ADRENOCEPTORS AND INTESTINAL FLUID AND ELECTROLYTE TRANSPORT IN THE RAT

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SUMMARY

Noradrenaline was found to significantly stimulate fluid and Na absorption across everted sacs of rat jejunum. Of a number of α_1 and α_2-adrenoceptor antagonists tested only prazosin significantly inhibited the stimulant effect of noradrenaline and further experiments revealed an antiabsorptive effect of prazosin alone. Theophylline reduced jejunal fluid and Na absorption and this effect was not reversed by α_2-adrenoceptor stimulation in contrast to previous findings *in vivo*. Evidence suggests the everted sac preparation is not appropriate to the study of intestinal fluid and electrolyte transport.

The investigation of Jejunal ion transport *in vitro* was continued using an Ussing chamber preparation. Selective α_2-adrenoceptor stimulation was found to depress electrogenic anion secretion, as evidenced by decreases in short circuit current. The use of the neurotoxin tetrodotoxin indicated that this was a direct epithelial effect. α_2-adrenoceptor agonists have considerable therapeutic value as antisecretory agents and the model of rat jejunum *in vitro* represents a convenient experimental model for research in this area. The selective β_2-adrenoceptor antagonist ICI 118551 decreased basal SCC and inhibited increases in SCC in response to isoprenaline or salbutamol indicating the presence of a β_2- adrenoceptor mechanism mediating both secretory tone and increases in secretory processes. Many intestinal secretagogues elicit electrolyte secretion via the stimulation of intramural secretory nervous pathways. If these pathways involve the activation of β_2-adrenoceptors then β_2-adrenoceptor antagonists may be useful in the treatment of diarrhoeal diseases.

A single pass lumen perfusion technique was used to investigate possible sympathetic tone over colonic fluid and electrolyte absorption in the rat colon *in vivo*. The technique employed appeared to lack the necessary resolution for this study and alternative approaches are discussed.

Key words:- Adrenoceptor Intestinal Jejunum Ileum Colon

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I dedicate this thesis to my parents, without whose support my years at university would barely have been possible and to my wife to be (at last), Caroline, for her years of support, encouragement, patience (and impatience) and many hours of assistance with the preparation of this thesis.

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INTRODUCTION TO THE THESIS

The manipulation of control mechanisms that directly influence intestinal absorption and secretion has obvious relevance to the treatment of diarrhoeal diseases, which are the most common cause of infant mortality in developing countries (Holmgren and Svennerholm, 1982), and to the treatment of constipation , which together with diarrhoeal disorders, is a major source of inconvenience in the western world.

The major first line antidiarrhoeal therapies are at present opiate based compounds and oral rehydration solutions. The antisecretory effects of opiate based compounds are still poorly understood. Originally it was accepted that they exerted their influence through an inhibition of gastrointestinal motility but now they are also thought to influence epithelial transport by a direct depression of secretory mechanisms (Kachur et al, 1980) and, for those that cross the blood brain barrier, by the stimulation of sympathetic outflow (Brown and Miller, 1984). The main problems with opiate therapy are tolerance and addiction liability. Oral rehydration solutions contain substrate (usually glucose) which stimulates substrate linked Na absorption.

The underlying physiological problems which result in constipation are obscure and the present treatments are indirect, involving largely the utilization of bulking agents or bowel stimulants. There obviously exists much room for improvement towards a more direct approach to the pharmacotherapy of diarrhoeal diseases and constipation.

Evidence now exists to suggest that adrenoceptor compounds may be of considerable therapeutic value in the treatment of intestinal disorders.

The initial aims of this investigation were to study adrenergic control of intestinal fluid and NaCl movement in the jejunum, ileum and colon of the rat using both *in vitro* and *in vivo* techniques since an understanding of the role of specific adrenoceptor subtypes in the control of intestinal transport processes is a prerequisite in the context of possible applications to the treatment of intestinal disorders.

Following the establishment of the early concept that the sympathetic division of the autonomic nervous system promoted absorptive processes whilst the parasympathetic division stimulated intestinal secretion (see Wright et al, 1940; Florey et al, 1941) work in this area came to a standstill for many years primarily due to a lack of techniques permitting close study of intestinal transport mechanisms. Renewed interest was slowly aroused by the development of the simple *in vitro* everted sac technique (Wilson and Wiseman, 1954) and the Ussing chamber preparation (Ussing and Zerahan, 1951) as adapted for the study of electrolyte transport across intestinal sheets (Schultz and Zalusky, 1964; Field et al, 1971). Using the everted sac technique, Aulsebrook (1965) demonstrated that noradrenaline stimulated Na transport across rat small intestine, whilst using the Ussing chamber preparation Field and McColl (1973) reported that noradrenaline promoted both Na and Cl absorption and inhibited Cl and HCO_3 secretion across rabbit ileum. Following the identification of distinct adrenoceptor subtypes there was a renewed interest in this field prompted by the realisation that drugs specific for appropriate adrenoceptor subtypes might prove to be useful in the treatment of intestinal disorders.

At the outset of this investigation there had been recent reports which appeared to show that noradrenaline induced stimulation of fluid absorption in the rat small intestine *in vivo* was mediated through an α_1-adrenoceptor mechanism under basal conditions (Levens et al, 1981a; Levens, 1983) or in conditions of haemorrhage or dehydration (Levens, 1984a; 1984b), but that the reversal of secretagogue induced fluid secretion was an α_2-adrenoceptor mediated event (Nakaki et al, 1982a; 1982b; Bunce and Spraggs, 1983a; 1983b). To further complicate matters, antisecretory effects of the β-adrenoceptor antagonist propranolol had been reported in the rat jejunun *in vivo* under both basal (Levens, 1983) and secretory (Donwitz and Charney, 1979) conditions. *In vitro* studies using everted intestinal sacs have suggested that noradrenaline stimulates basal levels of fluid absorption via an α_1-adrenoceptor mediated mechanism (Parsons et al, 1983; Cotterell et al, 1983). However, there had been no further attempts at an *in vitro* characterisation of adrenoceptor mechanisms in the rat small intestine and this was a major objective of the present investigation. The study began with a re-investigation of adrenoceptor mechanisms in everted jejunal sacs under both basal and secretory conditions.

The original aim of the study was to undertake a characterisation of adrenoceptor regulation of fluid and electrolyte transport both in the small intestine and in the colon of the rat. However as the study progressed it became clear that time would not permit a detailed study of colonic transport. In view of this, it was decided to undertake a limited extension of the study to investigate the possible role of sympathetic tone in the rat colon *in vivo*, the presence of which has been suggested by studies of diabetic diarrhoea in rats by Chang et al

(1983b). If adrenoceptor mediated tone is present in the rat colon *in vivo* then antagonists to the appropriate receptors should produce a decrease in basal fluid and electrolyte transport. It is thus possible that adrenoceptor antagonists may be potentially useful in the treatment of chronic constipation.

LITERATURE REVIEW

1. Active Transport of Sodium and Chloride in the Mammalian Small Intestine.

The present study is largely an investigation into adrenoceptor mediated control of the movement of fluid, and of the active transcellular transport of the principal components of the intestinal absorbate, that is sodium (Na) and chloride (Cl), across the jejunal epithelium of the rat *in vitro*. Although the majority of ionic movement across the small intestine is passive and occurs via the paracellular pathway it is the active transcellular transport of Na and Cl which provides the driving force for intestinal fluid movement. Before entering a discussion of the control mechanisms that exist for the active transport of sodium and chloride it is necessary to recognise those mechanisms that exist for the active movement of these ions in the mammalian small intestine. The 'direction' of active transport mechanisms for Na and Cl are shown diagrammatically in fig.1 and discussed below

a) Electrogenic Na Absorption.

The existence of an electrogenic process for Na absorption, a component of which is dependent upon co-transport with organic solutes (sugars or amino acids) has been extensively documented (for a recent review see Esposito, 1984). Na enters the cell down a steep electrochemical gradient generated by an ouabain sensitive Na/K-ATPase

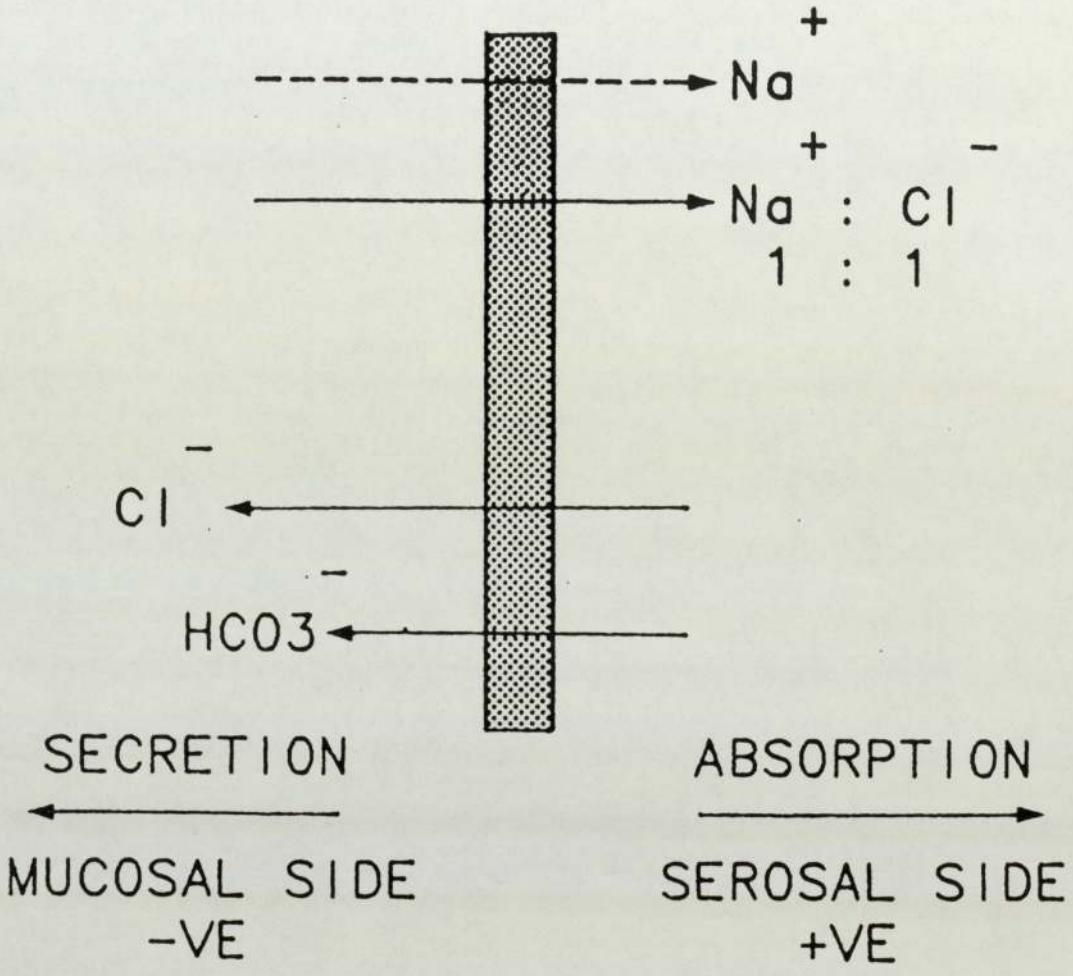

Fig. 1. Mechanisms of active transport for Na, Cl and HCO_3 in the mammalian small intestine. Na is absorbed by both an electrogenic mechanism and via neutral 1:1 absorption with Cl. Electrogenic secretory processes exist for both Cl and HCO_3. Electrogenic processes contribute to the transepithelial potential difference (mucosal side negative). Mechanisms shown in bold arrows are those proposed to be commonly influenced by neuroendocrine factors and/or neurotransmitters (see text for details).

dependent pump located in the basolateral cell membrane which actively extrudes Na from the cell into the serosal compartment (see Armstrong and Garcia-Diaz, 1984 for further discussion).

b) Electrically Silent NaCl Absorption.

Electrophysiological studies from a number of laboratories have reported the existence of a process for electrically silent 1:1 absorption of Na and Cl in villus cells of the small intestine from a number of mammalian species. Electrically silent absorption of Na and Cl across the apical membrane of epithelial cells was originally thought to be mediated through a single carrier mechanism (Frizell et al, 1979). However, more recent evidence obtained from studies performed with isolated membrane vesicles favours the hypothesis that electrically silent absorption of Na and Cl occurs via the coupling of Na/H and Cl/OH or Cl/HCO_3 exchange mechanisms (Knicklebien et al, 1985; Murer et al, 1983). The energy for electrically silent NaCl absorption is again thought to be derived from the basolaterally located Na/K-ATPase pump which delivers Na to the serosal compartment. Cl ions thus enter the cell against an electrochemical potential. The mechanism of exit of Cl ions is at present unclear. Electrodiffusion, Cl/HCO_3 exchange and electroneutral KCl symport have all been proposed as candidates mediating the exit of Cl ions across the basolateral membrane (see Armstrong and Garcia-Diaz, 1984 for a full discussion).

c) Electrogenic Cl Secretion.

The presence of an active transport mechanism for the secretion of Cl located largely in the intestinal crypt regions is well documented (see Rao and Field, 1983 and Armstrong and Garcia-Diaz, 1984 for recent reviews). Cl enters the cell across the contraluminal membrane apparently coupled to Na which is then extruded from the cell via the basolateral Na,K-ATPase pump. Cl thus accumulates intracellularly above electrochemical equilibrium. Under non-secretory conditions most of the entering Cl simply recycles across the contraluminal membrane although as mentioned previously the mechanism of basolateral Cl exit is unclear. In the presence of secretory stimuli such as cyclic AMP (cAMP) there is a rapid increase in the conductive Cl permeability of the apical membrane, resulting in net Cl secretion. Accompanying Na secretion is believed to be passive and probably via the paracellular pathway, driven by Cl accumulation in the crypt regions. Thus it is possible to account for net Na secretion without having to postulate the presence of a mechanism capable of mediating active Na secretion.

2. Intestinal Fluid and Electrolyte Transport - Neurohormonal Control Mechanisms.

The detailed physiological control of intestinal absorptive and secretory functions is poorly understood and complexed by the plethora of hormonal and neuroendocrine factors that have been shown to influence intestinal fluid and electrolyte transport (see table 1).

Neuroendocrine Agent	Absorptive (A) or Secretory (S) Influence
Noradrenaline/Adrenaline	A
Acetylcholine - Muscarinic	S
- Nicotinic	A
Dopamine	A
Enkephalin	A
Somatostatin	A
Neuropeptide Y	A
Serotonin (5 - Hydroxytrytamine)	S
Prostoglandin	S
Angiotensin - Low Dose	A
- High Dose	S
ATP/Adenosine	S
Vasoactive Intestinal Polypeptide	S
Substance P	S
Gastrin	S
Cholecystokinin	S
Secretin	S
Glucagon	S
Neurotensin	S
Bradykinin	S
Histamine	S
Gastric Inhibitory Peptide	S
Pancreatic Polypeptide	S
Motilin	S
λ-Aminobutyric Acid	S
Bombesin	S
Throtropin Releasing Hormone	S

Table 1. Neuroendocrine factors shown to influence intestinal fluid and electrolyte transport *in vitro* and/or *in vivo*. For comprehensive discussion of the possible physiological roles of these factors see Fondacaro (1986), Gaginella (1984), Hubel (1985), Tapper (1983), Turnberg (1983) and Turnberg (1984).

Glucocorticoids and aldosterone have been shown to enhance electrogenic Na absorption, primarily in the large intestine, in a number of species (see Turnberg, 1984). These hormones are believed to exert their effects via the synthesis of specific proteins involved in Na transport. It is likely that these 'classical' hormones influence salt and water transport in species where diurnal variation is prominant. More generally it is thought that they may have some importance in conditions of salt excess or depletion (Turnberg, 1983).

Much attention has recently been focused upon the neuroendocrine control of intestinal fluid and electrolyte transport which is thought to involve a complex interplay between the influences of the sympathetic and parasympathetic nervous systems, the enteric nervous system (ENS) and neuroendocrine factors released from endocrine cells interdispersed within the intestinal epithelium. Table 1 serves to highlight the complexity of the neuroendocrine control of intestinal function illustrating the wealth of factors shown to influence fluid and electrolyte transport. *In vitro* studies seem to indicate that those neuroendocrine factors that promote absorption seem to do so via both stimulation of neutral NaCl absorption and depression of electrogenic Cl secretion. Neuroendocrine factors promoting secretion all seem to stimulate electrogenic Cl secretion although some may also inhibit neutral NaCl absorption to some degree. Comprehensive discussions on the possible physiological roles and mechanisms of action of some of these factors can be found in a number of recent reviews (Fondacaro, 1986; Gaginella, 1984; Hubel, 1985; Tapper, 1983; Turnberg, 1983; Turnberg, 1984).

The general view seems to be that the extrinsic adrenergic and cholinergic nervous systems maintain overall control over absorptive and secretory functions. Numerous *in vivo* experiments performed in the late 19th and early 20th centuries involving sectioning or electrical stimulation of autonomic nerves or application of autonomic drugs first established the idea that the parasympathetic nervous system stimulated intestinal secretion, whereas the sympathetic nervous system promoted absorption of fluid and electrolytes (see the review of Florey et al, 1941) and experiments performed in recent years have tended to confirm these early concepts. Secretory effects of cholinoceptor agonists have been observed to be antagonized by atropine and are thought to be mediated via muscarinic receptors (Tapper et al, 1978). High concentrations of cholinoceptor agonists (e.g. carbachol) have, however, been reported to stimulate net NaCl absorption in the rabbit ileum *in vitro* (Tapper, 1978). These effects are inhibited by the nicotinic receptor antagonist hexamethonium and the α-adrenoceptor antagonist phentolamine and are believed to reflect stimulation of noradrenaline release through activation of pre-synaptic nicotinic receptors on sympathetic nerve terminals.

Studies reported by Crocker and Munday (1970) and Davies et al (1970) have shown that low concentrations of angiotensin stimulate fluid absorption whilst high concentrations of angiotensin inhibit fluid absorption across everted sacs of rat jejunum and colon *in vitro*. These effects of angiotensin are reproducible *in vivo* (Bolton et al, 1975; Levens et al, 1981a) and further studies by Levens and co-workers (Levens et al, 1979; 1981b; 1981c; Levens, 1983; 1985) have indicated that angiotensin enhances absorption of fluid and electrolytes *in vivo*

through enhanced release of noradrenaline from sympathetic nerve terminals. Angiotensin has also been reported to mediate reflex increases in fluid and electrolyte absorption from the rat small intestine in response to haemorrhage or dehydration (Levens, 1984a; 1984b). Secretion of fluid and electrolytes in response to administration of high doses of angiotensin *in vivo* is inhibited by indomethacin pretreatment and is believed to be mediated through stimulation of prostaglandin biosynthesis (Levens et al, 1981c; Levens, 1983).

Little is known of the functional significance of the various neurotransmitters and neuromodulators incorporated within the ENS. It is thought that the ENS together with local paracrine secretions provides a 'fine tuning' of electrolyte transport within discrete areas of the gut. Local control mechanisms probably respond to changing conditions in the luminal environment (e.g. dryness, pH) perhaps triggered by stimulating the release of paracrine secretions and/or the activation of luminal enteroceptor cells which initiate reflexes within the ENS.

It is also perhaps worthy of note that the recently identified atrial natriuretic factor (ANF) has been shown to directly inhibit Na/H exchange in isolated intestinal cells (Semrad and Chang, 1986).

3. Influences of Secretory Stimuli on Active Intestinal Electrolyte Transport.

A major impetus for the study of the control of electrolyte transport in the small intestine has been the realisation that the pathophysiology of a number of severe diarrhoeal diseases is characterised by massive

fluid and electrolyte secretion from an apparently morphologically normal small bowel mucosa. A large number of drugs, neuroendocrine factors and bacterial toxins have been shown to both stimulate active Cl secretion and/or inhibit absorption of Na and Cl. The initiation of secretory events appears to be dependent upon increases in intracellular calcium (Ca) levels which can be achieved through a number of different mechanisms. Intracellular Ca levels can be raised through influx of extracellular Ca, or release of Ca from intracellular stores mediated via activation of cyclic nucleotides or the phosphatidylinositol pathway (for reviews see Berridge, 1979; Powell and Fan, 1983; Rao and Field, 1983; Donowitz, 1983; Donwitz and Sharp, 1984; Fondacaro, 1986)

It has been proposed that in the mammalian small intestine neutral NaCl absorption occurs primarily in villus cells whilst electrogenic Cl secretion takes place predominantly in crypt enterocytes (see Field, 1980). Field (1980) and Powell and Fan (1983) have shown that elevations in intracellular Ca or cyclic nucleotides produced either directly or through the use of secretory agents (e.g. theophylline, bacterial toxins) inhibits coupled NaCl absorption in intact rabbit ileal epithelium or membrane vesicles prepared from villus cells from the same region. The weight of evidence available seems to support this model of secretagogue induced inhibition of active, neutral NaCl absorption. However, an interesting alternative hypothesis proposed by Naftalin and Simmons (1979), Holman and Naftalin (1979) and Holman et al (1979) claims that decreases in NaCl absorption occur primarily via Ca or cyclic nucleotide induced regurgitation of Na and Cl from lateral intercellular spaces initiated by changes in ionic permeability of the tight junction-intercellular space complex.

Ca stimulated electrogenic Cl secretion is believed to be achieved through increased apical membrane permeability to Cl in crypt cells, allowing the ion to escape down its thermodynamic gradient (see section 1c).

Recent studies by Cassuto and co-workers (Cassuto et al, 1981a; 1981b; 1982a; 1982b; 1982c) have indicated that the major proportion of the stimulation of secretory processes in response to cholera toxin and a number of other secretory stimuli is mediated through the stimulation of intramural reflexes, whilst the remaining fraction is mediated by the well accepted direct effect upon enterocytes. These authors have proposed that the neural effect is initiated by the release of 5-hydroxytryptamine from mucosal endocrine cells which then depolarizes afferent nerves of a reflex circuit that ultimately releases a secretory transmitter. Evidence for these proposals rests largely on the observations that the secretory effects of bacterial toxins and theophylline in rats and cats *in vivo* are substantially inhibited by the neurotoxin tetrodotoxin and the local anaesthetic lidocaine. It has further been postulated that a cholinergic synapse is a link in this reflex chain as secretagogue effects have been observed to be inhibited by hexamethonium (Cassuto et al, 1982b; 1982c; Eklund et al, 1985).

4. Fluid and Electrolyte Transport in the Small Intestine - Adrenoceptor Mediated Influences.

Aulsebrook (1965) reported that adrenaline or noradrenaline stimulated absorption of Na in everted sacs of rat jejunum *in vitro*. Hubel (1976) further demonstrated that noradrenaline enhanced net absorption of

fluid, Na and Cl in rat jejunum and of Na and Cl in rat ileum *in vivo*. Noradrenaline and adrenaline have also been reported to stimulate electrically silent NaCl absorption and inhibit Cl and HCO_3 secretion across sheets of rabbit ileum mounted in Ussing chambers *in vitro* (Field and McColl, 1973). Further studies (Durbin et al, 1982; Chang et al, 1982) have indicated that both the stimulation of neutral NaCl absorption and depression of electrogenic Cl secretion are α_2-adrenoceptor mediated events in the rabbit ileum. Similar effects upon isolated rabbit ileum have been reported in response to stimulated release of noradrenaline from sympathetic nerve terminals elicited by tyramine (Tapper et al, 1981) or nicotinic receptor stimulation (Tapper et al, 1978). The demonstration of α_2-adrenoceptors on basolateral membranes purified from epithelial cells of rabbit ileum (Chang et al, 1983) implies that the effects of α_2-adrenoceptor stimulation *in vitro* are exerted directly upon the epithelium.

Selective α_2-adrenoceptor stimulation has been shown to reverse secretagogue induced fluid secretion in the jejunum and ileum of anaesthetised rats (Bunce and Spraggs, 1983a; 1983b; Nakaki at al, 1982a; 1982b; Wahawisan et al, 1985) and to inhibit castor oil induced diarrhoea in conscious rats (Spraggs and Bunce, 1983). Studies in anaesthetised rats have further reported the inability of α_2-adrenoceptor agonists to stimulate basal levels of fluid absorption. In contrast studies by Levens (1983) have suggested that angiotensin stimulates fluid absorption in rat jejunum *in vivo* by enhanced release of noradrenaline from sympathetic nerve terminals which in turn interacts with post-synaptic α_1-adrenoceptors. Reflex increases in fluid absorption in response to haemorrhage or dehydration have also been

shown to be inhibited by angiotensin antagonists and the selective α_1-adrenoceptor antagonist prazosin (Levens, 1984a; 1984b;). *In vitro* the stimulation of fluid absorption across everted sacs of rat jejunum in response to noradrenaline has been proposed to be an α_1-adrenoceptor mediated event (Cotterell et al, 1983; Parsons et al, 1983). Binding studies have revealed the presence of both α_1 and α_2-adrenoceptors on basolateral membranes purified from epithelial cells of rat jejunum (Nakaki et al, 1983; Cotterell et al, 1984).

There have been few reports concerning β-adrenoceptor mediated influences upon electrolyte transport in the small intestine. Morris and Turnberg (1981) reported that in human volunteers administration of the β-adrenoceptor agonist isoprenaline stimulated fluid and electrolyte absorption whilst the β-adrenoceptor antagonist propranolol induced fluid and electrolyte secretion. In contrast Donowitz and Charney (1979) reported that propranolol inhibited cholera toxin induced fluid secretion in rat jejunum *in vivo* and Levens (1983) reported that propranolol significantly stimulated fluid and electrolyte absorption under basal conditions also in the rat jejunum *in vivo*.

Little detail is known concerning intracellular events associated with catecholamine influences upon active intestinal NaCl absorption. As mentioned earlier, in rabbit ileum α_2-adrenoceptor agonists have been shown to stimulate neutral NaCl absorption and depress electrogenic Cl secretion. Low Ca bathing solutions and plasma membrane Ca channel blockers have been shown to produce the same effects as α_2-adrenoceptor agonists in the rabbit ileum *in vitro* and it has been suggested that α_2-adrenoceptor agonists may act by decreasing Ca entry across the plasma membrane of enterocytes (Donowitz and Asarkof, 1982; Donowitz,

1983). In support of this proposal it has been reported that α_2-adrenoceptor agonists decrease Ca entry rabbit ileal basolateral membranes and decrease rabbit ileal Ca content (Donowitz et al, 1984). In contrast Parsons et al (1984) have shown Ca channel blockers to inhibit the stimulatory effect of noradrenaline in everted sacs of rat jejunum indicating that noradrenaline requires extracellular Ca in order to exert its influence. The effect of noradrenaline in this preparation has however been reported to be exerted via an α_1-adrenoceptor mediated mechanism (Cotterell et al, 1983; Parsons et al, 1983).

5. Colonic Fluid and Electrolyte Transport.

A characteristic of fluid and electrolyte transport in the mammalian colon is that it exhibits marked regional differences along its length (Engelhardt and Rechkemmer, 1983). The existence of active transport mechanisms similar to those reported in the small intestine have been identified in the colon with the additional proposal that in some species Cl absorption may be mediated by an independent Cl/HCO_3 exchange mechanism (for reviews see Binder, 1978; Phillips and Devroede, 1979; Powell, 1979 and Schultz, 1981).

There is still no clear understanding of the involvement of adrenoceptor mediated mechanisms in the control of colonic NaCl and fluid transport. Adrenaline has been shown to stimulate net NaCl absorption in both rabbit (Sellin and De Soignie, 1984) and rat colon (Racusen and Binder, 1979) *in vitro*. Findings from the latter study have further suggested that both α and β-adrenoceptor stimulation may mediate decreases in electrogenic anion secretory processes in the rat

colon. In contrast a number of reports have been made concerning antisecretory properties of propranolol reported in a number of species both *in vitro* and *in vivo* (Coyne et al, 1977; Conley et al, 1976; Taub et al, 1977). Durbin et al (1982) reported that α_2-adrenoceptor stimulation failed to influence NaCl transport in rabbit colon *in vitro* whilst α_2-adrenoceptor stimulation has been shown to decrease secretory processes in rat colon *in vitro* (Dharmsathaphorn et al, 1984). However Bunce and Spraggs (1983a) have failed to demonstrate any significant effects of α_2-adrenoceptor stimulation upon secretagogue induced fluid secretion in the rat colon *in vivo*. Clearly adrenoceptor control of fluid and electrolyte transport in the large intestine is an area of some confusion.

METHODS AND MATERIALS

1. The Everted Sac Technique.

This part of the investigation involved the measurement of fluid, Na and Cl transport across everted sacs of rat jejunum using a method similar to that described by Wilson and Wiseman (1954). Male Wistar rats (160 - 200g) housed in a room with a 12h light cycle (7 a.m. to 7 p.m.) were starved overnight and anaesthetised with sodium pentobarbitone (60 $mg.kg^{-1}$;i.p). The abdomen was opened by a midline incision and the proximal half of the small intestine removed and transferred to a glass trough containing ice-cold bicarbonate Krebs solution (composition (mM): NaCl 118.3, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.2 and glucose 10) gassed with 95% O_2/5% CO_2. The intestinal segment was immediately flushed through with Krebs solution and then carefully everted over a glass rod. A single 16cm segment of jejunum, 16-32 cm from the upper end of the small intestine, was excised for the preparation of either a single 16cm jejunal sac or two adjacent 8 cm sacs. For each pair of sacs, one sac was used for drug treatment with test drugs being present in both mucosal and serosal solutions, the other serving as a control. A cotton ligature was secured around one end of each of the sacs which were then blotted on moist filter paper and weighed (W1) on a Mettler H10 balance (Gallenkamp). Approximately 0.5 ml of Krebs solution (serosal fluid) together with a 0.05 ml air bubble for serosal oxygenation was introduced into each sac using a blunt needle and disposable syringe and the sac closed with a second

ligature and re-weighed (W2). Prepared sacs were incubated in 10mls of Krebs solution (mucosal fluid) contained in 50ml Erlenmeyer flasks, sealed with parafilm, gassed with 95% O_2 / 5% CO_2 and shaken at 80 oscillations/minute in a shaking water bath (Mickle Laboratory Engineering Co. Ltd.) at 37°C for one hour. At the end of the incubation period sacs were gently blotted on moist filter paper and weighed again (W3). The serosal contents were then drained into sample vials for Na and Cl determination and the empty sacs re-weighed (W4). Total serosal fluid transfer was calculated from the four weighings obtained by the following formula:-

$$\frac{(W3 - W2) - (W4 - W1)}{W1} = \text{Total serosal fluid transfer } (g.g \text{ wet } wt^{-1}.hr^{-1})$$

Sodium concentrations (mM) in the serosal and mucosal solutions were measured by flame photometry (Corning 435) and chloride concentrations (mM) were measured by precipitation against silver ions using an EEL 920 Chloride Meter. Total serosal ionic transfer was calculated from the following formula:-

$$\frac{(W3 - W4)\ IoS - (W2 - W1)\ IoM}{W1} = \text{Total serosal ion transfer } (mM.\ g \text{ wet } wt^{-1}.hr^{-1})$$

IoS = concentration of ion in serosal fluid

IoM = concentration of ion in mucosal fluid

Some experiments were performed using the everted sac method as described by Parsons et al (1984), in which results were expressed in terms of dry weight and test drugs present only in the serosal bathing

fluid. Additionally glucose was replaced by mannitol in the mucosal fluid and the incubation time was 30 minutes.

2. The Ussing Chamber Technique

a) General Principles

The Ussing Chamber technique was originally developed for the study of transepithelial electrical parameters and transepithelial ion fluxes across isolated sheets of frog skin (Ussing and Zerahan, 1951). Later the technique was adapted for investigation of the aforementioned parameters across sheets of intestinal tissue (Schultz and Zalusky, 1964). In this technique, the tissue is mounted vertically as a flat sheet between the two sides of a divided chamber. Each side of the tissue can be bathed and oxygenated independently and pharmacological agents can be selectively applied, in the case of the small intestine, to either mucosal or serosal bathing solutions. The volume of solution in each half chamber can be adjusted so that there is no hydrostatic pressure difference across the intestinal sheet. In addition to appropriate arrangements for oxygenating and circulating the bathing solutions in each half chamber, provision is made for the insertion of salt/agar bridges that are positioned as close as possible to the tissue and serve to monitor transepithelial potential difference (PD). For this purpose they are connected through electrodes to a suitable voltage recording device and also to a voltage clamp device, which is in turn, connected to reversible electrodes embedded in opposite ends of each of the half chambers. The voltage clamp can be used to apply a current

across the tissue which is equal and opposite to the tissues spontaneous PD. This short-circuit current (SCC) is thus a measure of transepithelial PD and can be monitored when connected to a suitable recording device. Under SCC conditions transepithelial PD is brought to zero and the transepithelial PD for each ionic species in the bathing media is abolished. Recorded SCC corresponds to the algebraic sum of the net fluxes of those ions that are actively transported and changes in SCC (or indeed any ionic flux) in response to the application of pharmacological agents reflect changes in active transport in the absence of electrochemical gradients. Unidirectional mucosal to serosal and serosal to mucosal fluxes for any solute can be measured under these controlled conditions by adding a radiolabelled form of the appropriate solute to each half chamber and measuring its rate of appearance in the contralateral fluid compartment.

In applying the SCC technique to the small intestine an important modification of the original technique of Ussing and Zerahan is necessary as the small intestine is a relatively 'leaky' epithelium of low resistance. During short circuiting the current passing electrodes pass a current enough to nullify the PD as sensed by the salt/agar bridges. The salt bridges however terminate at a small but finite distance from the tissue surface and it is necessary during short circuiting to compensate for the drop in electrical potential that occurs during the passage of the SCC between the ends of the bridges and the tissue surface as the resistance of fluid between the bridge tips and the tissue surface is of the same order as the resistance of the tissue. With highly resistive epithelia such as frog skin it is not necessary to compensate for fluid resistance which is negligible

compared to the resistance of the tissue. Manual and automatic methods for the compensation of fluid resistance are discussed further in sections c and d of this chapter.

b) Tissue Preparartion

Male Wistar rats (160-200g) housed in a room with a 12h light cycle (7a.m. to 7p.m.) were starved overnight and anaesthetised with pentobarbitone sodium (60mg.kg^{-1}; i.p). The abdomen was opened by a midline incision and a 2-3cm segment of jejunum taken from an area 20-30cm from the pyloric sphincter was removed and flushed through with ice cold Krebs solution (see section 1 for composition). The jejunal segment was then gently slid over a glass rod and secured with cotton ligatures. A partial thickness incision was then made beside the remaining mesentery using a blunt scalpel blade and the outer muscle layer carefully stripped away with fine forceps. The stripped intestinal segment was then cut away from the glass rod and mounted as a flat sheet between two Ussing-type perspex half chambers, the exposed area being 0.64cm^2. Muscle layers were removed to reduce tissue resistance not due to the epithelium and also to improve oxygenation to the serosal side of the epithelium. Normal Krebs solution was present in the serosal bathing medium whereas the mucosal bathing solution contained 10^{-2}M mannitol in place of glucose. This reduced the contribution of glucose coupled electrogenic Na transport to the tissues spontaneous PD. In some experiments Cl free and HCO_3 free bathing solutions were used where gluconate salts replaced Cl and/or HCO_3 salts. HCO_3 free solutions contained 5 x 10^{-3}M HEPES buffer and were gassed

with 100% O_2. Modified Krebs solutions were titrated to pH 7.4 with NaOH. Bathing solutions contained 10^{-4}M ascorbic acid in experiments where noradrenaline was used to prevent its oxidation.

c) Type I Ussing Chamber Design and Associated Electronics

The Ussing chamber model described in this section was designed and constructed in this laboratory and was a modification of an original design obtained from Dr. A. Garner (I.C.I. Pharmaceuticals Division). The chamber is shown diagrammatically in fig. 2 and consists of 6 interlocking perspex discs clamped together. The water jacketed reservoirs were fixed above the chamber and connected to it with siliconised rubber tubing. The water jacketing system was used to maintain the temperature of the bathing fluid at 37±0.5°C. Tissue was clamped between discs C and D, the facing surfaces of these sections having an aperture of 0.64cm^2. The facing surface of section C contained a ring of 8 stainless steel pins, of approximately 2mm exposed length, embedded in Silgard (Dow Corning Inc) and upon which the intestinal sheet was impaled serosal side up. The receiving surface of disc D contained a recess to receive the mounting pins. Visking membrane was placed between discs B and C, and between discs D and E to isolate the chamber area immediately adjacent to the tissue from particles disintegrating from the silver/silver chloride ($Ag/AgCl_2$) current passing electrodes embedded in the facing surfaces of discs A and F. $Ag/AgCl_2$ potential recording electrodes were placed in plastic pots containing Krebs solution and were in contact with the chamber via narrow tipped salt/agar bridges, and led from the Krebs containing pots,

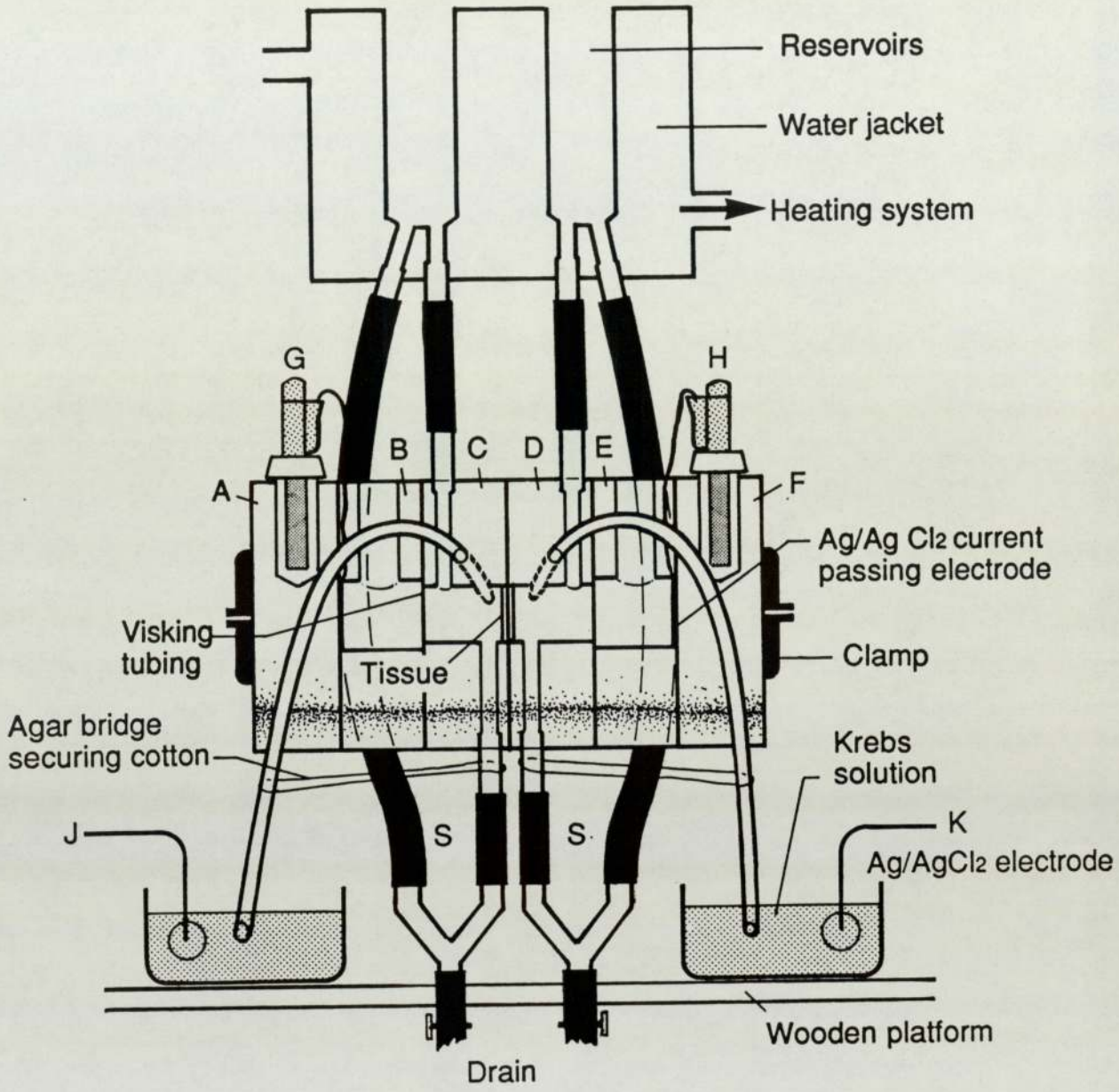

Fig. 2. Diagrammatic representation of type I Ussing chambers. The chamber system consisted of 6 interlocking perspex discs (A-F) connected to water jacketed reservoirs via siliconised rubber tubing. G, H, I, J represent references for electrode connections (see text for details). At the points (S) syringe needles leading from a 95% O_2/5% CO_2 supply were inserted into the rubber tubing to provide oxygenation and gas lift circulation.

through apertures in the perspex discs to lie close to the tissue surface. The composition of the Krebs solution contained both in the pots and the agar bridges was the same as that bathing the tissue except that glucose was omitted. The agar bridges were kept as short as possible in order to reduce their resistance, which, if too high, produced 'noise' on the tissue potential signal. In the pilot design for this system the $Ag/AgCl_2$ potential recording electrodes were inserted directly into the chamber and placed adjacent to the tissue. This design was soon identified as unsuitable since the electrodes were found to react with noradrenaline when added to the bathing solutions. The pots containing the recording electrodes and agar bridges were placed and secured in position on a thin wooden platform. Agar bridges were secured in position with cotton and the system was gassed and circulated with 95% O_2/5% CO_2 via syringe needles inserted into the rubber tubing as shown in fig. 2.

The potential recording $Ag/AgCl_2$ electrode 'K' was connected directly to a voltage clamp. Potential recording electrode 'J' was connected to the voltage clamp via a simple potentiometer (electrode offset compensator). This potentiometer was used to back off combined electrode and bridge junction potentials prior to tissue mounting. Junction potentials did not significantly alter during the course of the experiment unless the position of the agar bridges was altered, or if the $Ag/AgCl_2$ electrodes were removed from the Krebs solution for any length of time. Electrode leads leaving the voltage clamp apparatus were connected to the $Ag/AgCl_2$ current passing electrodes via connections at G and H. The voltage clamp apparatus was also connected to the following:-

i) a battery operated, high resolution, ultra high impedence digital millivoltmeter for potential recording. These meters were developed because of problems with noise on the potential signal when using mains operated millivoltmeters. The battery operated meter gave readings between +199.99 and -199.99 mV and gave resolution to 10μV. Further details of this device can be found in appendix I.

ii) a Teckman flatbed chart recorder with high impedence inputs (2 megohms) which was used to obtain continuous traces of short-circuit current over the required range (approximately 0-250 μamps). Current could be recorded directly on the chart recorder by placing a 1 Kohm precision resistor across the recorder's input terminals. Both the voltage clamp and the potentiometer were wired together and earthed via the chart recorder.

The voltage clamp device used in conjunction with the type I chambers was a copy of a design obtained from Dr C. Loat (Department of Physiology, Birmingham University). This voltage clamp was simple in its operation working through just one operational amplifier which fed back a current sufficient to nullify the potential recorded by the agar bridges from the tissue surface. However, this device which had been designed for short-circuiting preparations of highly resistive frog skin, did not incorporate any automatic compensation for the fluid resistance between the salt/agar bridge tips and the tissue surface.

This device did however possess a nulling point which could be easily adjusted with a small screwdriver to provide a constant current of variable magnitude. This enabled the voltage clamp device to be effectively used as a variable, constant current generator and allowed for experiments to be performed with intestinal tissue using the manual

method to correct for fluid resistance as described by Field et al (1971). In order to make corrections for fluid resistance a graph was drawn up prior to tissue mounting of current (μamps) passed through the bath (with Krebs solution present) versus potential difference (mV). Current recordings were obtained from the chart recorder and millivolt readings taken from the digital millivoltmeter. The variable current needed for construction of the graph was obtained by adjusting the null point on the voltage clamp. Once the tissue was mounted the SCC was determined by successive approximations and referenced to the current versus voltage graph. The correct SCC was that which resulted in the same PD with the tissue in place as with the tissue absent. SCC was adjusted during experiments at 3-5 minute intervals. Tissues were constantly short circuited throughout the course of the experiments except for a couple of seconds at 5 minute intervals in order to record the PD. This equipment was used for all the studies in which electrogenic anion secretory processes were measured by changes in recorded SCC. Two chambers were operational for the majority of these experiments.

d) Type II Ussing Chamber Design and Associated Electronics

A series of experiments were performed which involved the measurement of unidirectional fluxes of ^{22}Na and ^{36}Cl across isolated sheets of rat jejunum. It was felt that flux work would be difficult to perform with the type I Ussing chamber design and that improvements could be made upon this original design. In addition it was thought that rapid and accurate voltage clamping would be vital in experiments investigating

relatively small changes in unidirectional fluxes. Consequently type II Ussing chambers were developed for the measurement of unidirectional ion fluxes whilst experiments investigating changes in electrical parameters using the type I design continued. The type II chambers were based upon a design obtained from the laboratories of Professor L.A. Turnburg and Dr.G.I. Sandle (Hope Hospital, Manchester). In addition, an automatic voltage clamp was designed for use in flux experiments which automatically compensated for fluid resistance throughout the course of the experiments.

Type II chambers are shown diagrammatically in fig. 3 and contain some important modifications from the type I chamber design. The water jacketing system and bathing solution reservoirs were mounted directly upon each of two half chambers which could be simply clamped on the bench. This greatly improved the 'ease of handling' of the chambers when mounting the tissue and also cut out all the connecting rubber tubing which may have become contaminated with radioactivity. The gas lift system also fed directly into connections in the perspex chambers. Improvements were also made in the mounting pin arrangement. Pins were embedded directly into the perspex and fitted into holes on the facing half chamber. The design was additionally simplified by omitting the Visking tubing present in the type I chambers which was not seen to be an important design feature. Narrow tipped salt/agar bridges were introduced through apertures in the perspex and led to pots containing Krebs solution and $Ag/AgCl_2$ electrodes in a similar manner to the type I design. $Ag/AgCl_2$ current passing electrodes were embedded in opposite ends of the half chambers with silgard.

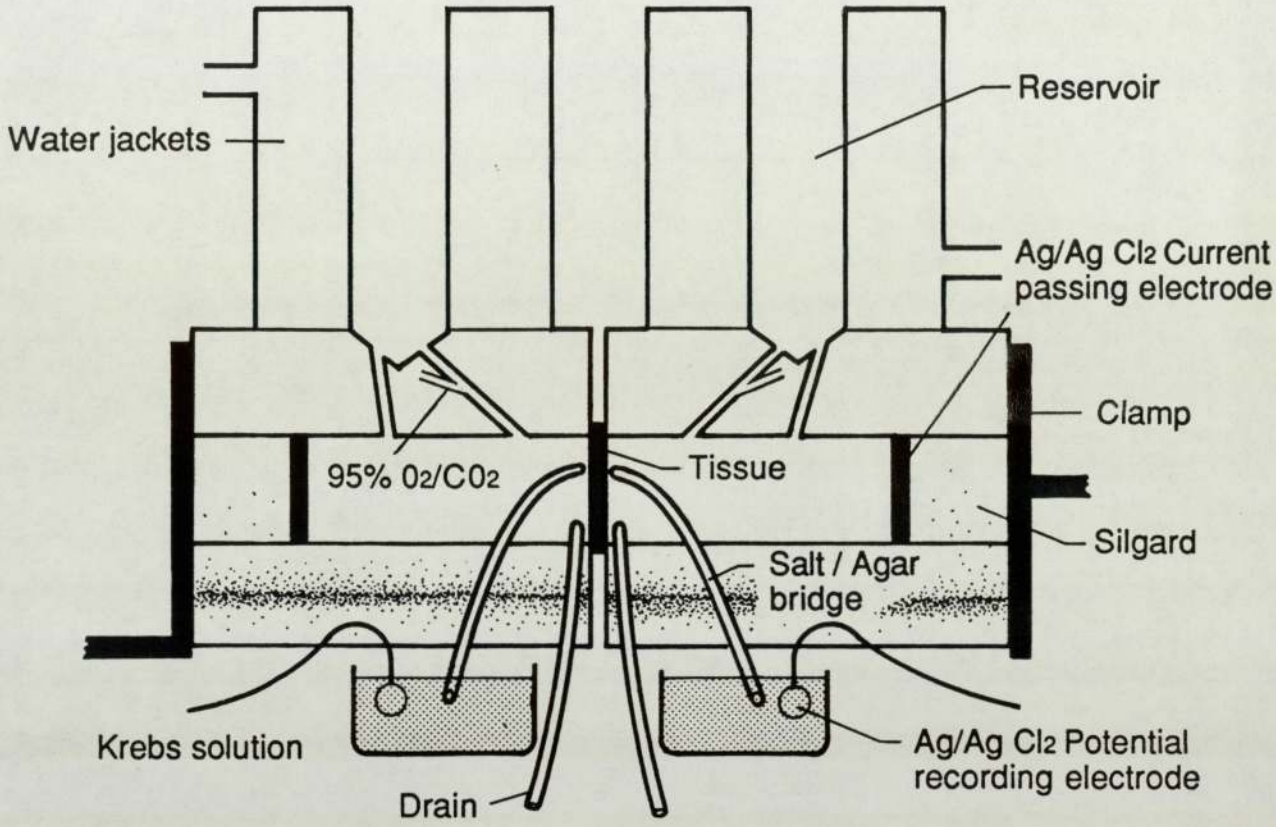

Fig. 3. Diagrammatic representation of type II Ussing chambers. The system comprised of two interlocking perspex half chambers with bathing fluid reservoirs and water jackets actually mounted on top of the chambers (see text for details).

The automatic voltage clamp employed in the flux experiments was very similar to that described by Rothe et al (1969). Fluid resistance was determined prior to tissue mounting by monitoring voltages generated by current flow through the Krebs solution between the bridge tips. Current flow was induced using a hand adjusted potentiometer to pass a varying current through the bath. A multiturn potentiometer built into the SCC circuitry was then used to just cancel out voltages injected into the bath, and when at this set point the clamp linked potentiometer would be operating to exactly cancel out the proportion of voltage signal resulting from fluid resistance. These clamps were designed and built in the department by Mr. D. Briggs and, to assist future readers, his description of their overall design and operation has been included in appendix I of this thesis. Due to limitations of time and resources only one flux chamber (with associated electronics) was available for radiosotope studies.

e) Unidirectional Flux Measurement of ^{22}Na and ^{36}Cl

Fluxes of Na and Cl were measured after the addition of 1.5μCi of ^{22}Na or 2.5μCi of ^{36}Cl to either the mucosal or serosal solutions of each chamber and measuring the rate of appearance in the contralateral fluid compartment. Isotopes were added 10 minutes after tissue mounting. After an equilibration period of 30 minutes, by which time SCC has stabilised, both the serosal and mucosal reservoirs were serially sampled at 10 minute intervals thus yielding results for 10 minute flux periods. 1ml samples were removed, the volume being replaced with unlabelled Krebs solution warmed to 37°C. 1ml samples of ^{22}Na were

collected in LP3 tubes and gamma emissions counted for 20 minutes in a Gamma Set 500 counter (ICN Instruments). Beta emissions from 0.8 ml samples of ^{36}Cl were measured by liquid scintillation counting in a 5ml volume of scintillant (NE260). Samples were counted for 20 minutes in a Beckmann LS-230 scintillation counter. All counts were corrected for background activity and unidirectional fluxes of Na and Cl were calculated using the following formula:-

$$\text{Ion flux } (\mu\text{equiv. hr}^{-1}.\text{cm}^{-1}) = \left(\frac{C_2}{P_2} - \frac{C_1}{P_1}\right) \frac{V}{At}$$

where - C_1 and C_2 = activities in consecutive samples from the unlabelled side (c.p.m.ml^{-1})

P_1 and P_2 = specific activities in consecutive samples from the labelled side (c.p.m.ml^{-1}.$\mu equiv^{-1}$.)

V = volume of bathing solution (mls.)

t = time interval between samples (hrs.)

A = area of tissue (cm^{-1}.)

3. Fluid Transport in Lumen Perfused Rat Colon *In Vivo*.

This part of the study was conducted in the laboratories of the Department of Pharmacology, Reckitt and Colman plc. A series of experiments were performed in which adrenoceptor regulation of fluid and NaCl absorption was examined in rat colon *in vivo* using a single pass lumen perfusion technique similar to that described by Bright-Asare and Binder (1973). It was thought that this technique would be more representative of the true state of passage of contents through the

lumen of the colon than a ligated loop preparation. Male Wistar rats (200-300g), either unfasted or 48 hour fasted, were anaesthetised with pentobarbitone sodium ($40mg.kg^{-1}$;i.p., $30mg.kg^{-1}$; s.c.) and a tracheostomy performed. Further s.c. administration of pentobarbitone sodium was given as required during the course of anaesthesia. Animals had received pre-treatment with indomethacin ($13mg.kg^{-1}$;i.p.) 60 minutes prior to the commencement of experimental procedures. This dose of indomethacin was empoloyed by Nakaki et al (1982b) to prevent the release of prostaglandins (which influence intestinal transport processes) as a result of mechancial stimulation. The abdomen was opened up by a midline incision and a length of colon (approximately 8cm) ligated proximally with polyethylene tubing and flushed out with 20 mls of warm isosmotic solution (composition ($g.l^{-1}$): NaCl 8.57, KCl 0.37 and glucose 1.0) from a 20 ml syringe. This physiological salt solution also served as the perfusion solution and has been employed previously in the investigation of intestinal fluid and electrolyte transport in the rat *in vivo* (Wahawisan et al, 1985). The distal end of the colonic segment was then cannulated with a further length of polyethylene tubing which was left extending for gravity drainage of the perfusate. Care was taken during cannulation not to impair the blood supply to the intestinal segment. The proximal cannula was connected to a Watson-Marlow peristaltic infusion pump set at a perfusion rate of 0.6 $ml.min^{-1}$.

The perfusion solution was pumped from a reservoir through coils of glass tubing placed in a heating water bath into the proximal cannula which was insulated with polystyrene for most of its length. The water bath temperature was adjusted so that the temperature of the perfusion solution as it entered the animal was 37±1°C. After the cannulation

procedure was completed the abdomen was sutured together, covered with gauze soaked in saline and left for a stabilisation period of 40 minutes. After this time samples of perfusate were collected over 20 minute collection periods. Fluid and electrolyte transport were determined on the basis of the concentration of a non-absorbable radioactive marker - ^{14}C- polyethylene glycol 4000 (^{14}C-PEG) in the influent and effluent perfustates. ^{14}C-PEG was added to all perfusion solutions so that there were approximately 10,000 to 12,000 c.p.m.ml^{-1} of perfusate. Beta emissions from 0.5 ml samples of influent and effluent solutions were counted in triplicate by liquid scintillation counting in 10 mls of scintillant (NE260) for 10 minutes on a Packard B2450 counter.

Na concentrations were measured by flame photometry using an Instrumentation Laboratories 943 flame photometer and Cl concentrations determined by titration against Ag ions with a Corning 925 chloride analyser. Net fluid, Na and Cl movement were calculated as follows:-

$$\text{Fluid transport } (\mu l.20\ min^{-1}.cm^{-1}) = \frac{PR\left(1 - \frac{(PEG_{In})}{(PEG_{Eff})}\right) \times 20}{L}$$

$$\text{Ion transport } (\mu equiv.20\ min^{-1}.cm^{-1}) = \frac{PR\left(I_{In} - \left(I_{Eff}\frac{(PEG_{In})}{(PEG_{Eff})}\right)\right) \times 20}{L}$$

where- PR = perfusion rate ($ml.min^{-1}$)

(PEG_{In}), (PEG_{Eff}) = ^{14}C-PEG c.p.m.0.5 ml^{-1} in influent and effluent solutions respectively.

L = colonic length (cm^{-1})

A positive value denotes net absorption and a negative value net secretion. Adrenergic agents were dissolved in dextrose and administered as a bolus dose via the femoral vein in experiments where they were employed. Details of experimental protocol can be found in the results section.

4. Statistical analysis

Statistical comparisons were made using paired or unpaired Student's 't' tests as indicated in the text. A number of results from the everted sac study were analysed using a one way analysis of variance technique. The principles of this technique are outlined in Appendix II and reasons for its use detailed in the text where appropriate.

5. Materials

The compounds employed in the present study were obtained from the following sources:-

Noradrenaline bitartrate	- Sigma
Propranolol hydrochloride	- Sigma
Corynanthine hydrochloride	- Sigma
Haloperidol	- Sigma
Isoproterenol	- Sigma
Phenylephrine hydrochloride	- Sigma

Somatostatin antagonist cyclo (7-Aminoheptanoyl L-phenylalanyl-D-tryptophyl-L-lysyl-threonyl [benzyl])	-	Sigma
Acetylcholine chloride	-	Sigma
Tetrodotoxin	-	Sigma
HEPES buffer	-	Sigma
Gluconic acid - sodium salt	-	Sigma
Gluconic acid - potassium salt	-	Sigma
Gluconic acid - calcium salt	-	Sigma
* Prazosin hydrochloride	-	Pfizer
* UK-14,304	-	Pfizer
* RX781094 (Idazoxan)	-	Reckitt & Colman
* RX811059A	-	Reckitt & Colman
* Cirazoline	-	Synthelabo
* Phentolamine mesylate	-	Ciba Geigy
Yohimbine hydrochloride	-	BDH
Theophylline hydrate	-	BDH
* BE2254	-	Biersdorf
* ICI 118551	-	ICI
* Practolol	-	ICI
* Salbutamol	-	Allen & Hanbury
* B-HT920	-	Boehringer Ingleheim
^{22}Na (200μCi.ml^{-1}) as aqeous NaCl	-	Amersham
^{36}Cl (113μCi.ml^{-1}) as aqeous NaCl	-	Amersham

^{14}C-Polyethylene glycol 4000 (50μCi.ml^{-1})	- Amersham
NE260 scintillant	- Nuclear Enterprises

* - denotes drugs generously donated as gifts

Stock solutions of prazosin (10^{-3}M) were dissolved in 5% glucose and 5% glycerol. Corynanthine (10^{-2}M) stock solution was dissolved in 10^{-2} ascorbic acid. BE2254 (10^{-2}M) was dissolved in 20% ethanol and haloperidol (10^{-2}M) in 1% tartaric acid. All other drugs were dissolved in distilled water.

RESULTS CHAPTER 1

ADRENOCEPTOR CONTROL OF FLUID AND ELECTROLYTE TRANSPORT IN EVERTED JEJUNAL SACS

Introduction.

The present study of adrenoceptor control of fluid and electrolyte transport in the rat jejunum began with an investigation of fluid transport using everted jejunal sacs. Antisecretory effects of adrenoceptor agonists in rat jejunum *in vivo* have been reported to be mediated via α_2-adrenoceptors (Bunce and Spraggs, 1983a; 1983b; Nakaki et al, 1982a; 1982b; Wahawisan, 1985). However in everted sacs of rat jejunum *in vitro* noradrenaline has been reported to stimulate basal levels of fluid absorption via an α_1-adrenoceptor mediated mechanism (Cotterell et al, 1983; Parsons et al, 1983). In the study of Cotterell et al (1983) it was of particular note that although the response to noradrenaline was interpreted as being mediated via α_1-adrenoceptors, the preferential α_1-adrenoceptor agonists phenylephrine and methoxamine (McGrath, 1982) were not observed to produce the same effects as noradrenaline. It was therefore considered worthwhile to further investigate adrenoceptor control of basal fluid and electrolyte transport and also to investigate adrenoceptor mechanisms under secretory conditions *in vitro*.

1. Effects of Noradrenaline on Fluid and Electrolyte Transport in Everted Sacs of Rat Jejunum.

The effects of noradrenaline on fluid and NaCl transport were initially investigated using single 16cm everted sacs of proximal jejunum taken 16-32cm from the pyloric sphincter and in adjacent 8cm sacs taken 16-24cm and 24-32cm from the pyloric sphincter. In adjacent sac experiments the proximal or distal sac alternately served as 'drug treated' with the adjacent sac serving as a control.

It is well established that the small intestine of the rat shows gradients in fluid transport along its length (Barry et al, 1961). Consequently for adjacent sac experiments it was first necessary to establish that the sacs exhibited similar levels of fluid and electrolyte transport. Preliminary experiments indicated that adjacent 8cm sacs did in fact exhibit similar levels of fluid and NaCl transport (table 2). However, large variations in transport levels were noted between individual animals; fluid transport ranging from 0.43 to 1.82g.g wet $wt^{-1}.hr^{-1}$.

Figs.4,5 and 6 show results for fluid, Na and Cl transport respectively from adjacent sac experiments where drug treated sacs contained varying concentrations of noradrenaline. Results are expressed as the means of the differences observed between adjacent sacs. Noradrenaline appeared to increase fluid, Na and Cl transport over the concentration range $2.5x10^{-6}M$ to $10^{-4}M$. However, only with concentrations of $2x10^{-5}M$ noradrenaline and above were there statistically significant increases in fluid and Na transport when

Parameter	Proximal Sac (n)	Distal Sac (n)	Mean Difference
Fluid Transport ($g.g.wet\ wt^{-1}.hr^{-1}$)	0.93±0.06 (22)	0.94±0.07 (22)	0.01±0.05
Na Transport ($\mu equiv.g.wet\ wt^{-1}.hr^{-1}$)	136±8 (22)	132±10 (22)	4±6
Cl Transport ($\mu equiv.g.wet\ wt^{-1}.hr^{-1}$)	76±5 (20)	69±7 (20)	7±5

Table 2. Resting fluid, Na and Cl transport in non-treated adjacent 8cm sacs of proximal jejunum.

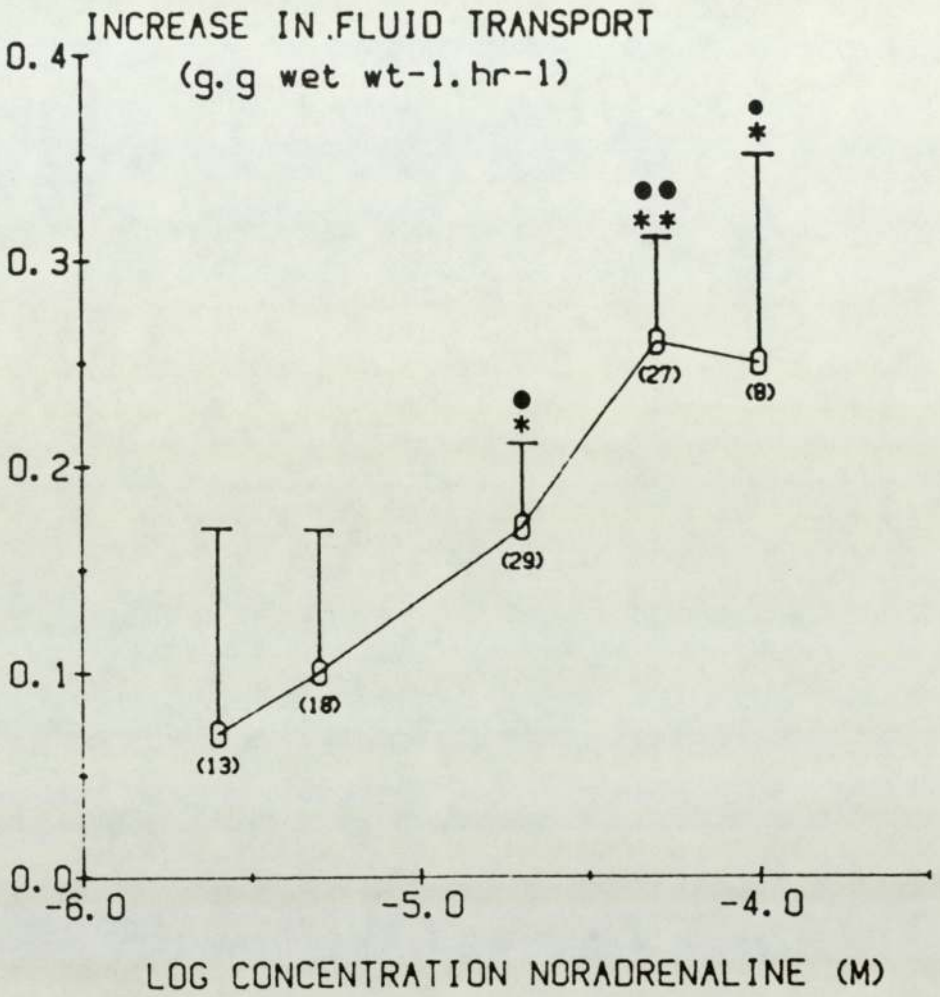

Fig 4. The effect of increasing concentrations of noradrenaline (2.5×10^{-6}M to 10^{-4}M) upon fluid transport (g.g wet $wt^{-1}.hr^{-1}$) in everted sacs of rat jejunum. Results are expressed as the means of individual adjacent sac differences (± S.E.M.) and were compared with control adjacent sac differences (0.01±0.05 g.g. wet $wt^{-1}.hr^{-1}$ (n=22)) using unpaired Student's 't'-tests (*) or one way anovar and 't'-test (●)(see Appendix II). NS:$p>0.05$; one symbol: $p<0.05$; two symbols: $p<0.01$. The number of values for each data point are shown in parentheses.

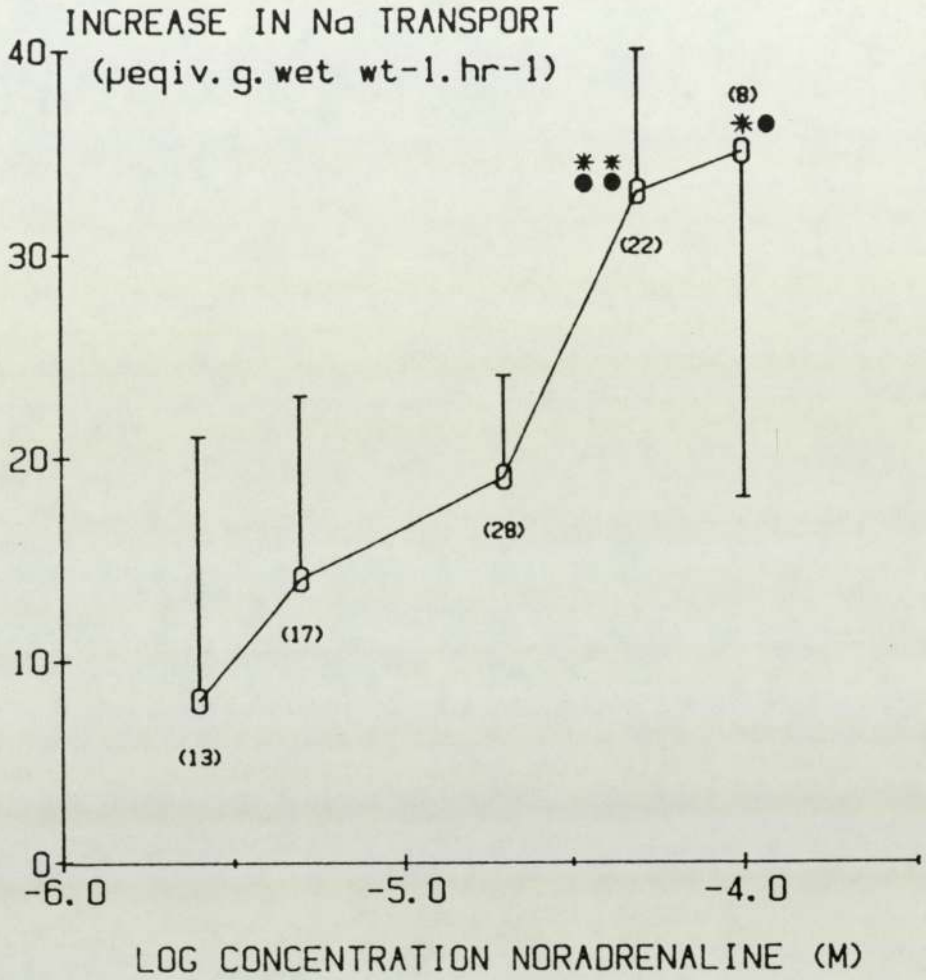

Fig 5. The effect of increasing concentrations of noradrenaline (2.5×10^{-6}M to 10^{-4}M) upon Na transport (μequiv.g wet wt^{-1}. hr^{-1}) in everted sacs of rat jejunum. Results are expressed as the means of individual adjacent sac differences (± S.E.M.) and were compared with control adjacent sac differences (4±6 μequiv.g wet $wt^{-1}.hr^{-1}$ (n=22))using unpaired Student's 't'-tests (*) or one way anovar and 't'-test (●) (see Appendix II). NS:$p>0.05$, one symbol:$p<0.05$, two symbols:$p<0.01$. The number of values for each data point are shown in parentheses.

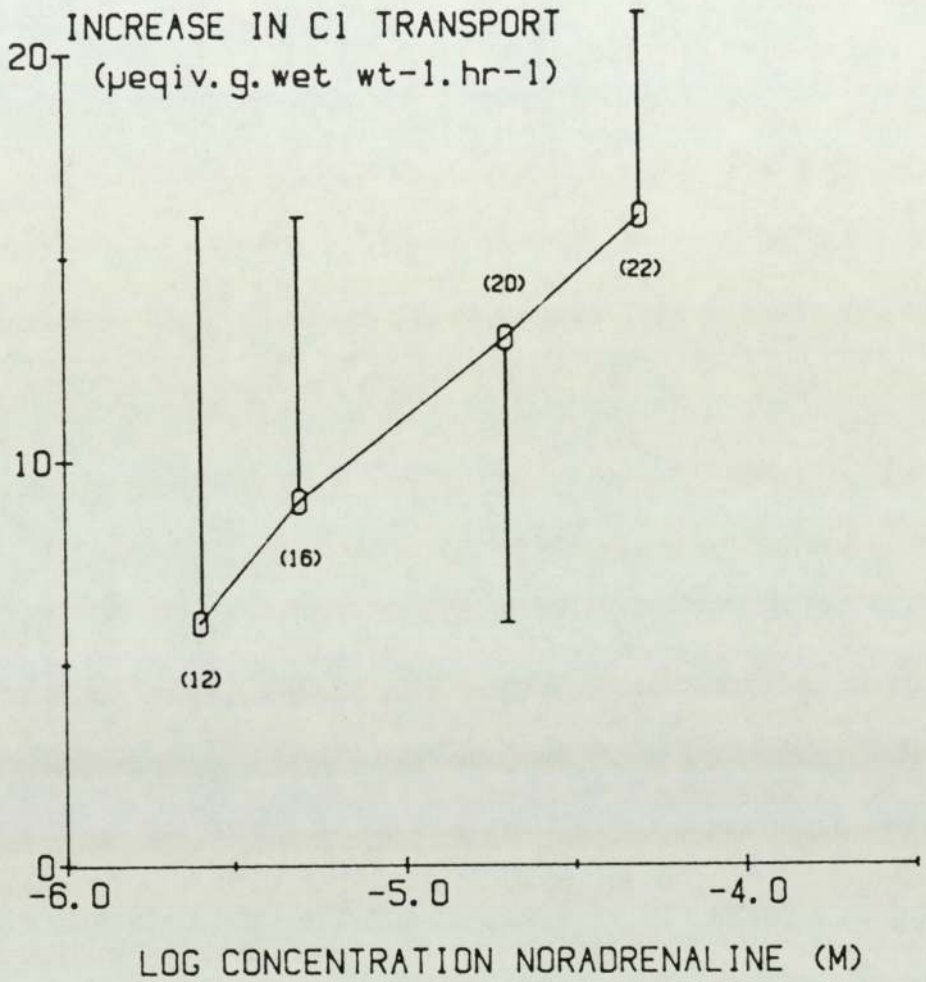

Fig. 6. The effect of increasing concentrations of noradrenaline (2.5×10^{-6}M to 5×10^{-5}M) upon Cl transport in everted sacs of rat jejunum. Results are expressed as the means of individual adjacent sac differences (± S.E.M.) and were compared with control adjacent sac differences (7±5 μequiv.g. wet wt^{-1}. hr^{-1} (n=20)) using unpaired Student's 't'-tests or one way anovar and 't'-test (see Appendix II). Noradrenaline did not significantly increase Cl transport at any point. The number of values for each data point are shown in parentheses.

compared to control adjacent sac differences. Although noradrenaline was seen to increase Cl transport, at no concentration was this significant.

In experiments employing single 16cm sacs no significant differences could be seen between levels of fluid, Na or Cl transport in control sacs and sacs containing $5x10^{-5}M$ noradrenaline (table 3) because of the large inter-animal variation in basal transport levels.

2. Interpretations and Implications of the Effects of Noradrenaline on Fluid and Electrolyte Transport in Everted Jejunal Sacs.

The initial results obtained from the present investigation highlighted the problem of inter animal variability in transport parameters in everted jejunal sacs and indicated that a paired sac protocol would have to be employed for further investigation of the stimulant effect of noradrenaline. In single 16cm sacs of rat jejunum no significant differences were observed between the means of control sacs and sacs incubated with $5x10^{-5}M$ noradrenaline for fluid, Na and Cl transport (table 3). However this concentration of noradrenaline appeared to produce a maximal effect upon fluid and Na absorption as determined by the paired sac protocol (figs.4 and 5).

Fluid transport levels in control 16cm sacs ranged from 0.77 to 1.67g.g wet $wt^{-1}.hr^{-1}$ and in noradrenaline ($5x10^{-5}M$) treated sacs from 0.53 to 1.55g.g wet $wt^{-1}.hr^{-1}$. It appeared that considerable variation in both the control and noradrenaline treated groups made the control group mean of similar magnitude to the noradrenaline group mean and this precluded the identification of a significant stimulant effect of noradrenaline. This type of problem has been reported previously by

Parameter	Control Sacs (n)	Treated Sacs (n)	Significance
Fluid Transport ($g.g.wet\ wt^{-1}.hr^{-1}$)	1.2±0.13 (8)	1.11±0.11 (8)	NS
Na Transport ($\mu equiv.g.wet\ wt^{-1}.hr^{-1}$)	149±17 (8)	137±14 (8)	NS
Cl Transport ($\mu equiv.g.wet\ wt^{-1}.hr^{-1}$)	71±10 (8)	64±9 (8)	NS

Table 3. Fluid, Na and Cl transport in single 16cm control and noradrenaline ($5x10^{-5}$ M) treated sacs. Statistical comparisons were made using unpaired Student's 't'-tests. Noradrenaline failed to induce a significant increase in any of the three parameters measured.

Crocker and Munday (1970) who used single 15cm jejunal sacs prepared from individual animals. Some animals had received pretreatment with 10ug of aldosterone and another group served as controls. Mean fluid transport was found to be similar in both groups of animals and the authors believed that this was due to high levels of fluid absorption in some animals in the control group. Further experiments showed that control levels of fluid transport could be reduced by bilateral adrenalectomy and nephrectomy but not by bilateral adrenalectomy alone. It was concluded that a factor was being produced by the kidney that caused high levels of fluid transport in some control animals. In a series of experiments performed with nephrectomised/adrenalectomised animals, pretreatment with 10μg aldosterone was clearly seen to stimulate fluid absorption. Crude kidney extract was also shown to stimulate jejunal fluid absorption, and the active principle was subsequently identified as angiotensin.

High levels of circulating angiotensin may have therefore been the reason for the high levels of fluid absorption observed in some control animals in the present investigation and one approach towards solving this problem may have been to perform a bilateral adrenalectomy/nephrectomy on each animal prior to experimentation or alternatively, to pretreat animals with an angiotensin converting enzyme inhibitor. However the former approach has not always proved successful in reducing basal levels of fluid transport (Willavoys, 1976) and as the paired sac protocol had allowed for the construction of concentration response curves for the stimulant effect of noradrenaline it was decided to proceed with the study using the paired sac approach.

Although the paired sac approach did improve the clarity of results it was apparent that data for the stimulant effect of noradrenaline was superimposed upon a large amount of intrinsic variation between the adjacent sacs. Differences in fluid transport between adjacent control sacs in a group of 22 animals ranged from 0.0 to 0.53 g.g wet $wt^{-1}.hr^{-1}$. compared with a mean maximum increase with $5x10^{-5}M$ noradrenaline of 0.26g.g wet $wt^{-1}.hr^{-1}$. This explains why only the highest concentrations of noradrenaline employed ($2x10^{-5}M$ or above) produced statistically significant increases in the transport of fluid and Na. Although Cl absorption appeared to increase with increasing concentrations of noradrenaline, the changes were not statistically significant at any of the concentrations of noradrenaline tested. A probable explanation for this observation is the lower resolution of the meter used for the measurement of Cl ions (by precipitation against silver ions) in comparison with the measurement of Na ions by flame photometry.

Statistical analysis of results was performed using Student's 't'-tests. Data points for adjacent sac differences obviously fell fairly evenly each side of the mean which permitted the application of parametric statistical techniques. In initial experiments, adjacent sac differences from the noradrenaline concentration-response curves for fluid, Na and Cl transport were compared with control adjacent sac differences using unpaired student's 't'-tests. Adjacent sac differences were also analysed using a one way analysis of variance technique (for details see appendix II) in an attempt to improve upon the resolution of Student's 't'-tests. This technique calculates an estimate of variability from a whole set of experimental data (e.g. the data for the noradrenaline concentration response curve), rather than on

individual means, and partitions it from any treatment effects. It was hoped that this technique would provide a more discriminating method for analising the data but in fact this was not the case because the calculated estimates of background variation were very large in comparison with the treatment effects and analysis with this technique proved similar to analysis with unpaired Students 't'- tests.

Alternative approachs to the problem of variability in the paired sac data were considered and it appeared that one possibility was to discard data where the paired control sac transport levels were high. This approach has been used by other workers (Willavoys, 1976). The rationale for this was that if a sac is transporting at a high level it might be operating at or near its maximal absorptive capacity and therefore the paired sac would be unable to show an increased absorption following application of exogenous noradrenaline. However, it was found that if data was discarded where control sac fluid transport exceeded 1.2g.g wet $wt^{-1}.hr^{-1}$ (this figure was simply chosen to serve as an example) that this made little difference to the pattern of results described thus far, and certainly not enough to warrant reproducing all the data here. It was also clear that the rationale for this approach was doubtful since a review of the original data showed that some noradrenaline treated sacs having a high paired control still exhibited a stimulatory response whereas some treated sacs having low paired control sac transport parameters did not show a stimulation in absorption in response to noradrenaline. Thus a further complication appeared to be a variable maximum capacity for absorption in tissues from individual animals. The underlying reason for this was not known and its investigation was thought to be outside the scope of this study.

Therefore, in the following experiments a paired sac protocol was employed with, in the case of noradrenaline, treated sacs containing $5x10^{-5}M$ noradrenaline alone or in combination with antagonist, and adjacent sacs serving as a control. Comparisons were made between the means of the differences from each group using unpaired Student's 't'-tests. Only the data for fluid transport has been presented as the Na data largely paralled the data for fluid transport without conveying any furthur useful information and Cl data contained a large amount of variability due to insufficient resolution of the chloride meter. A negative sign in front of an adjacent sac difference quoted in the text indicates that the compound under investigation has reduced, rather than stimulated fluid transport.

3. Noradrenaline Effects on Fluid Transport in the Presence of a Number of Adrenoceptor Antagonists.

The stimulant effect of the non-selective adrenoceptor agonist noradrenaline ($5x10^{-5}M$) on fluid transport was initially investigated in the presence of the selective α_1-adrenoceptor antagonist prazosin (Doxey et al, 1977) ($10^{-7}M$ and $10^{-5}M$) and the selective α_2-adrenoceptor antagonist idazoxan (Doxey et al, 1983) ($10^{-5}M$). It had previously been reported that prazosin inhibited the stimulatory effect of noradrenaline with an IC_{50} of $4.5x10^{-7}M$ (Cotterell et al, 1983). Consistent with this the results presented in fig.7 show that $5x10^{-5}M$ noradrenaline caused a significant increase in fluid absorption in the presence of prazosin ($10^{-7}M$) but not in the presence of prazosin ($10^{-5}M$). However, it was found on further investigation that $10^{-5}M$ prazosin alone significantly

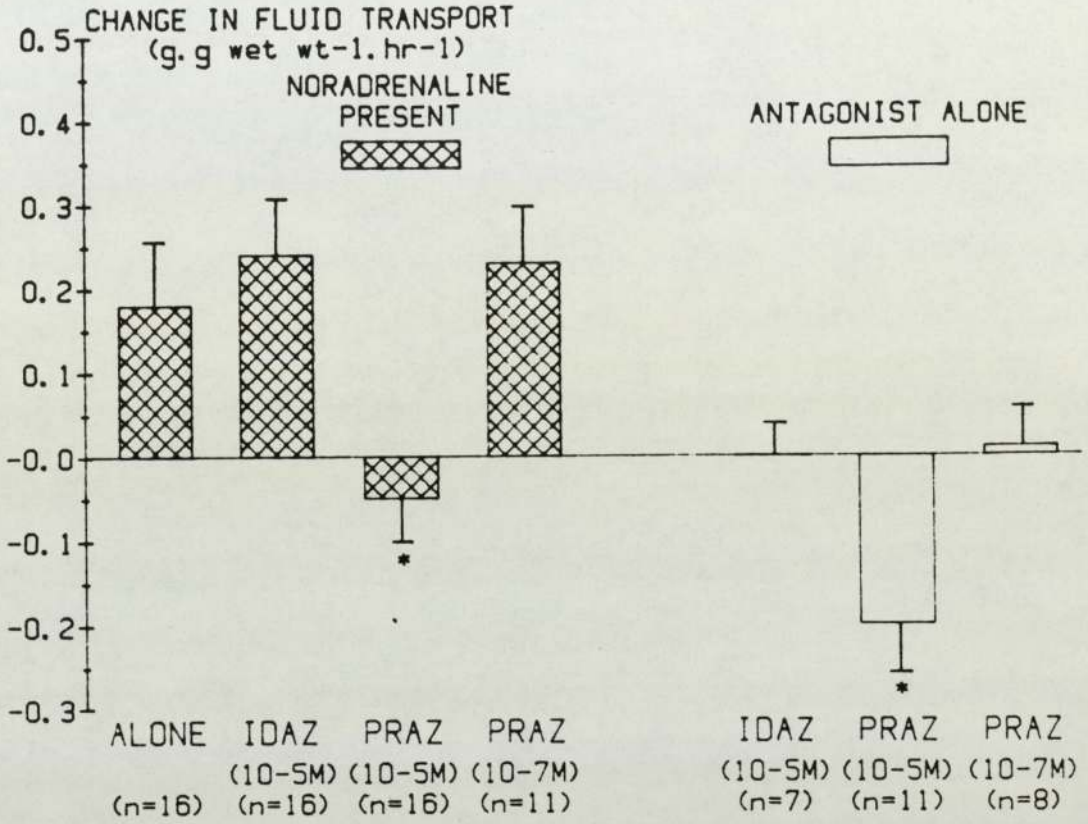

Fig 7. Changes in fluid transport induced by noradrenaline ($5x10^{-5}M$) alone and in the presence of prazosin (PRAZ) and idazoxan (IDAZ) in everted jejunal sacs. The noradrenaline response in the presence of antagonist was compared with the noradrenaline response alone, and the effects of antagonists alone were determined by comparing the antagonist response with differences in fluid transport observed between adjacent control sacs (0.01 ± 0.05 g.g. wet $wt^{-1}.hr^{-1}$.). Results are expressed as mean ± S.E.M. and all comparisons were made using unpaired Student's 't'-tests. * denotes $p<0.05$.

reduced basal levels of fluid transport whereas 10^{-7}M prazosin did not produce this effect (fig.7). This effect of prazosin could therefore account for its apparent inhibition of the stimulant effect of noradrenaline. Idazoxan (10^{-5}M) did not significantly affect the stimulant effect of noradrenaline or basal levels of fluid transport.

In the next series of experiments the effects of the α-adrenoceptor antagonist phentolamine (Furchgott, 1972), the β-adrenoceptor antagonist propranolol (Black et al, 1964), the selective α_2-adrenoceptor antagonist yohimbine (Starke et al, 1975) and the selective α_1-adrenocptor antagonists BE2254 (Gothert et al, 1981) and corynanthine (Shepperson et al, 1981) were determined upon the stimulant effect of noradrenaline ($5x10^{-5}$M). All of the antagonists were present in a concentration of 10^{-4}M. Results shown in fig.8 indicate that none of these antagonists at a concentration of 10^{-4}M significantly inhibited the stimulant effect of $5x10^{-5}$M noradrenaline although propranolol (10^{-4}M) did appear to reduce the effect of noradrenaline. Additionally, at this concentration none of the antagonists demonstrated any significant effect upon basal levels of fluid transport (fig.9).

Subsequently the effects of phentolamine (10^{-3}M), propranolol (10^{-3}M), the β-adrenoceptor antagonist timolol (Scriabine et al, 1973)(10^{-4}M), the dopamine antagonist haloperidol (Burt et al, 1976) (10^{-4}M) and a combination of the selective β_1-adrenoceptor antagonist practolol (Bieth et al, 1980) with the selective β_2-adrenoceptor antagonist ICI 118551 (O'Donnell and Wanstall, 1980) were investigated on the stimulant effect of noradrenaline ($5x10^{-5}$M) (fig.10). Only in the presence of propranolol (10^{-3}M) was there no significant stimulant effect of noradrenaline. However at this concentration propranolol did appear to reduce basal

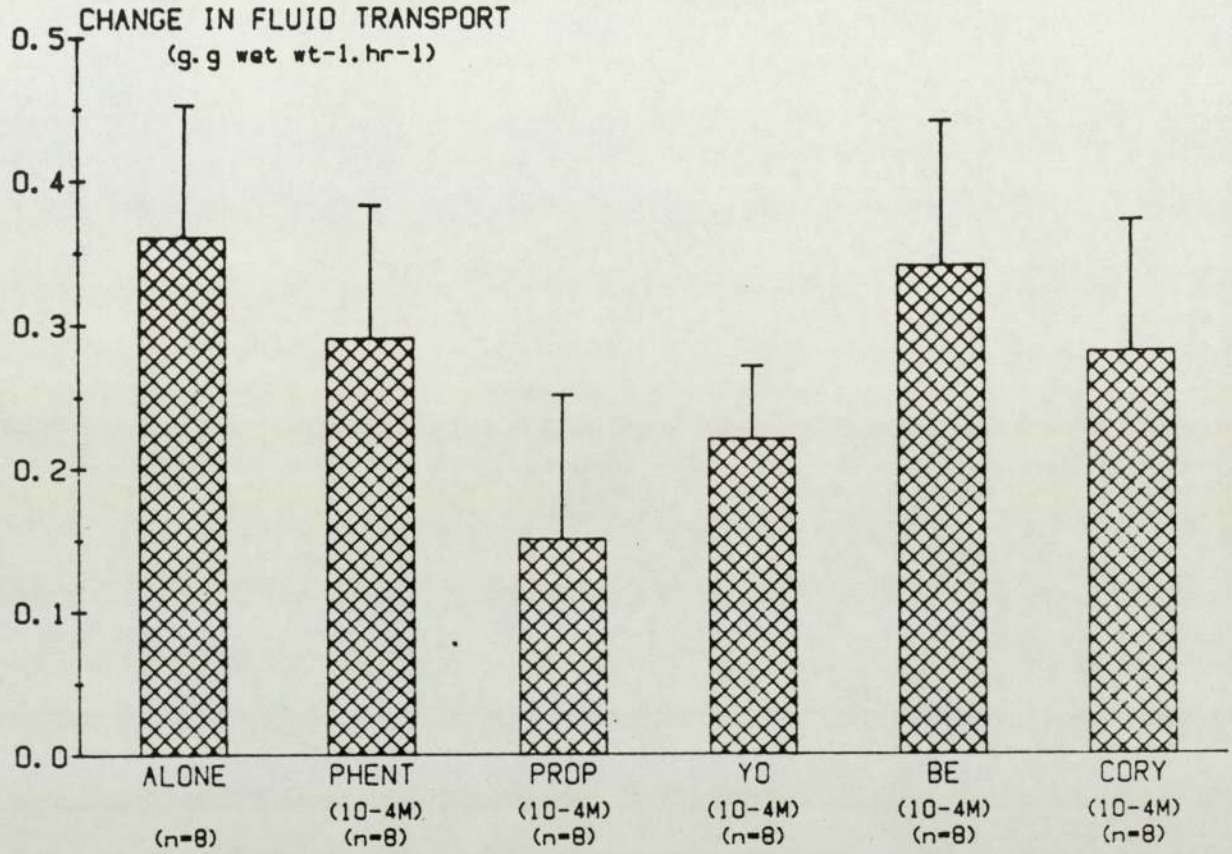

Fig 8. Changes in fluid transport induced by noradrenaline ($5x10^{-5}M$) alone and in the presence of phentolamine (PHENT), propranolol (PROP), yohimbine (YO), BE2254 (BE) and corynanthine (CORY) in everted jejunal sacs. For each treatment the antagonist concentration was $10^{-4}M$ and n=8. Results are expressed as mean ± S.E.M. The noradrenaline response in the presence of antagonist was compared with the noradrenaline response alone using unpaired Student's 't'-tests.

None of the antagonists tested produced any significant inhibition of the noradrenaline response.

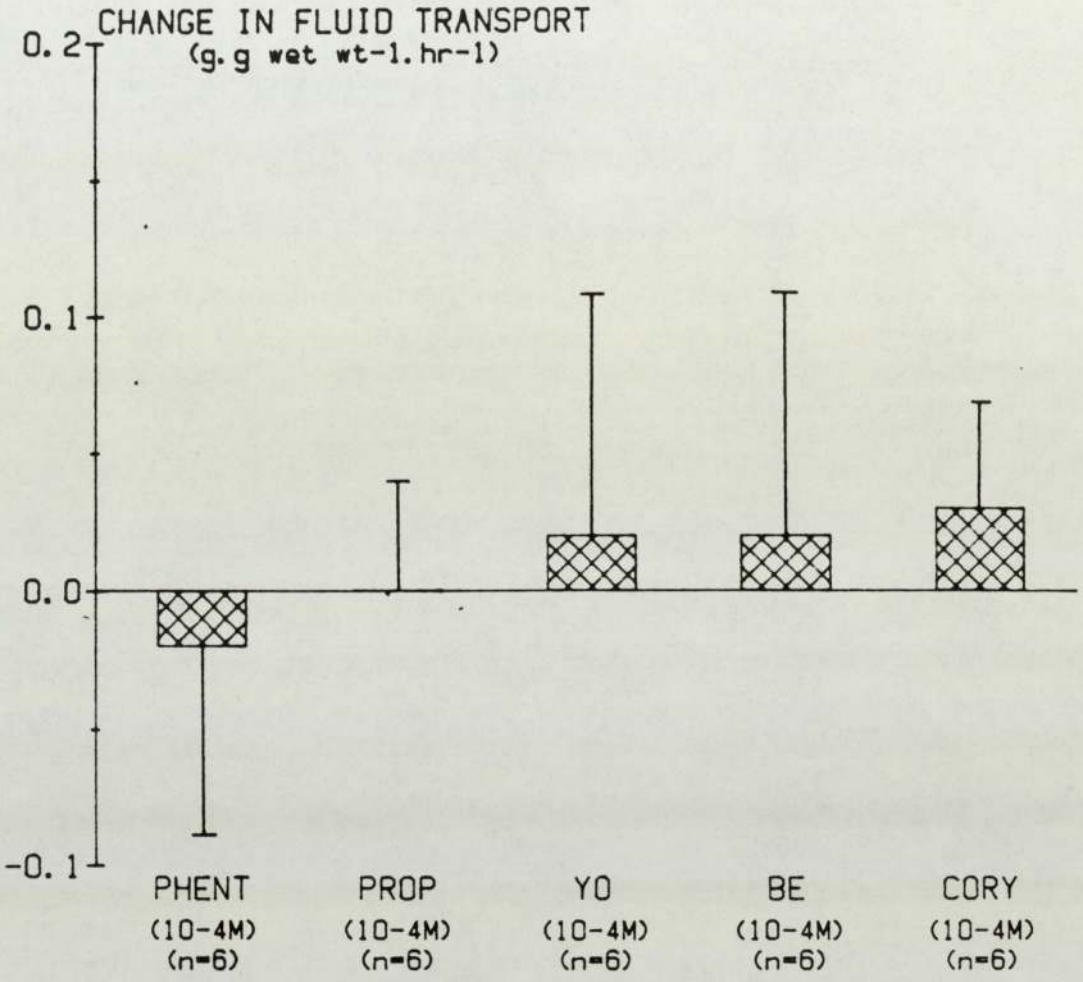

Fig 9. Changes in basal levels of fluid transport induced by phentolamine (PHENT), propranolol (PROP), yohimbine (YO), BE2254 (BE) and corynanthine (CORY) in everted jejunal sacs. For each treatment antagonist concentration was 10^{-4}M and n=6. Results are expressed as mean ± S.E.M. Adjacent sac differences from antagonist experiments were compared with transport differences between adjacent control sacs (0.01± 0.05 g.g. wet $wt^{-1}.hr^{-1}$.) using unpaired Student's 't'-tests. None of the antagonists tested produced any significant effect upon basal fluid transport.

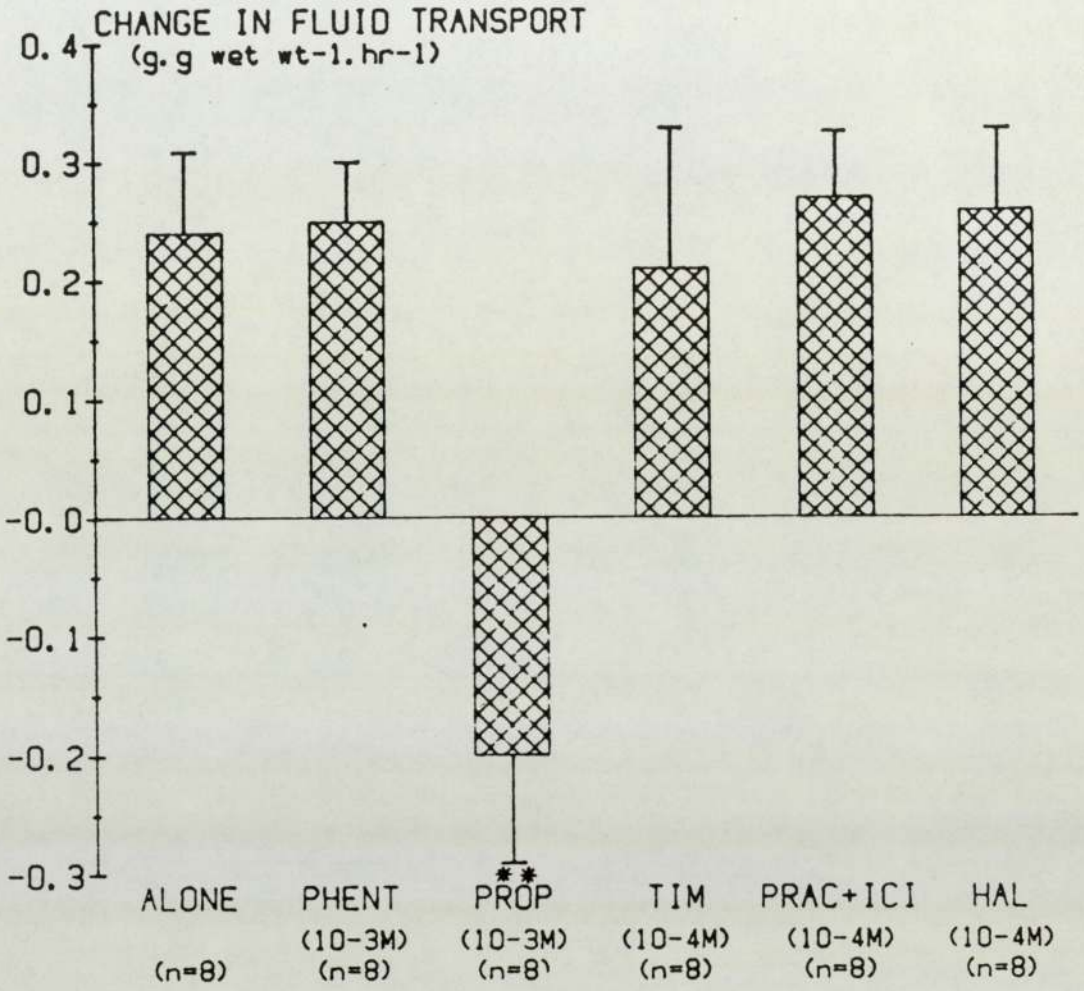

Fig. 10. Changes in fluid transport induced by noradrenaline ($5x10^{-5}$M) alone and in the presence of phentolamine (PHENT), propranolol (PROP), timolol (TIM), haloperidol (HAL) and practolol (PRAC) with ICI 118,551 (ICI) in everted jejunal sacs. Results are expressed as mean ± S.E.M. The noradrenaline response in the presence of antagonist was compared with the noradrenaline response alone by application of unpaired Student's 't'-tests. ** denotes $p<0.01$.

levels of fluid transport although this reduction was not quite significant at the 5% level (0.1>P>0.05). None of the other antagonists tested produced any significant effect on basal levels of fluid transport (fig.11). This part of the study has thus shown that of all the antagonists tested only prazosin (10^{-5}M) and propranolol (10^{-3}M) significantly inhibited the stimulant effect of noradrenaline on fluid absorption. However, at these concentrations both prazosin and propranolol alone apparently reduced basal levels of fluid absorption.

A small number of selected experiments were performed using the method of Parsons et al (1984). In these experiments test drugs were present only in the serosal bathing fluid and glucose was replaced by mannitol in the mucosal bathing fluid. In all experiments the incubation time was 30 minutes and results expressed in terms of dry weight. Increases in fluid transport in response to noradrenaline (10^{-3}M) alone and in the presence of phentolamine (10^{-3}M), BE2254 (10^{-4}M) and yohimbine (10^{-4}M) were determined. This concentration of noradrenaline was reported to produce maximal effects on fluid transport by Parsons et al (1984). The small number of results obtained from this study reinforced the findings of previous experiments since none of the antagonists tested inhibited the stimulant effect of noradrenaline. Because of the small number of experiments performed and the obvious presence of a response in each case, paired student's 't'-tests were applied to the data simply to confirm statistical significance (table 4).

The results presented in this section have consistently shown that at high concentrations, noradrenaline causes a significant stimulation of fluid absorption in everted sacs of rat jejunum. However, further investigation of this effect with selective adrenoceptor antagonists has

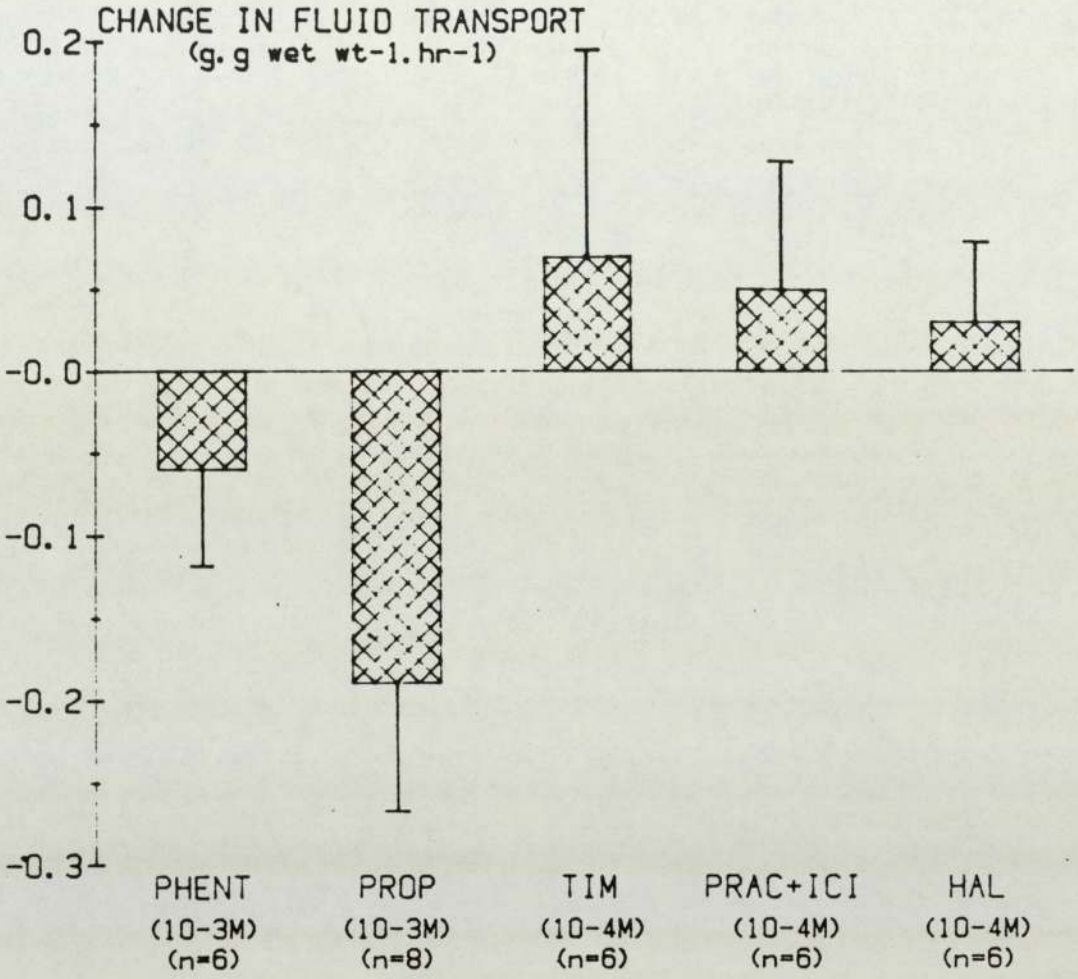

Fig. 11. Changes in basal levels of fluid transport induced by phentolamine (PHENT), propranolol (PROP), timolol (TIM), haloperidol (HAL) and practolol (PRAC) with ICI 118,551 (ICI) in everted jejunal sacs. Results are expressed as mean ± S.E.M. Adjacent sac transport differences in the presence of antagonist were compared with differences in fluid transport observed between adjacent control sacs (0.01±0.05 g.g. wet $wt^{-1}.hr^{-1}$.) using unpaired Student's 't'-tests. None of the antagonists tested produced any significant effect on basal levels of fluid transport.

Treatment	Treated	Control	P
NA alone	4.21±0.19	3.34±0.13	P<0.01
NA+PHENT (10^{-3} M)	4.08±0.77	2.96±0.62	P<0.01
NA+YO (10^{-4} M)	4.06±0.76	2.5 ± 0.51	P<0.01
NA+BE (10^{-4} M)	4.09±0.04	2.92±0.16	P<0.01

Table 4. Effects of phentolamine (PHENT), yohimbine (YO) and BE2254 (BE) upon noradrenaline (NA) (10^{-3} M) induced fluid absorption. Results are expressed as g.g dry wt^{-1}.30min^{-1}. n=4 for each treatment. Probability values were derived using paired Student's 't'-tests for each individual experiment.

not confirmed the suggestion that this is an α_1-adrenoceptor mediated event and the receptor which mediates the action of noradrenaline has not been identified. It is of note that the effect of noradrenaline was only shown at high concentrations and that it was also necessary to use high concentrations of antagonists. There must be doubt as to the selectivity of any of these compounds when used at such high concentrations and therefore caution is necessary in the interpretation of the results of the study in terms of adrenoceptor mechanisms.

4. Effects of Selective Adrenoceptor Agonists Upon Fluid Transport in Everted Sacs of Rat Jejunum.

Fig.12 shows changes in fluid transport induced by the preferential α_1-adrenoceptor agonists cirazoline (Roach et al, 1978) ($5x10^{-5}M$ and $5x10^{-4}M$) and phenylephrine ($10^{-3}M$), the selective α_2-adrenoceptor agonists UK-14,304 (Cambridge, 1981) (10^{-4} and $10^{-3}M$) and B-HT920 (Hammer et al, 1980) ($5x10^{-5}M$ and $5x10^{-4}M$) and the β-adrenoceptor agonist isoprenaline ($10^{-4}M$). Of all the agonists tested, only with a high concentration of cirazoline ($5x10^{-4}M$) was there a significant increase in fluid absorption when adjacent sac differences were compared with the difference in fluid absorption observed between adjacent control sacs. This observation taken together with the observed failure of phenylephrine to induce any change in fluid absorption again provides little support for the hypothesis that increases in fluid transport

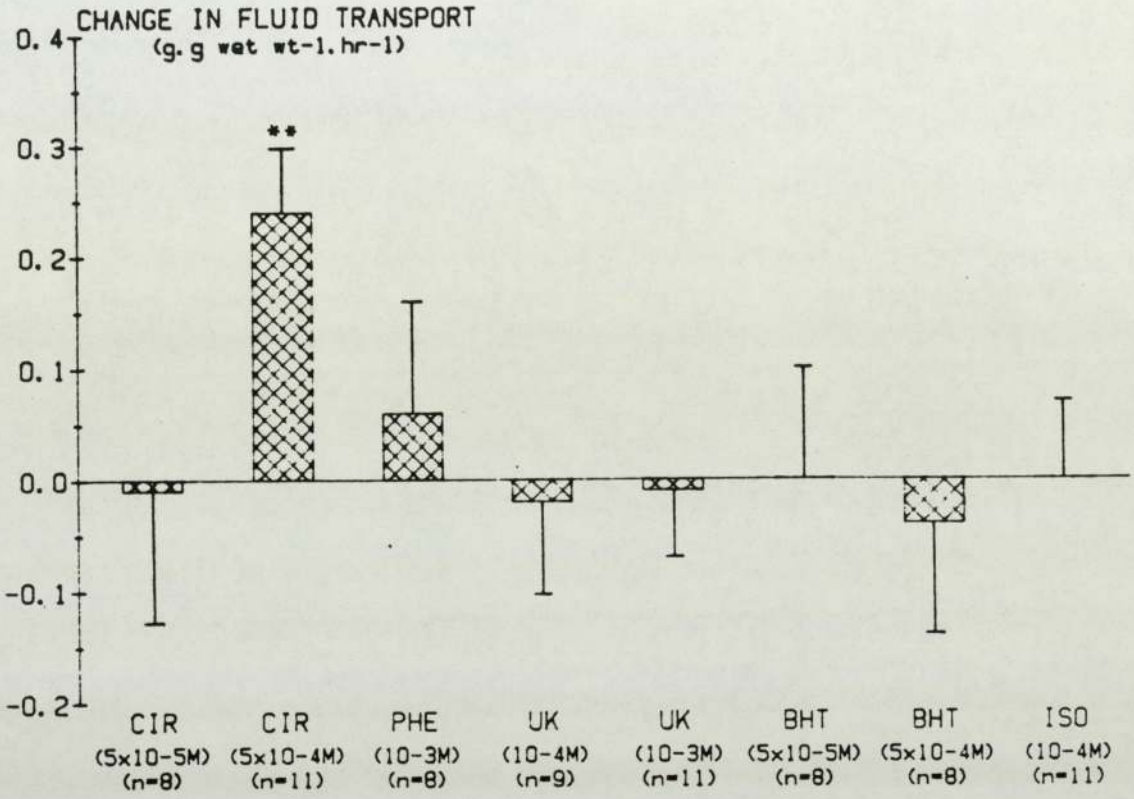

Fig. 12. Changes in fluid transport induced by cirazoline (CIR), phenylephrine (PHE), UK-14,304 (UK), B-HT 920 (BHT) and isoprenaline (ISO) in everted jejunal sacs. Results are expressed as mean ± S.E.M. Agonist effects were compared with differences in fluid transport observed between adjacent control sacs (0.01±0.05 g.g. wet $wt^{-1}.hr^{-1}$) using unpaired Student's 't'-tests. ** denotes $p<0.01$.

across everted sacs of rat jejunum under basal conditions are mediated through a simple α_1-adrenoceptor mediated mechanism.

5. The effects of adrenoceptor agonists on fluid absorption in theophylline treated everted sacs.

The purpose of this part of the study was to attempt to induce fluid secretion, or at least inhibit absorption, in everted sacs of rat jejunum *in vitro* using the secretagogue theophylline (see introduction section 3) and subsequently to examine the effects of adrenoceptor stimulation in the presence of theophylline. Secretagogue induced fluid secretion in rat jejunum *in vivo* has previously been reported to be reversed by specific α_2-adrenoceptor stimulation (Bunce and Spraggs, 1983a; 1983b; Nakaki et al, 1982a; 1982b).

Initial experiments investigated the effects of theophylline (10^{-3}M and10^{-2}M) upon basal levels of fluid transport. The paired sac protocol was again employed with treated sacs containing either 10^{-3}M or 10^{-2}M theophylline in both mucosal and serosal bathing solutions and the adjacent sac serving as a control. Both 10^{-2}M and 10^{-3}M theophylline caused a highly significant inhibition of absorption when adjacent sac differences were compared with control adjacent sac differences (0.01 ± 0.05g.g wet $wt^{-1}.hr^{-1}$.) using unpaired student's 't'-tests. Adjacent sac difference for 10^{-2}M theophylline was -0.5 ± 0.05g.g wet wt-1.hr^{-1}. (n=8), $P<0.001$ versus control sac differences, and for 10^{-3}M

theophylline was -0.34±0.1g.g wet $wt^{-1}.hr^{-1}$. (n=7), $P<0.01$ versus control sac differences.

Fig.13 shows the results of experiments where both adjacent sacs contained theophylline (either 10^{-3}M or 10^{-2}M) and one sac contained either cirazoline, UK-14,304 or noradrenaline, thus serving as a paired drug treated sac. Results clearly indicated that none of the agonists tested were able to induce a significant absorptive effect in theophylline treated sacs when compared to the adjacent sac containing theophylline alone using paired student's 't'-tests. Thus, the α_2-adrenoceptor mediated antisecretory effect upon fluid absorption observed *in vivo* is not reproducible in this *in vitro* preparation.

6. Interpretation of pharmacological data from the everted sac study and its implications.

The object of this study was to investigate adrenoceptor mechanisms mediating increases in fluid absorption in everted sacs of rat jejunum *in vitro* under both basal and secretory conditions.

While the results have confirmed that high concentrations of noradrenaline can stimulate fluid absorption in everted sacs, they provide little if any evidence to support the proposals of Cotterell et al (1983) and Parsons et al (1983) that noradrenaline stimulates basal levels of fluid absorption via an α_1-adrenoceptor mediated mechanism. The conclusions of Cotterell et al (1983) were almost entirely based on the observation that prazosin inhibited the stimulant effect of noradrenaline with an IC_{50} of $4.5x10^{-7}$M. It is therefore interesting that another selective, and potent α_1-adrenoceptor antagonist WB4101

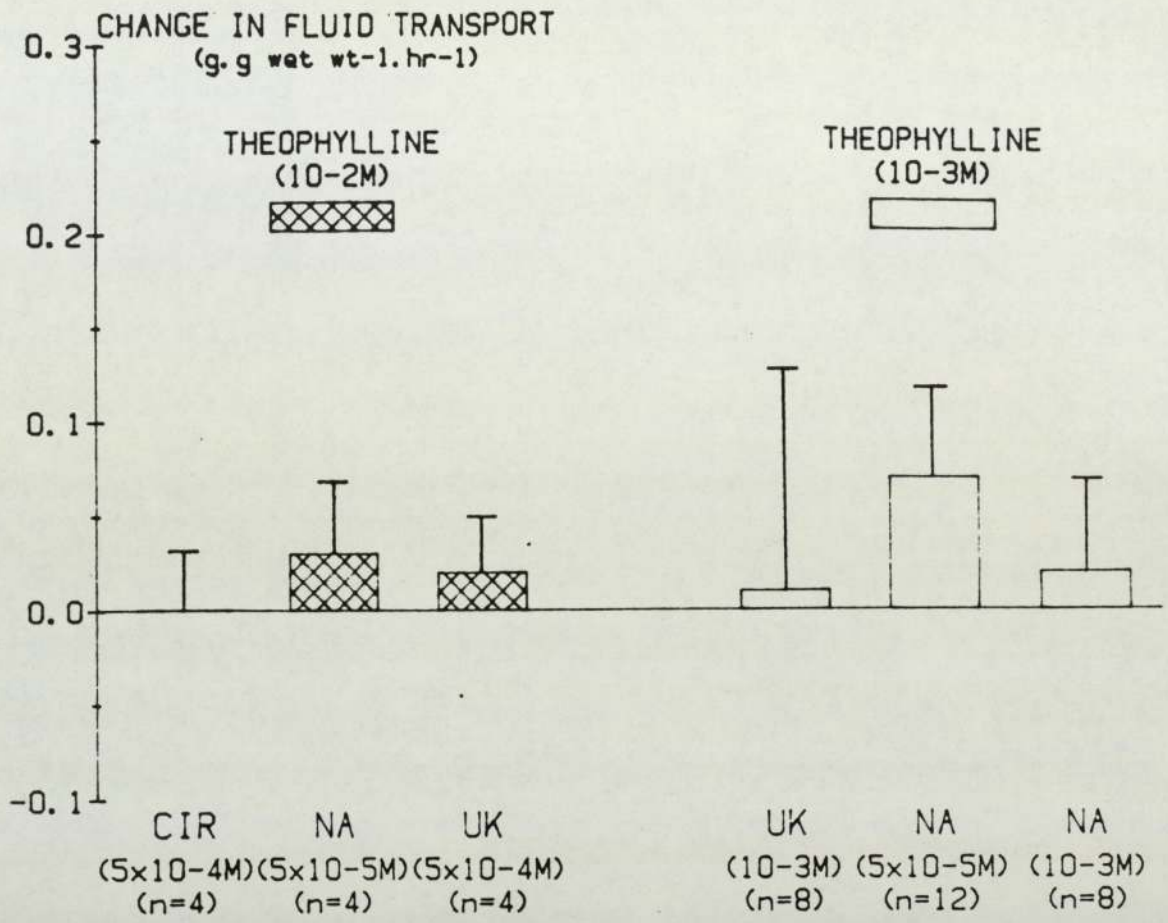

Fig. 13. Effects of varying concentrations of noradrenaline (NA), UK-14,304 (UK) and cirazoline (CIR) upon the antiabsorptive effects of theophylline (10^{-3}M and 10^{-2}M) in everted jejunal sacs. Results are expressed as mean adjacent sac differences (± S.E.M.) with treated sacs containing drug + theophylline and control sacs containing theophylline alone. Adjacent sac differences for each experiment were analysed using Student's 't'-tests for paired data. None of the agonists tested produced a significant response.

(Drew, 1982) which was also used in Cotterell's study was reported to have a much higher IC_{50} of $1.7x10^{-5}M$. Indeed, in a subsequent publication from the same group (Munday and Poat, 1983) the IC_{50} for WB4101 in this preparation was reported to be $1.7x10^{-4}M$. Cotterell et al (1983) also reported that the preferential α_1-adrenoceptor agonists phenylephrine and methoxamine failed to stimulate fluid absorption.

The results obtained in the present study were qualitatively similar to those obtained by Cotterell et al (1983). Thus prazosin ($10^{-5}M$) completely inhibited the stimulant effect of noradrenaline ($5x10^{-5}M$) upon fluid absorption and phenylephrine ($10^{-3}M$) failed to stimulate fluid absorption. However the selective α_1-adrenoceptor antagonists BE2254 and corynanthine failed to inhibit the stimulant effect of noradrenaline, as indeed did the non-selective α-adrenoceptor antagonist phentolamine, whereas prazosin alone ($10^{-5}M$) reduced basal levels of fluid transport. This property of prazosin could then account for its apparent inhibition of the stimulant effect of noradrenaline. Indeed it has been previously reported that prazosin alone inhibits absorption of NaCl across isolated sheets of rabbit ileum mounted in Ussing chambers *in vitro* (Burgess et al, 1984). Prazosin is known to inhibit phosphodiesterase activity producing a concomitant increase in cAMP levels (Sands and Jorgensen, 1979). It is therefore possible that this mechanism accounts for the prazosin induced inhibition of absorption in jejunal sacs since the antiabsorptive effects of cAMP are well documented (see introduction section 3). Parsons et al (1983) also reported that the α_1-adrenoceptor antagonist indoramin (Rhodes and Waterfall, 1978) like prazosin, inhibited the stimulant effect of noradrenaline in this preparation and interpreted this as evidence in

support of the hypothesis that α_1-adrenoceptors mediate increases in fluid absorption. However such a conclusion must be viewed with caution since indoramin is well known to have a number of pharmacological actions which are unrelated to its α_1-adrenoceptor blocking properties, and as a result is not commonly used in studies of adrenoceptor classification. It is interesting that one of these properties is a membrane stabilising action (Alps et al, 1971) similar to that observed with propranolol (Coltart et al, 1971).

In the present study the β-adrenoceptor antagonist propranolol (10^{-3}M) significantly inhibited the stimulant effect of noradrenaline, but as with prazosin, the same concentration of the antagonist alone appeared to inhibit basal levels of fluid absorption. It is unlikely that the effects of propranolol at this concentration were related to β-adrenoceptor blockade as the β-adrenoceptor antagonists timolol (10-4M) or a combination of practolol (10^{-4}M) and ICI 118551 (10^{-4}M) failed to produce any significant effects upon basal fluid transport. It is conceivable that the inhibitory effects of propranolol may have been a consequence of its its membrane stabilizing properties. A similar explanation might also account for the previously reported inhibitory influence of indoramin upon noradrenaline responses in this preparation. The possibility of high concentrations of noradrenaline stimulating absorptive processes via a dopaminergic mechanism was precluded by the observation that the dopamine antagonist haloperidol did not affect the stimulatory response to noradrenaline.

Agonist data also failed to lend much support to the proposal that α_1-adrenoceptors mediate increases in fluid transport. Only high concentrations ($5x10^{-4}$M) of the selective α_1-adrenoceptor agonist

cirazoline caused a stimulation of fluid absorption and phenylephrine failed to stimulate fluid absorption at a concentration of 10^{-3}M and Cotterell et al (1984) suggested that phenylephrine was ineffective in stimulating jejunal fluid absorption because it has a low efficiacy at transporting epithelia. However phenylephrine produces a full response at α_1-adrenoceptors mediating Na transport from proximal kidney tubules (Hesse and Johns, 1984).

Nakaki et al (1982a; 1982b) and Bunce and Spraggs (1983a; 1983b) reported that specific α_2-stimulation reversed secretagogue induced fluid secretion in the rat jejunum *in vivo*. In the present study theophylline was employed as a secretagogue to reduce absorption in everted sacs of rat jejunum *in vitro*, which it did effectively. Noradrenaline, cirazoline and the selective α_2-adrenoceptor agonist UK-14,304 all failed to reverse the antiabsorptive effects of theophylline in everted sacs *in vitro*, in contrast to the reported findings *in vivo*.

Obviously some explanations need to be offered as to why the stimulatory effects of noradrenaline *in vitro* cannot be reconciled with any receptor subtype and also as to why α_2-adrenoceptor stimulation *in vitro* failed to reverse the antiabsorptive effects of theophylline.

The everted sac technique has been criticised by a number of workers. Munck (1972a) provided evidence that inadequate serosal oxygenation brought about a state of anoxia in crypt regions which compromised secretory function in these areas. Munck (1972b) also demonstrated that the kinetics of intestinal absorption in everted sacs does not describe the transport function of a single membrane, largely because of the presence of connective tissue and smooth muscle layers which present a

considerable diffusion barrier thus antagonists may not have been able to block the stimulant effects of noradrenaline and indeed agonists unable to stimulate absorptive processes because of difficulties in accessing the receptor site from the serosal bathing fluid through the muscle and connective tissue layers. Noradrenaline has previously been shown to be inactive when present in the mucosal bathing solution alone confirming (Parsons et al, 1984) that adrenoceptor compounds can only access the receptor site from the serosal bathing solution. This is due to the location of adrenoceptors which are found on basolateral enterocyte membranes (Nakaki et al, 1983; Chang et al 1983; Cotterell et al, 1984).

Perhaps an alternative explanation for the ineffectiveness of adrenoceptor antagonists against noradrenaline is that anoxic conditions, due to poor serosal oxygenation, may cause some alteration in the receptor conformation so that although they can still respond to high concentrations of the natural agonist noradrenaline, they do not demonstrate the normal characteristics of the α_1 or α_2-adrenoceptor subtypes. This may also account for the observed inactivity of the selective α_1 and α_2-adrenoceptor agonists employed in this study.

If secretory function is compromised in the everted sac preparation then it may be assumed that the effects of theophylline are predominantly antiabsorptive. The observed effects of theophylline were apparently irreversible in this *in vitro* preparation, in contrast to observations *in vivo* (Bunce and Spraggs, 1983a; 1983b). This infers that receptor function is impaired in the everted sac preparation or that the effects of theophylline are only slowly reversible. Alternatively antiabsorptive influences of theophylline upon electrolyte movement *in*

vivo may be insignificant when compared to the stimulation of 'adrenoceptor reversible' secretory processes which are compromised in the everted sac preparation.

In summary it cannot be concluded from the present investigation that α_1-adrenoceptors do not play a role in the consistently observed stimulation of fluid absorption by noradrenaline in the rat jejunum. It does seem however, that the everted sac preparation is not appropriate for the study of adrenoceptor subtypes involved in mediating absorptive and/or secretory processes in the rat jejunum.

RESULTS CHAPTER 2

ADRENOCEPTOR CONTROL OF ELECTROLYTE TRANSPORT ACROSS JEJUNAL SHEETS

A. ELECTROGENIC TRANSPORT MECHANISMS

Introduction.

The experiments described in the previous chapter using the everted intestinal sac method had clearly shown that noradrenaline caused a stimulation of fluid transport but did not provide any clear insight into the role of adrenoceptor subtypes in this absorptive action. Because of the problems experienced with the everted sac technique it was decided to extend the investigation of adrenoceptor control of electrolyte transport processes *in vitro* by using the Ussing chamber preparation. Experiments described in this section were performed with the type I Ussing chamber design (see methods section 2c) with manual compensation for fluid resistance.

Active transport mechanisms for Na, Cl and HCO_3^- have been discussed in the introduction (pages 14-17) and are summarized in fig.1 (page 15). Electrogenic Na absorption and electrogenic Cl and HCO_3^- secretion make the mucosal side of the tissue negative with respect to the serosal side. In the Ussing chamber technique spontaneous transepithelial potential difference (PD) was nullified by manually adjusted short-circuit current (SCC). It is commonly accepted that decreases in SCC in response to application of neurotransmitters or neuroendocrine factors in the mammalian small intestine *in vitro* reflect decreases in electrogenic secretory processes. Decreases in transepithelial PD and

SCC across rabbit ileum *in vitro* in response to α_2-adrenoceptor stimulation have previously been directly correlated with decreases in electrogenic Cl secretion (Chang et al, 1982; Durbin et al, 1982).
In this part of the study changes in SCC in response to adrenoceptor agents were monitored as a reflection of changes in anion secretory processes.

1. <u>The Effects of Noradrenaline Alone and in the Presence of a Series of Adrenoceptor Antagonists Upon Basal Transepithelial PD and SCC.</u>

Control tissues showed that SCC (and PD) gradually declined with time, most obviously over the first 30-40 minutes after tissue mounting (fig 14). The gradient and time course of this initial decline varied from tissue to tissue.

Initial experiments were performed to determine the effects of noradrenaline on resting transepithelial SCC and PD across jejunal sheets bathed with Krebs solution. Preliminary experiments showed that addition of noradrenaline to the serosal bathing medium of jejunal sheets 40 minutes after tissue mounting caused an immediate sustained fall in SCC and PD towards zero. This effect was just maximal with a concentration of $5x10^{-6}$M noradrenaline although actual changes recorded across tissues presenting an area of only $0.64cm^2$ were too small to construct accurate concentration- response curves. Tissues having a basal SCC of less than 10 $\mu amps.cm^{-2}$ were discarded. Noradrenaline ($5x10^{-6}$M) caused falls in SCC and PD of -22 ± 4 $\mu amps.cm^{-2}$ and -0.5 ± 0.1mV. respectively which were maximal within 3 minutes of addition (fig.14). These changes in SCC and PD were highly significant ($P<0.001$

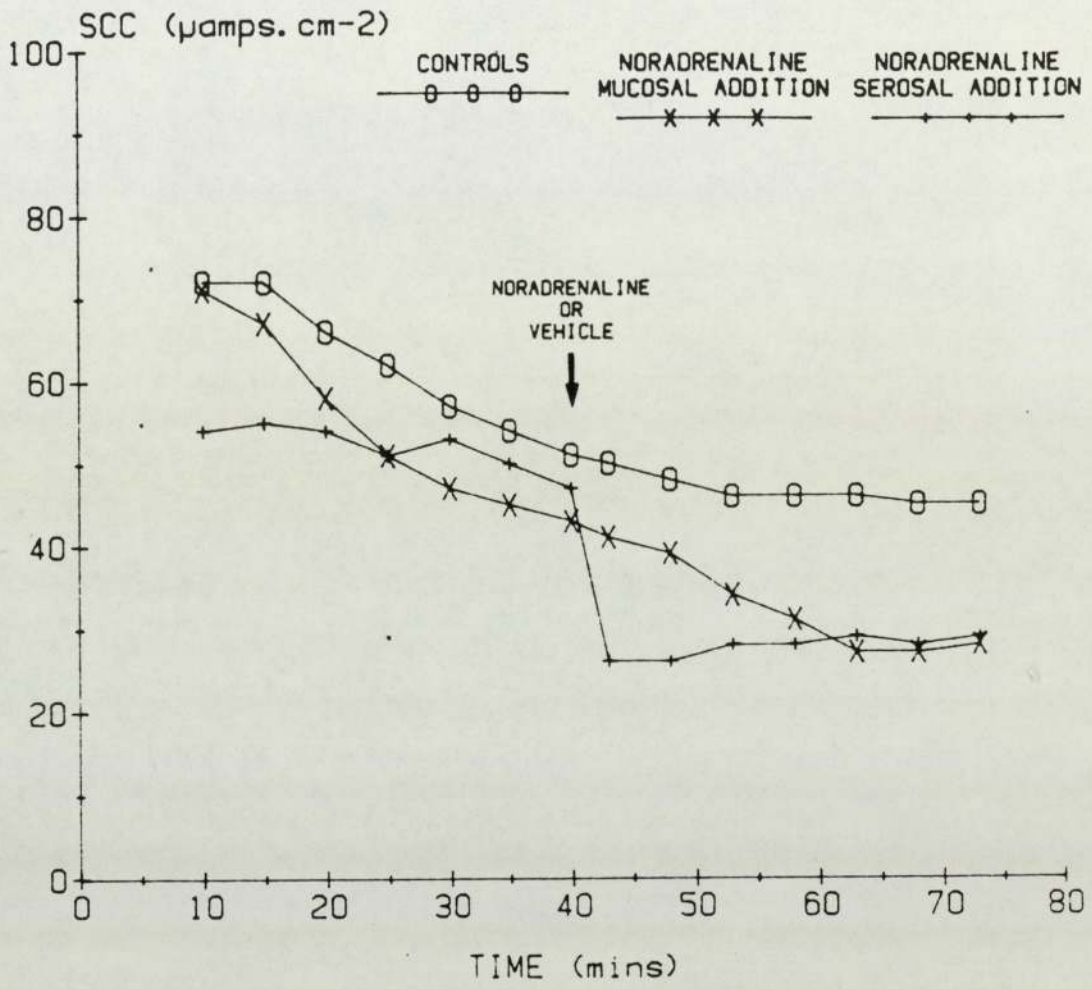

Fig. 14. Effect of serosal or mucosal addition of noradrenaline ($5x10^{-6}M$) on basal SCC. Serosal addition of noradrenaline was seen to induce an immediate sustained fall in SCC of $-22\pm4\mu amps.cm^{-2}$ (n=5) whilst addition of noradrenaline to the mucosal bathing solution did not elicit an immediate response (n=3). Control tissues received addition of distilled water containing ascorbic acid ($10^{-4}M$) to the serosal bathing medium (n=5).

and $P<0.01$ respectively) when compared with the changes in SCC and PD seen in control tissues (-1 ± 1 μamps.cm^{-2} and 0.0 ± 0.1mV (n=5) over the same 3 minute period. Addition of noradrenaline (5×10^{-6}M) to the mucosal bathing solution did not produce an immediate response although it did appear to perhaps produce a more gradual decline in SCC (fig.14); possibly reflecting diffusion of noradrenaline through to the serosal bathing medium. Addition of distilled water containing ascorbic acid (10^{-4}M), representative of vehicle addition, to the serosal bathing medium did not produce any change in SCC.

In subsequent experiments SCC was used as a marker of the noradrenaline response and the maximum change in SCC over the 3 minute period following addition of noradrenaline was recorded as the noradrenaline response. Fig.15 shows changes in SCC induced by serosal addition of noradrenaline (5×10^{-6}M) alone and in the presence of a number of adrenoceptor antagonists. Antagonists were added to the serosal bathing medium in 50μl or 100μl volumes 30 minutes after tissue mounting. Noradrenaline was added 10 minutes later. Prior addition of the α-adrenoceptor antagonist phentolamine (5×10^{-6}M) completely inhibited the SCC response to noradrenaline whilst noradrenaline was still seen to produce a change in SCC (-12 ± 2 μamps.cm^{-2} (n=5)) in the presence of the β-adrenoceptor antagonist propranolol (10^{-4}M). Fig. 15 clearly shows that the response to 5×10^{-6}M noradrenaline was significantly inhibited by the selective α_2-adrenoceptor antagonists idazoxan (10^{-6}M) and yohimbine (5×10^{-6}M) but not significantly affected by the selective α_1-adrenoceptor antagonists prazosin and corynanthine in concentrations up to 2.5×10^{-5}M and 10^{-4}M respectively. The SCC

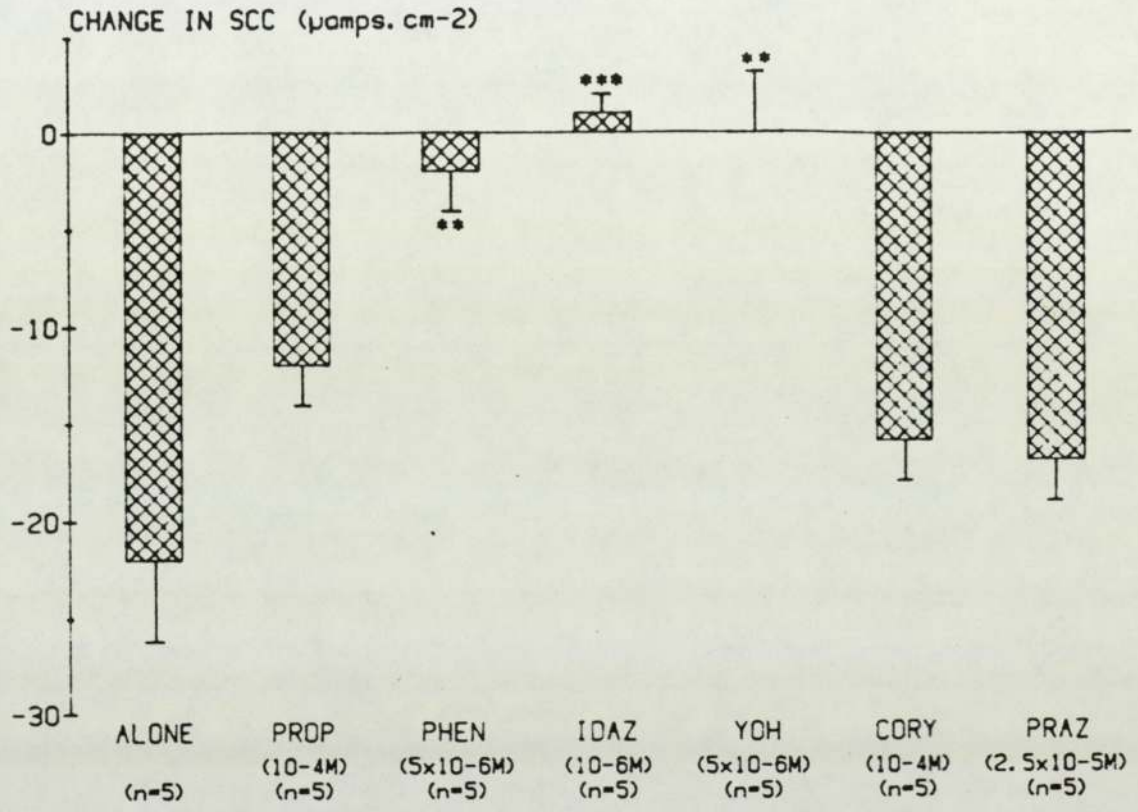

Fig. 15. Changes in SCC induced by serosal addition of noradrenaline ($5x10^{-6}$M) alone and in the presence of propranolol (PROP), phentolamine (PHEN), idazoxan (IDAZ), yohimbine (YOH), corynanthine (CORY) and prazosin (PRAZ). Results are expressed as mean ± S.E.M. SCC responses to noradrenaline in the presence of antagonist were compared to the noradrenaline alone response using unpaired Student's 't'-tests. Antagonists were added in a 50 or 100μl volume 30 minutes after tissue mounting and 10 minutes prior to the addition of noradrenaline. Two symbols:$p<0.01$, three symbols:$p<0.001$.

response to noradrenaline in the presence of propranolol (10^{-4}M) (-12±2 μamps.cm^{-2}) was considerably, but not significantly (P=0.06) lower than the SCC response to noradrenaline alone (-22±4 μamps.cm^{-2}). In all probability this was due to the ability of propranolol alone to induce an immediate, sustained fall in SCC (-15±3 μamps.cm^{-2} (n=5)) over the period 30-35 minutes after mounting), an effect observed with concentrations of propranolol as low as $5x10^{-7}$M and discussed further in section 7 of this chapter. Of the antagonists tested in this part of the investigation, propranolol was the only one to cause any obvious change in SCC when added alone.

2. Effects of UK-14,304 and phenylephrine on basal SCC.

Experiments were performed to examine changes in SCC in response to the selective α_2-adrenoceptor agonist UK,14-304 and the preferential α_1-adrenoceptor agonist phenylephrine. Agonist responses were also examined in the presence of idazoxan and corynanthine (fig.16). Again antagonists were added 30 minutes after tissue mounting to the serosal bathing medium and agonists were added 10 minutes later. UK-14,304 caused an immediate decrease in SCC and the maximum decrease of -18±1 μamps.cm^{-2} (n=5) was observed with a concentration of 10^{-7}M. Only a high concentration of phenylephrine, (10^{-4}M), was able to induce a decrease in SCC (-17±3 μamps.cm^{-2} (n=5)). The response to UK-14,304 (10-7M) was inhibited by idazoxan ($5x10^{-6}$M) but not significantly affected by corynanthine (10^{-4}M). Similarly decreases in SCC induced by phenylephrine (10^{-4}M) were inhibited by idazoxan ($5x10^{-7}$M) but not significantly affected by corynanthine (10^{-4}M). Phenylephrine (10^{-4}M)

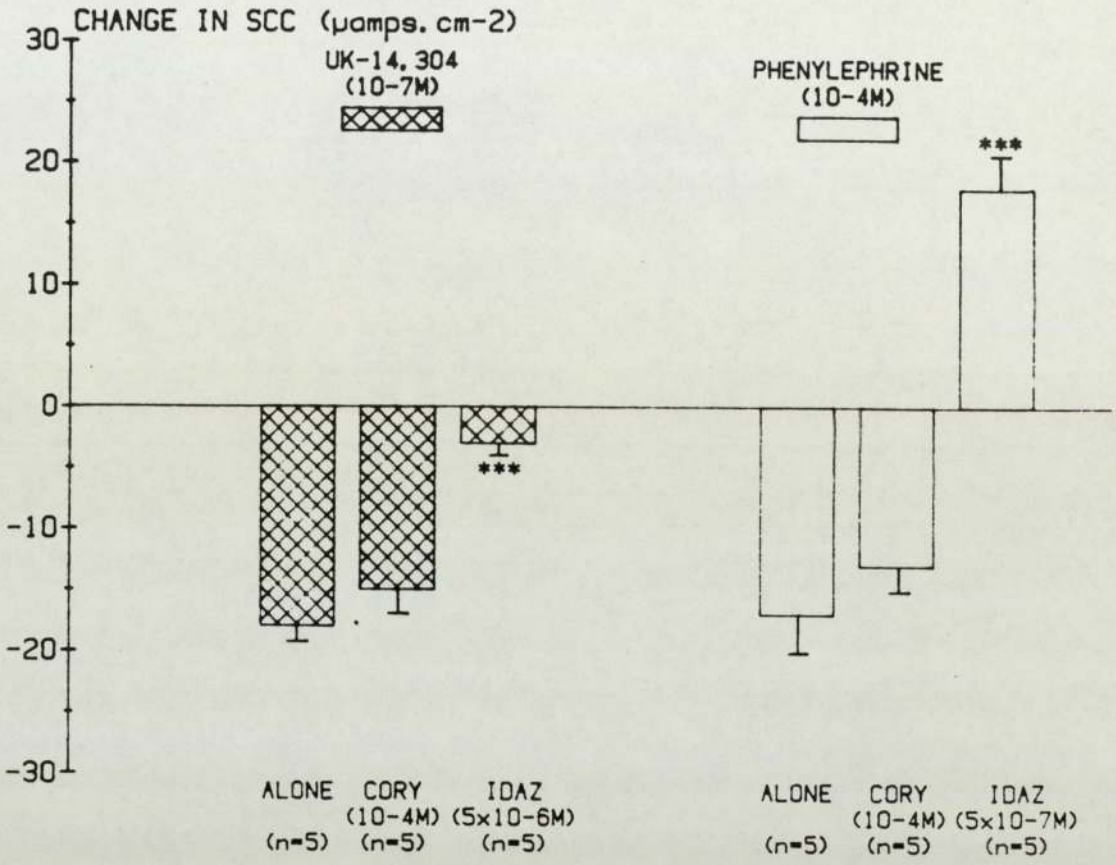

Fig. 16. Changes in SCC induced by UK-14,304 (10^{-7}M) and phenylephrine (10^{-4}M) alone and in the presence of corynanthine (CORY) or idazoxan (IDAZ). Results are expressed as mean ± S.E.M. Agonist responses in the presence of antagonist were compared with agonist responses alone using Student's 't'-tests for unpaired data. Three symbols: $p<0.001$.

was in fact seen to induce an immediate but poorly maintained increase in SCC of +18±3 μamps.cm^{-2} (n=5) after α_2-adrenoceptor blockade with idazoxan. No consistent increases in SCC in response to phenylephrine were observed at concentrations below 10^{-4}M.

3. Ionic Basis of Noradrenaline Induced Decreases in SCC.

Decreases in SCC across the mammalian small intestine *in vitro* in response to application of neurotransmitters or neuroendocrine factors commonly represents an antisecretory response upon serosal to mucosal anion movement. Conversely secretagogues generally increase SCC by stimulating electrogenic anion secretion. It was, however, considered worthwhile to perform some simple ion replacement experiments in order to provide some evidence in support of the proposal that noradrenaline decreases SCC across rat jejunum by depressing anion secretory processes.

Fig. 17 shows SCC recordings from rat jejunum made in HCO_3 free and Cl free Krebs solutions. Cl and HCO_3 salts were replaced by gluconate salts in these experiments. Serosal addition of noradrenaline ($5x10^{-6}$M) to rat jejunum bathed in HCO_3 free/Cl containing bathing solutions produced an immediate, sustained decrease in SCC of -20±4 μamps.cm^{-2} (n=5) which was not significantly different to the response observed in normal HCO_3 Krebs solution (-22±4 μamps.cm^{-2} (n=5)). In Cl free bathing media SCC recordings were low, and noradrenaline was only able to induce a small insignificant change in SCC of -4±2 μamps.cm^{-2}. These results are consistent with the hypothesis that most of the basal SCC recorded across rat jejunum represents electrogenic Cl secretion (Munck, 1972a)

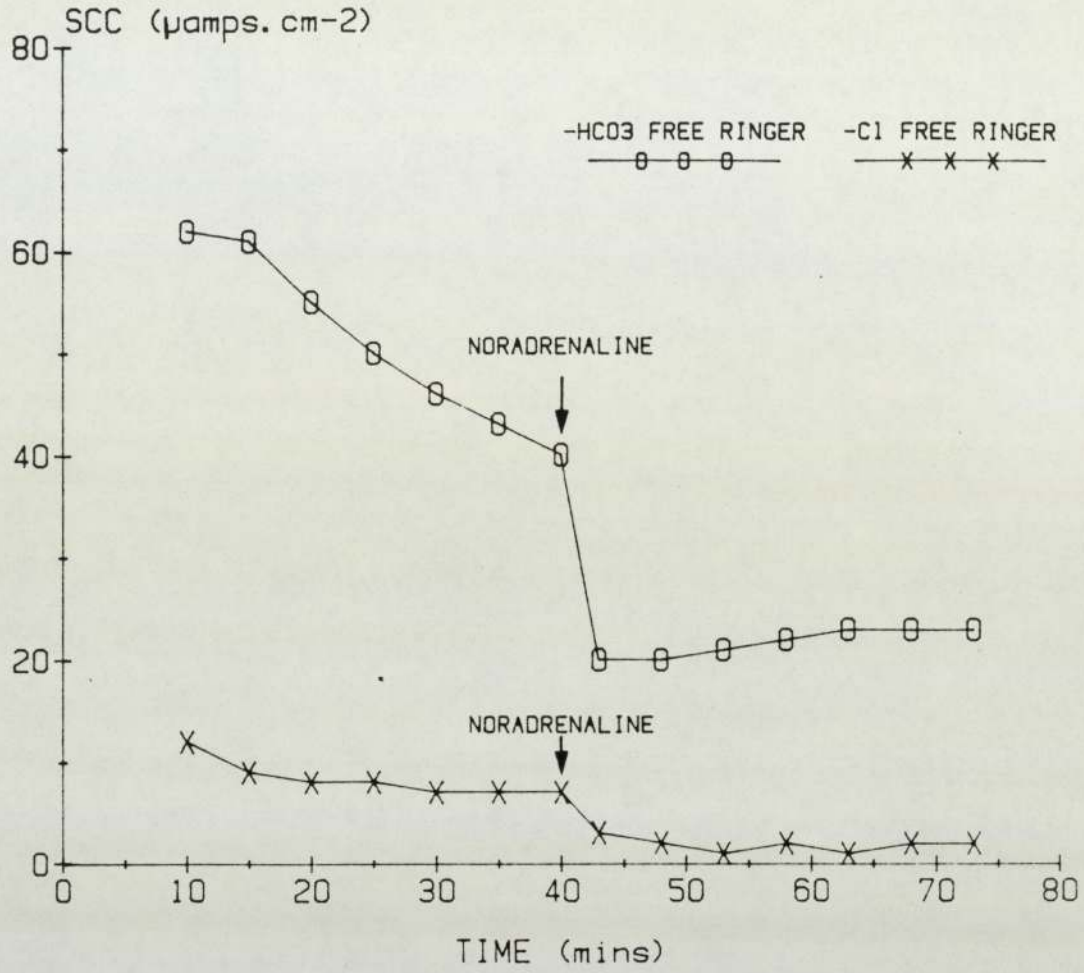

Fig.17. The effects of serosal addition of noradrenaline (5×10^{-6}M) to rat jejunum bathed with Cl or HCO_3 free Krebs solution. Results are expressed as the mean of 5 observations.

and that it is this process that is depressed by noradrenaline in normal HCO_3 Krebs solutions.

4. The Effects of Noradrenaline on Secretagogue Augmented SCC Levels.

Results obtained from the Ussing chamber study to this point indicated that α_2-adrenoceptor stimulation decreased basal SCC across rat jejunum probably reflecting a depression of electrogenic Cl secretion. It was considered worthwhile to further investigate adrenoceptor mediated changes in SCC under secretory conditions after SCC had been elevated by prior addition of the secretagogue theophylline. It is well documented that secretagogues stimulate electrogenic Cl secretion, thus increasing transepithelial SCC and PD in the mammalian small intestine. Theophylline raises intracellular cAMP concentrations by inhibiting the enzyme phosphodiesterase which cleaves cAMP. Preliminary experiments showed that addition of theophylline to the serosal bathing medium of jejunal sheets increased SCC in a concentration dependent manner (fig.18). Theophylline was dissolved in Krebs solution and cumulative additions were made (at 37°C) simultaneously to both mucosal and serosal bathing solutions.

($4x10^{-3}M$) theophylline produced a response that was approximately 80% of maximum and in subsequent experiments this concentration of theophylline was employed to elevate SCC levels. Fig.19 illustrates the SCC response to theophylline ($4x10^{-3}M$) and also the effects of cumulative addition of noradrenaline upon theophylline augmented SCC levels. The first addition of noradrenaline was made 60 minutes after tissue mounting and 30 minutes after the addition of theophylline by

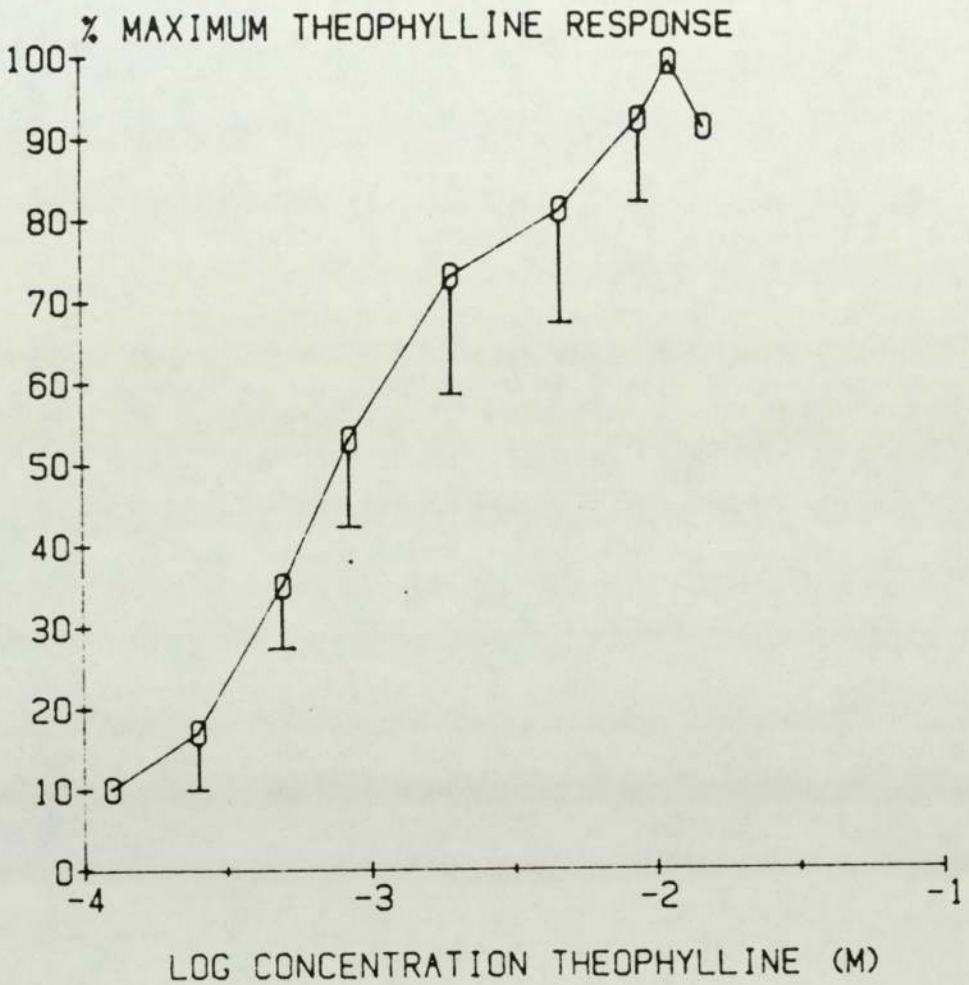

Fig. 18. Cumulative concentration-response curve for the effects of theophylline addition upon basal SCC. The first addition of theophylline was made 30 minutes after tissue mounting. 4×10^{-3}M theophylline was estimated to produce a response approximately 80% of maximum (n=3).

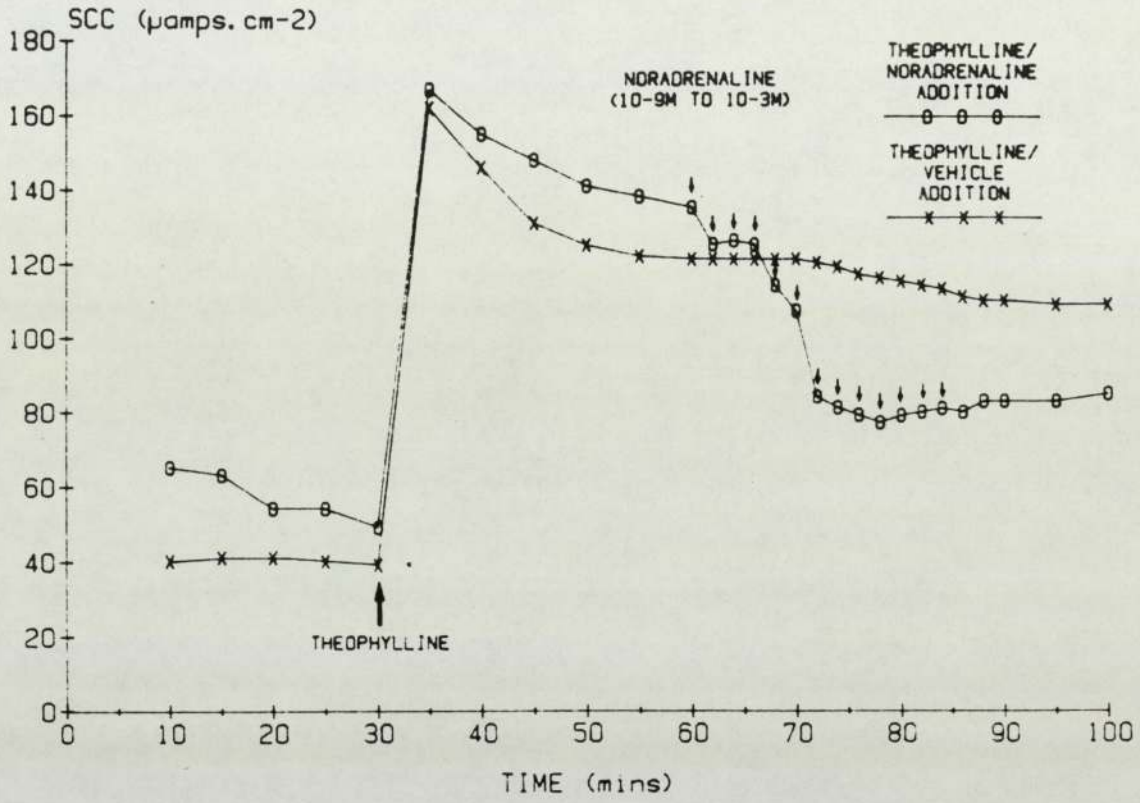

Fig. 19. Effects of theophylline addition ($4x10^{-3}M$) on basal SCC and the effects of cumulative serosal addition of noradrenaline on theophylline augmented SCC levels. Results are expressed as the mean of 5 observations.

which time the SCC had become fairly stable. Noradrenaline for addition was dissolved in Krebs solution and equal volumes of Krebs solution alone were added to the mucosal bathing solution with each serosal addition of noradrenaline. Although noradrenaline did not fully reverse the theophylline induced increase in SCC (maximal decrease in SCC of-58±11 μamps.cm^{-2}(n=5) being observed with noradrenaline representing an approximately 65% reversal of the mean theophylline response), it was possible to construct reproducible concentration response relationships to noradrenaline under these experimental conditions. Theophylline augmented SCC was defined as the difference in SCC between 30 and 60 minutes.

Fig.20 shows concentration response curves for the effect of noradrenaline alone and in the presence of 10^{-7}M and 10^{-6}M idazoxan on theophylline augmented SCC levels plotted as percentage maximum noradrenaline responses meaned from individual curves. Idazoxan at 10^{-7}M and 10^{-6}M produced a parallel rightward shift of the noradrenaline concentration-response curve indicating competitive antagonism, however only 10^{-6}M idazoxan produced a statistically significant change in the ED_{50} value for noradrenaline (from 1.2±0.2x10^{-7}M for noradrenaline alone to 6.1±0.7x10^{-6}M for noradrenaline in the presence of idazoxan $P<0.001$). Fig.21 shows concentration-response curves for noradrenaline alone and in the presence of prazosin (10^{-6}M). At this concentration prazosin did not significantly affect the noradrenaline concentration-response relationship. Antagonists were added to the serosal bathing fluid 10 minutes before the first addition of noradrenaline.

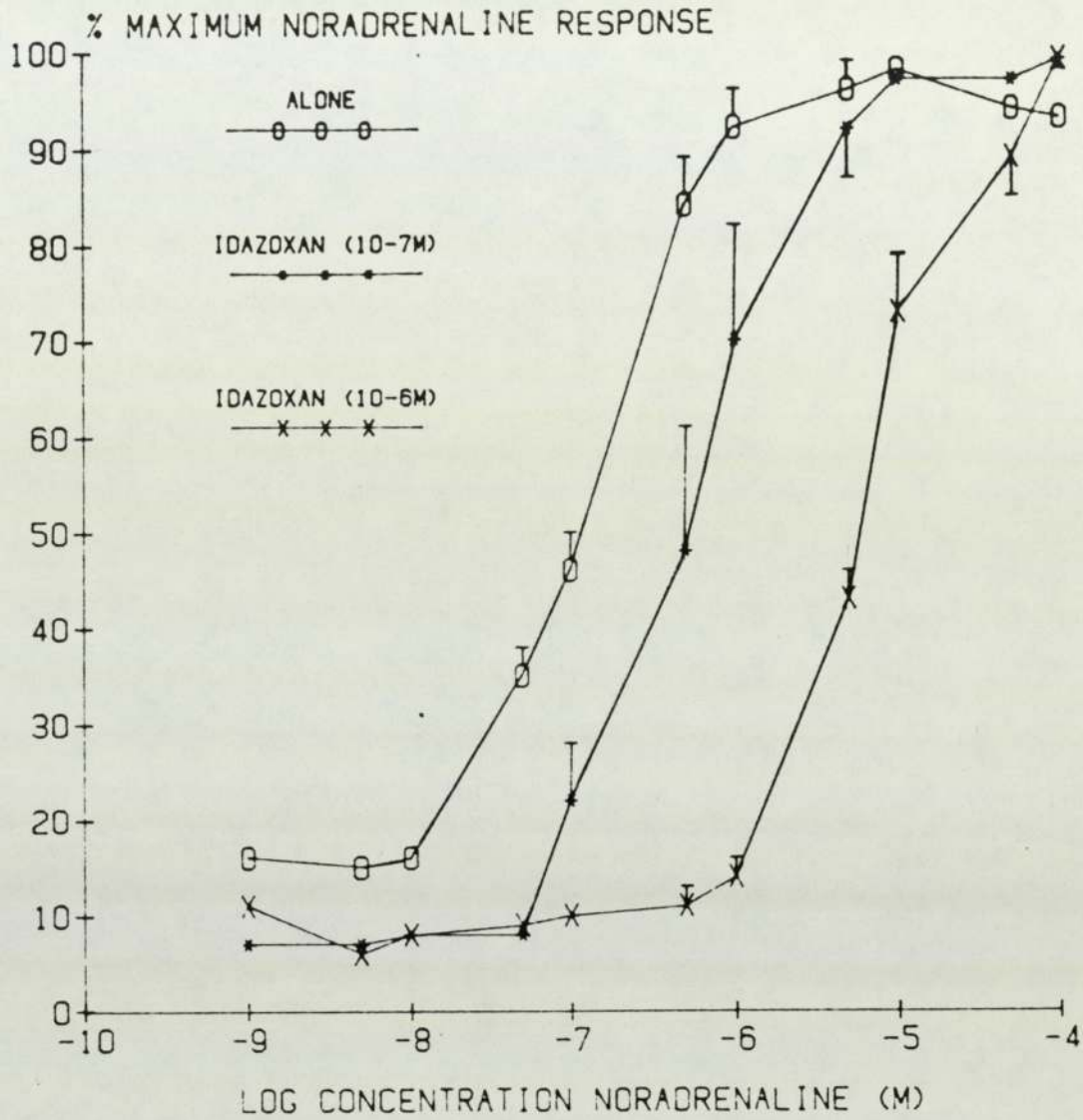

Fig. 20. Cumulative concentration-response curves for the effect of noradrenaline alone (n=5) and in the presence of 10^{-7}M (n=5) and 10^{-6}M (n=5) idazoxan on theophylline augmented SCC levels. SCC responses are plotted as percentage maximum noradrenaline responses meaned from individual curves and are shown as mean and S. E. of the mean.

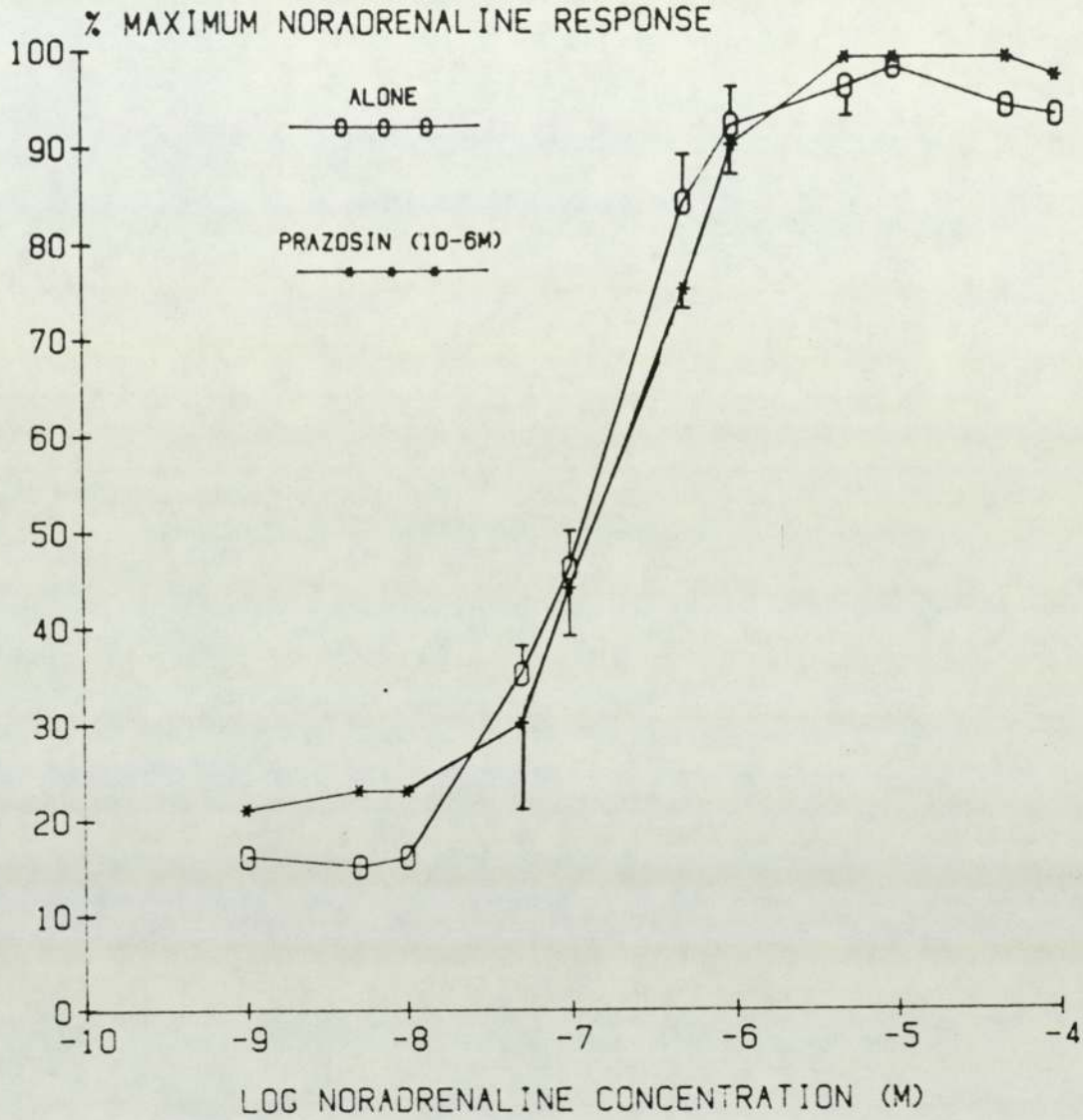

Fig. 21. Concentration-response curves for the effect of noradrenaline alone (n=5) and noradrenaline in the presence of prazosin (10^{-6}M) (n=5) on theophylline augmented SCC levels. SCC responses are plotted as percentage maximum noradrenaline responses and are shown as the mean and S. E. of the mean.

5. The Effects of Tetrodotoxin on Noradrenaline Induced SCC Responses.

Binding studies performed by Nakaki et al (1983) have demonstrated the presence of α_2-adrenoceptors on basolateral membranes of epithelial cells purified from rat jejunum. Consequently it is likely that the effects of α_2-adrenoceptor stimulation upon SCC across rat jejunum *in vitro* are a direct epithelial effect and not a nerve mediated event. To investigate this hypothesis, the effects of noradrenaline on SCC were studied after application of the neurotoxin tetrodotoxin. Addition of $5x10^{-6}M$ tetrodotoxin to the serosal bathing medium of jejunal sheets 30 minutes after tissue mounting caused an immediate sustained decrease in basal SCC of -27 ± 3 $\mu amps.cm^{-2}$ (n=4). Noradrenaline ($5x10^{-6}M$) was only able to cause a small decrease in SCC of -5 ± 1 $\mu amps.cm^{-2}$ (n=4) after prior addition of tetrodotoxin. It was thought that a likely explanation for these findings was that tetrodotoxin was removing some 'secretory tone' in this preparation thus removing the process(es) that are decreased by noradrenaline. It could not be inferred from these experiments that tetrodotoxin inhibited the effects of noradrenaline on SCC.

In a further series of experiments tetrodotoxin ($5x10^{-6}M$) was added to serosal bathing solutions 20 minutes after tissue mounting and theophylline was added 10 minutes later. Theophylline was able to produce increases in SCC after tetrodotoxin which may be attributed to an elevation of mucosal cAMP which in turn activates electrogenic secretory processes. The effects of noradrenaline ($10^{-5}M$) on theophylline augmented SCC levels were then compared in the presence and absence of tetrodotoxin. The magnitude of theophylline induced SCC

responses in the presence of tetrodotoxin (+82±13 μamps.cm^{-2} (n=8)) and in the absence of tetrodotoxin (+96±11 μamps.cm^{-2} (n=8)) were not significantly different from each other. Noradrenaline (10^{-5}M) reversed 68±10% (n=8) of theophylline augmented SCC in the presence of tetrodotoxin and 77±9% (n=8) of theophylline augmented SCC in the absence of tetrodotoxin. These percentage reversal figures were not significantly different indicating that the antisecretory activity of noradrenaline was not inhibited by tetrodotoxin and is thus not a nerve mediated event.

Somatostatin has been identified as one of the few neuroendocrine agents that stimulates absorptive rather than secretory processes (Dharmsathaphorn et al, 1980; Guandalini et al, 1980). Somatostatin has been found to be located in endocrine cells present within sheep jejunal mucosa (Harrison et al, 1986) and these cells are a likely location for somatostatin in other mammalian species. It was considered worthwhile to investigate the possibility that noradrenaline may stimulate absorptive processes through the stimulation of somatostatin release from mucosal endocrine cells. This possibility was investigated using the somatostatin antagonist cyclo(7-Aminoheptanoyl-phenylalanyl-D-tryptophyl-L-lysyl-threonyl[benzyl] (Fries et al, 1982). Somatostatin antagonist was added to the serosal bathing medium 30 minutes after tissue mounting and 10 minutes before the addition of $5x10^{-6}$M noradrenaline. Noradrenaline was seen to produce a normal response (a decrease in SCC of -19±3μamps.cm^{-2} (n=5)) in the presence of the somatostatin antagonist indicating that the effects of noradrenaline were not likely to be mediated through the release of somatostatin from mucosal endocrine cells.

6. β-Adrenoceptor Mediated Influences on Basal SCC.

This part of the study was initiated by the observation that addition of propranolol alone (10^{-4}M) elicited an immediate, sustained decrease in SCC (see section 1).

In initial experiments the effects of mucosal and serosal addition of the β-adrenoceptor agonist isoprenaline were investigated. Serosal addition of isoprenaline (10^{-6}M) 40 minutes after tissue mounting caused an immediate increase in SCC of +31±5 μamps.cm^{-2} (n=5) maximal within 2-3 minutes of addition whilst mucosal addition of isoprenaline (10^{-6}M) (n=3) did not produce this response (fig.22). The response to serosal isoprenaline appeared maximal at a concentration of approximately 10^{-6}M although, as with noradrenaline, concentration-response curves for the effect of isoprenaline could not be constructed. There were two reasons for this (a) the small magnitude of the response recorded across tissues presenting an area of only 0.64cm^{-2} and (b) because of the tendency of SCC to immediately and rapidly drop once the maximum value had been attained for a particular concentration. Fig.23a shows the magnitude of maximum changes in SCC induced by isoprenaline (10^{-6}M) alone and in the presence of phentolamine; propranolol; the selective β_1-adrenoceptor antagonist practolol and the selective β_2-adrenoceptor antagonist ICI 118551. All antagonists were added to the serosal bathing medium 10 minutes before the addition of isoprenaline and were present in a concentration of 10^{-6}M. Results showed that phentolamine and practolol did not significantly affect the response to serosal isoprenaline whilst both propranalol and ICI 118551 significantly ($P<0.001$) inhibited the response to isoprenaline providing evidence for the involvement of

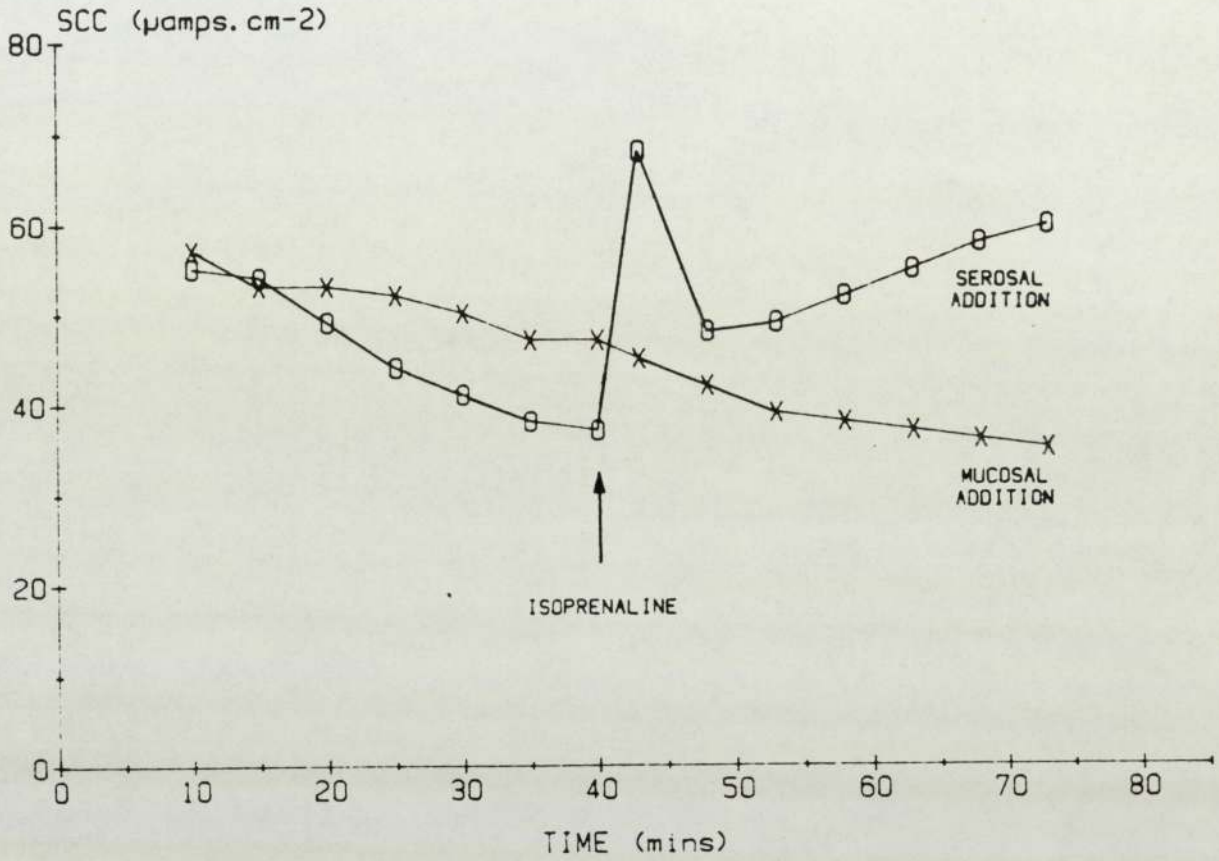

Fig. 22. Effect of serosal or mucosal addition of isoprenaline (10^{-6}M) on basal SCC. Serosal addition of isoprenaline caused an immediate increase in SCC of +31±5μamps.cm^{-2} (n=5) maximal within 3 minutes of addition. Addition of isoprenaline to the mucosal bathing solution (n=3) was without effect.

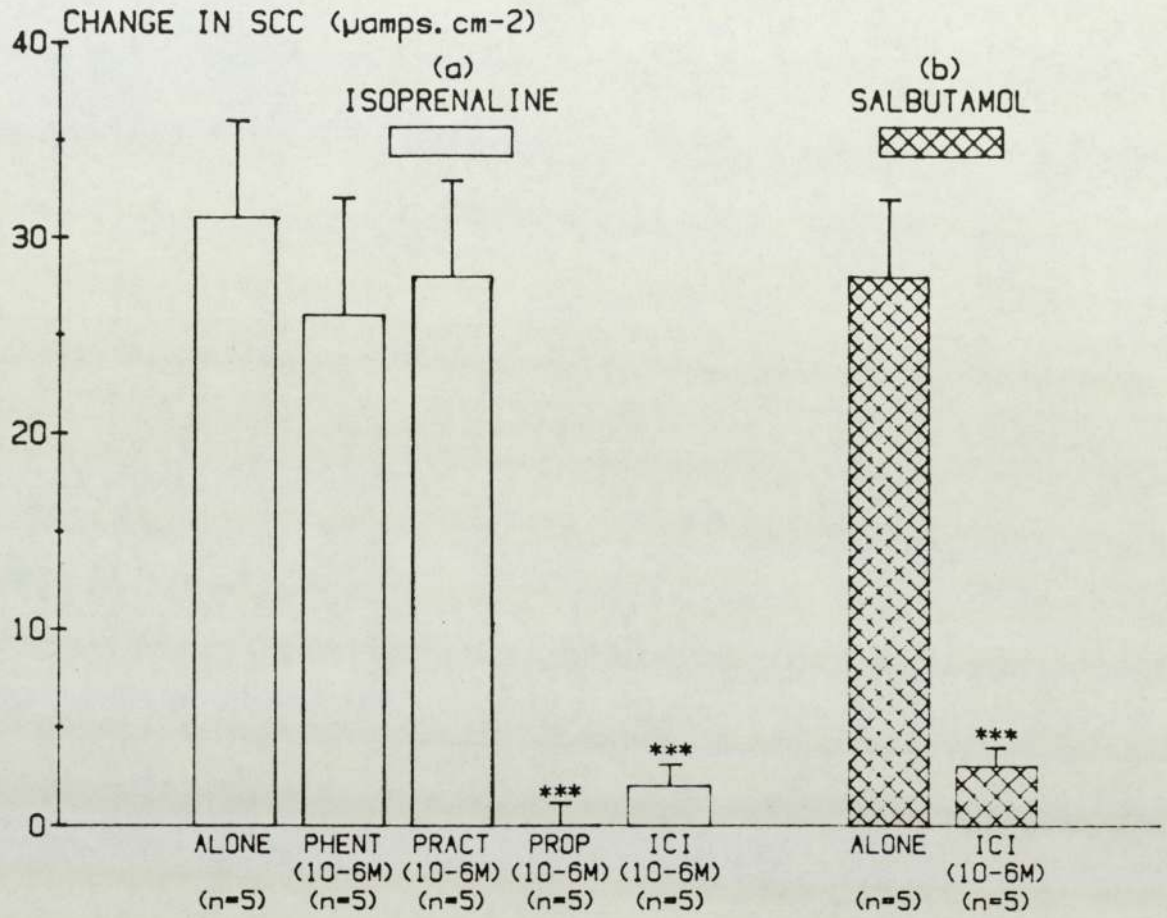

Fig. 23. Changes in SCC induced by (a) isoprenaline (10^{-6}M) and (b) salbutamol (10^{-6}M) alone and in the presence of phentolamine (PHENT), practolol (PRACT), propranolol (PROP) and ICI 118,551 (ICI). Agonist responses in the presence of antagonist were compared with the respective agonist response alone using unpaired Student's 't'-tests. All antagonists were tested at a concentration of 10^{-6}M and added to the serosal bathing medium 10 minutes before the addition of agonist. Three symbols: $p<0.001$.

β_2-adrenoceptors in isoprenaline induced increases in SCC. Isoprenaline induced increases in SCC in the presence of tetrodotoxin ($5x10^{-6}M$) (+23±9 $\mu amps.cm^{-2}$ (n=4)) were not significantly different from responses to isoprenaline alone (+31±5 $\mu amps.cm^{-2}$ (n=5)). The β_2-adrenoceptor agonist salbutamol (Farmer et al, 1970) ($10^{-6}M$) induced a similar increment in SCC to that observed with isoprenaline. This effect of salbutamol was inhibited by ICI 118551 (fig 23b) providing further evidence for a β_2-adrenoceptor mechanism mediating increases in SCC.

Fig. 24 shows histogrammatically the magnitude of immediate changes in SCC induced by addition of the antagonists tested in this part of the study alone and also the change in SCC induced by tetrodotoxin. Tetrodotoxin ($5x10^{-6}M$), propranolol ($10^{-6}M$) and ICI 118551 ($10^{-6}M$) were all seen to induce decreases in SCC. In contrast to the effects of tetrodotoxin and ICI 118551, the propranolol induced decrease in SCC was not significantly different from the change in SCC seen in tissues which had not received antagonist.

It seems likely that a small increase in the number of experimental observations would have resulted in significance within the propranolol data which was in fact already close to significance ($0.1>P>0.05$). Again unlike the decrease in SCC seen with propranolol (-14±5 $\mu amps.cm^{-2}$ (n=5)), the observed decrease in SCC with ICI 118551 (-11±2 $\mu amps.cm^{-2}$ (n=10)) was significantly different to that seen with tetrodotoxin (-23±4 $\mu amps.cm^{-2}$ (n=4)). As has already been stated a decrease in SCC in the mammalian small intestine commonly represents a decrease in electrogenic secretory processes and these results may be indicative of the presence of secretory tone in this preparation which may be partially maintained through a β_2-adrenoceptor mediated mechanism.

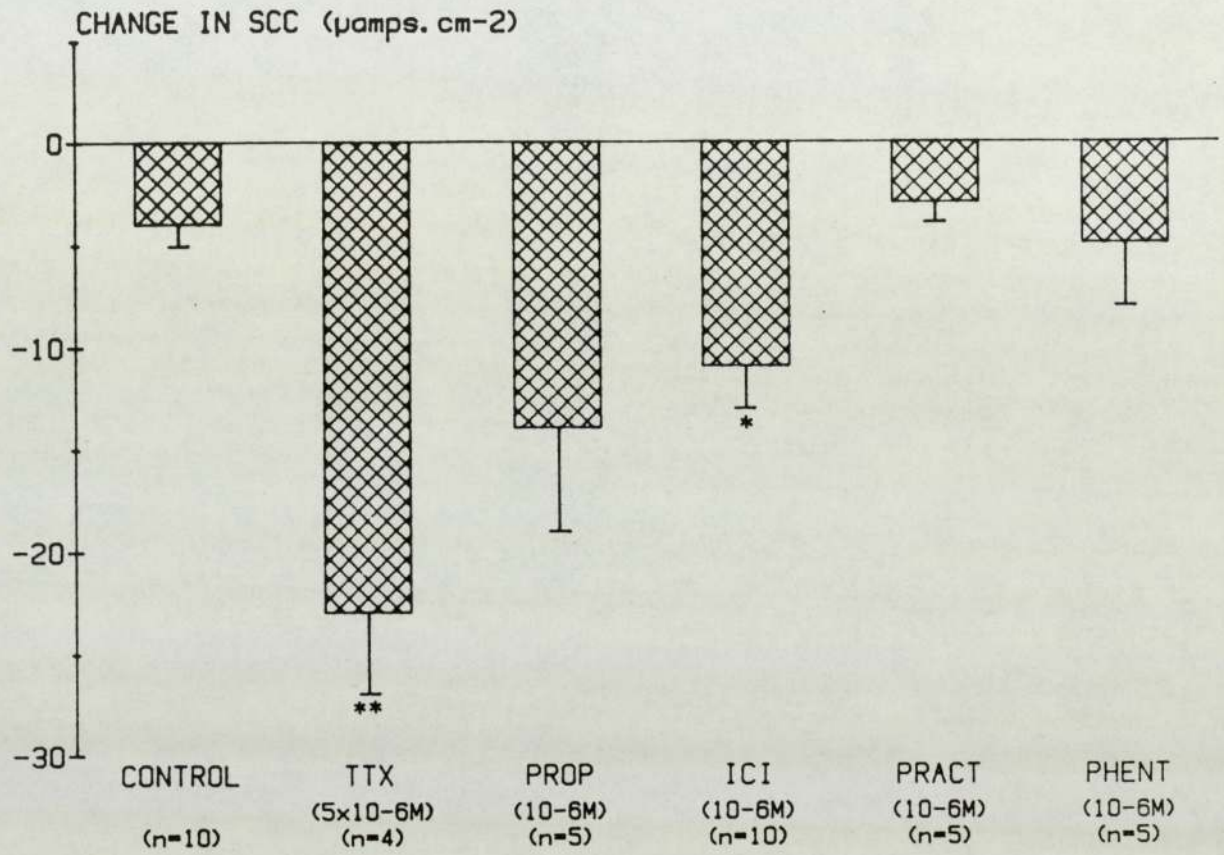

Fig. 24. Changes in SCC induced by tetrodoxin (TTX), propranolol (PROP), ICI 118,551 (ICI), practolol (PRACT) and phentolamine (PHENT). In each case unpaired Student's 't'-tests were used to compare changes in SCC over the period 30-35 minutes with changes in SCC over the same period in tissues which had received no addition at 30 minutes. *: $p<0.05$, **: $p<0.01$.

7. Ionic Basis of Isoprenaline Induced Increases in SCC.

An increase in SCC is a response characteristic of secretagogue addition to the mammalian small intestine *in vitro* which generally reflects an increase in electrogenic Cl secretion. In the present study it was considered to be worthwhile to perform some simple experiments to investigate the ionic basis of isoprenaline induced increases in SCC. Responses to serosal isoprenaline (10^{-6}M) were investigated in Cl free and HCO_3 free solutions where these ions were replaced by the appropriate gluconate salts. Fig.25 shows responses to serosal isoprenaline (10^{-6}M) in normal, Cl free and HCO_3 free Krebs solutions. SCC responses to isoprenaline in both Cl free (-3 ± 1 μamps.cm^{-2} (n=5)) and HCO_3 free ($+5\pm2$ μamps.cm^{-2} (n=5)) solutions were significantly smaller ($P<0.001$) than the isoprenaline response in normal Krebs solutions ($+31\pm5$ μamps.cm^{-2} (n=5)). The absence of a response to isoprenaline in HCO_3 free solutions seems to contradict the hypothesis that isoprenaline induces an increase in SCC by stimulating electrogenic Cl secretion. However, some interesting observations were made when SCC, PD and resistance were compared in normal and HCO_3 free solutions 40 minutes after tissue mounting and just prior to the addition of isoprenaline (table 5). It was noted that PD was higher, although not significantly ($0.1>P>0.05$) in HCO_3 free Krebs solutions when compared to the PD in normal Krebs solutions. Perhaps surprisingly, however, was the observation that SCC was not also elevated in HCO_3 solutions with the result that significantly higher tissue resistances were observed in HCO_3 free Krebs solutions when compared to normal Krebs solutions. These observations are discussed further in the following section.

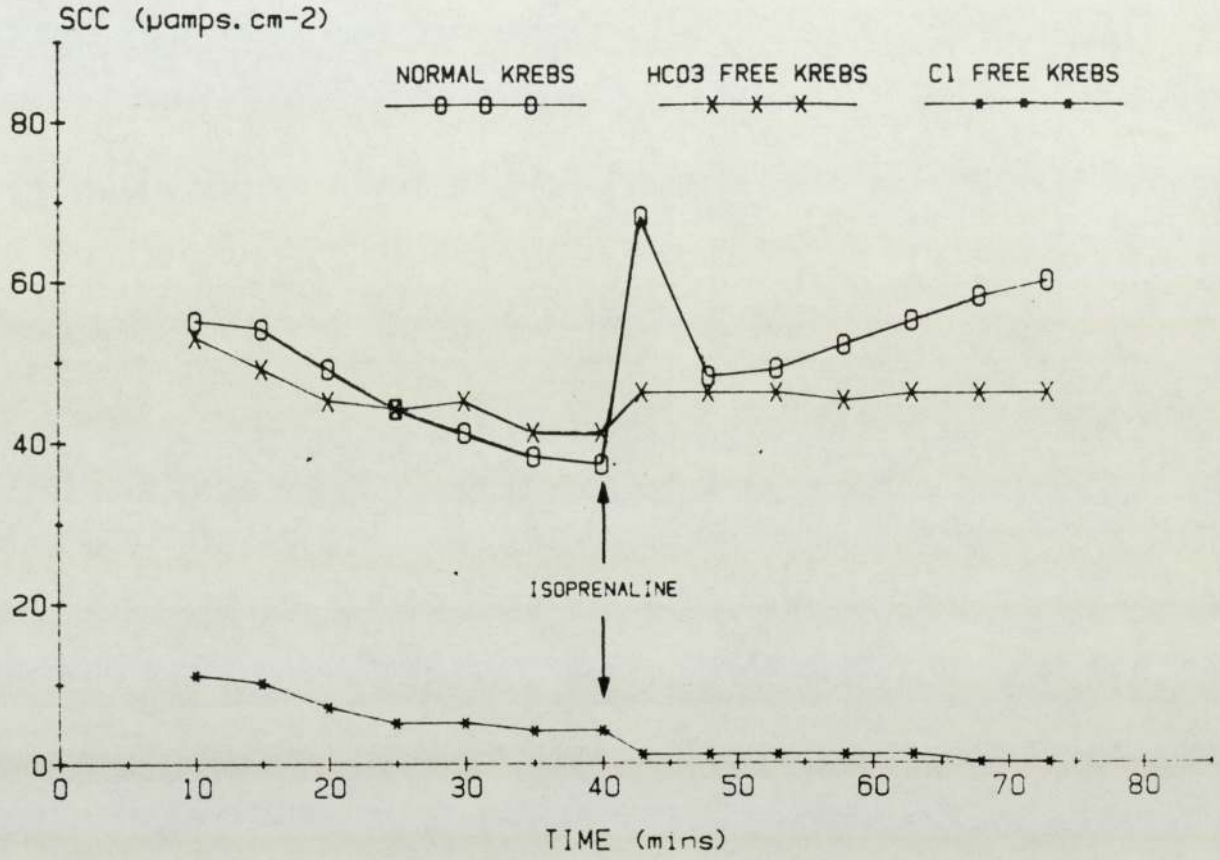

Fig. 25. Short-circuit current recordings made from rat jejunum bathed in normal, Cl free and HCO_3 free Krebs solutions. Isoprenaline (10^{-6}M) was added serosally at 40 minutes to each bathing medium. Results are expressed as the mean of 5 experimental observations.

Parameter	Normal Krebs	Bicarbonate Free	p
Short Circuit Current (μamps)	37±5	41±8	NS
Potential Difference (mV)	0.9±0.1	1.6±0.4	NS
Resistance (ohms)	23.0±1.0	38.0±3.0	<0.01

TABLE 5.

Short-circuit current, potential difference and resistance measurement taken at 40 minutes from tissues bathed in normal and bicarbonate free Krebs solutions. Results were compared using Student's 't'-test for unpaired data.

8. A Discussion of Electrogenic Transport Mechanisms in the Rat Jejunum *In Vitro*.

Initial experiments performed with the type I Ussing chamber design clearly showed that serosal addition of noradrenaline to stripped sheets of rat jejunum caused an immediate sustained decrease in transepithelial PD and SCC. This effect is consistent with an antisecretory response upon serosal to mucosal electrogenic anion movement in the mammalian small intestine. Mucosal noradrenaline did not produce an immediate response which is consistent with the location of α_2-adrenoceptors on basolateral epithelial cell membranes (Nakaki et al, 1983). This response was inhibited by the selective α_2-adrenoceptor antagonists yohimbine (Stake et al, 1975) and idazoxan (Doxey et al, 1983) but was not significantly inhibited by the selective α_1-adrenoceptor antagonists prazosin (Doxey et al, 1977) and corynanthine (Shepperson et al, 1981) . Addition of antagonists prior to noradrenaline enabled any effects of antagonists alone on SCC to be revealed. Contrary to the findings obtained with the everted sac model, prazosin alone did not appear to produce any effects on transport processes. It is therefore likely that in concentrations of approximately 10^{-5}M prazosin predominantly exerts an antiabsorptive influence upon electrically silent absorption rather than eliciting a 'secretory' response.

The involvement of α_2-adrenoceptors in mediating decreases in SCC was confirmed by the use of the selective α_2-adrenoceptor agonist UK-14,304 (Cambridge, 1981) and the preferential α_1-adrenoceptor agonist phenylephrine (McGrath, 1982). UK-14,304 (10^{-7}M) mimicked the effects of noradrenaline ($5x10^{-6}$M) whilst only a high concentration of

phenylephrine (10^{-4}M) was able to elicit a decrease in SCC. Responses to both agonists were blocked by the selective α_2-adrenoceptor antagonist idazoxan (Doxey et al , 1983) and not significantly affected by the selective α_1-adrenoceptor antagonist corynanthine indicating an interaction with α_2-adrenoceptors. High doses of phenylephrine have been reported to elicit an antisecretory response upon fluid absorption in the rat jejunum *in vivo* through activity at α_2-adrenoceptors (Bunce and Spraggs, 1983b). It was noted that after α_2-adrenoceptor blockade with idazoxan , phenylephrine (10^{-4}M) did in fact induce increases in SCC. This finding is further discussed later.

The major part of the recorded SCC across rat jejunum has been reported to represent electrogenic Cl secretion (Munck, 1972a). Ion replacement experiments involving the replacement of either Cl or HCO_3 ions with gluconate ions confirmed this finding. In Cl free bathing solutions the recorded SCC was low and also noradrenaline failed to produce a response. The latter observation is consistent with the hypothesis that noradrenaline acts to decrease electrogenic Cl secretion as has also been reported in the rabbit ileum (Chang et al, 1982; Durbin et al, 1982). A full response to noradrenaline was observed in HCO_3 free solutions indicating that any effect of noradrenaline on HCO_3 secretory mechanisms was of minor significance under basal conditions.

It has been reported by Bunce and Spraggs (1983a) and Nakaki et al (1982a; 1982b) that α_2-adrenoceptor stimulation does not influence basal levels of fluid absorption in the rat jejunum *in vivo*. It is thus likely that the antisecretory effects of α_2-adrenoceptor agonists under basal conditions are too small to affect bulk fluid transport. α_2-adrenoceptor mechanisms were further investigated after SCC had been elevated by

prior administration of the secretagogue theophylline which activates anion secretory mechanisms in the mammalian small intestine. Under these conditions noradrenaline was seen to reverse secretagogue augmented SCC levels in a concentration-dependent manner similar to the α_2-adrenoceptor mediated antisecretory effects upon fluid absorption in the rat jejunum *in vivo* reported by Bunce and Spraggs (1983a; 1983b), Nakaki et al (1982a; 1982b) and Wahawisan et al (1985). Idazoxan at concentrations of 10^{-7}M and 10^{-6}M produced parallel rightward shifts of the noradrenaline concentration-response curve indicative of competitive antagonism at α_2-adrenoceptors. A pA_2 value (the negative logarithm of the antagonist concentration that produces a competitive two fold shift of the agonist concentration-response curve) of 7.6 for idazoxan was estimated using the method of Furchgott (1972). This value compares favourably with the antagonist potency of idazoxan at post junctional α_2-adrenoceptors in rat and cat vascular smooth muscle (see Medgett et al, 1984). The selective α_1-adrenoceptor antagonist prazosin (10^{-6}M) did not significantly deflect the noradrenaline concentration response relationship.

Experiments involving prior addition of the neurotoxin tetrodotoxin or the somatostatin antagonist cyclo(7-Aminoheptanoyl-phenylalanyl-D-trptophyl-L-lysyl-threonyl[benzyl]) (Fries et al, 1982) showed that neither of these agents blocked the antisecretory effects of noradrenaline, indicating that the effects of noradrenaline were neither nerve mediated nor mediated via the release of somatostatin from mucosal endocrine cells. It was additionally noted that the theophylline response after tetrodotoxin was not significantly different to the theophylline response alone. Consequently in this *in vitro* preparation

the effects of the secretagogue were predominantly via a direct mucosal mechanism and not via the activation of intramural secretory pathways.

In the initial series of SCC experiments it was noted that propranolol (10^{-4}M) caused a decrease in SCC. Upon further investigation it was found that 10^{-6}M propranolol produced a similar response to that produced by the selective β_2-adrenoceptor antagonist ICI 118551 (O'Donnell and Wanstall, 1980) (10^{-6}M). Tetrodotoxin was also observed to produce a decrease in SCC which in fact appeared to be larger than that induced by either propranolol or ICI 118551. This data may be interpreted as revealing the presence of nerve mediated secretory tone in this preparation, a portion of which appears to be mediated through β_2-adrenoceptors. An absorptive effect of propranolol alone upon fluid absorption has been previously reported in the rat jejunum *in vivo* (Levens et al, 1981c; Levens, 1983). A β_2-adrenoceptor mechanism mediating increases in SCC was identified using the non-selective β-adrenoceptor agonist isoprenaline and the preferential β_2-adrenoceptor agonist salbutamol (Farmer et al, 1970). Increases in SCC produced by these agents were blocked by the selective β_2-adrenoceptor antagonist ICI 118551 but not by the selective β_1-adrenoceptor antagonist practolol (Bieth et al, 1980). The effects of isoprenaline were not blocked by tetrodotoxin indicating the liklihood of a direct mucosal influence. Ion replacement experiments failed to clarify the ionic basis of isoprenaline induced increases in SCC since isoprenaline failed to produce a response in either Cl or HCO_3 free bathing solutions. It was however noted that PD was elevated , though not significantly, in HCO_3 free solutions. Similar observations have been made by Binder et al (1973) in rabbit ileum and Powell et al (1972) in guinea pig ileum. It

was shown in these studies that in HCO_3 free bathing solutions increases in PD were due to increases in electrogenic Cl secretion. If this interpretation is true for rat jejunum then isoprenaline may be unable to further stimulate an already enhanced Cl secretion in HCO_3 free solutions. However SCC was not elevated in HCO_3 free bathing solutions with a consequently significant increase in tissue resistance under these conditions. The reason for this is unknown.

The presence of a β_2-adrenoceptor mechanism mediating increases in SCC provides a possible explanation for the increases in SCC observed with 10^{-4}M phenylephrine after α_2-adrenoceptor blockade with idazoxan. This effect of phenylephrine was not observed with concentrations of phenylephrine much below 10^{-4}M and probably reflects the documented β_2-adrenoceptor agonist activity of phenylephrine (Lefevre et al, 1977). It appears that under normal conditions the α_2-adrenoceptor activity of phenylephrine predominates over its β_2-adrenoceptor activity.

The presence of this β_2-adrenoceptor mechanism also provides a possible explanation for the incomplete reversal of theophylline augmented SCC by noradrenaline. At high concentrations noradrenaline may begin to activate secretory processes via its activity at β_2-adrenoceptors cancelling out any furthur α_2-adrenoceptor effects. However, if this were the case, then the concentration-response curves to noradrenaline after idazoxan might not have been expected to be parallel with the control curve. Alternatively it is conceivable that β_2-adrenoceptor influences might only start to affect SCC once a critical level of α_2-adrenoceptor stimulation has been attained.

To briefly summarize, evidence has been provided here for an α_2-adrenoceptor mechanism which mediates decreases in electrogenic Cl

secretion and also for a β_2-adrenoceptor mediated mechanism which mediates increases in SCC, almost certainly reflecting increases in anion secretory processes. There appear to be no apparent α_1-adrenoceptor mediated influences upon electrogenic anion transport in the rat jejunum. It is possible however that α_1-adrenoceptor mediated influences upon jejunal fluid transport reported both *in vitro* (Cotterell et al, 1983; Parsons et al, 1983) and *in vivo* (Levens et al, 1981c; 1983) reflect activity upon electrically silent NaCl transport processes.

B. RADIOISOTOPE FLUXES ACROSS ISOLATED JEJUNAL SHEETS.

Introduction.

As documented in the literature review there is evidence to suggest the existence of an α_1-adrenoceptor mechanism that mediates fluid and electrolyte absorption in the rat jejunum both *in vitro* and *in vivo*. However in the present study (see chapter 1) it was not possible to confirm the presence of an α_1-adrenoceptor mechanism mediating fluid absorption in the rat jejunum *in vitro* using the everted sac technique.

Results presented in the previous section clearly show that specific α_2-adrenoceptor stimulation mediates decreases in electrogenic secretory mechanisms in the rat jejunum *in vitro*. It is, however, possible that an α_1-adrenoceptor mechanism is present in the rat jejunum that mediates increases in electrically neutral NaCl absorption. Coupled NaCl transport is known to exist in the rat jejunum (Murer et al, 1983), as it does in the rabbit ileum where it is stimulated by specific α_2-adrenoceptor stimulation (Chang et al, 1982; Durbin et al, 1982). The aim of this part of the study was to attempt to characterize the receptors mediating noradrenaline induced increases in mucosal to serosal NaCl movement. It was further thought a worthwhile exercise to attempt to directly correlate decreases in SCC with decreases in serosal to mucosal Cl movement to confirm the results of the ion replacement experiments described previously (see chapter 2A).

This part of the study necessitated the measurement of unidirectional isotope fluxes and experiments described here were performed with the type II Ussing chambers and employed an automatic voltage clamp device.

As discussed in methods section 2d, the type II chambers were seen as an improvement upon the prior design cutting out all connecting tubing and greatly ameliorating handling, also the automatic voltage clamp enhanced the accuracy of voltage clamping. Due to limitations in time and resources the work described here was performed with only one chamber and clamp.

1. Noradrenaline Effects Upon Mucosal to Serosal NaCl Absorption.

In the following experiments ^{22}Na was chosen as a marker of absorptive processes as any stimulation of electrically silent mucosal to serosal NaCl movement would be reflected in a stimulation of ^{22}Na fluxes. Measuring gamma emissions from ^{22}Na, as a marker of absorptive processes, eliminated quenching difficulties that would have arisen with liquid scintillation counting of beta emissions from ^{36}Cl, in experiments where noradrenaline was present (see Field and McColl, 1973).

In initial experiments 1.5 μCi of ^{22}Na was added to the mucosal bathing solution 10 minutes after tissue mounting. After an equilibration period of 30 minutes, by which time SCC had stabilized, ^{22}Na fluxes were measured over 5 consecutive 10 minute flux periods. 1ml samples were removed from both the mucosal and serosal reservoirs and replaced with an equal volume of unlabelled Krebs solution warmed to 37°C. Fluxes of ^{22}Na for each 10 minute flux period were calculated as described in methods section 2e. When noradrenaline was used, it was added to the serosal bathing solution in a 50μl volume in the middle of flux period 3. 50μl of distilled water was simultaneously added to the

mucosal reservoir. Consequently, flux periods 1 and 2 served as control periods, period 3 as the addition period and periods 4 and 5 (period 4 commencing 5 minutes after the addition noradrenaline) as test periods with noradrenaline present. Noradrenaline was added in a concentration of $5x10^{-4}M$, this concentration having previously been reported to stimulate mucosal to serosal movement of ^{22}Na across rat jejunum by Munday and Poat (1983) and also observed to stimulate fluid absorption in everted jejunal sacs in the present study. Table 6 shows flux measurements for mucosal to serosal movement of ^{22}Na. Flux periods 1 and 2 were combined and referred to as period A. Flux periods 4 and 5 were combined and referred to as period B. In control tissues no significant difference was observed between periods A and B for ^{22}Na fluxes. Noradrenaline ($5x10^{-4}M$) was only able to induce a small but insignificant increase in ^{22}Na flux in period B under these basal conditions.

It was noted that control period A was in fact significantly different ($P<0.01$) between the two experimental groups reflecting inter-animal variation. Analysis of individual results indicated that those animals with a low control period A did not necessarily demonstrate a stimulatory response to noradrenaline. This is analogous to the situation in the paired everted sac study where noradrenaline treated sacs having low paired control sac fluid transport levels did not necessarily show a stimulatory response to noradrenaline.

Following these negative findings it was decided to investigate the effects of noradrenaline ($5x10^{-4}M$) upon mucosal to serosal ^{22}Na movement in the presence of theophylline ($2x10^{-2}M$). Theophylline is known to inhibit coupled NaCl transport in mammalian tissues through an elevation

n	Period	Treatment	^{22}Na Flux (m-s)
	A	Control	9.93±0.36
	B	Control	10.53±0.64
5	B-A		+ 0.6±0.75
	P		NS
	A	Control	16.97±1.4
	B	(Noradrenaline (5×10^{-4} M)	17.62±2.06
7	B-A		+0.66±2.45
	P		NS
	A	Control in Theophylline (4mM)	11.12±2.22
	B	Control in Theophylline (4mM)	10.99±1.19
4	B-A		- 0.14±1.33
	P		NS
	A	Control in Theophylline (4mM)	10.77±1.21
	B	Noradrenaline (5×10^{-4} M) in Theophylline (4mM)	11.81±0.78
7	B-A		+1.04±1.45
	P		NS

Table 6. Mucosal to serosal fluxes of ^{22} Na in normal and theophylline (4mM) containing Krebs bicarbonate solutions. Results are expressed as $\mu equiv.hr^{-1}.cm^{-2}$. Period A is the average of two 10 minute flux periods sampled before the addition of noradrenaline (5×10^{-4} M) or 50µl of distilled water containing 10^{-4} m ascorbic acid. Period B is the average of two 10 minute flux periods beginning 5 minutes after the addition of noradrenaline or vehicle. Comparisons were made using Student's 't'-tests for paired data.

of mucosal cAMP levels (see literature review section 3). It was hoped that if NaCl absorption was depressed in period A then the chances of seeing a clearer response to noradrenaline in period B would be enhanced. Results again shown in table 6, for fluxes of ^{22}Na in thoephylline containing bathing solutions mirrored the findings under basal conditions. Noradrenaline ($5x10^{-4}M$) was only able to induce a small insignificant stimulatory effect upon mucosal to serosal movement of ^{22}Na.

2. Decreases in SCC and Electrogenic Cl Secretion.

As has been stated in the introduction to this section decreases in SCC in the mammalian small intestine in response to specific α_2-adrenoceptor stimulation have been directly correlated with decreases in electrogenic Cl secretion. In this part of the study it was attempted to directly correlate decreases in SCC in response to UK-14,304 ($10^{-6}M$) in the presence of theophylline ($4x10^{-3}M$) (employed to elevate electrogenic secretory processes) with decreases in serosal to mucosal movement of ^{36}Cl. UK-14,304 was employed rather than noradrenaline to avoid problems that would have occurred with noradrenaline quenching of beta emissions from ^{36}Cl.

The experimental protocol was similar to that employed for the measurement of mucosal to serosal movement of ^{22}Na. 2.5 μCi of ^{36}Cl was added to the serosal bathing medium 10 minutes after tissue mounting and serosal to mucosal ^{36}Cl fluxes were measured over 5 consecutive 10 minute flux periods beginning 30 minutes later. 1ml samples were taken from both the mucosal and serosal reservoirs and replaced with an equal

volume of unlabelled Krebs solution warmed to 37°C. Fluxes of ^{36}Cl were calculated as described in methods section 2e. In test experiments involving addition of UK-14,304 (10^{-6}M), UK-14,304 was added to the serosal bathing solution in a 50µl volume 1 minute before the beginning of flux period 4. An equal volume of distilled water was added simultaneously to the mucosal bathing solution. Consequently flux periods 1 and 2 served as control periods, period 3 as the addition period and periods 4 and 5 as test periods with UK-14,304 present. Table 6 shows flux measurements for serosal to mucosal movement of ^{36}Cl. Flux periods 1 and 2 were combined and referred to as period A and flux periods 4 and 5 combined and referred to as period B. No significant difference was observed between flux periods A and B for ^{36}Cl movement in either control tissues or tissues that had received addition of UK-14,304 (10^{-6}M). Thus these experiments had failed to show directly a correlation between decreases in SCC and decreases in serosal to mucosal ^{36}Cl movement.

Samples with or without UK-14,304 present were 'spiked' with equal amounts of ^{36}Cl (which had a calculated counting efficiency of 99%) and the increments in counts per minute measured. Increments were similar in both UK-14,304 containing and UK-14,304 free samples indicating that UK-14,304 did not exert any quenching influence on beta emissions from ^{36}Cl.

3. Discussion of Results.

Results obtained from this study failed to show a stimulatory effect of noradrenaline ($5x10^{-4}$M) upon mucosal to serosal movement of ^{22}Na and

n	Period	Treatment	^{36}Cl Flux (s-m)	SCC
	A	Control	11.87±0.36	3.33±0.26
	B	Control	11.25±0.33	3.57±0.27
4	B-A		-0.62±0.34	+0.25±0.13
	P		NS	NS
	A	Control	11.24±0.6	5.42±0.58
	B	UK-14,304 (10^{-6}M)	11.1 ±0.9	3.03±0.46
4	B-A		-0.14±0.84	-2.39±0.26
	P		NS	P<0.01

Table 7. Serosal to mucosal fluxes of ^{36}Cl in theophylline containing Krebs bicarbonate solutions. Period A is the average of two 10 minute flux periods sampled before the addition of UK-14,304 (10^{-6}M) or distilled water as a 50µl volume. Period B is the average of two 10 minute flux periods beginning 1 minute after the addition of UK-14,304 or distilled water, Flux data and SCC are both expressed as µequiv.hr^{-1}.cm^{-2}. Average SCC for each 10 minute flux period was calculated from three time points at 0, 5 and 10 minutes. Significances were determined using Student's 't'-tests for paired data.

also failed to directly correlate decreases in SCC in response to UK-14,304 (10^{-6}M) with decreases in electrogenic ^{36}Cl secretion.

Munday and Poat (1983) have claimed to show a significant effect of noradrenaline ($5x10^{-4}$M) upon mucosal to serosal movement of ^{22}Na across stripped sheets of rat jejunum. However they failed to show a significant stimulatory effect of either angiotensin or tyramine. Both of these agents have been shown to stimulate release of noradrenaline from pre-synaptic nerve terminals in rat jejunum *in vivo* and to stimulate fluid absorption via subsequent interaction of post-synaptic α_1-adrenoceptors (Levens et al, 1981c; Levens, 1983; 1984a; 1984b). Both angiotensin and tyramine have also been shown to stimulate fluid absorption across everted sacs of rat jejunum *in vitro* (Crocker and Munday, 1970; Munday et al, 1980). Munday and Poat (1983) explained the lack of effect of angiotensin and tyramine in Ussing chamber preparations by claiming that the stripped sheets of rat jejunum contained little endogenous noradrenaline and also that any endogenous noradrenaline released would be rapidly inactivated due to the small tissue to medium ratio in the chambers. Tyramine has however been shown to stimulate NaCl absorption across isolated sheets of rabbit ileum (Tapper et al, 1981).

Although the two explanations in the previous paragraph may help explain the negative findings reported with angiotensin and tyramine, they do not explain the negative findings of this study with noradrenaline.

However, the reported increase in neutral NaCl absorption in response to noradrenaline in rabbit isolated tissues is small - representing an approximately 10% increase over background (Sjovall, 1985). There is

evidence that the passive background flux in the rat jejunum is higher than that in rabbit ileum (K.Moriarty personnel communication). Therefore a likely explantion for the failure of noradrenaline (and possibly angiotensin and tyramine) to significantly stimulate mucosal to serosal movement of ^{22}Na is simply that the effect *in vitro* is too small to be detected against the background flux of ^{22}Na.

Theophylline did not appear to obviously reduce control fluxes of ^{22}Na and this observation coupled with the failure of noradrenaline to reverse theophylline induced decreases in fluid absorption in everted sacs (see chapter 1) make it unsurprising that noradrenaline also failed to significantly stimulate ^{22}Na absorption in theophylline containing bathing media.

UK-14,304 ($10^{-6}M$) also failed to significantly decrease Cl secretion as evidenced by fluxes of ^{36}Cl. The average change in SCC over the experimental test period after UK-14,304 ($10^{-6}M$) was calculated as 2.39 ± 0.26 $\mu equiv.cm^{-2}$ representing approximately 20% of the calculated background serosal to mucosal ^{36}Cl flux. Again background variability could have masked a change in ^{36}Cl flux of this magnitude. Indeed discrepancies between the effects of noradrenaline upon SCC and ^{36}Cl movement in the rabbit ileum have been encountered previously (Field and McColl, 1973) and put down to the problem of detecting small changes against a relatively large background.

RESULTS CHAPTER 3

ADRENOCEPTOR MEDIATED TONE IN THE RAT COLON *IN VIVO*

Introduction.

The work described in this section was performed in the department of phamacology, Reckitt and Colman plc. The aim of this part of the study was to elucidate whether basal fluid and electrolyte transport in the rat colon *in vivo* is under tonic adrenoceptor mediated neural control. Studies performed by Chang et al (1983b) indicated that diabetic diarrhoea in rats was due to the destruction of sympathetic nerve endings supplying the ileum and colon. In diabetic rats, ion absorption was reduced in the ileum and colon but not in the jejunum implying that the adrenergic nervous system exerts a tonic influence over ileal and colonic, but not jejunal transport. If the adrenergic nervous system does normally exert a tonic influence over colonic fluid and electrolyte transport, then blockade of the appropriate receptors *in vivo* should result in a demonstrable decrease in fluid and electrolyte transport. Fogel and Kaplan (1984) demonstrated a tonic influence over colonic fluid and electrolyte transport in the rat that appeared to be mediated through δ-opiate receptors. The technique employed in this study was a single pass lumen perfusion technique similar to that described by Bright-Asare and Binder (1973). This technique was chosen for use in the present study in preference to the isolated loop techniques of Levens et al (1980) or Bunce and Spraggs (1982) since it was thought to be more representative of the passage of material through the gut. Specific

antagonists to receptors mediating adrenergic tone (if present) in the human intestine may be useful in the treatment of constipation.

1. An Attempt to Measure Colonic Fluid and Electrolyte Transport *In Vivo.*

Male Wistar rats pretreated with indomethacin (13 $mg.Kg^{-1}$.s.c.) to prevent endogenous prostaglandin formation as a result of mechanical stimulation were anaesthetized and set up for single pass lumen perfusion with isosmotic solution as described in methods section 3. Initial experiments were performed to validate the use of ^{14}C-PEG as a radioactive marker upon which calculations of colonic fluid and ion transport were based. 20mls of isosmotic solution containing ^{14}C-PEG was perfused through the colon at a rate of 0.5 $mls.min^{-1}$ followed by a furthur 20 mls of unlabelled solution. Percentage recovery of ^{14}C-PEG was then calculated by counting 0.5ml samples of influent and effluent solutions using the formula:-

$$\% \text{ recovery} = \frac{\text{effluent c.p.m. x 2}}{\text{influent c.p.m.}} \times 100$$

Throughout the study all samples were counted in triplicate with the mean c.p.m. value used for subsequent calculations. Recovery of ^{14}C-PEG from solutions perfused through the colon was calculated to be 97±1% (n=4) which indicated that ^{14}C-PEG could be used as an accurate marker of colonic fluid transport with little absorption or adsorption occurring. Influent and effluent fluid samples containing ^{14}C-PEG were further 'spiked' with a known amount of ^{14}C-PEG. Increments in c.p.m.

were observed to be similar in both influent and effluent solutions indicating that any contamination in effluent samples was not producing a significant amount of quenching of the beta emissions from ^{14}C-PEG during liquid scintillation counting.

Results shown in table 8 show measurements of colonic fluid, Na and Cl transport over 7 consecutive 20 minute periods beginning after a 40 minute equilibration period in unfasted and 48h fasted rats. It appeared that similar levels of fluid and electrolyte transport were recorded in both fasted and unfasted animals. It was decided to use fasted rats for future experimentation since this facilitated the flushing out of colonic contents and therefore reduced the likelihood of tissue damage due to distention. To obtain a preliminary indication as to whether this technique could register differing levels of fluid and electrolyte transport, two animals were perfused with a solution containing hypertonic mannitol (110mM) which is known to induce osmotic secretion in the rat colon *in vivo* (Donowitz and Charney, 1979). There did appear to be obvious differences between the levels of fluid and electrolyte absorption recorded in animals perfused with normal isosmotic solutions and those perfused with hypertonic mannitol (see table 8).

The protocol for subsequent experiments was as follows:- After a 40 minute equilibration period fluid and electrolyte transport was measured over 2 consecutive 20 minute control periods and transport parameters expressed as the mean of these 2 periods (period A). Animals were then given a single injection of adrenoceptor antagonist dissolved in dextrose (or dextrose alone for controls) and after 5 minutes fluid and electrolyte transport were measured over two consecutive 20 minute test periods. Period B represents the mean values of these two periods.

Fluid Transport ($\mu l.20min^{-1}.cm^{-1}$.)	n	20 MINUTE PERFUSION PERIODS 1	2	3	4	5	6	7
Fasted rats	4	67.35±27.8	66.26±21.0	59.92±13.1	67.32±12.21	74.84±25.79	74.12±25.76	66.2±34.82
Unfasted rats	4	76.92±26.44	74.65±26.82	76.25±21.81	36.0 ±31.54	47.0 ± 31.54	56.74±29.16	44.37±19.88
Fasted rats mannitol (110mM) perfused	2	0.72±12.23	8.91±12.38	15.43±4.29	16.1±4.16	0.57±25.98	-	-
Na Transport ($\mu equiv.20min^{-1}.cm^{-1}$)								
Fasted rats	4	71.17±44.03	63.00±41.09	54.57±30.7	73.74±19.83	83.27±36.2	80.83±44.07	63.55±64.14
Unfasted rats	4	86.94±34.26	94.63±39.12	94.7±46.49	31.12±42.25	39.41±59.1	56.26±52.44	30.51±39.38
Fasted rats mannitol (110mM) perfused	2	-98.73±34 97	-95.69±3.23	-73.2±9.34	-72.25±9.53	-84.92±56.66	-	-
Cl Transport ($\mu equiv.20min^{-1}.cm^{-1}$)								
Fasted rats	4	-9.81±59.95	9.96±33.56	-7.65±6.19	28.13±2.55	17.42±45.66	19.72±46.48	5.43±56.29
Unfasted rats	4	43.85±27.01	54.77± 19.44	819.47±906.46	-16.99±45.05	12.61±50.89	-6.65±63.18	-3.1±43.82
Fasted rats mannitol (110mM) perfused)	2	-139.21±40.02	-127.17±3.85	-117.59±15.74	-116.6±15.93	-139.42±60.23	-	-

Table 8. Fluid, Na and Cl absorption in rat colon *in vivo* as measured by single pass lumen perfusion. Negative values indicate net secretion.

Antagonists were administered intravenously via cannulation of the left femoral vein, the cannulation procedure being performed prior to the 40 minute equilibration period.

Table 9 shows the results obtained from this study with adrenoceptor antagonists. In control animals (receiving dextrose only between periods A and B) similar levels of fluid, Na and Cl transport were recorded in periods A and B. Bolus administration of either phentolamine ($5mg.Kg^{-1}$) or propranolol ($5mg.Kg^{-1}$) did not produce any significant effect upon fluid or electrolyte transport. It did become apparent however that a great deal of variability existed in transport parameters between individual animals, as can be seen by comparing the measurements made in control periods from the phentolamine and propranolol groups. In addition variability between the adjacent 20 minute control periods contributing to period A was often discouragingly large. At this point a check was made to determine whether a significant decrease in absorption in period B could actually be registered with this experimental protocol. This was done by perfusing normal isosmotic solution through the colon during period A and solution containing 110 mM mannitol during period B. As has been mentioned, mannitol was certain to produce an anti-absorptive effect in each case. Mannitol was seen to produce a significant decrease in fluid transport but not electrolyte transport. The effect upon fluid transport was not highly significant ($0.05>P>0.01$) highlighting the problem of period to period variability within each individual animal which it seemed would mask all but the largest of effects. It was,however, considered worthwhile to conclude this investigation by examining the effects of the selective α_1-adrenoceptor antagonist prazosin ($1mg.Kg^{-1}$) and the selective α_2-adrenoceptor

n	Period	Treatment	PARAMETER Fluid Transport ($\mu l.20min^{-1}.cm^{-1}$)	Na Transport ($\mu equiv.20min^{-1}.cm^{-1}$)	Cl Transport ($\mu equiv.20min^{-1}.cm^{-1}$)
	A	Control	75.09±15.82	74.65±14.55	63.44±38.67
	B	Control	64.44±17.64	72.71±24.44	65.64±42.11
10	P		NS	NS	NS
	A	Control	41.33±16.71	3.44±21.82	7.56±22.08
	B	* Mannitol (110mM)	18.27±12.93	-25.86±34.49	-31.85±21.02
5	P		< 0.05	NS	NS
	A	Control	6.96±8.48	-1.67±16.52	2.69±44.88
	B	Propranolol ($5mg.Kg^{-1}$)	-3.42±16.41	-15.32±28.17	-26.17±25.47
5	P		NS	NS	NS
	A	Control	77.25±34.48	93.98±48.82	53.31±47.14
	B	Phentolamine ($5mg.kg^{-1}$)	59.61±29.33	77.65±42.26	42.18±40.3
4	P		NS	NS	NS
	A	Control	15.07±18.36	-16.31±32.73	-24.07±30.9
	B	Prazosin ($1mg.kg^{-1}$)	6.24±16.48	-0.68±35.61	-29.28±26.91
5	P		NS	NS	NS
	A	Control	83.38±18.17	102.48±31.7	77.28±29.63
	B	Idazoxan ($5mg.kg^{-1}$)	50.74±17.63	56.35±27.87	38.00±22.62
6	P		<0.01	<0.05	NS
	A	Control	45.88±11.99	32.74±19.16	5.78±19.68
	B	Idazoxan ($5mg.kg^{-1}$)	31.7±11.13	11.16±21.39	-16.32±18.17
6	P		NS	NS	NS
	A	Control	40.47±8.64	39.32±11.23	18.17±7.88
	B	RX811059A ($1mg.kg^{-1}$)	36.44±6.94	31.99±8.22	14.4±19.42
4	P		NS	NS	NS

Table 9. Fluid, Na and Cl transport in rat colon *in vivo* measured by single pass lumen perfusion. Period A is the mean of two 20 minute perfusion periods beginning after an initial 40 minute equilibration period. At the end of Period A animals received i.v. administration of antagonist or vehicle as indicated in Period B. Period B is the mean of two 20 minute perfusion periods beginning 5 minutes after the end of Period A. * indicates group received perfusion of salt solution containing mannitol during Period B. Statistical comparisons were made between Periods A and B for each experiment using Student's 't'-tests for paired data.

antagonists idazoxan ($5mg.Kg^{-1}$) and RX811059A (Doxey et al, 1984) ($1mg.Kg^{-1}$) upon transport parameters. Prazosin did not produce any significant effect upon colonic fluid or electrolyte transport, but in one group of 6 animals idazoxan did appear to produce a significant anti-absorptive influence upon colonic fluid, Na and Cl transport. This effect was, however, not reproducible in another group of 6 idazoxan treated animals and could not be mimicked RX811059A.

2. Discussion.

As mentioned in the introduction to this section, evidence exists to suggest that the sympathetic nervous system exerts a tonic influence upon colonic fluid and electrolyte transport in the rat. If this is the case then antagonism of the appropriate receptors involved should produce a reduction in transport parameters. Unfortunately results obtained in this study using the single pass lumen perfusion technique with specific adrenoceptor antagonists have proved inconclusive. After some initially encouraging results (table 8) it became clear that the technique was indeed posing problems, the major problem being inherant variability from period to period within individual animals. The technique employed was chosen in preference to a ligated loop preparation as it was thought to be more representative of the passage of contents through the lumen and had been previously used to demonstrate opiate mediated absorptive tone in the rat colon (Fogel and Kaplan, 1984). Unfortunately use of the technique revealed an inherent and major source of error. It was observed that equal volumes of fluid were not always collected over the 20 minute perfusion periods, due

largely to the apparent secretion of mucus with consequent blockage and distension of the colon, and also movement of the colon *in situ*. This meant that fluid was present in the colon for variable periods of time with a consequent non-uniformity in the time for absorptive and secretory processes to act resulting in period to period variability in measured absorption. As the colon is attached to the posterior abdominal wall for much of its lengh it was not possible to expose the colon outside the animal which might have aided the the passage of fluid through the lumen . A ligated loop preparation would not have presented these problems associated with 'transit time' of fluid through the gut.

Results obtained with mannitol perfusion during period B indicated that with the technique as described here only the largest of anti-absorptive responses would stand any chance of generating a significant result. In one group of 6 animals an apparently significant inhibitory effect of the α_2-adrenoceptor antagonist idazoxan was observed. This effect was however not reproducible since it was not observed with a second experimental group. Furthermore, no significant effect was observed with the other α_2-adrenoceptor antagonist employed; RX811059A. Results were not improved if period B was designated as only the first 20 minute test period instead of the mean of two. Combining two 20 minute periods may have contributed to the difficulty in demonstration of effects since the influence of intravenously administered antagonists may have been declining during the second 20 minute test period. A further source of difficulty in this study, even if the technique had been more reliable, was the liklihood that varying degrees of sympathetic tone would be present within individual animals.

On the basis of results obtained from the present study it is not possible to claim a demonstrable inhibitory effect upon fluid and electrolyte absorption as a result of α_2-adrenoceptor blockade, however, there is some evidence to suggest that α_2-adrenoceptors do play a role in the regulation of absorptive and/or antisecretory processes in the rat colon. α_2-adrenoceptor stimulation produces an antisecretory response in the rat colon *in vitro* (as evidenced by decreases in SCC) (Dharmsathaphorn et al, 1984) but not however in the rat colon *in vivo* (Bunce and Spraggs, 1983a). There is also circumstantial evidence which suggests that α_2-adrenoceptor blockade does inhibit intestinal fluid and electrolyte transport, since in a number of studies rats pretreated with idazoxan have been observed to rapidly develop loose stools (J.Lord personnel communication). α_2-adrenoceptor antagonists could reduce fluid absorption *in vivo* by at least two peripheral mechanisms:- 1) a blockade of epithelial α_2-adrenoceptors normally mediating absorptive tone and 2) a blockade of pre-junctional α_2-adrenoceptors which exert an inhibitory influence upon the cholinergic stimulation of motility i.e. inhibitory influences upon motility would be removed and transit time of luminal contents decreased providing less time for the absorption of fluid in the colon. Overall there would appear to be good reason to re-investigate the role of α_2-adrenoceptor sub-types in the regulation of colonic absorption, perhaps using the isolated loop techniques of Bunce and Spraggs (1982) or Levens et al (1980).

GENERAL DISCUSSION

1. Perspectives

It has been known for many years that extrinsic nerves that release noradrenaline can influence transepithelial intestinal electrolyte transport and more recent studies have confirmed this concept. Much of this work has been recently summarised by Sjovall (1985) whose own work has shown that stimulation of sympathetic nerves enhances intestinal fluid and electrolyte transport both under basal and secretory conditions and that these effects are inhibited by the α-adrenoceptor antagonist phentolamine.

Following the identification of distinct sub-populations of α-adrenoceptors (Langer, 1977; Starke, 1977) there was increased interest in the sympathetic control of fluid and electrolyte transport in the small intestine since it was recognised that selective adrenoceptor compounds might be of considerable therapeutic value in the treatment of severe diarrhoeal diseases, many of which are characterised by massive secretion of fluid and electrolytes from the small intestine. The elucidation of the role of specific adrenoceptor sub-types in the control of intestinal fluid and electrolyte transport is necessary in order to maximise the efficacy of drug treatment of these diseases.

Prior to the present study it had been reported that selective α_1-adrenoceptor stimulation increased basal levels of fluid absorption across everted sacs of rat jejunum (Parsons et al, 1983; Cotterell et al, 1983) whilst in the rat jejunum *in vivo* secretagogue induced fluid secretion was reported to be reversed by selective α_2-adrenoceptor

stimulation (Nakaki et al, 1982a; 1982b; Bunce and Spraggs, 1983a; 1983b). Subsequent studies have revealed the presence of both α_1 and α_2-adrenoceptors on basolateral membranes purified from epithelial cells of rat jejunum (Nakaki et al, 1983; Cotterell et al, 1984). Again in the rat jejunum *in vivo* Levens and co-workers have reported that angiotensin stimulates fluid absorption via pre-synaptic release of noradrenaline from sympathetic nerve terminals which subsequently interacts with post-synaptic α_1-adrenoceptors (Levens et al, 1981b; 1981c; Levens, 1983). This mechanism has since been reported to mediate the reflex increases in fluid absorption from the rat jejunum in models of haemorrhage and dehydration (Levens, 1984a; 1984b). From a mechanistic viewpoint, studies performed with isolated sheets of rabbit small intestine have shown that selective α_2-adrenoceptor stimulation enhances electrically silent NaCl absorption and inhibits electrogenic Cl secretion (Chang et al, 1982; Durbin et al, 1982).

The broad aims of the present study were to characterise adrenoceptor mechanisms mediating changes in intestinal fluid and NaCl transport in the rat. The study commenced with the jejunum since (a) there was evidence for adrenoceptor mediated influences upon intestinal fluid and electrolyte transport in this tissue and (b) the manipulation of fluid and electrolyte transport in the small intestine has relevance to the treatment of diarrhoeal disease. The rat was studied because it is a relatively cheap experimental animal.

2. The Influences of Adrenoceptor Compounds Upon Fluid Absorption in Everted Jejunal Sacs

The initial technique employed for the investigation of adrenoceptor control of jejunal fluid and NaCl transport was the everted sac technique of Wilson and Wiseman (1954). There were a number of reasons for selecting this *in vitro* technique; it is quick, simple, allows for the direct measurement of fluid transport and is widely employed in intestinal transport studies where it has been reported that noradrenaline stimulates fluid and Na transport (Aulsebrook, 1965; Parsons et al, 1984) through an α_1-adrenoceptor mediated mechanism (Parsons et al, 1983; Cotterell et al, 1983). However in the present study this technique was found to be unsuitable for the characterisation of receptor mechanisms. The pharmacological data obtained using this technique failed to support the rather tenuous evidence from previous studies (Parsons et al, 1983; Cotterell et al, 1983) that a selective α_1-adrenoceptor mechanism mediates increases in fluid absorption across everted sacs of rat jejunum. The conclusions of Cotterell et al (1983) were based on the observation that the selective α_1-adrenoceptor antagonist prazosin inhibited the stimulant effect of noradrenaline. However both the present study and that of Burgess et al (1984) have shown prazosin alone to exert an antiabsorptive influence upon intestinal fluid and electrolyte transport. The conclusions of Parsons et al (1983) were only supported by the reported inhibition of the stimulant effect of noradrenaline by the selective α_1-adrenoceptor antagonist indoramin which has many properties unrelated to α_1-adrenoceptor blockade and for this reason is not commonly used in

receptor classification studies. The present observations that the selective α_1-adrenoceptor antagonists BE2254 and corynanthine failed to inhibit the stimulant effect of noradrenaline, that phenylephrine failed to reproduce the stimulant effect of noradrenaline and that only a high concentration of cirazoline was effective in stimulating fluid transport made it impossible to implicate α_1-adrenoceptors in the control of intestinal fluid and electrolyte transport in this preparation.

It has previously been reported that secretory mechanisms are compromised in the everted sac preparation (Munck, 1972a) and this affords a likely explanation for the observation that the antisecretory effects of α_2-adrenoceptor stimulation reported in the rat jejunum *in vivo* could not be reproduced in everted sacs *in vitro* in the presence of theophylline, whose effects in this preparation are likely to be predominantly antiabsorptive rather than secretory. Thus it appears that antiabsorptive influences of theophylline are not reversed by α_2-adrenoceptor stimulation in this preparation. Generally the everted sac preparation appears to be inappropriate to the study of receptor mechanisms mediating increases in fluid and NaCl transport. In the present study a stimulation of fluid transport in response to noradrenaline under basal conditions has been identified but attempts to characterise the receptors involved using selective adrenoceptor antagonists has been unsuccessful. It is possible that anoxic conditions in the region of receptor sites produces conformational changes such that receptors do not conform to either α_1 or α_2-adrenoceptor subtypes. Alternatively a major problem could be access to the receptor sites which have been reported to be located on basolateral epithelial cell membranes (Nakaki et al, 1983; Cotterell et al, 1984). Access to these

receptor sites via the serosal bathing medium requires passage through the smooth muscle and subepithelial tissue which may present an insurmountable barrier to many of the synthetic adrenoceptor compounds.

3. α_2-Adrenoceptors and Electrogenic Secretory Mechanisms

The next major step in the present study was the development of the Ussing chamber technique. This *in vitro* system has been successfully used in the investigation of neuroendocrine control of active ion transport in a number of species and it was hoped that this technique would allow for adrenoceptor classification studies concerning the absorptive and secretory mechanisms in the rat jejunum.

The pharmacological characterisation of adrenoceptor mediated changes in electrogenic secretion as evidenced by changes in SCC was fairly straightforward. Decreases in basal SCC in response to noradrenaline were inhibited by selective α_2-adrenoceptor antagonists and reproduced by low concentrations of the selective α_2-adrenoceptor agonist UK-14,304, but only high concentrations of the preferential α_1-adrenoceptor agonist phenylephrine. The effects of both UK-14,304 and phenylephrine were inhibited by α_2-adrenoceptor blockade with idazoxan. Experiments involving the replacement of Cl ions in the bathing media supported the hypothesis that most of the basal SCC recorded across rat jejunum represents electrogenic Cl secretion (Munck, 1972a) and that it is highly probable that the electrical effects of α_2-adrenoceptor stimulation reflect a depression of this process. In the presence of a secretory stimulus (theophylline) which elevates SCC predominantly via the stimulation of electrogenic Cl secretion (Rao and Field, 1983),

noradrenaline was seen to decrease SCC in a concentration dependent manner. The concentration-response relationship to noradrenaline showed parallel rightward displacement in the presence of idazoxan, indicating competitive antagonism, with an estimated PA_2 of 7.6 for idazoxan which is consistent with figures reported for idazoxan at other peripheral post junctional α_2-adrenoceptors (Medgett et al, 1984). Experiments employing the neurotoxin tetrodotoxin supported the hypothesis that the antisecretory effects of α_2-adrenoceptor stimulation *in vitro* are exerted directly upon the epithelium. This observation is consistent with the demonstration of α_2-adrenoceptors on basolateral epithelial cell membranes purified from rat jejunum (Nakaki et al, 1983; Cotterell et al, 1984).

It was of note that the effects of theophylline in this *in vitro* preparation were reversed by α_2-adrenoceptor stimulation which is analogous to the findings of other workers *in vivo* and in contrast to the results obtained with the everted sac preparation providing further, strong, circumstantial evidence to suggest that transport mechanisms in the everted sac preparation were not operating normally. Antisecretory effects of α_2-adrenoceptor stimulation have also been reported in rabbit duodenum, jejunum and ileum *in vitro* (Durbin et al, 1982; Chang et al, 1982) and rat colon *in vitro* (Dharmsathaphorn et al, 1984). Indeed α_2-adrenoceptor agonists show considerable promise therapeutically as a new approach to the treatment of diarrhoeal diseases. In 1982, McArthur and co-workers reported that a patient with secretory diarrhoea secondary to a VIP-producing bronchogenic carcinoma responded to treatment with the α_2-adrenoceptor agonist clonidine with a 50% decrease in stool volume. Treatment with α_2-adrenoceptor agonists appears to

produce little clinically significant hypotension but chronic depression does appear to be a problem. The development of α_2-adrenoceptor agonists that do not cross the blood brain barrier is thus of interest as is the exploitation of the reported subtle differences between central and gastrointestinal α_2-adrenoceptors as observed in both the rabbit ileum and rat colon *in vitro* (Dharmsathaphorn et al, 1984). The model of rat jejunum *in vitro* provides a relatively cheap source of material for the study of antisecretory properties of adrenoceptor agonists and indeed other compounds.

4. α_1-Adrenoceptors and Absorptive Processes in the Rat Jejunum- Experimental Findings and Wider Implications

Reasonable evidence exists to suggest that in the rat jejunum *in vivo* an α_1-adrenoceptor mechanism mediates increases in basal fluid and electrolyte transport (Levens et al, 1981a; Levens, 1983) and reflex increases in fluid absorption in response to haemorrhage (Levens, 1984a) or dehydration (Levens, 1984b). Increases in fluid absorption in response to haemorrhage or dehydration are believed to be mediated via release of angiotensin which in turn enhances noradrenaline release from sympathetic nerve terminals synapsing with α_1-adrenoceptors. Pharmacological characterisation of these receptors, which is a difficult task in the complex *in vivo* situation, has not been performed by Levens and co-workers and the conclusions that α_1-adrenoceptors are involved is based on the observations that responses to noradrenaline or angiotensin are blocked by prazosin or phentolamine. As has been discussed, prazosin alone has been shown to influence electrolyte

transport *in vitro* via mechanisms unrelated to blockade of α_1-adrenoceptors(Burgess et al, 1984).

Clearly there is room for further pharmacological investigation in this area and attempts have been made in the present study to demonstrate an α_1-adrenoceptor mediated influence in the simpler *in vitro* situation which is more appropriate to studies of receptor characterisation. As has been discussed, the everted sac study did not allow for the identification of specific adrenoceptor mechanisms. and the Ussing chamber study has shown that α_1-adrenoceptors do not participate in mediating electrogenic antisecretory processes in the rat jejunum. Consequently if an α_1-adrenoceptor mechanism is present at the jejunal mucosa it is likely that it influences electrically silent NaCl absorption. In support of this, the effects of angiotensin *in vivo* have been shown to occur in the absence of any changes in transmural potential difference (Levens et al, 1979).

In the present study mucosal to serosal fluxes of ^{22}Na were recorded across isolated sheets of rat jejunum and the effects of noradrenaline upon electrically silent NaCl absorption were investigated under both basal and secretory conditions by monitoring changes in mucosal to serosal ^{22}Na movement. However, no significant stimulation of Na absorption in the presence of noradrenaline was recorded. Similarly preliminary experiments showed that a significant stimulation could not be obtained with either the selective α_1-adrenoceptor agonist cirazoline or the selective α_2-adrenoceptor agonist UK-14,304. It was thought that a likely explanation for this was the fact that the experiment was attempting to demonstrate a relatively small stimulatory effect against

large, passive background fluxes apparently present in the rat small intestine. Additionally any stimulation of Na absorption in response to noradrenaline is likely to be variable in magnitude, in a similar manner to the observed effects of noradrenaline in everted sacs which probably reflects only absorptive processes, the secretory processes being compromised.

Despite the failure to demonstrate an α_1-adrenoceptor mediated influence upon NaCl absorption *in vitro*, strong circumstantial evidence does suggest that such a mechanism is present at a mucosal level. Radioligand binding studies by Cotterell et al (1984) have demonstated the presence of α_1-adrenoceptors on jejunal epithelial cells although such binding studies cannot be taken as confirmation of a functional role. Further the stimulant effect of angiotensin *in vivo* occurs in the absence of any changes in intestinal blood flow (Bolton et al, 1975) and is reproducible in everted sacs of rat jejunum *in vitro* (Crocker and Munday, 1970). It must be stressed, however, that there has been no further successful investigation of the mechanism of action of angiotensin *in vitro*. It is possible that in the *in vitro* situation angiotensin exerts its influence via a direct mucosal mechanism whilst the observed stimulation of absorption by angiotensin *in vivo* is mediated by the enhanced release of noradrenaline. In this respect it would be interesting to see if a stimulant response to noradrenaline was present in everted sacs taken from animals that had undergone chemical sympathectomy with 6-hydroxydopamine. In the *in vivo* situation it is conceptually possible that the arm of the sympathetic nervous system working through post-junctional α_1-adrenoceptors mediates adjustments in absorptive processes in response to changes in extracellular fluid

volume, this process being reinforced by the pre-synaptic action of angiotensin, whilst the α_2-adrenoceptor system which mediates antisecretory influences functions to maintain overall control over the plethora of local intestinal secretory mechanisms.

Munday and Poat (1983) failed to demonstrate any significant effects of angiotensin or tyramine upon transepithelial ^{22}Na fluxes or electrical parameters but did report that noradrenaline significantly stimulated mucosal to serosal ^{22}Na movement and also significantly reduced transepithelial SCC. The failure of angiotensin or tyramine to influence electrolyte transport was interpreted as being indicative of a lack of endogenous noradrenaline. However, on the basis of the results obtained in the present study, it is possible that a stimulatory influence of angiotensin or tyramine on mucosal to serosal ^{22}Na transport was not observed by these workers because of the problem of large background fluxes. Angiotensin may not have produced an electrical response simply because it acts to selectively release noradrenaline to receptors mediating neutral NaCl absorption. However, decreases in transepithelial SCC in response to tyramine, which stimulates release of noradrenaline from sympathetic nerve terminals, have been reported in the rabbit ileum *in vitro* (Tapper et al, 1981). An alternative explanation to that offered by Munday and Poat (1983) for the non-effect of tyramine upon electrical parameters in the rat jejunum, that of a lack of endogenous noradrenaline, is that tyramine may release noradrenaline which acts additionally at β-adrenoceptors mediating electrogenic secretory events which 'cancel out' the antisecretory effects of noradrenaline at α_2-adrenoceptors. This suggestion is supported by two observations from the present study -

i) incidental observations in a number of tissues (data not presented) that high concentrations of noradrenaline (>$5x10^{-4}$M) do not apparently influence basal SCC (or even slightly increase SCC) and ii) that noradrenaline does not fully reverse the effects of theophylline. The implication is that in each case high concentrations of noradrenaline activate β-adrenoceptors which mediate secretory events which antagonise the antisecretory effects of noradrenaline at α_2-adrenoceptors. This hypothesis would be supported if a decrease in SCC in response to tyramine was observed in the presence of a β-adrenoceptor antagonist.

5. β_2-Adrenoceptors and Jejunal Electrolyte Secretion

There have been only a few conflicting reports concerning β-adrenoceptor mediated influences upon intestinal NaCl and fluid transport. Antisecretory or absorptive effects of the β-adrenoceptor antagonist propranolol have been reported in both the rat jejunum *in vivo* (Donowitz and Charney, 1979; Levens, 1983) and the colon of a number of mammalian species, both *in vitro and in vivo* (Coyne et al, 1977; Conley et al, 1976; Taub et al, 1977). In contrast however, administration of propranolol to human volunteers has been reported to result in an increase in secretion of fluid and electrolytes in the small bowel (Morris and Turnberg, 1981). In the rat colon *in vitro* β-adrenoceptor stimulation with isoprenaline has been reported to stimulate net NaCl absorption (Racusen and Binder, 1979) and in the rabbit colon *in vitro* it has been reported to inhibit Cl secretion (Gaginella, 1984).

In the present study the interest in β-adrenoceptor mediated mechanisms was aroused by the observation that propranolol induced an immediate sustained decrease in SCC in concentrations as low as $5x10^{-7}M$. Similar effects were observed with low concentrations of the selective β_2-adrenoceptor antagonist ICI 118551 and larger decreases in SCC observed with the neurotoxin tetrodotoxin, the latter observation almost certainly indicating the presence of nerve mediated secretory tone in this *in vitro* preparation. Thus a proportion of secretory tone may be mediated via β_2-adrenoceptors. Further it was shown that the non selective β-adrenoceptor agonist isoprenaline and the preferential β_2-adrenoceptor agonist salbutamol both induced increments in basal SCC, indicative of a secretory response, and that these effects were inhibited by ICI 118551. In rat jejunum *in vitro* it appears that β_2-adrenoceptors mediate both a portion of secretory tone and also increases in secretory processes. The effects of isoprenaline were not inhibited by tetrodotoxin indicating an epithelial effect of β_2-adrenoceptor stimulation. Secretory tone mediated by spontaneously active neurons originating from the submucosal plexus in stripped preparations of rat colon *in vitro* has been previously reported (Andres et al, 1985; Bridges et al, 1986).

Cassuto and colleagues (Cassuto et al, 1981a; 1981b; 1982a; 1982b; 1982c; Eklund et al, 1985) have recently provided evidence that the majority of the secretory effects of bacterial toxins and a number of other secretagogues are mediated via the activation of neuronal reflexes. It has been further postulated that these reflexes are initiated in many cases via the release of 5-hydroxytryptamine from mucosal endocrine cells and that a cholinergic synapse is a link in the

reflex chain. The neurotransmitter(s) released at the final enterocyte synapse are at present unknown but on the basis of observations made in the present study, and of other reports of the antisecretory effects of propranolol in models of stimulated secretion *in vivo* (Donowitz and Charney, 1979; Coyne et al, 1977; Conley et al, 1976; Taub et al, 1977) a possible candidate is noradrenaline synapsing with β_2-adrenoceptors located on enterocytes or endocrine cells releasing a paracrine secretory factor. Such a scheme would explain the apparent antisecretory or absorptive influences of propranolol. However, much of the attention concerning adrenoceptor control of fluid and electrolyte transport has focused on the antisecretory effects of α-adrenoceptor stimulation and relatively little comment appears to have been made in recent literature concerning the possible use of β-adrenoceptor antagonists as an antisecretory therapy in diarrhoeal diseases. This may be due at least in part to the observation of Morris and Turnberg (1981) that propranolol appeared to stimulate fluid and electrolyte secretion in human volunteers. However, the present investigation has provided the first evidence to suggest that β_2-adrenoceptors may be involved in mediating secretory processes and highlights the possible usefulness of selective β_2-adrenoceptor antagonists in the treatment of secretagogue induced fluid loss.

6. Adrenoceptor Subtypes and the Control of Intestinal Electrolyte Transport Mechanisms

A general scheme for adrenoceptor mediated influences upon jejunal electrolyte transport is summarised in fig 26 embracing sites of action

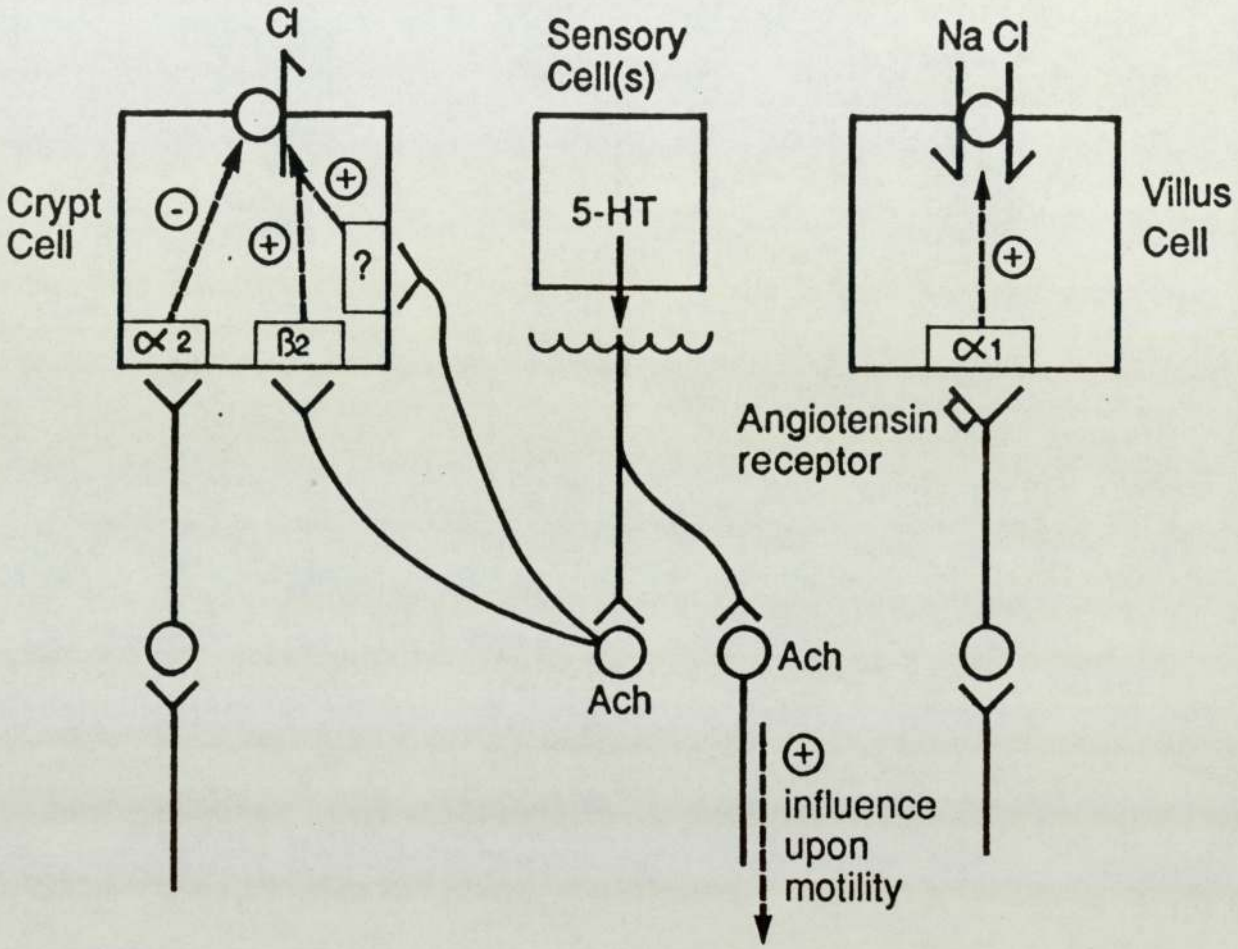

Fig. 26. Diagrammatic representation of the likely localization and influences upon Na and Cl transport of adrenoceptor subtypes in the rat jejunum. Adrenoceptors are represented as being present directly upon enterocytes, it is possible however, that adrenoceptors are present on mucosal endocrine cells that release paracrine agents which elicit absorptive or secretory responses as appropriate. + indicates a stimulatory influence and - an inhibitory influence. α_1-adrenoceptors maybe located on cells mediating neutral absorption of NaCl, the nervous influence being reinforced by presynaptic release of noradrenaline in response to angiotensin stimulation. $\alpha_2$adrenoceptors mediate an antisecretory influence upon electrogenic Cl secretion. β_2-adrenoceptors appear to mediate secretory processes and normally mediate a tonic influence. Recent work (see text for details) has shown that a large portion of the secretory response to a number of bacterial toxins and other secretagogues are mediated via neuronal reflexes which in many cases appear to be initiated via the release of 5-HT and involve at some level a cholinergic synapse. One of the final synapses for this reflex circuit may involve β_2-adrenoceptors located on epithelial cells.

for α_1-adrenoceptors mediating neutral NaCl absorption, α_2-adrenoceptors mediating antisecretory influences upon electrogenic Cl secretion and β_2-adrenoceptors mediating electrogenic secretion. The physiological roles of these adrenoceptor mediated mechanisms in the control of intestinal fluid and electrolyte transport is unclear. It is possible that sympathetic nerves synapsing with α_1-adrenoceptors which mediate increased fluid absorption under basal conditions (Levens et al, 1981a; Levens 1983) and in conditions of haemorrhage (Levens 1984a) or dehydration (Levens 1984b) modulate absorptive processes in response to extracellular fluid volume depletion. The α_2-adrenoceptor mediated system may function more specifically to maintain overall control over the secretory functioning of the intestine. This would however require some sort of sensory input at some level providing information about the status of secretory processes. This may be achieved through the modulation of release of noradrenaline from sympathetic nerve terminals by as yet unidentified factors. As discussed in the previous section, β-adrenoceptors may mediate anion secretion whose level of activity is dictated by input from the enteric nervous system. Activity may vary in discrete areas of intestine according to conditions in the local luminal environment. In each case adrenoceptors appear to be located on epithelial cells . These cells may be enterocytes or possibly endocrine cells releasing paracrine secretory or absorptive agents as appropriate. Indeed the idea that α_2-adrenoceptors are located on endocrine cells which release an antisecretory agent is tempting bearing in mind the low density of α_2-adrenoceptor ligand binding to basolateral membrances purified from rat jejunum (Cotterell et al. 1984). Somatostatin is one of the few naturally occuring compounds known to inhibit secretory

processes, and it has recently been localised to mucosal endocrine cells of sheep jejunum (Harrison et al, 1986). In the present study the possibility that noradrenaline may have been inhibiting electrogenic secretory processes via the stimulation of release of somatostatin from mucosal endocrine cells was tested by investigating the effects of noradrenaline on SCC in the presence of a somatostatin antagonist. Noradrenaline was observed to produce a full antisecretory response in the presence of somatostatin antagonist which suggested that the effects of noradrenaline were not mediated via somatostatin release. However, until α_2-adrenoceptors are posotively localised to enterocytes as opposed to endocrine cells the possibility remains that noradrenaline may inhibit anion secretion via the stimulation of release of some as yet unidentified neuroendocrine intermediate from mucosal endocrine cells.

7. Sympathetic Tone and Colonic Fluid and Electrolyte Transport

The possible existence of adrenoceptor mediated absorptive tone in the rat colon *in vivo* has been highlighted by the observations of Chang et al (1983b) that in diabetic rats where sympathetic nerve endings have been destroyed, diarrhoea develops largely due to colonic fluid secretion. Dharmsathaphorn et al (1984) identified the existence of an α_2-adrenoceptor mechanism which mediated antisecretory processes in the rat colon *in vitro*. This observation raises the possibility that any sympathetic tone present may be mediated via α_2-adrenoceptors. If this is the case then it might be expected that α_2-adrenoceptor antagonists would reduce basal levels of fluid and electrolyte transport in the rat

colon *in vivo*. This line of investigation seemed to provide for a concise investigation that hopefully would quickly answer questions concerning adrenoceptor mediated tone in the colon. Furthermore, selective α_2-adrenoceptor antagonists may be of some potential therapeutic value in the management of chronic constipation if α_2-adrenoceptor blockade proves to consistently reduce colonic fluid transport. Possible sites of action for the antiabsorptive effects of α_2-adrenoceptor antagonists are:-(i) the removal of inhibitory influences upon secretory mechanisms through blockade of α_2-adrenoceptors located on epithelial cells and (ii) the blockade of inhibitory α_2-adrenoceptors located pre-synaptically on cholinergic neurons which normally stimulate colonic motility and reduce intestinal transit time.

Unfortunately the results of this investigation proved to be rather inconclusive (see chapter 3). The technique selected for the measurement of colonic fluid and electrolyte transport *in vivo*, a single pass lumen perfusion technique (Bright-Asare and Binder, 1973) did not appear to be sufficiently precise to answer questions concerning adrenoceptor mediated tone. This is yet another area that needs to be re-adressed, perhaps using an alternative *in vivo* technique such as the isolated loop techniques of Bunce and Spraggs (1982a) or Levens et al (1980). Ligated loop techniques do not necessitate the 'even' passage of fluid through the gut during the course of an experiment which is *a priori* with a perfused system. Details of the problems raised by the lumen perfusion technique employed in the present study have been discussed in chapter 3.

8. Avenues For Future Research

A number of possible lines of investigation can be highlighted that would clarify some of the questions raised by the experimental observations of the present study and provide further information concerning the functions of the intestine in respect to the control of transepithelial fluid and electrolyte transport and volume homeostasis:-

(i) An attempt to characterise receptors associated with the stimulation of basal fluid and electrolyte transport by noradrenaline *in vivo*. This has been reported to be an α_1-adrenoceptor mediated event as it is inhibited by the selective α_1-adrenoceptor antagonist prazosin. As has been seen from the everted sac investigation, classification of a response as being α_1-adrenoceptor mediated on the sole basis of blockade by prazosin is dubious, although the *in vivo* studies of Levens and co-workers indicated that prazosin did not influence basal levels of fluid absorption. Because of a shortage of time it was not possible to follow this line of investigation in the present study, particularly in view of the difficulties that had already been encountered in trying to measure the stimulatory effect of noradrenaline upon basal levels of absorption in both the everted sac and Ussing chamber studies.

(ii) It may be possible to characterise adrenoceptors associated with the stimulation of absorptive processes using an approach that makes use of cell culture techniques. For example sodium and chloride uptake into cultured enterocytes could be measured using radiolabelled ions under basal conditions, in the presence of noradrenaline and in the presence of noradrenaline and selective adrenoceptor antagonists. This type of approach has recently been used by Semrad and Chang (1986) to positively

identify an inhibitory effect of atrial natriuretic factor upon ^{22}Na uptake into isolated chicken enterocytes.

(iii) Further research is necessary to establish the mechanism of action of angiotensin *in vitro*. Again in this respect use could be made of cell culture techniques as attempts to identify effects of angiotensin upon transepithelial Na transport in isolated sheets of rat jejunum mounted in Ussing chambers have proved unsuccessful (Munday and Poat, 1983). Alternatively the mechanism of action of angiotensin *in vitro* could be investigated using other mammalian species such as the rabbit.

(iv) The natriuretic hormone atrial natriuretic factor has as stated been demonstrated to reduce ^{22}Na uptake into isolated chicken enterocytes (Semrad and Chang, 1986). Further studies into the influences of this peptide in both *in vitro* and *in vivo* systems will provide new information concerning its role in the control of transepithelial fluid and electrolyte transport and volume homeostasis.

(v) The observations of Nakaki et al (1982a;1982b) that phenoxybenzamine or prazosin alone inhibit secretagogue induced fluid secretion in the rat jejunum *in vivo* are worthy of further investigation, initially in respect as to whether this effect is related to the adrenoceptor blocking properties of these compounds.

(vi) On the basis of results obtained in the present study which indicated that β_2-adrenoceptors mediate secretory processes in the rat jejunum, and other reports that many secretagogues initiate secretory processes via activation of neuronal reflexes, and further that propranolol exhibits antisecretory properties *in vivo*, it would have been interesting to investigate the effects of selective β_2-adrenoceptor

antagonists upon secretagogue induced fluid secretion *in vivo*. The use of β_2-adrenoceptor antagonists may represent a new approach in the treatment of diarrhoeal diseases. This approach may have the advantage of actually directly blocking the secretory pathways activated by intestinal secretagogues.

(vii) A micro-autoradiographical study of the location of mucosal α_2-adrenoceptors would provide information concerning the binding of α_2-adrenoceptor ligands to epithelial cells and it may be possible to identify if the binding is to enterocytes or to the endocrine cells of the jejunal mucosa. This type of investigation would indicate if α_2-adrenoceptor stimulation inhibited secretory processes by a direct effect upon enterocytes or by by the release of an intermediate from mucosal endocrine cells.

CONCLUSIONS

This investigation has failed to confirm previous claims that noradrenaline stimulates fluid absorption in everted sacs of rat small intestine *in vitro* via an α_1-adrenoceptor mediated mechanism. This classification was largely based on the observation that the selective α_1-adrenoceptor antagonist prazosin inhibited the stimulatory effect of noradrenaline. However, this study revealed an anti-absorptive influence of prazosin *per se*. The everted sac investigation further highlighted the general inappropriatness of this technique for receptor classification studies.

Using a modified Ussing chamber preparation the presence of an α_2-adrenoceptor mechanism mediating anti secretory effects upon anion secretion through a direct mucosal influence was identified *in vitro*. Thus antisecretory effects of α_2-adrenoceptor agonists reported in rabbit small intestine *in vitro* (and rat small intestine *in vivo*) are reproducible in this *in vitro* model of rat jejunum. A β_2-adrenoceptor mechanism mediating both increases in anion secretion and also a portion of secretory tone was also identified.

These findings have confirmed the potential of α_2-adrenoceptor agonists as antisecretory agents and further suggested that β_2-adrenoceptor antagonists may be useful in the treatment of diarrhoeal conditions where secretagogues exert their influences via the activation of enteric secretory pathways. The model of rat jejunum *in vitro* would

appear to be appropriate for further pharmacological investigation in these areas.

APPENDIX I

ELECTRONIC DEVICES ASSOCIATED WITH THE MONITORING OF TRANSEPITHELIAL PD AND SCC FROM INTESTINAL TISSUE MOUNTED IN USSING CHAMBERS

The following equipment was designed and built in the Department of Pharmaceutical Sciences, Aston University by Mr.D. Briggs.

1) Electrode Offset Compensator (Potentiometer)

This device was needed to make adjustments to correct for slight differences in potential between the voltage measurement electrodes. It was comprised of a stable mercury battery (1.35v) which passed a small current through a large value resistor (3.3 Mohms) onto a bridge network of fixed resistors and a multi-turn potentiometer. The connections are such that, when the battery is switched on, the offset voltage obtainable between the wiper of the potentiometer and the mid-point of the resistor network is evenly adjustable between +3 and -3 mV. Across the entire adjustment range, the net impedence of the circuit, as seen by the voltage clamp, remains close to 10 Kohms.

2) High Resolution Digital Millivoltmeter

The circuitry for this device is based on the use of a digital multimeter integrated circuit (R.S. Components Ltd., Northants). This chip uses a sophisticated combination of analogue and digital techniques to obtain 4½ digits of conversion. It also provides the control signals for the liquid crystal display and requires very little additional circuitry to make the complete meter. The display gives a reading between +199.99 and -199.99 mV with a resolution of 10μV. The conversion technique used guarantees a zero reading for a zero signal input, which makes the meter useful for nulling the voltage clamp. To provide additional accuracy, a quartz crystal (100KHz) is used to drive the converter clock. The display is updated at the end of each conversion, of which there are approximately 2 each second. The meter runs on a single 9V battery monitoring circuit which illuminates a 'Low Battery' annunciator on the display when the battery voltage drops below about 6.5v.

3) Automatic Voltage Clamp (Short-Circuit Current Amplifier)

a) Circuitry

This voltage clamp design is very similar to that described by Rothe et al (1969). The circuitry for the device breaks down into three main sections. These are:-

(i) the voltage sensing differential amplifier;
(ii) the current measurement amplifier; and
(iii) the current generator.

Signals from the potential recording electrodes feed into the inputs of two high input impedence operational amplifiers which are arranged in an instrumentation amplifier configuration. This amplifier multiplies, by a factor of approximately 5, the difference between the potentials, before

feeding two signals into a following differential amplifier. This amplifier also amplifies the signal, having a gain of about ten. This differential amplifier produces a single-sided output, one referenced to ground, which reflects the voltage difference across the membrane.

The current measurement amplifier is used to maintain the overall potential of the bath at a controlled level, and it also sucks any current injected into the bath out again. Current measurement is achieved by forcing the amplifier to use a 4.7 Kohm resistor in the current path. The amplifier always tries to maintain the bath end of this resistor at circuit ground potential. When current is passed into the bath, the voltage rises, and the amplifier has to draw more out again. This arrangement turns the current passing into a voltage signal, also referenced to the circuit ground, but inverted (i.e. a positive current produces a negative voltage).

The output from both signals of these circuits are addded together by the current generator. The resultant signal is the clamp command signal. This is amplified by the current generator, which is also inverting, to give an integral control current which is passed out to the tissue bath.

b) Operation

When no clamp current is flowing, the voltage amplifier senses the difference in voltage across the membrane. The resultant signal can be used as a command signal for the current generator. If the current path was completed and the short-circuit clamp switched on, this would have the effect of reducing the voltage across the membrane to zero. However, when current flows through the saline in the bath, small voltages are deveoloped as a direct consequence of the resistance of the saline itself. The current generator actually clamps the voltage sensing electrodes, and not the tissue. The electrodes do not normally change position during an experiment, so if the resistance in the saline was known, it should be possible to compensate for it. This is determined in the following way:-

The bath is assembled without any tissue in it, but with all other conditions as for an experiment. A hand adjusted potentiometer is then used to pass a varying current through the bath (with the clamp current disconnected). While monitoring the output voltage of the current generator, the proportion of the current signal (as measured by the current measurement amplifier) that is added to the voltage command signal can be varied using a 'built in' multiturn potentiometer.

The object of this adjustment is to cancel out (just) the voltage changes which are being produced by the first potentiometer. When this has been done, then the proportion of the voltage signal which resulted from the resistance of the saline is exactly cancelled out. The multi-turn dial is then locked in position and the first potentiometer removed from the circuit. Then, with a piece of tissue in place, a similar amount of saline will be present between the voltage sensing electrodes, and a similar proportion of the voltage seen will be produced by current flowing in the clamp circuit. When the clamp is operating, any current that is passed through the tissue goes through an accurate 1 Kohm resistor first. The current will induce a voltage across the resistor, and this is measured by another differential amplifier (which can be used to monitor the voltage difference as well). The current or voltage signal is fed out to the high resolution digital millivoltmeter. This

output signal is a low impedence (approximately 1 Kohm) single ended signal, referenced to circuit ground. During operation of the clamp, the voltage signal obtained will not be zero, but can be used to check the clamp efficiency if the saline resistance is known. The automatic voltage clamp is mains powered.

APPENDIX II

ONE WAY ANALYSIS OF VARIANCE

The one way analysis of variance techniques described here is taken from Snedecor and Cochran (1967) and is for application to data where n for each treatment is of unequal number. Data is entered from a table (see table 10) and an F value calculated from the table as indicated, where -

(1) Is the correction for the mean (c), $C = \frac{(\Sigma X)^2}{n}$

This is the sum of every observation in the table squared then divided by the total number of observations in the table.

(2) Is the total sums of squares (total deviance) = $\Sigma (X^2) - C$
This is the sum of the squares of each individual observation - C.

(3) Is treatment sums of squares = $\Sigma \left(\frac{TT_1^2}{n_1} + \frac{TT_2^2}{n_2} + \frac{TT_3^2}{n_3} \ldots . \right) - C$

This is the sum of every treatment total squared divided by n for that treatment - C.

(4) Is the error sums of squares, which is (2) - (3).

A significant F ratio (referenced to statistical tables) simply indicates that there is a significant treatment effect within the table. Tests for a significant difference between two means are performed by calculating a t value from the following formula.

$$t = \frac{\text{difference between the two means compared}}{\sqrt{\text{Error mean square} \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}}$$

n	Treatment 1	Treatment 2	Treatment 3	Treatment 4	.....
	↓	↓	↓	↓	
Means of Data	.....				

Variation	Sums of Squares (SS)	Degrees of Freedom (DF)	Mean Square (Variance Estimate)	F Ratio
Total	(2)	Total number of observations - 1	--	
Treatment	(3)	Number of treatments applied - 1	$\frac{\text{Treatment SS}}{\text{Treatment DF}}$	$\frac{\text{Treatment MS}}{\text{Error MS}}$
Error	(4)	Total DF - Treatment DF	$\frac{\text{Error SS}}{\text{Error DF}}$	

Table 10. One way analysis of variance (Anovar) table.

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