in Rt and the subsequent fall in Rt;

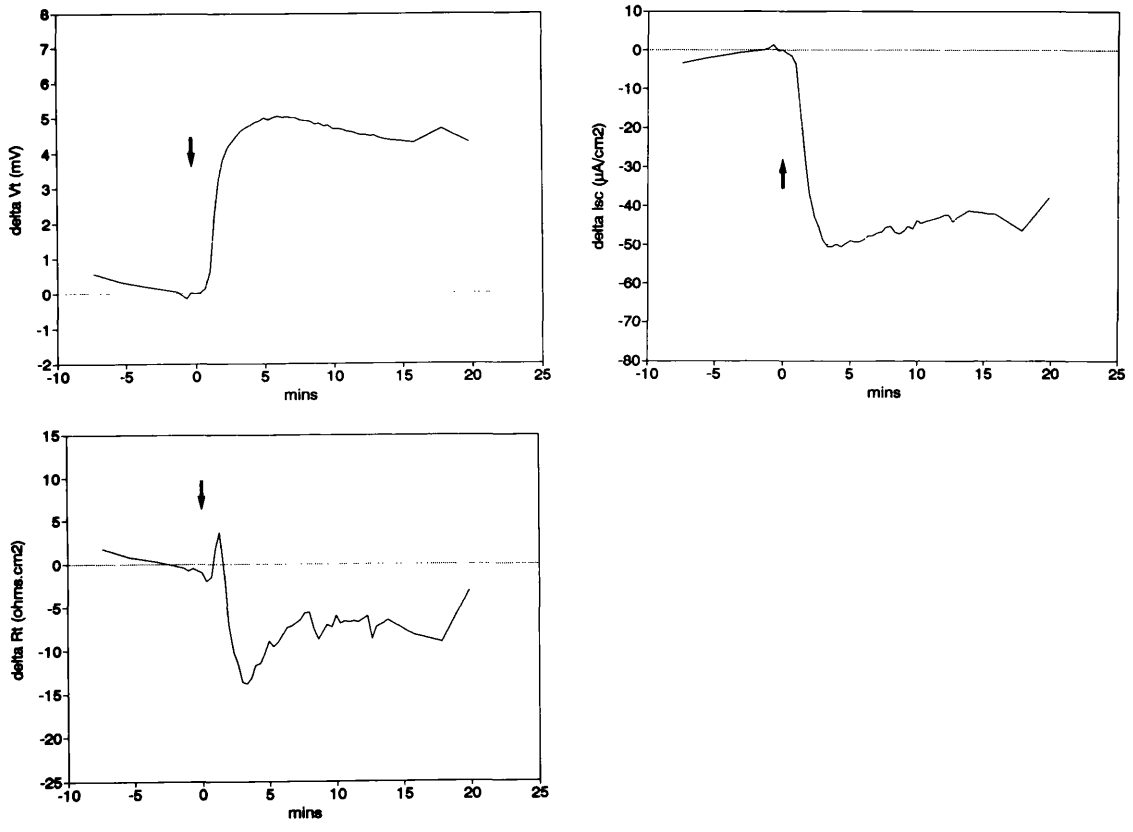


Fig.4.10. Effect of *E.coli* STa enterotoxin (50MU/ml) added to the mucosal bathing solution at Time O (arrow) of human ganglionic rectosigmoid colon (4mth♀ with imperforate anus).

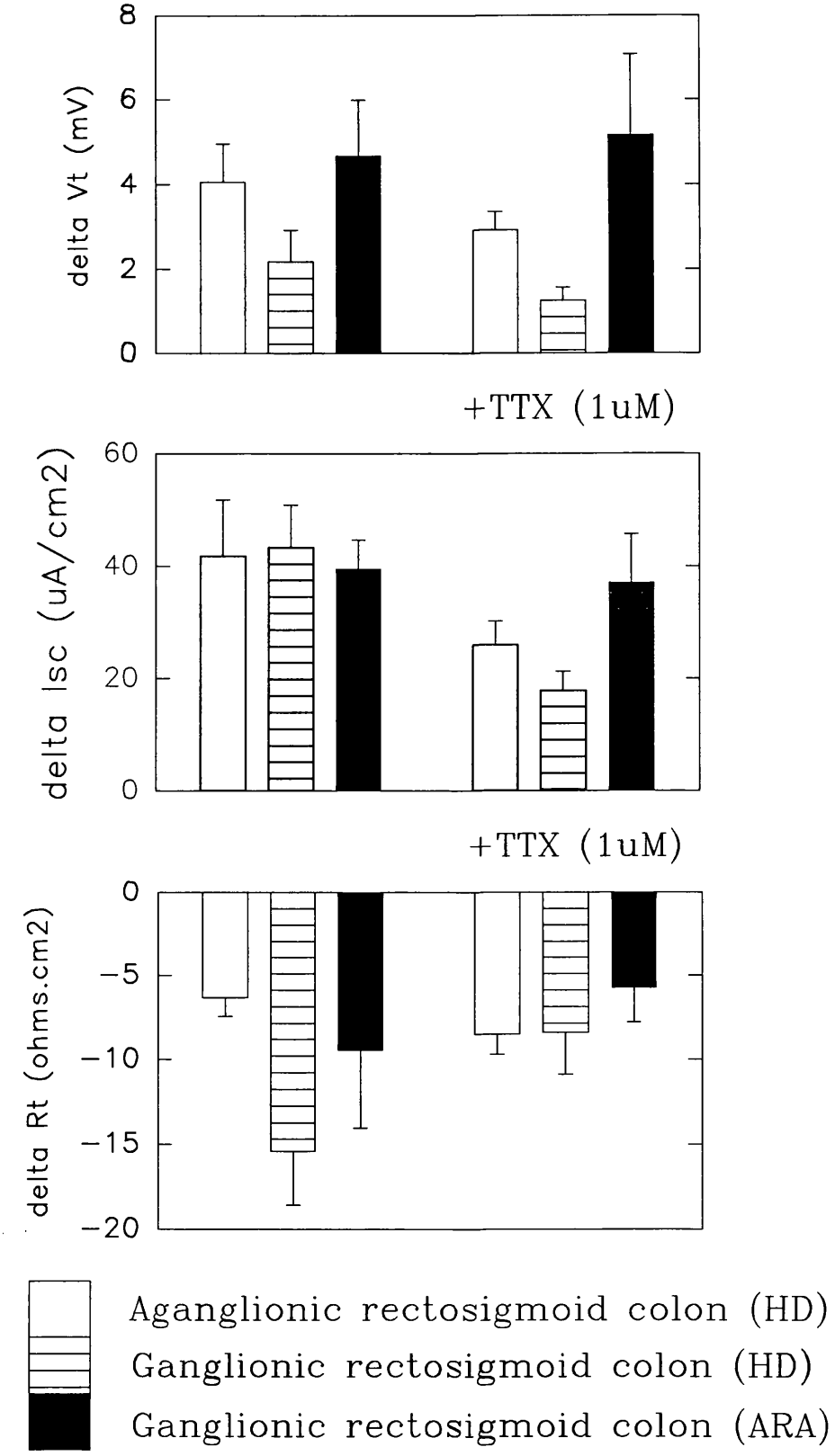


Fig.4.11. Comparison of response of human colon to *E.coli* STa enterotoxin and the effect of serosal TTX.
Results given as mean±SEM.

PHOSPHODIESTERASE INHIBITORS: RECTOSIGMOID COLON

Effect of theophylline

The effect of theophylline , an inhibitor of cell phosphodiesterase activity, was investigated. Theophylline (2mM) was added to the mucosal bathing solution.

Control ganglionic colon (ARA): A rise in the Vt and Isc together with a fall in Rt occurred, taking 7-12 mins to reach maximum steady state. A typical example, showing the time course of action is shown in Fig.4.12. The addition of vancomycin (5mg/ml) to the mucosal bathing solution had no effect on the increases in the Vt, Isc or Rt (n=3). This concentration was based on measured concentrations obtained in faeces after oral dosage (Gotz & Rand, 1982)

Second exposure to TTX in the presence of TTX (9μM) resulted in responses that were not significantly different from those obtained without TTX (Table 4.10; Fig.4.13).

*Aganglionic colon (HD):*The response of aganglionic rectosigmoid colon to theophylline was significantly reduced in all three electrical parameters ($p<0.05$, K-W ANOVA) compared with both ganglionic rectosigmoid from the normally innervated colon.

When tested in the presence of TTX (9μM) in Period 2 the response of the aganglionic tissues, although giving the smallest increases, had increased and were similar to the control ganglionic tissue (Table 4.10; Fig.4.13).

*Ganglionic colon (HD):*The ganglionic rectosigmoid colon from children with HD gave increases in Vt and Isc to theophylline (Vt: $+5.4\pm1.4$mV, Isc: $+87.7\pm18.9\mu/cm^2$, Rt: $-24.5\pm8.9\Omega\cdot cm^2$, mean increase$\pm$SEM, n=13) which were not significantly different from the values obtained from the control ganglionic colon described above. The response to second challenge with theophylline in the presence of serosal TTX (10^{-6}M) was not significantly different from the control ganglionic tissues (Table 4.10; Fig.4.13).

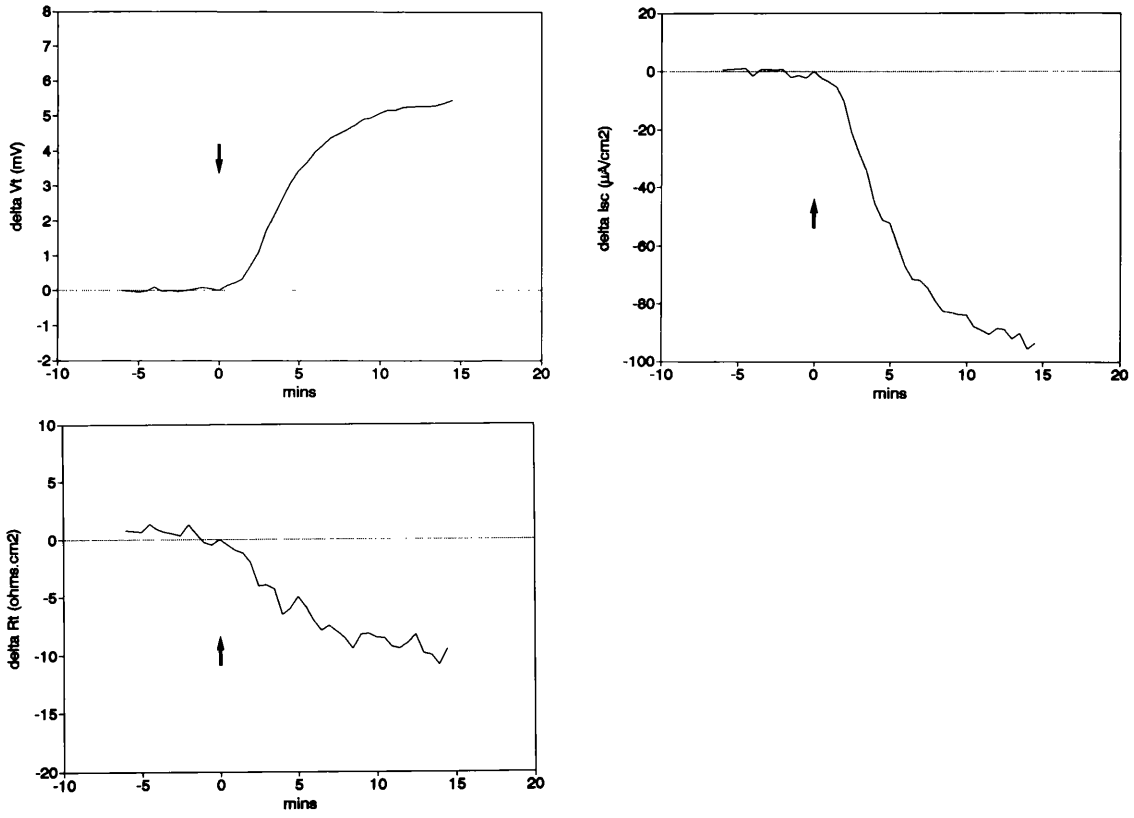


Fig.4.12 Effect of mucosal theophylline (2mM) on Vt, Isc and Rt of ganglionic rectosigmoid colon (3mth♀ with imperforate anus).

Table 4.9 Response of aganglionic and ganglionic colon to 2mM theophylline

	N	Vt(mV) (n)	Isc(μA/cm^2) (n)	Rt($\Omega\cdot$cm^2) (n)	Time (mins)
Aganglionic (HD)	9	+3.1±0.3* (13)	+55.5±6.4¶ (13)	-9.9±1.5§ (13)	13.6
Ganglionic (HD)	7	+6.5±1.2 (9)	+105.9±18.4 (10)	-32.2±8.8 (9)	13.8
Ganglionic (ARA)	10	+6.1±1.1 (11)	+106.8±13.1 (10)	-24.5±3.2 (11)	14.3

Results are given as change in Vt, Isc and Rt and expressed as mean ± SEM. Other abbreviations are as given in footnote to Table 4.1.
* $P<0.01$; ¶ $P=0.005$; § $P<0.01$ versus Ganglionic (ARA)(K-S 2-sample test).

Table 4.10 Response of aganglionic and ganglionic colon to 2mM theophylline in the presence of 10^{-6}M TTX

	N	Vt(mV) (n)	Isc(μA/cm^2) (n)	Rt($\Omega\cdot$cm^2) (n)	Time (mins)
Aganglionic (HD)	6	+2.3±0.3 (9)	+39.9±4.2 (8)	-7.4±1.9 (8)	17.9
Ganglionic (HD)	3	+3.9±0.3 (4)	+51.2±5.3 (4)	-14.0±4.4 (4)	14.8
Ganglionic (ARA)	7	+5.7±1.3 (8)	+73.8±14.9 (8)	-9.0±1.8 (8)	15.0

Results are given as change in Vt, Isc and Rt and expressed as mean ± SEM. Other abbreviations are as given in footnote to Table 4.1.

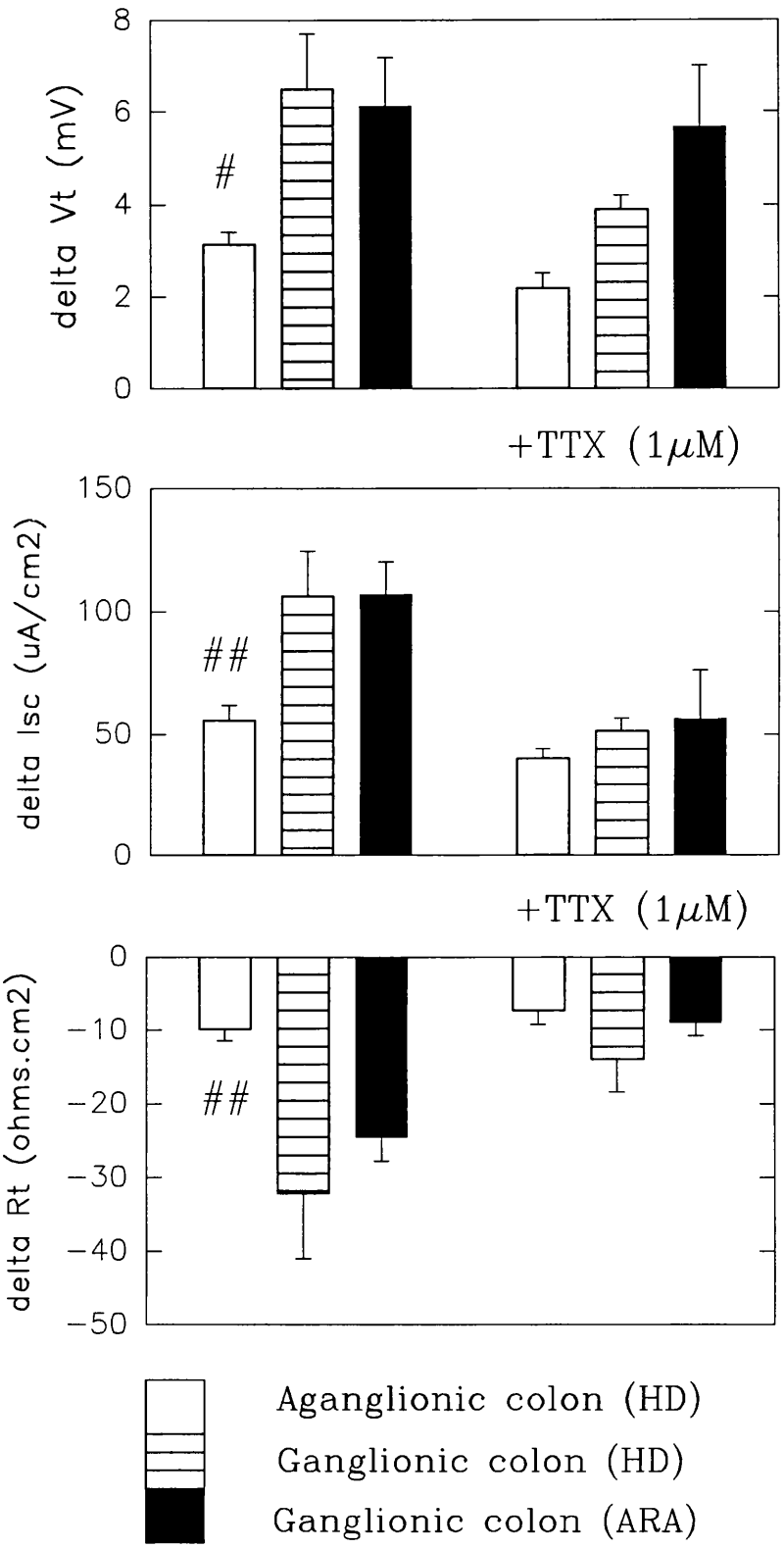


Fig.4.13. Comparison of the response of paediatric rectosigmoid colon to mucosal theophylline (2mM) and the effect of TTX (10^{-6}M). Results given as mean±SEM.
P=0.02 (K-W ANOVA)
P<0.01 (K-W ANOVA)

Effect of prior exposure to acetylcholine

Caffeine, as a model methylxanthine, is recognised as having a number of different effects on cellular events: phosphodiesterase inhibition, mobilisation of $[Ca^{++}]_i$ and inhibition of the purine P2 receptor activity. Experiments were carried out to determine whether the prior exposure to acetylcholine was involved in the response of aganglionic and ganglionic colon to theophylline.

In cases when sufficient ganglionic rectosigmoid colon was available for two tissues to be examined simultaneously, one tissue was first challenged with 10^{-4}M acetylcholine (serosal bathing solution) and the other tissue with physiological saline. As soon as the Vt had returned to baseline values after washing out the acetylcholine (20-30 minutes) both tissues were exposed to theophylline (mucosal bathing solution). Tissues exposed to acetylcholine reacted with significantly greater increases in Isc to theophylline ($151.6\pm15.4\mu A/cm^2$ n=3) than the saline controls ($79.0\pm11.9\mu A/cm^2$ n=3; $p<0.05$ paired t-test) (Fig. 4.14).

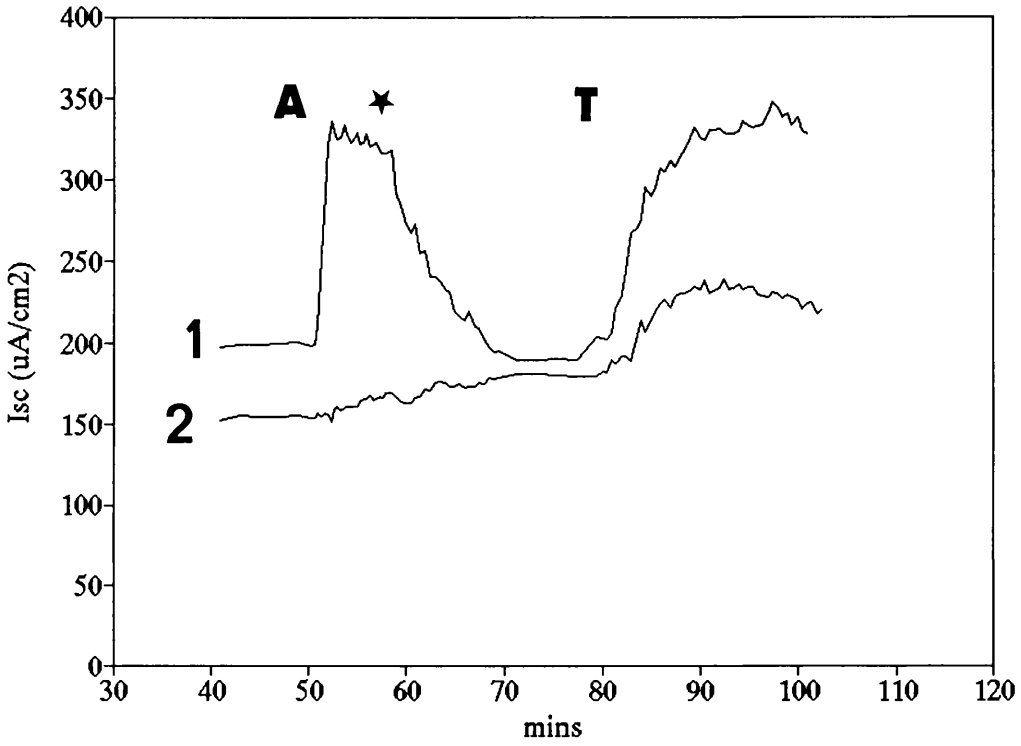


Fig.4.14. Effect of acetylcholine on the response to theophylline. Serosal 10^{-4}M acetylcholine (**A**) was added to **1** only. 20 minutes after solution changes (*), BOTH tissues were challenged with 2mM mucosal theophylline (**T**). The ganglionic rectosigmoid colon was taken from a 12mth♀ with imperforate anus.

Effect of 3-isobutyl-1-methylxanthine

Further studies were carried out to examine the response of aganglionic colon to cAMP-mediated secretagogues. IBMX is a more potent phosphodiesterase inhibitor than theophylline without significant effects on intracellular $[Ca^{++}]_i$ mobilisation. Tissues were challenged with either 1mM or 0.2mM IBMX in the mucosal bathing solution.

Control ganglionic colon (ARA): Addition of both concentrations of IBMX resulted in an immediate rise in Vt and Isc together with a fall in Rt (Table 4.11). The were responses to both concentrations were not significantly different indicating that maximal concentrations were used (Fig.4.15).

Aganglionic colon (HD): No difference was seen in the response of aganglionic colon to both concentrations of IBMX and they were similar to those of the control ganglionic colon (Table 4.11).

Ganglionic colon (HD): The ganglionic rectosigmoid colon from children with HD gave increases in Vt and Isc to 1mM IBMX which were not significantly different from the values obtained from the control ganglionic colon described above (Table 4.11). The response of 1 further tissue to the lower concentration was also in the same order of magnitude as that of the control colon.

The data for each tissue group are given in Table 4.11 and compared in Fig.4.15.

Table 4.11 Response of human aganglionic and ganglionic rectosigmoid colon to IBMX

	1mM			0.2mM		
	Vt(mV)	Isc(μA/cm^2)	Rt($\Omega\cdot$cm^2)	Vt(mV)	Isc(μA/cm^2)	Rt($\Omega\cdot$cm^2)
Aganglionic (HD)	+11.9±0.9 (3)	+157.4±4.3 (3)	-21.0±4.7 (3)	+8.3±1.7 (8)	+113.9±15.4 (8)	-28.0±2.9 (8)
Ganglionic (HD)	+12.9 (1)	+191 (1)	-55.0 (1)	+4.1±1.3 (3)	+39.4±5.3 (3)	-9.5 (3)
Ganglionic (ARA)	+10.2±1.2 (3)	+170.7±12.6 (3)	-26.0±6.0 (3)	+5.6±1.6 (7)	+138.8±31.6 (7)	-11.9±2.3 (7)

Results are given as change in Vt, Isc and Rt and expressed as mean ± SEM. Abbreviations are as given in Table 4.1. Results taken from 5-6 patients for 1mM and 1-3 children for period 2. No significant difference was found between the response of the three groups of tissue

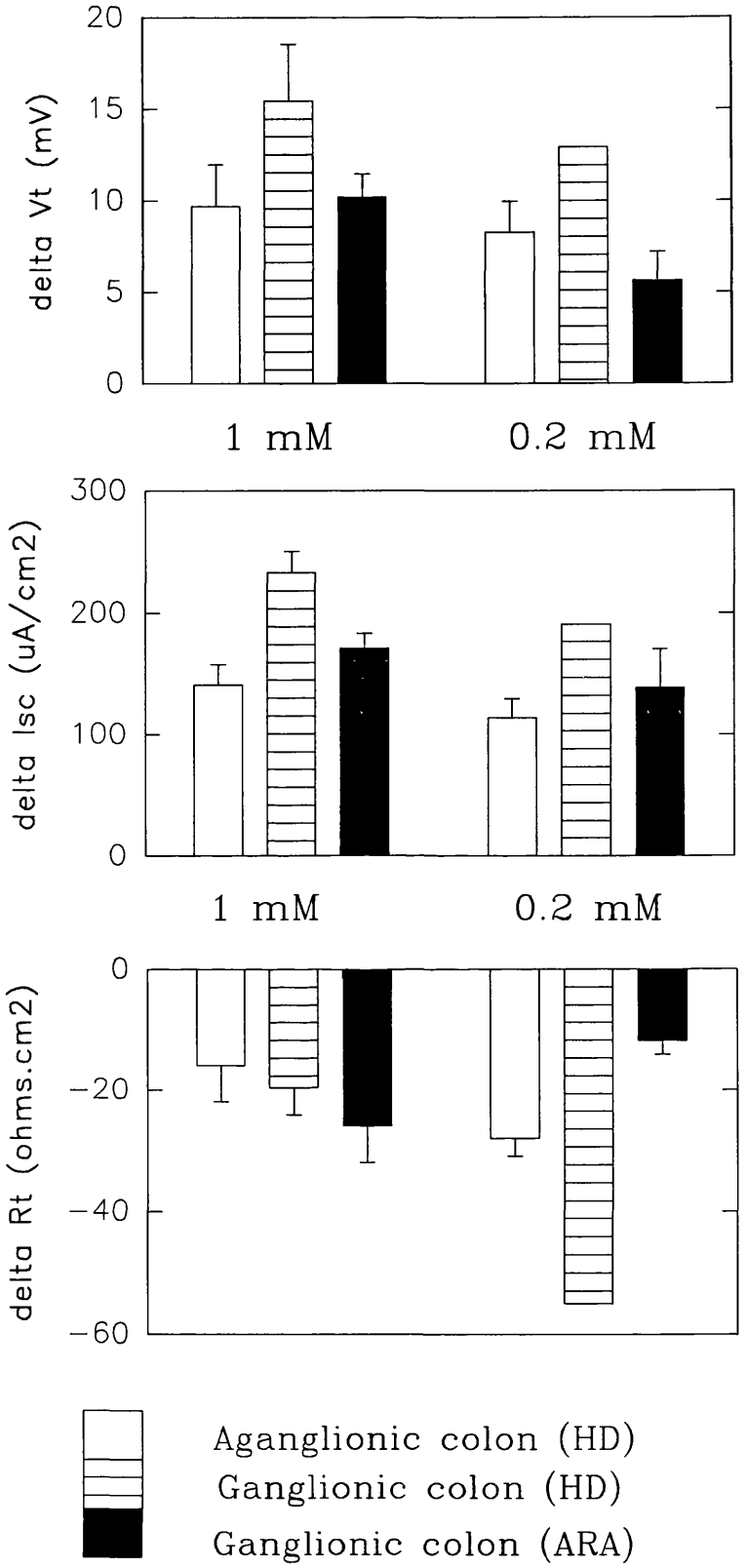


Fig.4.15. Comparison of response of paediatric rectosigmoid colon to 0.2mM and 1mM IBMX. Results given as mean±SEM.

Transverse colon

BASAL ELECTRICAL PROPERTIES: TRANSVERSE COLON

The basal electrical values of the proximal transverse colon in children with HD and children with anorectal anomalies are given in Table 4.12. The basal values were similar between the two groups of children (as shown in Fig.4.16) and were not significantly different from the values obtained for the rectosigmoid colon.

HISTOLOGY

All tissues from both groups were ganglionic in both the myenteric and submucosal plexus when examined for NADH-diaphorase activity.

Acetylcholinesterase patterns were indistinquishable from the ganglionic rectosigmoid colon. Occasional fibres were visible in the mucosal layer but increased acetylcholinesterase staining activity within the muscularis mucosae or lamina propria was not seen in any of the tissues.

NEURALLY MEDIATED SECRETION: TRANSVERSE COLON

Effect of ATXII

ATXII (10^{-6}M) added to the serosal bathing solution resulted in rises in Vt ($+2.4\pm0.5$mV) and Isc ($+44.5\pm11.9\mu A/cm^2$) together with a fall in Rt ($-7.4\pm1.5\Omega\cdot cm^2$) in the ganglionic transverse colon from 3 children with Hirschsprung's disease (mean$\pm$SEM; n=5 tissues from 3 children). Serosal TTX (10^{-6}M) reversed the changes (-5.0 ± 0.9mV; $-84.4\pm27.9\mu A/cm^2$; $+5.0\pm2.0\Omega\cdot cm^2$ mean$\pm$SEM of 3 children). Insufficient numbers of corresponding control tissues from children with ARA were available to study the response to ATXII.

Table 4.12 Basal electrical properties of human paediatric transverse colon

	N	Vt(mV) (n)	Isc(μA/cm^2) (n)	Rt($\Omega\cdot$cm^2) (n)
Hirschsprung's disease	14	+11.3±1.7 (19)	-104.5±12.9 (18)	+109.8±13.2 (18)
Controls	10	+10.0±2.1 (12)	-90.4±17.1 (12)	+117.9±13.7 (12)

Results are given as mean ± SEM. Abbreviations as given in Table 4.1
No significant differences were found between the five groups of tissues.

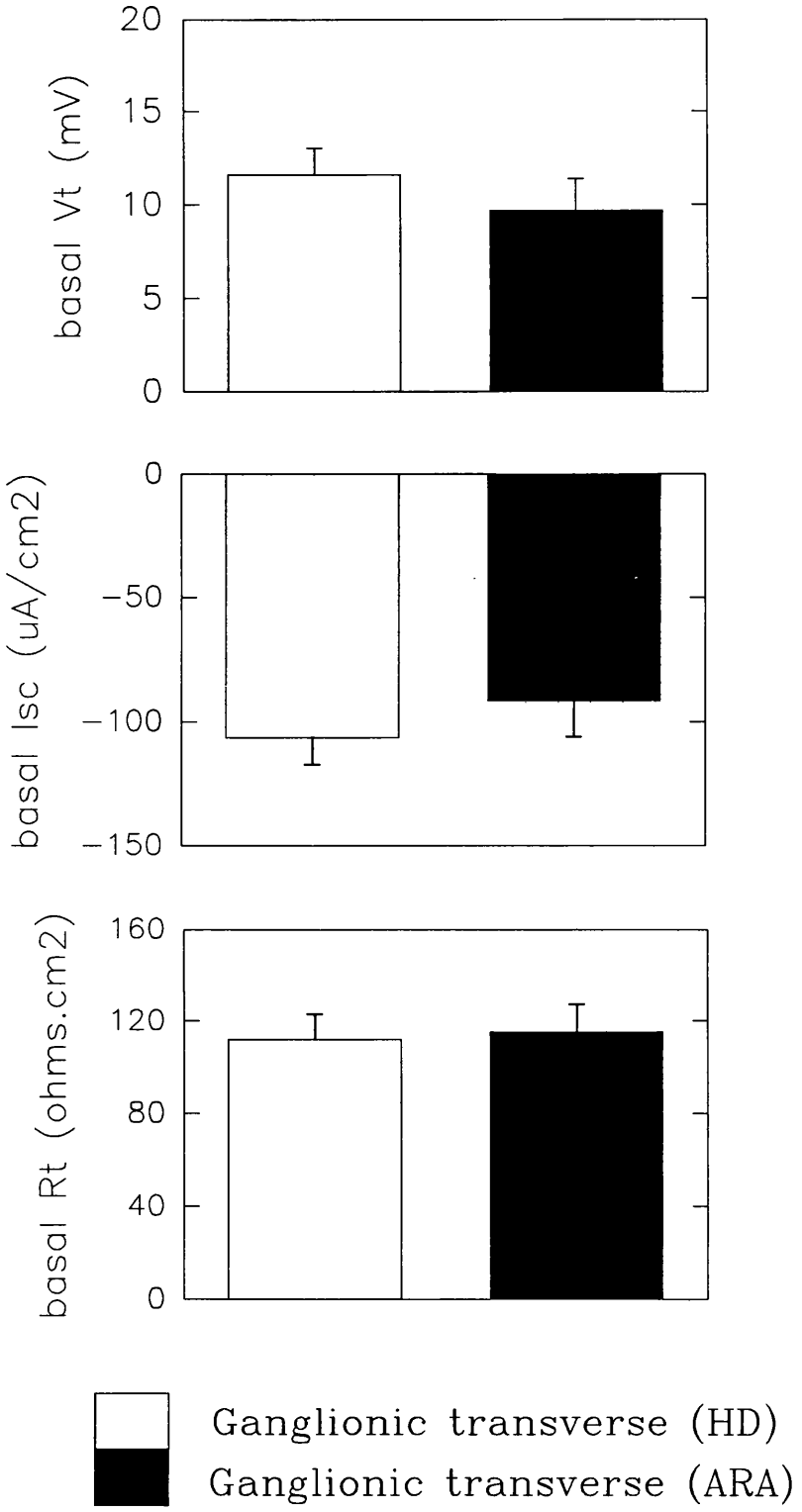


Fig.4.16. Basal electrical properties of human transverse colon. Results given as mean±SEM.

Effect of Iloprost

When added to the serosal bathing solution Iloprost resulted in increases in Vt and Isc in ganglionic transverse colon from children with Hirschsprung's disease that were not significantly different from those seen in the tissues from the control children (Data given in Table 4.13). The responses of both groups of tissues were comparable to those of the rectosigmoid colon. Addition of TTX to the serosal bathing solution resulted in a sharp reduction in the Vt and Isc.

CHOLINERGIC AGONISTS: TRANSVERSE COLON

Effect of acetylcholine

Control ganglionic colon (ARA): Addition of acetylcholine (900 & 9μM) to the serosal bathing solution of the control ganglionic transverse colon gave transient increases in Vt and Isc coupled with a fall in Rt (data in Table 4.14). Similar results were found in the transverse colon from children with HD (Table 4.14). The response of both groups of tissues including time course were similar to those seen in the ganglionic rectosigmoid colon.

Tissues were challenged with carbachol and, although the control transverse colon gave higher readings, the mean changes in Vt, Isc and Rt were not significantly different using non-parametric analysis of variance.

Table 4.13 Response of human transverse colon to Iloprost (10^{-6}M)

	N	Vt(mV) (n)	Isc(μA/cm^2) (n)	Rt($\Omega \cdot$cm^2) (n)	Time (mins)
Hirschsprung's disease	3	+7.4±2.0 (5)	+153.4±22.7 (5)	-22.8±6.6 (5)	7.4
Controls	3	+6.2±3.8 (7)	+120.5±26.6 (7)	-22.3±6.4 (7)	7.4

Results are given as change in Vt, Isc and Rt and expressed as mean ± SEM. Other abbreviations are as given in footnote to Table 4.1. No significant differences were found between the response of the two groups of children.

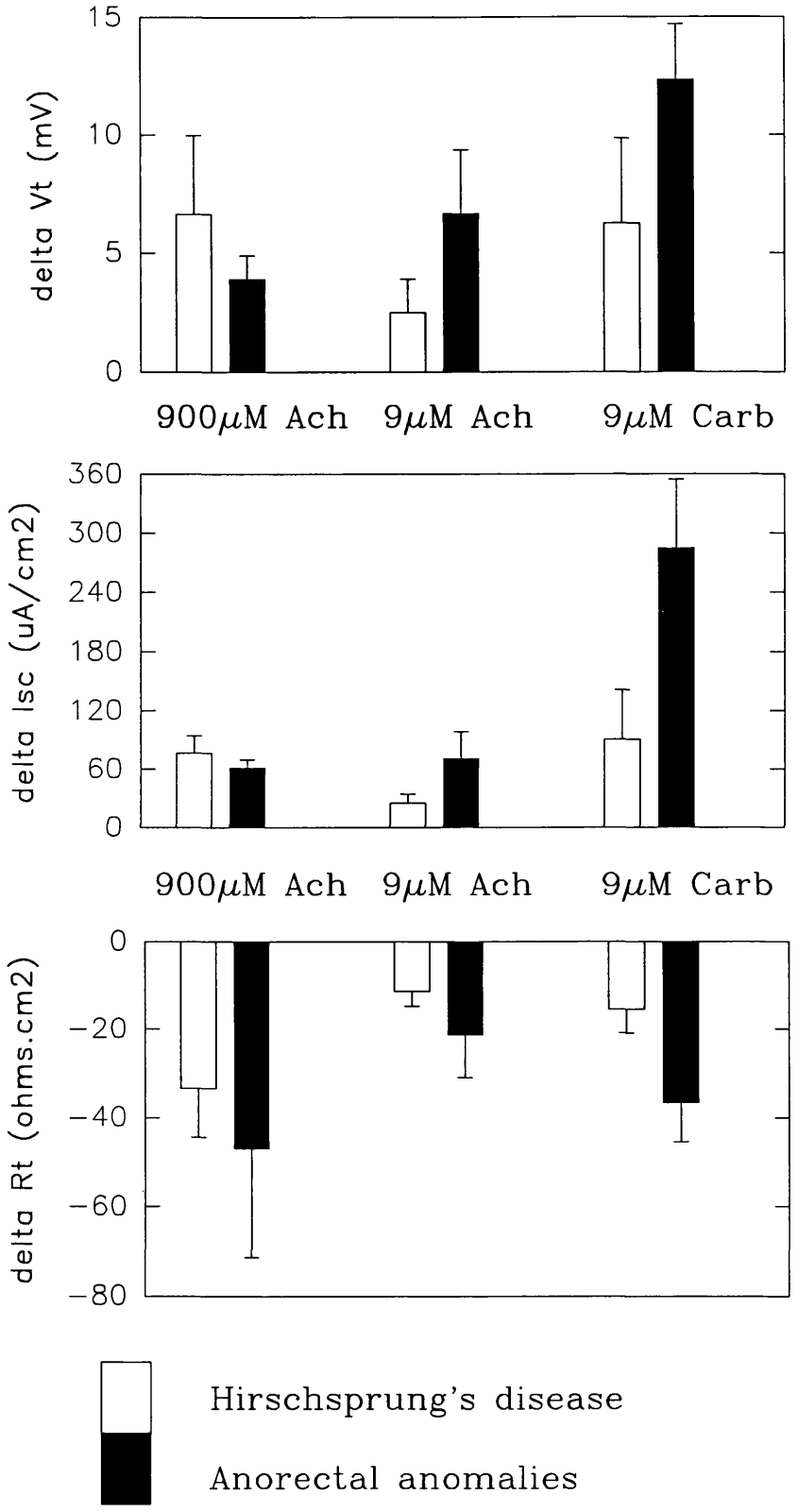


Fig.4.17. Comparison of response to cholinergic agonists in human paediatric transverse colon.
Results given as mean±SEM.

Table 4.14 Response of human paediatric transverse colon to acetylcholine ($9x10^{-6}M$)

	Ach 900μM			Ach 9μM		
	Vt(mV)	Isc($\mu A/cm^2$)	Rt($\Omega \cdot cm^2$)	Vt(mV)	Isc($\mu A/cm^2$)	Rt($\Omega \cdot cm^2$)
HD	+6.5±3.4 (11)	+76.6±17.5 (11)	-33.4±11.1 (10)	+2.5±1.4 (6)	+24.9±9.7 (6)	-11.3±3.5 (6)
Controls	+3.9±1.0 (3)	+61.0±8.9 (3)	-47.0±24.4 (3)	+6.7±2.7 (6)	+70.9±27.9 (6)	-21.3±9.6 (6)

Results are given as change in Vt, Isc and Rt and expressed as mean ± SEM. Abbreviations are as given in Table 4.1. Results taken from 2-4 children (900μM) and 4-5 children(9μM Ach).

ESCH COLI STa ENTEROTOXIN: TRANSVERSE COLON

Responses to mucosal *Esch coli* STa enterotoxin (50MU/ml) were similar in tissues from children with HD and the control children both in with and without serosal tetrodotoxin (10^{-6}M). The response of the tissues to a second challenge of mucosal STa in the presence of serosal TTX (10^{-6}M) were not significantly different from first challenge without TTX (Table 4.15). The increases in Vt and Isc were unaltered by the addition of 5mg/ml vancomycin to the mucosal bathing solution (n=3). The responses were not significantly different from those of the ganglionic rectosigmoid colon.

PHOSPHODIESTERASE INHIBITORS: TRANSVERSE COLON

Effect of theophylline

Responses to mucosal theophylline (2mM) were similar in tissues from children with HD and the control children both with and without serosal tetrodotoxin (10^{-6}M) (Data summarised in Table 4.16). Responses to a second challenge of mucosal theophylline in the presence of serosal TTX (10^{-6}M) were not significantly different from those seen with first challenge without TTX (Fig.4.18).

The increases in Vt and Isc were unaltered by the addition of 5mg/ml vancomycin to the mucosal bathing solution (n=4). The responses were not significantly different from those of the ganglionic rectosigmoid colon.

Table 4.15. Response of human paediatric transverse colon to 50MU/ml *E.coli* STa enterotoxin

	Period 1			Period 2 +TTX(10^{-6}M)		
	Vt(mV)	Isc(μA/cm^2)	Rt($\Omega\cdot$cm^2)	Vt(mV)	Isc(μA/cm^2)	Rt($\Omega\cdot$cm^2)
HD	+7.1±2.5 (6)	+64.1±18.1 (6)	-21.8±5.6 (6)	+7.9±5.2 (2)	+47.6±28.1 (2)	-5.5±4.5 (2)
Controls	+6.4±2.2 (6)	+88.7±26.7 (6)	-31.7±14.1 (6)	+3.4±2.4 (3)	+47.8±34.2 (3)	-16.3±13.2 (3)

Results are given as change in Vt, Isc and Rt and expressed as mean ± SEM. Abbreviations are as given in Table 4.1. Results taken from 5-6 patients for period 1 and 2-3 children for period 2.

Table 4.16 Response of human paediatric transverse colon to 2mM theophylline

	Period 1			Period 2 +TTX(10^{-6}M)		
	Vt(mV)	Isc(μA/cm^2)	Rt($\Omega\cdot$cm^2)	Vt(mV)	Isc(μA/cm^2)	Rt($\Omega\cdot$cm^2)
HD	+4.0±0.7 (11)	+101.5±12.8 (11)	-29.6±6.4 (10)	+3.9±0.8 (7)	+76.7±23.9 (7)	-17.3±3.1 (7)
Controls	+7.7±2.6 (6)	+75.3±12.6 (7)	-27.4±9.7 (7)	+6.2±1.9 (4)	+58.2±12.1 (5)	-19.7±6.6 (6)

Results are given as change in Vt, Isc and Rt and expressed as mean ± SEM. Abbreviations are as given in Table 4.1. Results taken from 5-6 patients.

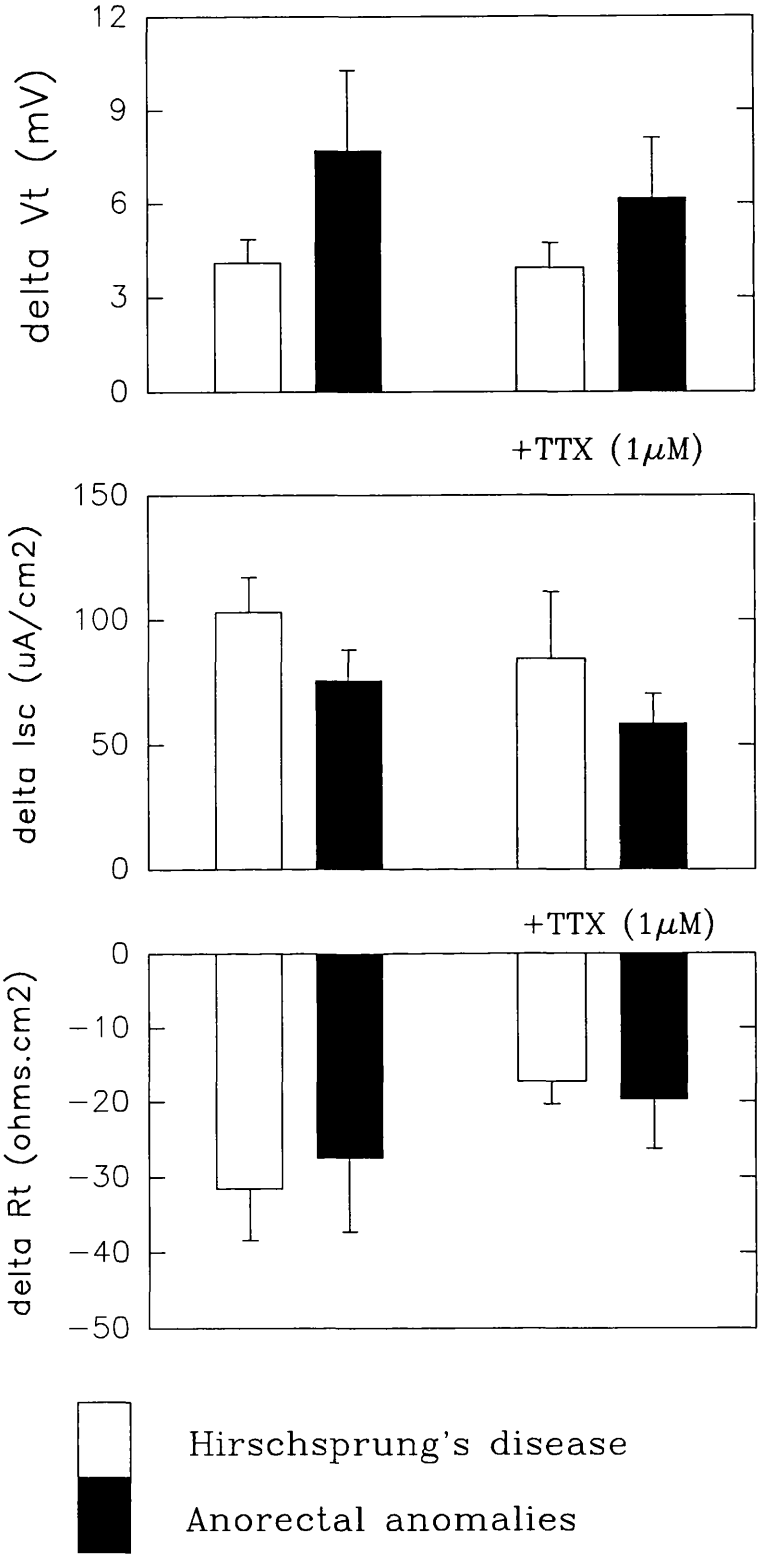


Fig.4.18. Comparison of response of human paediatric transverse colon to mucosal theophylline (2mM) with and without the presence of 10^{-6}M serosal TTX. Results given as mean±SEM.

Discussion:

Tissue viability

The period of time in which the major vascular supply was occluded varied between tissues and can not be corrected for. To examine the length of time that tissues could maintain electrical viability experiments were carried out on 5 tissues in which sufficient tissue was available to examine the basal properties and response to theophylline in tissues mounted immediately and then in tissues held in oxygenated Ringer's solution on ice for up to 5 hours after removal from the patient. Although there was a reduction in basal values, the differences in basal short-circuit current were not statistically significant (tissues mounted in the chambers immediately after excision: Isc=147.1μA/cm2; mean$\pm$SEM, n=5; tissues held on ice for up to 3-5 hours: Isc= 90.4$\pm$23.1μA/cm2 mean$\pm$SEM; n=5; $p>0.05$ students t-test). Similarly, the responses of the two groups of tissues to 2mM theophylline added to the mucosal bathing solution were not significantly different (increase in Isc = 64.1$\pm$19.5μA/cm2 and 79.5$\pm$19.5μA/cm2 in the immediate and stored tissues, n=5 for each group).

In addition, reduced viability of the aganglionic colon was considered unlikely to account for the reduced responses to the Iloprost and acetylcholine for the following reasons: the basal values and response to IBMX and STa enterotoxin were comparable to control tissues and to the proximal ganglionic tissue from Hirschsprung's disease; and the ganglionic rectosigmoid colon also demonstrated a response to Iloprost comparable to the control tissues which can act as an internal control.

Basal readings

The initial fall in Vt seen once the tissues were mounted in the Ussing chambers has been described by many workers in the initial studies on animal intestinal epithelia. Work on rat distal colon has shown that TTX abolishes this initial fall in Vt which is due to the stretch mediated secretion of chloride (Schultze *et al.*, 1989).

The gradual rise in Rt during the first hour of equilibration might be due to the

reactivation of the tight junctions as the cells rewarm up to 37°C from the chilled transport medium. The regulation of tight junction action is thought to be maintained by a metabolic mechanism which would require the cell to be actively metabolising. Chilled electrolyte solutions have a higher electrical resistance (the resistance of the bicarbonate-Ringers solution used to calibrate the solution resistance of the chambers was always higher when first filled from room temperature and fell as it reached 37°C). This excludes the possibility that the increase in Rt was due to changing temperature of the Ringers solution.

Segmental heterogeneity, principally reflecting Na^+ transport, exists between the proximal and distal colon in both the rat and human colon (Hubel *et al*, 1987; Sandle *et al*, 1986, Sandle & McGlone, 1987; Sandle, 1989). Consequently, the transverse and rectosigmoid colon were treated separately. The basal electrical values in the rectosigmoid and transverse colon were similar and both were comparable to the reported data on adult colon (Hubel *et al*, 1987; Sandle *et al*, 1986, Sandle & McGlone, 1987; Sandle, 1989; Sellin & DeSoigne 1987; Grady, Duhamel & Moore 1970; Escobar, Galindo & Parisi, 1990; Hawker, Mashiter & Turnberg 1978; Kuwahara *et al*, 1989; Rask-Masden & Hjelt, 1977; Sandle *et al*, 1990; Wills *et al.*,, 1984)

Studies in animal and human colon indicate that certain developmental changes in enterocyte tissue enzymes occur in the colon, particularly in the immediate pre and post natal period (Potter, 1990). Jenkins *et al* (1988, 1990), using *in vivo* rectal dialysis, found evidence of fully developed sodium transport in preterm neonates but reduced anion exchange (probably reflecting net bicarbonate secretion) in children of 1-12 months of age. We found no significant correlation between the basal Vt and age. Vt readings obtained in Ussing chamber preparations of human colon primarily reflect active sodium transport (Sandle *et al*, 1986; Sandle & McGlone, 1987). Consequently, the data presented here support the *in vivo* data that human electrogenic sodium transport is fully developed at birth.

We hypothesed that an altered response to secretory stimuli might exist in

Hirschsprung's disease as a result of the changes in the innervation to the colon and that these changes would be seen in the electrical parameters. The similar basal electrical parameters between ganglionic and aganglionic tissues and the apparent absence of effect of TTX indicates that basal electrogenic ion transport is not influenced by the ENS in either normally innervated or aganglionic colon.

Iloprost has been shown to elicit Cl^- secretion in the distal colon of the rat (Diener *et al*, 1988). The secretion was inhibited by 50μM atropine indicating that Iloprost acted via the ENS and did not affect the colonocyte directly. In these studies 1μM TTX prevented any effect of Iloprost on human colon and reversed the increase in Vt and Isc if they were added after Iloprost. The responses were not affected by the addition of 1μM atropine. Preliminary use of 10^{-4}M atropine did reduce the secretory response but at this comparatively high concentration nonspecific effects were seen, and addition of 10^{-4}M atropine to normally ganglionic colon induced increases in Vt and Isc alone before the addition of Iloprost (data not shown).

From the results presented here the likely site of action of Iloprost is on the ganglion nerve cell bodies rather than the nerve fibres as the principal difference between the tissues is the presence or absence of ganglion cells. The reversal or inhibition by TTX and equal density of nerve fibres supplying the lamina propria in aganglionic and ganglionic tissues suggests that Iloprost acts directly on the ganglion cell bodies and not on either the nerve fibres or postsynaptically.

The reduced or absent response to acetylcholine and carbachol in aganglionic rectosigmoid colon was probably due, at least in part, to reduced concentrations of acetylcholine reaching the basolateral muscarinic receptors due to enzymatic breakdown by the abundant tissue acetylcholinesterase activity. It is possible that the reduced responses reflect reduced muscarinic receptor density, but studies in other types of tissue show increases in muscarinic receptor density in response to reduced exposure to acetylcholine (Nathanson, 1989). Receptor density analysis in human Hirschsprung's disease are needed to clarify the extend to which both events occur.

The reduced response of the proximal ganglionic colon in the children with Hirschsprung's disease to 9μM acetylcholine is of particular interest. The reduced secretory response was not seen at the higher acetylcholine concentration. It is possible that these tissues are from the "transitional zone" although the histological acetylcholinesterase activity was considered to be indistinguishable from that of control ganglionic colon. The functional assay used here is of greater sensitivity and indicates that the normality of the proximal bowel cannot be adequately assessed by acetylcholinesterase activity and presence of ganglion cells. Histological studies have also demonstrated abnormal neuropeptide staining patterns in the proximal ganglionic colon in children with Hirschsprung's disease (Romanska *et al*, 1991).

A possible source of variability in response of the aganglionic colon to cholinergic activation was the differences in response of tissues from total colonic aganglionosis and Hirschsprung's disease. Differences have been described in the extent of the acetylcholinesterase staining patterns in the colon between these two forms of aganglionosis (Meier-Ruge, 1974). The changes in muscarinic receptor density and activation have not been studied in the present study but this information might help to clarify the differences in innervation in the two forms of aganglionosis.

Because of the wide variation in the response of aganglionic colon to carbachol the values of the responses were adjusted to obtain identical values for the number of tissues and children and expressed as median and range.

The reduced response to theophylline in aganglionic rectosigmoid colon indicates that the neural abnormalities in Hirschsprung's disease can indirectly influence the response of the colonocytes to certain secretory stimuli. Theophylline and other methylxanthines have been used previously as agents to raise cAMP concentrations but it is becoming clear that methylxanthines act on several different cell signalling mechanisms including inhibition of cellular cAMP and cGMP phosphodiesterases, inhibition of purine P2 receptors and mobilisation of Ca^{++} from intracellular stores. Coupled with the fact that secondary messenger pathways do not necessarily

act in isolation but may act in concert with other key signalling pathways (Houslay, 1991) makes it difficult to determine which events might be responsible. A number of possibilities were considered:

i) Changes in the ability of theophylline to permeate the mucosal colonocytes: This was considered unlikely as methylxanthines are highly permeable and that the tissue resistance values were not different between the different tissue groups.

ii) Excessive inhibitory innervation to the mucosa: support for this viewpoint can be drawn from the finding that second challenge with theophylline in the presence of TTX (which would block any inhibitory (absorptive) innervation) was greater than primary challenge. However, an alternative interpretation is that as the electrical responses to second exposure of acetylcholine increased so did the $[Ca^{++}]_i$ and consequently the response to second challenge with theophylline was increased (similar events appear to have occurred in the response of the aganglionic colon to acetylcholine where responses increased to values comparable with the ganglionic control tissues). In addition, there was no enhanced reaction seen to STa enterotoxin in the presence of TTX which would have blocked any inhibitory (absorptive) neural influences.

iii) altered regulation of intracellular cAMP and $[Ca^{++}]_i$.

The reduced response to theophylline of the aganglionic rectosigmoid colon compared with ganglionic colon could arise through a reduced or absent $[Ca^{++}]_i$ mobilising effect by the prior challenge with acetylcholine. This was supported by the experiments showing a greater increase in Isc of the colon which had been challenged with acetylcholine.

We found no relationship between response to STa enterotoxin and age. We have taken care to differentiate between transverse and distal colon and although not reaching statistical significance, the transverse colon did give greater changes in Vt, Isc and Rt to STa enterotoxin. Receptors for STa have been identified in human small and large intestine and both fall with age over the first 24 months. The binding of STa to cell receptors results in increased levels of guanylate cyclase in

the small and large intestine (Guarino *et al*, 1987). In the small intestine the greatest increases in guanylate cyclase were in the youngest (1day old) and also fell with age. Whether or not a similar effect existed in the colon was difficult to interpret as the authors found no age related changes in response to STa but tested enterocytes only from children over 6 months of age. Our findings do not support the view that the reduced receptor density corresponds with functional response.

The neural abnormalities in the colon of children with Hirschsprung's disease were found not to alter the response to STa enterotoxin. The addition of TTX did not alter the response of the normally-innervated rectosigmoid or transverse colon. The experimental design was such that tissues were challenged twice with certain agonists, first without and then, after a period of $\approx$60 minutes during which there were two sets of solution changes, in the presence of serosal TTX. This introduces the possibility that tachyphylaxis, priming or depletion of intracellular components may occur. Nevertheless, comparisons were made between the response of the ganglionic and aganglionic tissue groups and as the response of the abnormally innervated tissues was not significantly different from the control tissues and that the response to second challenge of STa in the absence and presence of TTX were not statistically different in both tissues, it is likely that the conclusions are valid.

In rat distal colon a substantial basal neural tone can be demonstrated (Andres *et al*, 1985) and cholinergic agonist mediated secretion is also reduced by TTX (Diener *et al*, 1989). The present data on human colon suggest that there is no such tonic neural involvement in basal electrogenic ion transport or in secretion induced by luminal secretagogues in human paediatric colon.

The response of the transverse colon to neurally-mediated agonists (Iloprost), cholinergic agonists (acetylcholine and carbachol) resulted in no significant differences between the proximal transverse colonic mucosa from children with Hirschsprung's disease and the appropriate controls (ARA). Although the numbers of tissues are small compared to those studied from the rectosigmoid colon, these data give no indication that neural abnormalities either extend to the proximal

transverse colon or, if they do, that they alter electrogenic mucosal ion transport. Furthermore, the transverse colon from children with Hirschsprung's disease was shown to react to ATXII. Although it is not possible to compare the response to that of transverse colon from children with ARA, this does support the Iloprost data that neurally-mediated secretion is intact in the ganglionic proximal transverse colon of children with Hirschsprung's disease.

The responses to neurotransmitters such as acetylcholine and indirect neural stimulation with Iloprost show that human paediatric distal colon does secrete in response to neural activation but that this is separate to continuous tonic neural release of secretory neurotransmitters.

In summary, the *in vitro* electrical properties of paediatric transverse and rectosigmoid colon indicate that 1) the electrogenic ion transport mechanisms in both the rectosigmoid and proximal transverse colon of children are similar and 2) they are comparable to reported adult data; 3) basal net electrogenic ion transport of the transverse and rectosigmoid colon are unaffected by the neural abnormalities in the colon in Hirschsprung's disease; 4) aganglionic and ganglionic colon from children with Hirschsprung's disease are able to respond to certain secretagogues which act directly on the colonocyte and 5) this response does not have a neural component; 6) the response of aganglionic rectosigmoid colon to acetylcholine and Iloprost is either absent or attenuated when compared with ganglionic control tissues (The hypertrophied acetylcholinesterase positive nerve bundles contribute to the reduced secretion to acetylcholine); 7) the proximal ganglionic rectosigmoid colon in children with Hirschsprung's disease has an attenuated ability to respond to acetylcholine. This indicates that the neural abnormalities in Hirschsprung's disease exert an inhibitory effect on the secretory mechanisms in response to cholinergic stimuli.

Chapter 5.

Enterotoxic activity in the stools of children with Hirschsprung's disease and diarrhoea

5.i Introduction

5.ii Methods
-reverse passive latex agglutination for cholera toxin
-gene probe analysis
-sorbitol fermentation
-Ussing chamber studies
-fibroblast cytotoxicity

5.iii Results
-rabbit colon
-rat colon
-piglet colon
-response to stool filtrates

5.iv Discussion

5.i Introduction

The data given in the previous chapter provide evidence that the human ganglionic colon proximal to the aganglionic region in Hirschsprung's disease is capable of electrogenic secretory responses to bacterial enterotoxins such as *Esch.coli* STa enterotoxin and other secretagogues such as theophylline and IBMX when challenged from the lumenal surface. Such events indicate that both active secretion and reduced absorption can occur in the colon and can be involved in human diarrhoea.

Microbial gastroenteritis accounted for almost 50% of the episodes of diarrhoea in children with Hirschsprung's disease and diarrhoea (data given in Chapter 2). In addition to the recognised enteric pathogens, enterotoxins have been described in a growing number of Enterobacteriaceae which have not previously been associated with diarrhoea, eg *Citrobacter spp, Klebsiella aerogenes, Enterobacter spp* (Guarino *et al.*, 1989; Rubino, 1989). The presence of enterotoxic activity has also been demonstrated in *Bacteroides fragilis* isolated from humans with diarrhoea (Myers *et al.*, 1987). Plasmids have been decribed coding for enterotoxin production which can transfer the property from one species to another amongst Enterobacteriaceae (Guarino *et al.*, 1987; 1988). This phenomenon opens up the possibilities of infection with an enterotoxin-producing strain through transmission of a plasmid rather than by acquisition of a recognised enteric pathogen. The range of possible enterotoxic organisms is then widened considerably.

To examine the possibility that "enterotoxins" produced by the colonic flora were involved in the aetiology of diarrhoea in children with Hirschsprung's disease studies were carried out on the enterotoxigenic potential of members of the colonic flora. A number of different methods were used to detect the enterotoxins: gene probe analysis for the heat-labile, heat-stable enterotoxin and the vero-toxin gene, cytotoxicity in cultured cell lines, immunological detection of cholera toxin and "cholera toxin-like" cross-reacting antigens, and electrogenic secretion in Ussing

chambers.

An antisecretory action of mucosal vancomycin on electrogenic secretion was also investigated as a cellular basis for the often dramatic clinical improvement seen in children with Hirschsprung's disease and diarrhoea.

5.ii Methods

Production of Cholera-like enterotoxin

The production of enterotoxins which will cross react with antisera raised against cholera toxin was tested for using the VET-RPLA (reverse passive latex agglutination) test (Unipath Ltd UK). Strains of Esch.coli were grown in 15ml of tryptone soy broth (Oxoid) for 18 or 48 hours at 37°C on a rotary shaker. Bacteroides spp were grown in 5ml Wilkins-Chalgren broth for 18 and 48 hours at 37°C (unshaken). Cells were then incubated at 37°C for 5hours in the presence of 10,000 units/ml polymyxin B. After centrifugation at 10,000rpm (minifuge) the supernatants were then tested according to the manufacturer's instructions. Briefly, serial double dilutions of the culture supernatants were incubated with anti-cholera toxin treated latex particles in a V-well microtitre plate overnight at RT. Inhibition of the formation of a button of latex particles at the bottom of the V-well indicates the presence of cholera toxin. A strain of *Esch.coli* known to possess plasmids coding for the heat labile enterotoxin (*Esch coli* F251 LT+ kindly supplied by Dr M Woodward, MAFF, Weybridge) were used as positive controls along with the purified enterotoxin supplied by the manufacturers. Titres of agglutination greater than 1 in 4 were considered to be positive.

Ussing chamber studies

The "Clamp" computer-based voltage clamp was used as described in the human colon studies in Chapter 4. Tissues were not challenged with more than one secretagogue.

Animals

The piglets and Wistar rats were obtained from the same source as those described in Chapter 3. New Zealand-White rabbits were obtained from Froxfield Ltd, Hampshire.

The enterotoxic activity was tested using the descending colon removed from 6-8wk old piglets. Animals (unstarved) were anaesthetised using open mask delivery of

halothane with nitrous oxide and oxygen. The abdomen was opened along the midline and the descending colon identified. The length of descending colon extending from the base of the spiral colon down to the rectum was clamped at both ends using bowel clamps. After clamping of the major vascular supply the colon was cut and removed into ice cold DMEM buffered with ≈0.2% final concentration of sodium bicarbonate (7.5% stock solution, Gibco) and transported immediately to the laboratory. The colon was then opened along the mesenteric aspect and the contents washed in DMEM, pinned out on wax (mucosa down) and the outer muscle layers were removed by microdissection at 50x magnification, continuously bathed in chilled DMEM. The mucosa/submucosa preparations were then placed in the 4 chambers and allowed to equilibrate in the bicarbonate Ringer' solution. The complete process from removal of the tissue to mounting in the chambers took 10-20 minutes. The rabbit and rat distal colon was taken form animals anaesthetised with intramuscular sodium pentobarbitone (Sagatal, May & Baker). Tissues were mounted unstripped.

Production of stool culture broth filtrate for enterotoxin assay

Stools obtained from children with Hirschsprung's disease and idiopathic diarrhoea were stored at -27°C suspended in Brain Heart Infusion (Oxoid) + 0.05% cysteine hydrochloride (as a reducing agent) + 10% glycerol (cryoprotectant). Samples were thawed at 37°C and then inoculated into 500ml Dulbecco's minimal essential medium (DMEM, Gibco) supplemented with 7.5% sodium bicarbonate, 0.01% w/v cysteine hydrochloride and 10% v/v newborn calf serum. The modified DMEM (rDMEM) was rendered partially selective for gram negative anaerobes by the addition of the GN anaerobe supplement (Oxoid-Unipath) containing haemin 2.5mg, menadione 0.25mg, sodium succinate 1.25mg, nalidixic acid, 5mg and vancomycin 1.25mg. The pH was adjusted to 7.3.

Cytotoxic activity of stool culture filtrates

The stool culture filtrates were tested for cytotoxic activity in fibroblast (HEL:human embryonic lung) and HEp2 cell lines. 200μl of the culture filtrates

were added to the cell culture tubes contianing 2ml maintenance medium (Dulbecco's minimal essential medium, buffered with sodium bicarbonate) and incubated on a roller at 36°C for up to 7 days. A culture supernatant of a toxigenic (toxin A and B) strain of *Clostridium difficile* supplied by Dr S. Borriello was used as the positive control.

Chemicals

Purified cholera toxin (CT) was obtained from Calbiochem and reconstituted in 0.9% saline. All other chemicals were obtained from Sigma unless otherwise indicated.

5.iii Results

Incidence of cholera-like enterotoxins

The production of cholera-like enterotoxins were screened in isolates of *E.coli* and *Bacteroides spp* isolated from, the stools of children with Hirschsprung's disease and idiopathic diarrhoea. 22 strains of *Esch.coli* and 12 strains of *Bacteroides spp* were examined using the VET_RPLA kit.

None of the isolates gave agglutination titres of greater than 1 in 2 when tested after 18 and 48 hours incubation. The toxigenic LT+ control strain of *Esch coli* gave titres ranging from 1 in 4 to 1 in 32. The purified enterotoxin supplied as a positive control gave titres of $\geq$1 in 32.

Sorbitol fermentation by *Esch coli*

All of the 22 strains of E.coli were able to ferment sorbitol when cultured on sorbitol MacConkey No 3 agar (Oxoid-Unipath CM813).

Gene probe analysis for bacterial enterotoxins

An independent study of the incidence of enterotoxins was carried out by Dr Martin Woodward, Bacteriology Department, Ministry of Agriculture, Fisheries & Food, Central Veterinary Laboratory, Weybridge. 25 coliforms (*Esch.coli* 14, *Klebsiella aerogenes* 5, *K oxytoca* 2, *Proteus mirabilis* 2, *Citrobacter koseri* 1, *Aeromonas hydrophila* 1) isolated from children with Hirschsprung's disease and diarrhoea were analysed for the presence of genes coding for heat labile (LT) heat stable (STa, STb) enterotoxins and verotoxin (VT) using specific gene probes as described by Woodward *et al* (1990).

None of the 25 isolates carried genes for the LT, ST or Vt toxins. The enterotoxic isolate (F251) used in the VET-RPLA test was positive for LT as well as STa and STb.

The lack of enterotoxigenicity was therefore supported by the gene probe analysis by Dr Woodward, MAFF.

USSING CHAMBER STUDIES

Validation of piglet rectosigmoid colon: response to theophylline and *Esch coli* STa enterotoxin

Preliminary studies were carried out to determine the sensitivity of the colonic mucosa from piglets, rabbits and rats to theophylline and STa enterotoxin for use in detecting electrogenic secretory events in the Ussing chambers.

Rabbit colon

The addition of 2mM theophylline to the mucosal bathing solution of unstripped preparations of rabbit distal colon failed to induce any change in either the Vt, Isc or Rt (n=4).

Rat colon

Unstripped preparations of the distal colon of adult Wistar rats were challenged with 2mM theophylline to the mucosal bathing solution. A prompt increase of the Vt ($+3.1\pm0.5$mV, n=16), Isc ($+37.1\pm9.0\mu A/cm^2$, n=12) and fall in Rt ($-7.5\pm1.5\Omega\cdot cm^2$, n=12) were seen.

The response to 50MU/ml *Esch. coli* STa enterotoxin added to the mucosal bathing solution failed to induce any significant change in the electrical values after 20 minutes' exposure to the toxin.

Piglet distal colon

Muscle-stripped preparations of the distal descending colon (distal to the spiral colon) of 6-9 week old piglets were examined. This length of colon is equivalent to the rectosigmoid colon of man.

The addition of 2mM theophylline to the mucosal bathing solution at 90 minutes resulted in a gradual increase in Vt ($+2.0\pm0.2$mV; n=7) and Isc ($+19.2\pm2.5\mu A/cm^2$; n=7) which took 20 minutes to reach maximum values. The addition of vancomycin (5mg/ml) to the mucosal bathing solution once values had started to level out (approximately 15mins after addition), had no significant effect on the response of the Vt and Isc to theophylline (n=4).

The response to *Esch coli* STa enterotoxin resulted in prompt increases in Vt and Isc which reached maximum values after a mean of 2.2 mins (increase in Vt: $+3.5\pm0.6$mV, n=12; increase in Isc: $+33.9\pm5.7$ μA/cm^2 n=12) as well as a gradual rise in Rt (increase in Rt: $+25.4\pm5.2\Omega\cdot$cm^2, n=12). The addition of mucosal vancomycin (5mg/ml) had no significant effect on the electrical values (n=4) compared with untreated tissues. A typical example is shown in Fig 5.1

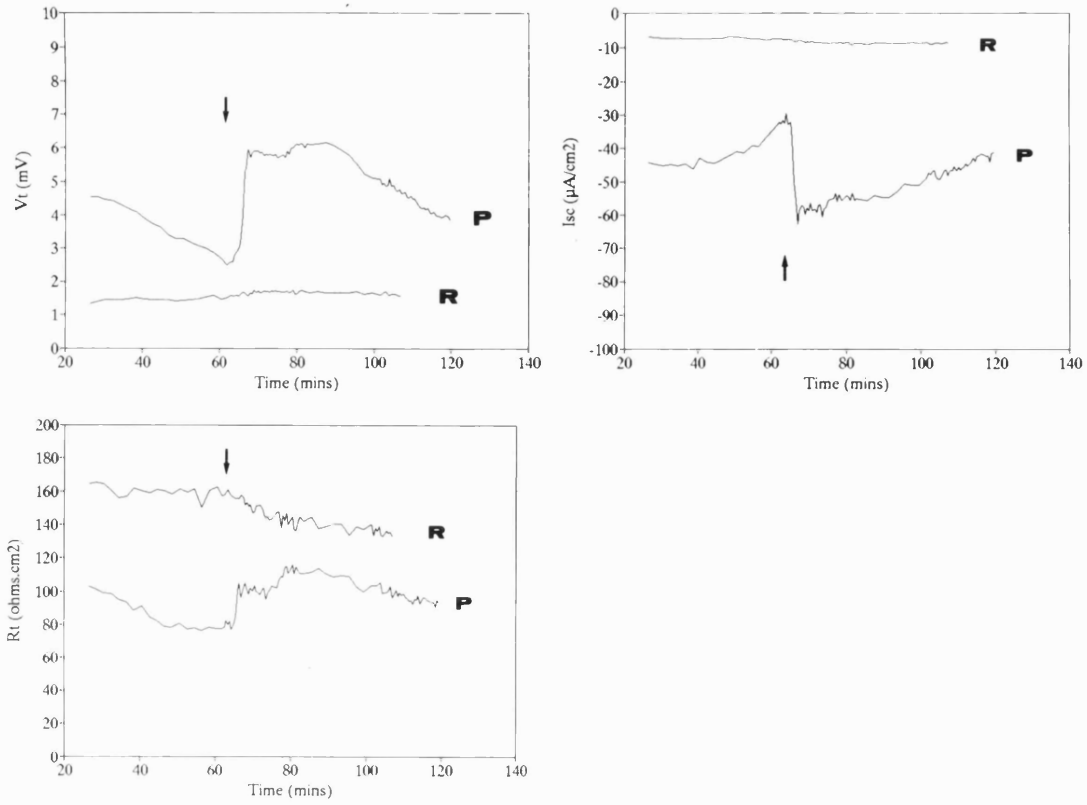


Fig 5.1 Typical examples of the increases in electrical properties of piglet (**P**) and rat (**R**) distal colon to 50MU/ml STa enterotoxin.

The response of the pig colon to CT was examined. CT (5μg/ml) was added to the mucosal bathing solution once the electrical values had stabilised (after 60 minutes). The tissues were maintained for 3 hours in the presence of CT. No significant changes were seen in the Vt, Isc or Rt during the entire three hour period. Tissues were challenged with 2mM theophylline (mucosal bathing solution) at the end of this period. Table 5.1 compares the maximum increase in Vt and Isc to theophylline of the tissues that had seen CT and control tissues. No significant differences were found between the three groups.

Table 5.1. Response of piglet descending colon to 2mM theophylline of distal colon after prior exposure to cholera toxin, stool culture- filtrates or saline.

	Vt(mV) (n)	Isc(μA/cm^2) (n)
Cholera toxin	+1.1±0.4 (5)	+20.9±7.9 (5)
Stool filtrate	+0.8±0.1 (3)	+15.6±5.2 (3)
Saline	+1.2±0.5 (5)	+11.4±3.2 (5)

Results are given as mean±SEM. Vt = transepithelial potential difference (serosal side positive); Isc = calculated short-circuit current; n = number of tissues. No significant difference exists between the three groups.

Stool culture filtrates

The presence of enterotoxigenic activity in the stool broth cultures was examined using piglet descending colon. Four stool samples were selected from children with idiopathic diarrhoea who had not received antibiotics and from whom no recognised enteropathogen had been isolated.

Preliminary experiments showed that addition of 10-100μl BHI to 10ml mucosal bathing solution resulted in an immediate increase in Vt and Isc of piglet and human distal colon. No such secretory events occurred after addition of the 10-500μl rDMEM to the serosal bathing solution.

No changes were seen in the electrical properties of the piglet colon exposed 200μL of any of the four stool culture filtrates for up to three hours. Similarly, no response was seen to the rDMEM broth culture controls. An example of the trace of the Vt to one of the stool culture filtrates is shown in Fig. 5.2. Tissue viability was confirmed by the increase in Vt and Isc to 2mM theophylline added to the mucosal bathing solution at the end of the experiment after $>$3hrs (increase in Vt: 1.2 ± 0.5mV; Isc: $+11.4\pm3.2\mu A/cm^2$; n=5).

Cytotoxic activity of stool culture filtrates

No cytotoxicity was detected in the HEp2 or HEL cell lines after 7 days from any of the 4 stool culture filtrates. In contrast, the *C.difficile* control supernatant resulted in the cells of both cell lines rounding up and detaching from the glass tubes after overnight incubation.

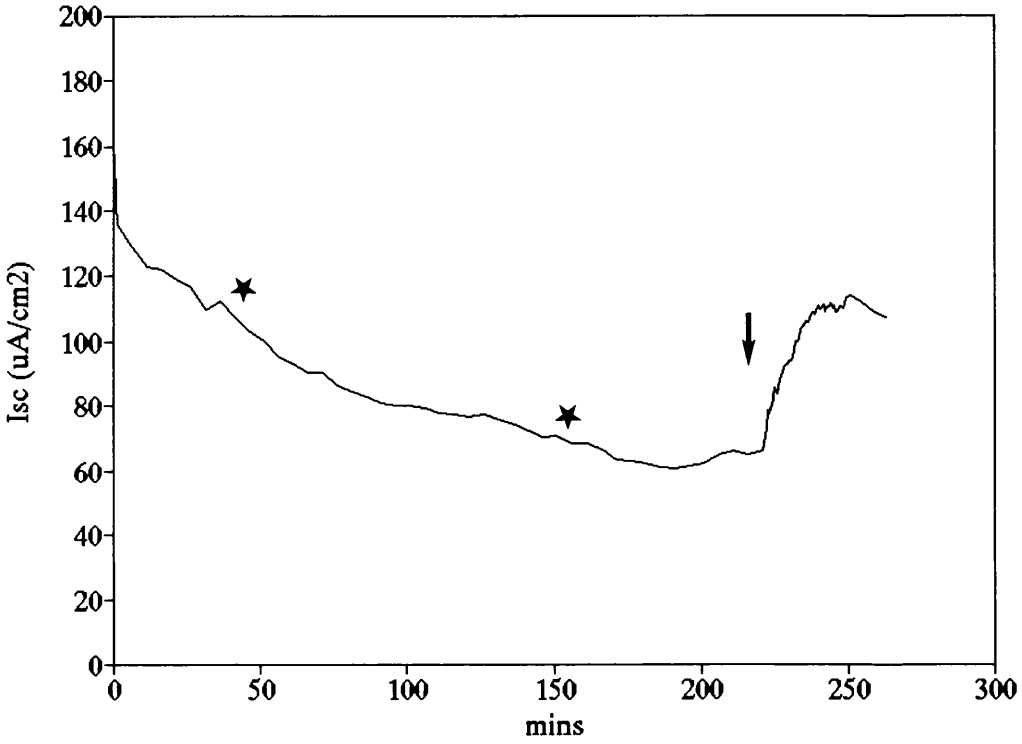


Fig.5.2 Typical trace of the transepithelial potential difference of piglet distal colon challenged with 2 challenges of 100μl of stool culture filtrate (*) in the mucosal bathing solution from a child with Hirschsprung's disease and diarrhoea. Mucosal theophylline (2mM) was added at ↓.

5.iv Discussion

A surprisingly small number of suitable stool samples were available for the Ussing chamber experiments due to the high proportion of samples that were excluded for the following reasons: antibiotic treatment before sample collection, ileostomy samples from children with long segment or total colonic aganglionosis, presence of a recognised enteropathogen and the presence of *C difficile*.

There is a recognised correlation between production of Vero enterotoxin and inability to ferment sorbitol in strains of *Esch coli* O157 which are associated with Haemolytic Uraemic Syndrome and haemorrhagic colitis (Smith & Scotland, 1988). All the strains of Esch coli were able to ferment sorbitol. The results of the gene probe analysis support the conclusion that verotoxin was not present in these strains.

The failure to detect any enterotoxins in the Ussing chamber studies on the colonic flora from children with Hirschsprung's disease and diarrhoea does not exclude the possibility that enterotoxic activity was present acting via electrically silent mechanisms. However, the additional testing for toxicity by other methods (HEL cell line cytotoxicity, gene-probe analysis for LT, St and VT enterotoxins and CT-like enterotoxin RPLA screening) also failed to detect any enterotoxic activity.

Heat stable enterotoxic activity has been described in species *Enterobacter sp., Klebsiella pneumoniae, Yersinia enterocolitica* in addition to *Eschericia coli*. Unidentified enterotoxins have also been demonstrated in strains of *Esch.coli* associated with enteroinvasive (Fasano *et al.*, 1990) or enteroaggregative (Savarino *et al.*, 1991) serotypes. The incidence of enterotoxin-producing klebsiellae from children is likely to be low. Guarino *et al* (1987) found only 2 strains reactive in the suckling mouse assay from the stools of 237 children with diarrhoea and none in 179 children without diarrhoea. The enterotoxic culture material of the *Esch coli* strains reported by Fasano *et al* (1990) induced an increase in potential difference of 0.65 to 1.25 ± 0.3mV and increases in Isc of $20\text{-}50 \pm 10 \mu A/cm^2$ after 3hours in rabbit ileum when measured in Ussing chambers. The secretory activity was

substantiated by the other studies in rabbit ileal loops and suckling mouse assays. Such small voltage changes without any recorded fall in tissue resistance indicate that the enterotoxic activity was either produced or released in small quantities or was of low potency. The extent to which these enterotoxins would induce secretory changes in the colon is unknown.

All of these studies have used either rabbit small intestine or suckling infant mouse methods to detect the secretory activity. The choice of animal tissue as well as the site of intestine will have profound influences on the sensitivity of the assay as well as its relevance to human disease. The small intestine appears to be a more sensitive tissue to enterotoxic action than the colon. The piglet jejunal loop test, for example, is the only biological assay that will detect *Esch coli* STb enterotoxin. The use of a range of different methods for detection of enterotoxins will clearly enhance the success rate. It is possible that the colon is more refractory to bacterial secretory enterotoxins than the small intestine. The failure of the rabbit colon to respond to theophylline and the rat colon to STa enterotoxin highlights the differences in arrangement of ion transport mechanisms in different animals. A concentration of 200U/ml Sta enterotoxin was used by Nobles *et al* (1991) to elicit maximal increases in rat distal colon. A gradient in the response was also demonstrated with the proximal colon being significantly more sensitive. The present study indicates Sta enterotoxin and theophylline but not CT are able to induce electrogenic changes in piglet rectosigmoid colon. Argenzio & Whipp (1981) investigated the response of the pig spiral colon to *Esch. coli* STa enterotoxin, theophylline and CT using an *in vivo* isolated loop technique. All three secretagogues reversed Na^+ and Cl absorption and caused HCO_3 and Cl secretion. The time course of action with 6.25μg/ml CT was slow with significant differences between the control and test loops only starting after 90 minutes. Ussing chamber studies were carried out and reported (Argenzio & Whipp, 1983) on the effect of theophylline and STa enterotoxin but not CT. It is tempting to speculate that small, delayed responses were also seen with CT which made the Ussing chamber technique unsuitable.

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Chapter 6.

General discussion

6.i Diarrhoea in children with Hirschsprung's disease

a) reduced colonic absorptive surface
    -effect of surgical resection
    -diversion colitis
b) Gastroenteritis & unrecognised enterotoxic organisms
    -method to detect toxins
c) defective neural control of secretion

6.ii Distension-induced secretion:
    -defective neural response

6.iii Summary and Conclusions

6.iv Future studies
    -electrically silent mechanisms
    -neurotransmitter sensitivity

6.i Diarrhoea in Hirschsprung's disease

This study has been concerned with the aetiology of enterocolitis in children with Hirschsprung's disease. Although standard texts on paediatric gastroenterology list a huge number of possible causes, diarrhoea can be broadly simplified into two underlying mechanisms: increased secretion and decreased absorption. Examples are listed below:

Net effect	Aetiological agent	Example
Increased secretion		
	enterotoxins	choleragen
	luminal metabolites	fatty acids bile salts laxatives
	neurotransmitters	neuroendocrine-disorders eg VIPoma
Decreased absorption		
	loss of surface area	resection
	mucosal damage	IBD food allergy
	osmotic	sugar-intolerance

(IBD: inflammatory bowel disease)

Taking into account the underlying abnormality and treatment of Hirschsprung's disease along with the findings of this study, three causes of diarrhoea can be considered:

a) reduced absorptive capacity of a shortened intestine

b) microbial gastroenteritis,

c) defective neural control of secretion

Of these three mechanisms, the first two are readily accomodated in the table given above, but the last mechanism does not fall into recognised mechanisms of diarrhoea.

a) Reduced colonic absorptive surface

The surgical treatment of Hirschsprung's disease relies on the resection of the aganglionic colon. Consequently diarrhoea in these children could be due to loss of absorptive colon (Read, 1982). Surprisingly little information exists on the incidence of diarrhoea in relation to extent of residual colon after resection in children. Diarrhoea, with chronic fluid and nutrient depletion is a well recognised complication in patients after resection of the terminal (but not proximal) ileum (Nuguid, Bacon & Boutwell, 1961; Hill, Mair & Goligher, 1975). The length of residual colon correlates directly with the output volume in adults with ileal and partial colonic resection (Cummings, James & Wiggins, 1973). The consequences of ileorectal anastomosis are reviewed in the literature principally in children with long-segment Hirschsprung's disease (Nixon, 1985; Tariq, Brereton & Wright, 1991, Levy & Reynolds, 1992). A high incidence of diarrhoea is reported in such children. It is likely that resection of the distal ileum during ileorectal anastomosis will account for a proportion of episodes of diarrhoea.

A review of 50 children who had undergone total colectomy (34 of whom had Hirschsprung's disease) found that frequent "pulpy" stools occurred in 20 children, continence disturbances in 16 and severe recurrent enteritis in 14 (Kraeft *et al.*, 1985). Almost all children had normal growth rate. The loss of the resorptive function of the colon would explain the passage of pulpy stools. In children with an intact colon the excess fluid secreted from the small intestine will be retrieved by the colon.

At the pullthrough operation great effort is made to avoid resecting more colon than is possible. Adaptation by the colon after resection has not received much study, though work on small bowel adaption after resection in rats has shown that considerable compensation occurs over the following 6 months with a 250% increase in electrogenic Na^+-glucose uptake due to increased villus and microvillus surface area (Schulzke *et al.*, 1992). Similar increases in the ileum are seen in man (reviewed by Reicken *et al.*, 1989). The likelihood is that compensatory

mechanisms occur in the colon but the need to retain a proportion of the colon is necessary to avoid incontinence of semi-solid stools.

The problems of incontinence will be also be related to impaired anal sphincter function as this will be damaged during the pullthrough procedure (Cass, 1986). This view is supported by the similar problems of post-operative incontinence in children after posterior sagittal anorectoplasty for anorectal anomalies (Langemeijer & Molenaar, 1991).

An alternative possible cause of diarrhoea in children who have undergone corrective surgery is diversion colitis. In patients in whom the faecal stream has been diverted, usually as a colostomy in the distal colon, inflammatory changes have been described, associated with diarrhoea. The mechanism is not understood but convincing evidence has accumulated to implicate an epithelial starvation of short chain volatile fatty acids as the likely cause. Short chain volatile fatty acids, in particular butyrate, have been shown to act as the prefered metabolic substrate for isolated colonocytes *in vitro*. It is proposed that the concentrations of these volatile fatty acids produced by the colonic anaerobic flora are reduced through the lack of luminal substrate and the colonocytes are then starved of their principal metabolic fuel (Roediger, 1988). Resolution of symptoms and histological changes have been demonstrated in adults given short chain fatty acid enemas or reversal of the diverted colostomy. Theoretically, children in whom a diverting colostomy has been created run the risk of diversion colitis. Similarly, prolonged antibiotic treatment could induce similar changes through severe reductions in the colonic anaerobic flora. The incidence of diversion colitis in this age group is unknown but it might have important implications in the treatment of diarrhoea in children with colostomies.

b) Microbial gastroenteritis

Most reviews on diarrhoea in children state that viral gastroenteritis is the most frequent cause. This is supported in part by the study of diarrhoea in children with

Hirschsprung's disease in Chapter 2 where microbial enteropathogens were the found in almost 50% of cases. However, idiopathic diarrhoea accounted for 30% of cases.

As mentioned above, in children with extensive colonic resection enteritis is an important problem. Children with an intact colon who have developing gastroenteritis will remain asymptomatic under conditions where the excess fluid secreted by the small intestine is adequately reabsorbed by the colon. This compensation will be compromised in children with extensive colonic resection and might explain their increased frequency of symptomatic enteritis.

The colonic dysmotility in aganglionic colon was considered to offer an abnormal environment in which the microbial flora could exert enterotoxigenic action. The stasis would permit a prolonged exposure of the colonic luminal contents on the distended colonic mucosa. In this situation any potential secretory stimulus produced by bacteria which, under normal conditions would be insufficiently enterotoxic, would be able to induce secretion through prolonged exposure time.

Unrecognised enterotoxigenic bacteria were not found in the studies described in Chapter 5. Nor was enterotoxic activity demonstrated in the stool culture filtrates from children with Hirschsprung's disease associated enterocolitis. In addition to the small number of samples examined, the failure to find a microbial aetiology is limited by factors relating to the source material; where antibiotic treatment of the children before adequate stool samples are obtained will eliminate possible pathogens, and the total numbers of the pathogen may increase before symptoms appear and then fall at the onset of diarrhoea (as seen in rotavirus infections).

The methods of screening for enterotoxic activity will also have selective effects by nature of their methodology. Three methods were considered at the outset of the project by which secretory enterotoxins could be identified: animal intestinal loop tests, infant mouse assays and Ussing chamber studies. *In vitro* experiments primarily measure active transport processes and do not yield details of passive transport events. In contrast, *in vivo* studies show net transport of both active and

passive mechanisms without enabling any clear differentiation between the two.

In choosing the method, preliminary experiments were carried out using ileal and colonic loops in neonatal and adult piglets (Thornbury *et al.*, 1990). Although massive secretion could be induced by use of 5μg/ml cholera toxin in both the colon and ileum after 12-18 hours it was found that a small degree of secretion could often occur in the adjacent distal loop. It is possible that a signalling pathway exists running along the vascular supply to adjacent loops. This type of mechanism has been suggested by the studies of Lundgren *et al.*, descibed earlier (Chapter 1). Also, reaction to the CT was not consistently observed. All commercially supplied pigs are vaccinated against LT^+ *Esch coli* and the transfer of maternal antibodies to newborn piglets occurs via colostrum which is taken up through the intestine during the first weeks of life. It was considered likely that this could account for variability in the response to CT as well as interfere with any antigenically similar enterotoxins.

The infant mouse assays were not used as they are suited more to studies of the small intestine and would not easily permit specific studies of secretion in the colon. Ussing chambers permit the use of any animal or human tissues from any site in the intestine (in this case proximal and distal colon) and enable a more exact monitoring of the electrogenic secretory events. Further more the effect of specific inhibitors can be easily investigated. Obviously, a more complete picture of the colonic secretory processes will only be obtained if both *in vitro* and *in vivo* methods are employed.

The absent effect of CT on the electrical properties *in vitro* indicates that the colon might be relative unresponsive to microbial enterotoxins and play little part in the pathophysiology of gastroenteritis. The contribution of the colon in causing fluid output in diarrhoea has only comparatively recently been considered. Teleologically, the colon is not the primary site of enterotoxic activity as most enteropathogenic microorganisms will reach and colonise the small intestine. However, the response to *Esch.coli* STa enterotoxin both in Ussing chambers as shown in Chapter 5 and in

receptor binding and guanylate cyclase activation studies in man show that STa receptors exist in the colon (Guarino *et al.*, 1987). Similarly, the ganglioside GM1 cholera toxin receptors are present on most eukaryotic cells and colonic dysfunction has been documented in adults suffering from cholera (Speelman *et al.*, 1986). Studies in pigs have also convincingly demonstrated colonic secretory events induced by microbial enterotoxins (Argenzio & Whipp, 1981, 1983; Argenzio, 1985) and have also highlighted the important resorptive function of the colon in neonatal piglet viral gastroenteritis (Argenzio *et al.*, 1984).

Epithelial invasion by bacteria with cytotoxic rather than secretory toxins (eg Verotoxin-producing *Esch coli*) offers a further example of the colon as a site of microbial "gastroenteritis". Although, Verotoxin production and sorbitol fermentation by *Esch coli* was investigated, colonic invasion by bacteria remains a possible mechanism of action in the aetiology of diarrhoea in children with Hirschsprung's disease.

The clinical experience of the paediatric surgeons at the Hospitals for Sick Children, London, is that oral vancomycin treatment in children with Hirschsprung's disease suffering from idiopathic enterocolitis results in a prompt improvement and this was noted in the study by Thomas (Thomas *et al*, 1986). It is possible that the clinical efficacy of vancomycin in the treatment of Hirschsprung's disease diarrhoea is due to the (incidental) treatment of small bowel bacterial overgrowth by reduction in luminal bacterial counts. No antisecretory effect of vancomycin was seen either in the human or pig colon to theophylline or STa enterotoxin. Neither has any colonic pharmacological effect of vancomycin such as prokinetic activity similar to that of erythromycin been reported. The clinical management of children with Hirschsprung's disease usually involves rectal washouts to relieve obstruction and constipation. This procedure may be the more important procedure carried out rather than any intrinsic effect of vancomycin.

c) Defective neural control of secretion

The Ussing chamber studies in Chapter 4 showed that the basal electrogenic ion transport was unaffected by the neural abnormalities in aganglionic colon as well as in the proximal ganglionic colon. It was hypothesed that the neural abnormalities would result in excessive secretion through defective inhibitory neural regulation or through chronic release of secretory neurotransmitters by the hypertrophied nerve fibres. In contrast to this, evidence was found that the aganglionic colon has a reduced net secretory potential rather than excessive secretory action. This would imply that that the underlying neural abnormalities in aganglionic colon are not a direct cause of diarrhoea.

The response of the proximal ganglionic left colon in children with Hirschsprung's disease was not significantly different in its response to Iloprost from the control ganglionic colon in this study and indicates that neurally mediated secretion in response to the distension will occur. Experimental obstruction in the distal ileum of dogs has been clearly shown to result in excess fluid and electrolyte secretion in the section of ileum immediately proximal to the obstruction while net fluid flow in the obstructed tissue remains unaffected (Shields, 1965). This is compatible with the hypothesis that an overflow diarrhoea in response to the megacolon is the source of the explosive diarrhoea seen after rectal examination of children with obstructed Hirschsprung's disease (Nixon, 1982).

6.ii The effect of impaired neurally-mediated secretion in Hirschsprung's disease

The absent secretory response to Iloprost has implications for the role of both the ENS and of prostaglandins in regulation of colonic function. In addition to stimulating electrolyte secretion, prostaglandins have a wide range of effects on other gastrointestinal function: growth, development and repair, smooth muscle contraction (motility) blood flow and synthesis and secretion of mucous (Powell, 1991). Prostaglandins are synthesised by the subepithelial tissues. The epithelium

can only degrade prostaglandins and does not possess any notable capacity to synthesis them. This arrangement is in keeping with the proposed regulatory role that prostaglandins play in gastrointestinal function.

Increased levels of prostaglandins have been found in cholera and in inflammatory bowel diseases and treatment with prostaglandin synthesis inhibitors such as indomethacin and aspirin have been reported to reduce intestinal fluid losses in both cases (Powell, 1991). However, interpretation is clouded by the secretory effect prostaglandins have on the ENS and their underlying basal secretory influence under normal conditions let alone in disease states. Nevertheless, the defective secretory response of aganglionic colon to Iloprost strongly indicates that the net effect of the neural abnormalities in Hirschsprung's disease is reduced secretory responses to appropriate stimuli.

The pathophysiology of Hirschsprung's disease is largely due to the defective anorectal coordination of the muscle and sphincters (Schärli, 1982). The aganglionic anorectum is characterised by uncoordinated motility patterns, absent adaptation to increased bulk (due to the failure of the muscle to relax) and a defective reflex relaxation of the anal internal sphincter to rectal distension. The absent secretory response to neural secretagogues in aganglionic colon corroborates with one of these classical features of Hirschsprung's disease: absent rectal adaptation reflex to distension. Rectal loading with faeces induces reflex urge to evacuate and probably also stimulates mucosal secretion. Mechanical pressure and distension of the intestine are recognised stimuli of mucosal secretion (Florrey, Wright & Jennings, 1941) and smooth muscle activity (Greenwood & Davison, 1987). Distension of the mucosal/submucosal preparations of distal colon in Ussing chambers results in transient electrogenic secretion mediated by the action of different prostaglandins on the enteric nervous system (Schulzke *et al.*, 1989; Diener & Rummel, 1990). The distension of the colonic wall is thought to release prostaglandins from the submucosal tissues (eg fibroblasts, vascular endothelium or the muscularis mucosae). Different prostaglandins act on different tissues: Prostacyclin acts

exclusively via the neural tissues whereas PGE2 acts on both the neural tissue and directly on the colonocyte (Diener *et al.*, 1988b). It seems likely that this phenomenon facilitates the movement of the faeces along the colonic lumen. The absent neurally mediated secretion in aganglionic colon indicates that the constipation and intestinal obstruction result not just from abnormal muscle functioning but from abnormal mucosal secretion also. The net result will excacerbate the stasis of luminal contents in the colon with resulting megacolon.

The use of rectal washouts in cases of Hirschsprung's disease with colonic obstruction can be seen to have a theoretical basis in alleviating obstruction and preventing distension-induced secretion. Certainly, its effectiveness is validated by the number of children with chronic constipation who are managed by rectal washouts and suppositories for years before a diagnosis of Hirschsprung's disease is made.

6.iii Summary and Conclusions

The findings of this study can be summarised as follows:

1) A proportion of children with Hirschsprung's disease suffer from recurrent episodes of diarrhoea. The most frequent identifiable cause in this study period was microbial gastroenteritis. Idiopathic recurrent diarrhoea (Hirschsprung's disease-associated enterocolitis) accounted for 30% of cases of diarrhoea.

2) No significant differences were found in the isolation rate and toxigenicity of *C difficile* from children with Hirschsprung's disease with or without diarrhoea. The isolation rates were not significantly different from those of children without Hirschsprung's disease although colonisation persisted in a proportion of cases.

3) No significant ablation of colonic ganglia with benzalkonium chloride was observed in the neonatal pig.

4) Basal electrogenic colonic ion transport was unaffected by the neural abnormalities in the distal colon of children with Hirschsprung's disease

5) Neurally-mediated secretion to Iloprost and acetylcholine was defective in human aganglionic colon.

6) Neurally-mediated secretion was intact in the proximal ganglionic colon in children with Hirschsprung's disease.

7) Secretion induced by direct stimulation of the colonocytes by enterotoxins and other secretagogues was unimpaired in aganglionic colon.

8) No detectable enterotoxic activity was present in the colonic flora of children with Hirschsprung's disease and diarrhoea.

Conclusions

No evidence of excessive electrolyte secretion was found in the distal colon of children with Hirschsprung's disease but there was evidence of an overall reduced secretory potential.

Diarrhoea in these children is more likely to occur due to i) secretory events in the small intestine and ii) distension induced secretion in the ganglionic proximal colon.

6.iv Future studies

Several groups have described changes in neuropeptidergic innervation of the colon of children with Hirschsprung's disease eg increased mucosal NPY fibres (Hamada *et al.*, 1987; Larsson *et al.*, 1988) together with reduced or or absent mucosal SP fibres (Bishop *et al.*, 1981). With improvements in immunohistological staining methods, it appears likely that specific patterns of peptide co-localisation will differentiate nerve fibre types eg Substance P co-localised with calcitonin gene-related peptide are thought to act as sensory nerve fibres in the intestine. Changes in their density have been described in inflammatory bowel disease (Mantyh *et al.*, 1991). The next few years should see an increasing understanding of the changes in innervation that occur to different stimuli within the intestine.

Further studies on the response of human aganglionic colon to different neuropeptides are needed to elucidate details of the neuropeptides which are normally involved in regulating human colonocyte secretion and absorption and their extrinsic or intrinsic origin.

The reasons for the absent secretion in response to Iloprost remain unknown. Identification of the normal site of action of Iloprost in ganglionic colon is necessary perhaps by studying its effects on isolated ganglion cells.

Non-electrogenic ion transport in aganglionic colon was not studied in this project. Regulation of electrically silent processes such as bicarbonate secretion may be controlled by the ENS and in aganglionic colon might result in excessive secretion and merits investigation.

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