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EFFECTS OF pH MANIPULATION ON CONTRACTILITY OF RAINBOW TROUT INTESTINAL SMOOTH MUSCLE *IN VITRO*

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfillment of the Requirements

for the Degree of

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Faculty of Veterinary Medicine

University of Prince Edward Island

John R. Underhay

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ABSTRACT

Salmonids are exposed to a number of factors that impact on the acid-base status of the animal. Extreme imbalances have been shown to impact on gastrointestinal motility. Changes in pH are known to affect cardiac and skeletal muscle in fish, and mammalian intestinal smooth muscle preparations. It is not known if pH changes affect fish intestinal smooth muscle. This study was conducted to determine if the contractility of rainbow trout intestinal muscle is sensitive to pH alterations and to begin the investigation into the mechanisms by which pH affects the contractility. Isolated duplicate or triplicate segments of rainbow trout intestine were suspended in organ baths containing modified Krebs-Henseleit solution. The pH was adjusted by varying the concentration of CO_2 aerating the solution or with HCl. Contractility was determined as response to the administration of 5-hydroxyamine (5-HT), KCl, and transmural stimulation (TS) within the pH range of 6.3 to 8.5. Optimum pH for proximal segments was 7.85 while the range was much wider for distal segments. When associated with decreasing pH, CO_2 causes a greater inhibition than decreasing the pH in 100% O_2, particularly when the tissues are stimulated electrically. This effect is ameliorated as the fish attain sexual maturity, although the mechanisms involved are not clear. The Cl^-/HCO_3^- exchanger - inhibited by 4-acetamido-4'-isothiocyanto-stilbene-2-2'-disulfonic acid (SITS) - appears to be involved in recovery from acidotic stress, particularly at pH levels below 6.5. The role played by the Na^+/H^+ exchanger - inhibited by 5-(N-ethyl-N-isopropryl) amiloride (EIPA) - is still unclear. Although contractility is inhibited in the presence of EIPA, whether the inhibition is due to blockage of ion transport, or to cytosolic effects is unknown. Lactic acid does not inhibit contractility induced by 5-HT or KCl; however, at physiological concentrations, an inhibition of electrically induced contractions occurred. It can be concluded that the effects of pH on rainbow trout intestinal smooth muscle contractility are exerted primarily on the nerves innervating the gut rather than on the muscle itself.

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For Sally, Joshua, Sara Lise and Mitchell
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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
EIPA	5-(N-ethyl-N-isopropryl)amiloride
GH	Growth hormone
GI	Gastrointestinal
HCl	Hydrochloric acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethane sulfonic acid)
KCl	Potassium chloride
KHS	Krebs-Henseleit solution
MES	2-(N-morpholino)ethane sulfonic acid
NANC	Non-adrenergic non-cholinergic
pH_e	Extracellular pH
pH_i	Intracellular pH
SID	Strong ion difference
SITS	4-acetamido-4'-isothiocyanto-stilbene-2-2'-disulfonic acid
TAPS	N-tris(hydroxymethyl)methyl-3-aminopropane sulfonic acid
TS	Transmural stimulation
VIP	Vasoactive intestinal peptide

1. CHAPTER 1 LITERATURE REVIEW

... the examination of material elements and means is not to be regarded as final, but as preparatory to the conception of the total form.

Aristotle, Parts of Animals

1.1 Acid-base regulation

Maintaining an acid-base equilibrium presents a greater challenge to fish than it does to terrestrial animals, for both environmental and physiological reasons. The gill epithelium of aquatic animals must be exposed to large amounts of water to compensate for the lower oxygen concentration of water compared to that of air. At the same time, the chemical composition of water is highly variable. Thus, the physiology of fish is modulated by the environment to a much greater extent than that of land animals (reviewed by Heisler, 1993). A particular concern for fish is acid-base regulation.

1.1.1 Buffering systems

Acid-base disruptions are the result of the introduction of H^+ or OH^- into body fluids as metabolic by-products or from the consequence of environmental disturbances (Heisler, 1986). Buffering of the surplus ions may occur in the intracellular regions where the system involves protein residues. Alternatively, the buffering may occur in the extracellular fluids and the circulatory system where the bicarbonate/carbon dioxide ratio is altered.

The two buffering systems are differentiated as being 'open' or 'closed', based on whether or not the concentrations of the components are able to fluctuate. The bicarbonate system consists of CO_2, H_2CO_3, HCO_3^- and CO_3^{2-} (Equation 1), and is considered to be open because the $[CO_2]$ varies as the gas readily diffuses across membranes (Heisler, 1986).

$$H_2O + CO_2 \rightleftarrows H_2CO_3 \rightleftarrows HCO_3^- + H^+ \qquad \text{(Eq 1)}$$

In terrestrial animals, regulation of $[CO_2]$ is easily accomplished by hyperventilating, as the Pco_2 of plasma is much higher than that of the surrounding air, facilitating diffusion. Removal of the CO_2 from the system drives the equilibrium to the left, decreasing the amount of free H^+. The Pco_2 of water, however, is almost the same as that of the plasma of fish; therefore, increasing water flow over the gills will not offer any relief to the fish (Heisler, 1989). In fish, bicarbonate buffering functions as a closed system, and is of little use to fish under normal circumstances.

Non-bicarbonate buffering occurs within the intracellular space where surplus H^+ ions are neutralized by protein residues such as histidine, cysteine and terminal NH_2 moities (Heisler, 1986). This is considered to be a closed system as the total protein concentration remains relatively constant (Equation 2).

$$H^+ + Base^- \rightleftarrows HBase \qquad \text{(Eq 2)}$$

As transepithelial and transmembranous transfer of strong ions is rapid in fish, non-bicarbonate buffering is efficient and the principal method of acid-base regulation utilized by fish (Cameron, 1989).

1.1.2 Branchial ion flux

Branchial ion transfers occur both through apical exchange mechanisms and by diffusion through paracellular channels (McDonald and Prior, 1988). Increasing the base load in the plasma of rainbow trout (*Oncorhynchus mykiss*) results in a net increase in inward Cl^- flux primarily by augmenting the surface area of gill filament chloride cells (Goss and Perry, 1994). This is accomplished, not by increasing the number of chloride cells, but by altering the morphology of the adjacent pavement cells which expand and block a number of apical Cl^-/HCO_3^- exchangers on the chloride cells (Perry and Laurent, 1993). The exposed surface area of the chloride cells can be experimentally altered by the infusion of cortisol and growth hormone (GH) (Perry and Goss, 1994). Similar examination of the integument did not find a cortisol stimulated proliferation in skin chloride cells (Iger at al., 1994).

The ionic membrane flux is explained by the Strong Ion Difference (SID) in which quantitative evaluation of acid-base regulation can be interpreted utilizing three independent variables - Pco_2, [SID] and total protein (Stewart, 1983). Strong ions are electrolytes that dissociate completely in physiological solutions, and the strong ion difference is the net electrical charge represented by the arithmetical difference between the total cation and the total anion concentrations in the solution. The most common physiological strong ions are Na^+, Cl^-, K^+ and, in some circumstances, lactate. To maintain the principle of electrical neutrality, the concentrations of H^+ and HCO_3^- are regulated by shifting reaction equilibria within the system. The strong ion difference has been experimentally verified to be an effective estimator for acid-base status in physiological systems (Kowalchuk and Scheuermann, 1994).

1.2 Response to acid and alkaline stress

Imbalances in the acid-base regulation of salmonids can arise from a number of sources. Plasma alkalosis can be caused by high external ammonia (Cameron and Heisler, 1983), hypercapnia (Gilmour and Perry, 1994), and acute alkaline exposure (Wright and Wood, 1985; Wilke *et al.*, 1994). In contrast, high internal ammonia (Cameron and Heisler, 1983) or hyperoxi (Gilmour and Perry, 1994; Gilmour *et al.*, 1994) induce acidosis. Acidosis can also be induced by increasing water temperature (Cameron, 1989) or by decreasing water pH (Packer and Dunson, 1970; Neville, 1979; Packer, 1979; Booth *et al.*, 1982; Fryer *et al.*, 1988). The acid-base disturbances of environmental acidification are dependent on the concentration of Ca^{2+} in the water (reviewed by Wood and McDonald, 1982, Wood, 1989). Exercise induced lactacidosis will also decrease the plasma pH (Tang *et al.*, 1989; Wood, 1991; Wang *et al.*, 1994).

1.2.1 Water acidification

One of the primary causes of acid-base disturbances in freshwater fish is the acidification of lakes and streams. Along with atmospheric deposition transported downwind from industrial areas, localized natural and anthropogenic sources of acidification occur. These include: the production of carbonic acid from CO_2; the hydrolysis of minerals; decomposition and nitrification of ammonium produced by bacterial decomposition of vegetation and from fertilizers; the oxidation of sulfur in dry soil; the action of organic acids from decaying humus; the release of H^+ from plant roots in exchange for Mg^{++} and Ca^{2+}; and from runoff in newly deforested areas (reviewed by Mason, 1989).

These anthropogenic and natural sources combine to decrease pH levels in fresh water below the survival threshold for many organisms,

particularly on base poor substrates that are found over much of the Precambrian Shield (Gorman *et al.*, 1986). The impact of this has been expressed by Trembley and Richard (1993), who estimate that more than 10,000 fish populations in Quebec lakes have been lost to the effects of acidification since 1900. These effects are not isolated to Canada, as analyses have also been conducted in the United States (Haines *et al.*, 1986) and Europe (Bulger *et al.*, 1993) with similar results. Nor are these losses confined to vertebrates; disruptions throughout the food chain are a result of this acidification. Macroinvertebrates are also affected by acidification; reductions in taxonomic richness, abundance and diversity of molluscs, crustaceans, ephemeroptera and diptera was found in low pH waters in northeastern France (Guerold *et al.*, 1993). The effects of acidification on the growth of fish populations involve food source depletion, physiological compensatory complications and physical damage.

1.2.1.1 Results of acid stress

Manifestations of acid stress are myriad in salmonids. One of the most obvious is a decrease in feeding (Dively *et al.*, 1977; Lacroix and Townsend, 1987) and a concomitant reduction in growth rate (Menendez, 1976). In soft water (low $[Ca^{2+}]$) the primary physiological response to sublethal acid exposure is ionic imbalance. Whole body levels of Na^+, K^+, and Cl^- are decreased (Packer and Dunson, 1970; Booth *et al.*, 1982; Lacroix and Townsend, 1987; Audet *et al.*, 1988; Curtis at al., 1989; Farmer at al., 1989); however, this change is a removal of ions from the muscle and not a change in plasma levels (Audet *et al.*, 1988; Booth *et al.*, 1982). In Atlantic salmon (*Salmo salar*), the impairment of ionic regulation was found to be associated with an inhibition of branchial ATPase activity (Johnston *et al.*, 1984; Saunders *et al.*, 1983). An

increase in hematocrit and plasma concentrations of protein and glucose (Lacroix and Townsend, 1987; Audet *et al.*, 1988; Farmer *et al.*, 1989) indicates a decrease in blood volume that eventually leads to circulatory collapse (Wood, 1989). Audet and Wood (1988) determined that sublethal acid exposure decreases the ability of rainbow trout to respond to further acid stress.

Morphologically, tissue damage of fish exposed to either acid or alkaline environments occurs below pH 5.2 and above pH 9.0. At both extremes, mucous cells hypertrophy and an increase in mucus secretion and epithelial necrosis and sloughing is apparent on the gills, nares and integument (Daye and Garside, 1976). The tissue response of rainbow trout exposed to acid stress is similar to that of fish exposed to other environmental stressors (Iger *et al.*, 1994), suggesting that many of the responses are related to increases in cortisol or other aspects of the generalized stress response as described by Pickering (1992). Olfaction in mature male Atlantic salmon is impaired by exposure to acid water, interfering with pheromonal communication essential for reproduction (Moore, 1994); however, whether the decreased response is due to epithelial damage or other factors is not yet known.

1.2.1.2 Amelioration by calcium

In hard water, the high concentration of Ca^{2+} ameliorates the effects of acidification both in the water and in the fish itself. In the water, the effect is to drive the dissociation of water away from the production of free H^+ ions. On the fish, the effects are several, including making the membranes less permeable to both ions and water, and inhibiting the ionic exchangers thereby reducing the net efflux of Na^+ and Cl^- (McDonald *et al.*, 1983). In soft water, branchial losses of Na^+ and Cl^- are approximately equal; in hard water, however, there is a greater relative Na^+ loss and increased uptake of H^+ (Wood, 1989). These

interactions have the result of maintaining the ionic balance within the plasma of the fish while initiating a chronic metabolic acidosis (McDonald *et al.*, 1980). The ameliorating effects of calcium are also apparent in a decrease in critical swimming speed and an increase in exercise induced mortality in acidified soft water when compared with hard water (Graham and Wood, 1981).

1.2.2 Effects of akalinization

Alkaline environments are more likely to be the result of slow changes in the geography of a region than the result of relatively rapid anthropogenic disturbances. Rainbow trout exposed to extreme alkaline conditions demonstrate much of the same physical damage that is associated with extremely acidic water (section 1.2.1.1). The physiological accommodations required, however, are somewhat different. Cutthroat trout (*Oncorhynchus clarki henshawi*) transferred from pH 8.4 to pH 9.4 exhibited a negative metabolic acid load, which was relieved within 24 hours, and an indefinite decrease in arterial Pco_2 (Wilkie *et al.*, 1994). Rainbow trout exposed to pH 9.5 decrease ammonia efflux and Na^+ uptake as much as 80% compared with normal conditions (Wright and Wood, 1985). During $NaHCO_3$ infusion, rainbow trout utilize elevated Cl^- influx, depressed Cl^- efflux and extensive intracellular buffering to maintain ionic homeostasis (Goss and Perry, 1994). Cutthroat trout, however, decrease their ammonia production, and increase Cl^- influx (Wilkie *et al.*, 1994). This is accomplished by an increase in the exposed surface area of the branchial chloride cells and an increase in the Cl^-/HCO_3^- exchange activity, assisted by a relatively high NaCl concentration in the lake (Wilkie *et al.*, 1994).

1.2.3 Sources of acidosis in fish

Besides the ionic and acid-base irregularities caused by external acidification or alkalinization, there are several other sources of acid-base disturbances in trout.

1.2.3.1 Water temperature

Increasing water temperature inversely affects internal pH. The dissociation of water into H^+ and OH^- ions is enhanced at higher temperatures, causing a drop in the environmental pH. Greater metabolic activity coupled with hypoxia at elevated temperatures, as dissolved O_2 drops, leads to a decrease in plasma pH. This is primarily due to the increase in anaerobic metabolism resulting in chronic lactacidosis (Cameron, 1989). Exhaustive exercise increases plasma lactate levels, a condition exacerbated by higher temperatures; post-exercise recovery time, however, is not affected by temperature (Kieffer *et al.*, 1994).

1.2.3.2 Hyperoxia, hypoxia and hypercapnia

The fall in arterial Po_2, under hypoxic conditions, triggers the release of catecholamines (Perry and Reid, 1994). The resultant increase in oxygen carrying capacity described by Heath (1987) is caused by the release of sequestered erythrocytes, the activation of the Na^+/H^+ exchanger on existing erythrocytes and an enhancement of gill O_2 diffusing capability (Perry and Reid, 1994). Tang and Boutilier (1988) suggested that an increase in the level of plasma cortisol was the result of acidosis. The activation of the Na^+/H^+ exchanger by circulating catecholamines suggests that plasma acidosis is the result, rather than the cause, of elevated chromaffin tissue activity.

Adding to the acid-base imbalance is the production of lactic acid as the end product of anaerobic metabolism (Cameron, 1989). Acidosis is also the physiological response to hyperoxic and hypercapnic conditions. In hyperoxia, respiratory rate decreases, Pco_2 increases and plasma pH drops (Randall and Cameron, 1973). Hypercapnia causes an increase in free HCO_3^- and H^+ and a new steady state pH is maintained for as long as the hypercapnia occurs (Cameron, 1989).

Acidotic stress places a strain on cardiac muscle of rainbow trout, reducing force and elongating the action potential (Höglung and Gesser, 1987). The effects are ameliorated somewhat, but not completely, by the increased plasma levels of Ca^{2+} and adrenaline that occur during acidosis (Farrell *et al.*, 1986).

1.2.3.3 Exercise

Exhaustive exercise in fish causes a decrease in plasma pH resulting from a combination of free H^+ ions and elevated Pco_2 (reviewed by Wood, 1991). The lactate released into the blood, however, is much less than the total produced in the muscle, suggesting *in situ* oxidation (Wang *et al.*, 1994) rather than gluconeogenesis via the Cori cycle that occurs in the liver in mammals (Stryer, 1975). As described above (section 1.2.3.1), increased temperature causes an increase in the amount of lactic acid produced, but does not affect recovery time. In addition, saltwater acclimated rainbow trout are more efficient in regulating exercise induced acid-base disturbances than sibling fish acclimated to freshwater probably due to the more efficient ionoregulatory mechanisms that accompany smoltification (Tang *et al.*, 1989). Environmental acid potentiates the acidotic stress of exercise, regardless of water hardness (Graham and Wood, 1981).

1.3 Rainbow trout gastrointestinal tract

1.3.1 Trout gastrointestinal anatomy

The basic anatomy of the rainbow trout gastrointestinal tract (GI) tract follows the conventional vertebrate pattern in both cross-sectional and longitudinal organization. A cross-section of the intestinal wall shows the muscularis longitudinalis (longitudinal muscle) immediately inside the serosa. Internal to this is the muscularis circularis (circular muscle) followed by the strata granulosum and compactum (loose and dense connective tissue respectively). On the basement membrane lies the luminal epithelium which may be folded and ciliated depending upon the location within the GI tract (Yasutake and Wales, 1983) (Fig. 1.1).

Following the entire tract in a cranio-caudal direction, we find first, an oral cavity ringed with teeth, followed by a pharynx, and a short esophagus. Caudal to this is the stomach, consisting of three parts, a cardiac region, a mid or transitional region and a pyloric region ending in a pyloric sphincter (Yasutake and Wales, 1983). The intestine is also divided into two or three sections: (1) proximal section with blind, absorptive caeca, (2) mid-intestine - the region between the last pyloric caeca and (3) the distal section which is identified visually by a reddish color and annular folds (Fig 1.2). Histologically, the proximal and middle regions are similar, and debate has continued over the proper nomenclature (Yasutake and Wales, 1983); the principal difference being the presence of the caeca in the proximal section which increase the effective surface area and aid in digestion of lipids (Buddington and Diamond, 1987, Ostos Garrido *et al.*, 1993). The distal section is visually, histologically, and functionally distinct. Separated from the mid-intestine by the ileorectal valve, the posterior section is designed to facilitate chyme retention and nutrient

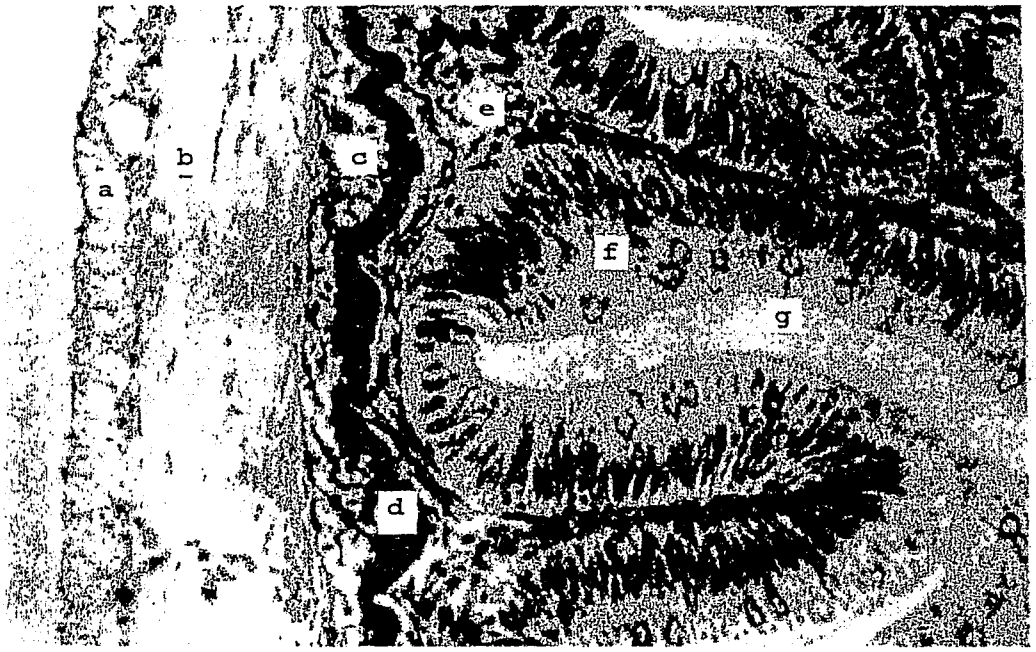


Fig. 1.1. Cross-section of the proximal intestine of rainbow trout showing the layers of the intestinal wall and a mucous cell. (a) muscularis longitudinalis; (b) muscularis circularis, (c) stratum granulosum, (d) stratum compactum, (e) lamina propria, (f) lamina epithelialis, and (g) mucous cell. From Yasutake and Wales, 1983.

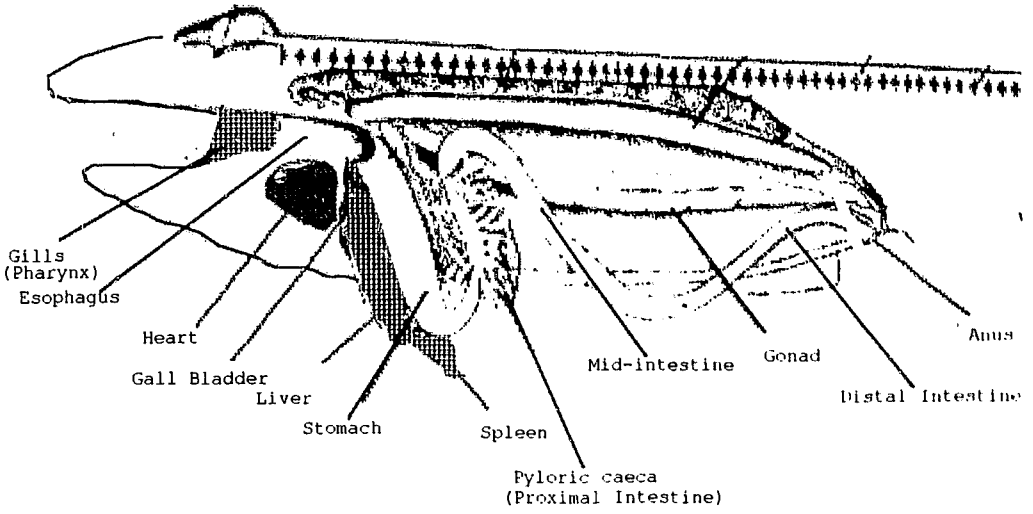


Fig. 1.2. Stylized representation of the digestive system of a rainbow trout with closely associated organs included for reference. From Yasutake and Wales, 1983.

absorption without interfering with defecation (Ezeasor and Stokoe, 1980).

1.3.2 Digestion and growth

Protein macromolecules are absorbed by pinocytosis and digested via vacuolated cells (Abaurrea *et al.*, 1993) and possibly degraded by eosinophilic granulocytes (Dorin *et al.*, 1993) in the posterior intestine. McCarthy *et al.* (1994) suggest that individual growth rates in rainbow trout are, at least partially, dependent on individual rates of protein turnover. Commercially available fish feeds contain 25-45% crude protein (Murai, 1992), and the dietary energy retained is in the order of 45-55% (Cowey and Cho, 1993); therefore, factors that impact on protein digestion will undoubtedly affect growth.

Rainbow trout have an optimum temperature for growth of approximately 15°C, (Sumpter, 1992), and a preferred temperature close to 11.5°C (McCauley *et al.*, 1974), although these values may vary with environmental acclimation. This discrepancy may partially be the result of decreased food consumption at higher temperatures, as feeding frequency is dependent on gastric evacuation time which decreases with increasing temperature (Grove *et al.*, 1978). These effects are at least partially due to receptor mediated events in intestinal smooth muscle contractility, as agonists demonstrate greater potency at 15°C than at either 5 or 10°C (Burka *et al.*, 1990, 1993*a*,*b*). Since sublethal acid stress is also associated with decreased appetite (Dively *et al.*, 1977), smooth muscle effects may also be a consequence in acid stressed fish. This may be related to a decrease in gastrointestinal motility caused by a generalized stress response and the increased concentration of circulating catecholamines (Åtland and Barlaup, 1991, Pickering, 1992). These chemicals decrease smooth muscle contractility by increasing the

production of cyclic AMP, thereby exerting a modulating effect on the tissue (Burka *et al.*, 1992). The muscle itself may be sensitive to alterations in physiological pH, either in the intracellular contractile mechanisms, or in the events leading up to the contraction.

1.3.3 Fish GI motility

There has been considerable research into the endogenous control of GI motility in fish; much of the work, however, points to species specific responses to many of the exogenously applied agents.

1.3.3.1 Innervation of the gut

Extrinsic innervation of the gastrointestinal tract of salmonids can be separated, as in most vertebrates, into sympathetic and parasympathetic divisions. Sympathetic innervation is via the coeliac ganglion, part of the sympathetic chain, and located at the level of the third and fourth spinal nerves, from which anterior splanchnic nerves arise that innervate the gut, swim bladder, spleen and liver (reviewed by Nilsson and Holmgren, 1993). The vagus nerve supplies parasympathetic innervation in the esophagus and stomach, while the intestine is innervated by the anterior splenic nerve and posterior nerves that arise from the urophysis (Burnstock, 1959).

An intrinsic enteric nervous system was also identified by Burnstock (1959). This system is ubiquitous among the vertebrates (Jensen and Holmgren, 1994) and consists of two neuronal networks - the myenteric (Auerbach's) and the submucosal (Meissner's) plexi (Fänge and Grove, 1979). In mammals, the myenteric plexus functions primarily to control GI motility, while the submucosal neurons are principally involved in intestinal ion transport and modulation of GI blood flow (Suprenant, 1994). Quantitative comparisons of enteric

nerve cell numbers have demonstrated the presence of fewer cells in fish than in mammals (Burnstock, 1959).

1.3.3.2 Control of GI motility

A large number of both exogenous and potentially endogenous neurotransmitters have been shown to inhibit, initiate or potentiate contractions of salmonid gastrointestinal smooth muscle. These include acetylcholine, adrenaline, nicotine, serotonin (5-HT), eserine and barium (Burnstock, 1958). More recently, Holmgren (1983) examined some putative non-adrenergic non cholinergic (NANC) neurotransmitters and validated and expanded the work of Burnstock (1958). In addition, sensitivities to adenosine, adenosine triphosphate (ATP), vasoactive intestinal peptide (VIP), neurotensin, bombesin, gastrin/ cholecystokinin, substance P, somatostatin and glucagon were also found Holmgren, 1983). Immunocytochemical analysis of gastric mucosal endocrine cells suggested that these same transmitters modulate both secretion and motility (Barrenechea *et al.*, 1994). However, since antisera used in these studies were mostly designed for mammalian preparations, any negative results do not necessarily mean the absence of a modulatory agent. There is also the possibility that substances unique to these species, and as yet unknown, may be involved.

In the smooth muscle of the cardiac stomach, many of the interactions among the known neuronal and hormonal modulators have been elaborated, and some are summarized in Fig 1.3. Substance P is released by enteric neuro[illegible], and acts directly on the smooth muscle fibers to initiate contractions, or on other neurons to release 5-HT. This release of 5-HT is inhibited by VIP. Circulating somatostatin inhibits the release of acetylcholine from cholinergic neurons. The activity of acetylcholine is potentiated by bombesin.

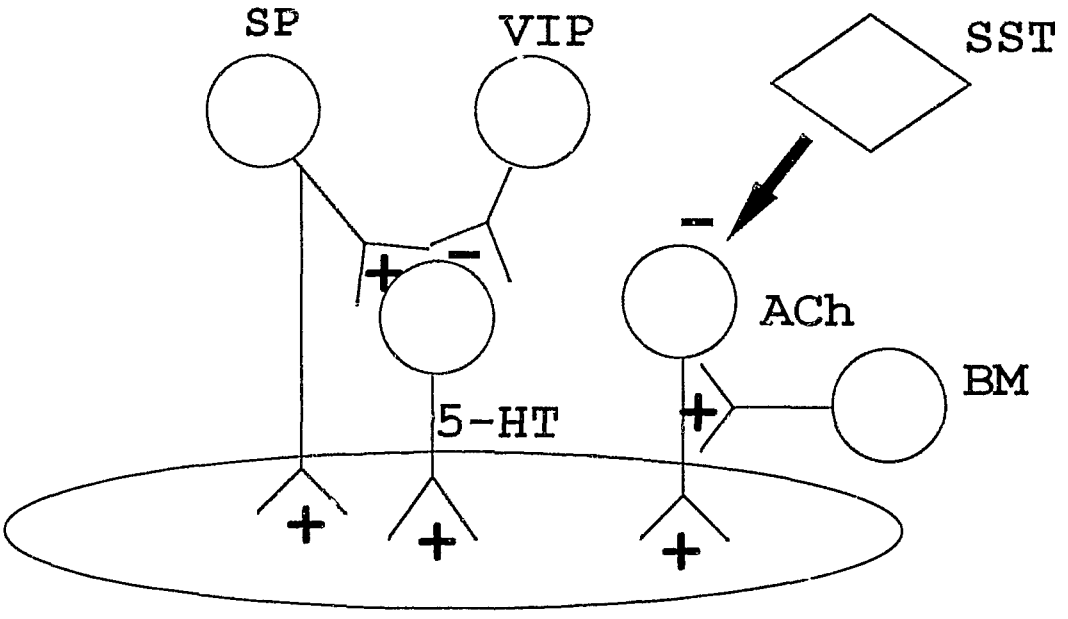


Figure 1.3. Schematic of proposed neuronal and hormonal control of gastric smooth muscle of rainbow trout, (*Oncorhynchus mykiss*). ACh, acetylcholine; BM, bombesin; 5-HT, 5-hydroxytryptamine; SP, substance P; SST, somatostatin; VIP, vasoactive intestinal peptide. + indicates excitation; -, inhibition. Redrawn from Jensen and Holmgren, 1994.

The interactions among these have been examined in relation to artificially induced gastric distension (Grove and Holmgren, 1992). Ignoring the possible contribution of central nervous modulation, the vagal nerve had been severed in these experiments, the enteric interactions have been interpreted in the following manner. The initial rapid expansion is inhibited by the release of acetylcholine. Maintenance of muscle tone and contractions involved in the mixing action are mediated by 5-HT. Somatostatin acts to inhibit contractions necessary to control gastric evacuation, while inhibition by VIP is responsible for the long term relaxation required to accommodate a meal.

1.4 Smooth muscle contractility

Smooth muscle contractions are modulated largely through the regulation of calcium, either by release from intracellular stores or by the entrance into the cell of calcium ions across the cellular membrane (reviewed by Jiang and Stephens, 1994). Calcium stimulates the activation of the calmodulin-myosin light chain kinase complex and the attendant phosphorylation of myosin required for the cycling of actin-myosin crossbridges (reviewed by Allen and Walsh, 1994). These covalent crossbridges have four states - free and attached, phosphorylated and dephosphorylated - allowing for an exceptional economy in force maintenance (Murphy, 1994). This is the mechanism that allows smooth muscle to maintain tone without exhausting the tissue.

The release of calcium from the sarcoplasmic reticulum (SR) is regulated by second messengers such as inositol triphosphate (reviewed by Berridge, 1993). Calcium also enters the cytosol through plasmalemma ion channels such as the L-type calcium channel (Tsien and Tsien, 1990). Modulation of calcium levels is via a buffer barrier

consisting of superficially located SR that maintains the calcium equilibrium required for steady state concentrations within the cell (Chen and van Breeman, 1993). Calcium release from the SR is also regulated by a feedback mechanism that involves the calcium induced degradation of the inositol triphosphate receptor on the SR (Magnusson *et al.*, 1993).

1.5 Intracellular pH

Smooth muscle is not homogeneous. The methods by which each tissue maintains homeostasis are specific to the lifestyle of the organism and the function of the tissue under consideration. Providing a pH range within which cellular activities can be sustained is imperative to life, and organisms have evolved mechanisms to regulate physiological pH. These mechanisms can operate at the organism level (Section 1.2) or at the cellular level within the organism.

Mammalian smooth muscle exists in an environmental pH of approximately 7.4 and maintains an internal pH (pH_i) that ranges from 7.08 to 7.20 depending on the species, the tissue, the method of measurement and the temperature of the muscle (Wray, 1988). Regulation of pH is accomplished by passive diffusion of metabolic gases, particularly CO_2, or by ion transport mechanisms (reviewed by Roos and Boron, 1981). The active transport of hydrogen ions in exchange for sodium is ubiquitous in prokaryotes and eukaryotes, both plants and animals (Padan and Schuldiner, 1993). Thomas (1984) describes two other ion exchangers involved in intracellular pH regulation: the Na^+ dependent and independent Cl^-/HCO_3^- exchange (Fig. 1.4). All of these mechanisms may not be found in a single cell, and if more than one is present, the relative importance of each may be related to the physiological function of the cell (Aickin, 1994*c*). For example, Mair

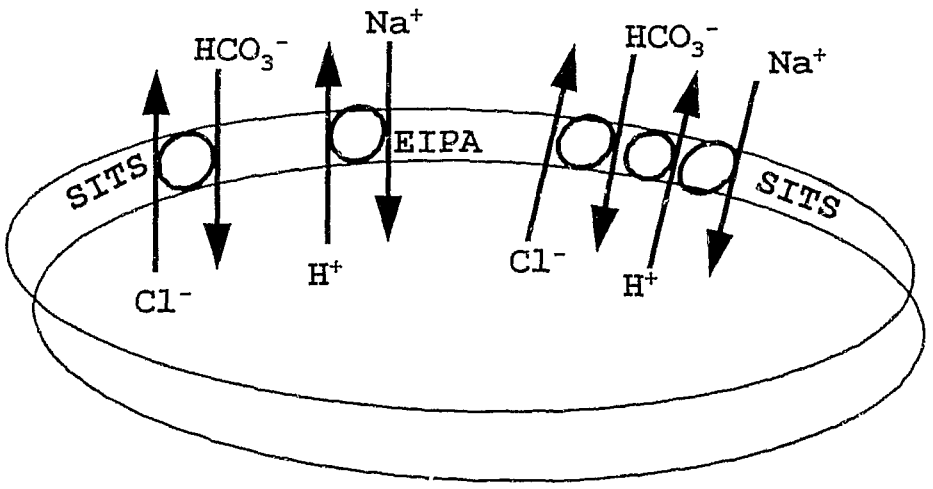


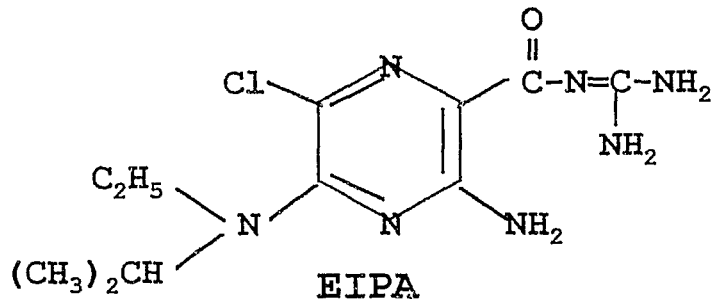
Fig 1.4. Representation of the three primary exchange mechanisms involved in intracellular pH regulation. Pharmacological exchange blockers are noted. (SITS: 4-acetamido-4'-isothiocyanto-stilbene-2,2'-disulfonic acid; EIPA: 5-(N-ethyl-N-isopropyl)amiloride. Redrawn from Thomas (1984).

et al. (1993) determined that the Na^+/H^+ and the Na^+ dependent Cl^-/HCO_3^- exchange both function in crayfish neurons, while only the Na^+ independent Cl^-/HCO_3^- exchange was found in cardiac myocytes from the marine clam *Mercenaria campechiensis* (Ellington, 1993). In smooth muscle from the guinea pig ureter, pH_i is returned to normal by activation of the Cl^-/HCO_3^- exchange following experimentally induced alkalosis (Aickin, 1994*b*), while only the Na^+/H^+ exchange is involved in recovery from acidosis (Aickin, 1994*a*).

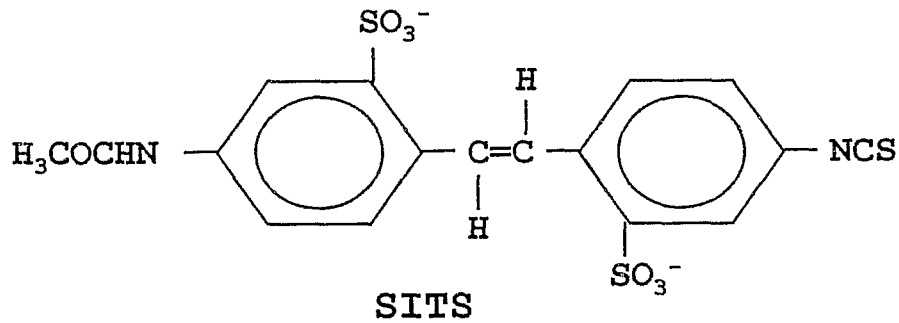
The relative importance of each of the exchange mechanisms can be clarified by the use of pharmacological blocking agents. The Na^+/H^+ exchange is blocked by amiloride and its analogs, such as EIPA (5-(N-ethyl-N-isopropyl)amiloride) which exhibits greater selectivity for this specific ion channel (Kleyman and Cragoe, 1988) (Fig 1.4). Blocking of the Na dependent and independent Cl^-/HCO_3^- exchanges can be accomplished with stilbene sulfonic acid derivatives such as SITS (4-acetamido-4′-isothiocyanto-stilbene-2-2′-disulfonic acid) (Thomas, 1984) (Fig 1.4). The latter two exchangers can be differentiated by the relative ability of tissues to regulate pH_i in the presence or absence of sodium. SITS binds covalently to free amines on the exchanger by the Edman Reaction (Roos and Boron, 1981). This reaction and the chemical structures of EIPA and SITS are presented in Fig. 1.5.

1.5.1 Na^+/H^+ exchange

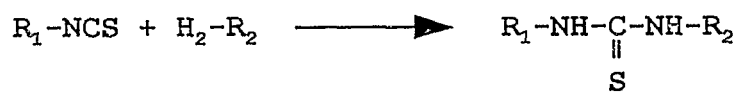
The function of the Cl^-/HCO_3^- exchange has been well documented, but little work has been done on its regulation and expression. Much time and effort, however, has been dedicated to understanding the Na^+/H^+ exchange. Alfonso *et al.* (1994 *a,b*) determined that rat mast cell Na^+/H^+ exchange is activated by phosphorylation through pathways involving protein kinase C and Ca^{2+}. This increased activity



EIPA



SITS



Edman Reaction

Fig 1.5. Structural formulas for ion exchange blockers. Na^+/H^+ exchange: EIPA (5-(N-ethyl-N-isopropyl)amiloride; Cl^-/HCO_3^- exchange: SITS (4-acetamido-4'-isothiocyanto-stilbene-2-2'-disulfonic acid; Edman Reaction by which SITS binds to the anion exchanger. From Roos and Boron, 1981.

can be mediated by hormonal stimulation and chronic acidosis (Fliegel and Frölich, 1993). In human lymphocytes, prolonged metabolic acidosis enhances Na^+/H^+ exchange activity and increases levels of Na^+/H^+ exchange mRNA (Quednau *et al.*, 1994). L-glutamine and L-asparagine stimulate exchange in porcine jejunal enterocytes (Rhoads *et al.*, 1994).

Altering the activity of the Na^+/H^+ exchange can influence smooth muscle contractility indirectly by affecting calcium metabolism. Increasing Na^+/H^+ exchange activity by decreasing pH_i in guinea pig cardiac myocytes slows Ca^{2+} efflux through the Na^+/Ca^{2+} exchange due to the unfavorable Na^+ gradient generated by the excess H^+ ions (Terracciano and MacLeod, 1994). Ca^{2+} is mobilized from the sarcoplasmic reticulum by Na^+ (Borin *et al.*, 1994); however, whole cell Ca^{2+} decreases due to modulation of L-type Ca^{2+} channels (Klöckner and Isenberg, 1994). Protons interact with specific sites on the channel proteins to regulate Ca^{2+} influx (Pietrobon *et al.*, 1989; Prod'hom *et al.*, 1989). A direct effect of pH_i on the Ca^{2+} dependent aspects of contraction must also be considered (Iino *et al.*, 1994), for example, the phosphorylation of myosin light chain kinase increases the Ca^{2+} concentration required for further contractions (Stull *et al.*, 1993).

1.5.2 Intracellular pH in trout

In trout, the physiological impact of acidotic stress on smooth muscle function is unclear. Butler and Day (1993) found that in brown trout (*Salmo trutta*) there is no relationship between changes in plasma pH from pH 7.85 to pH 8.0 (due to temperature changes) and changes in intracellular pH of red (pH 7.2), white (pH pH 7.2) or cardiac (pH 7.4) muscle. In contrast, hypercapnic acidosis (pH 6.9)

in rainbow trout decreases force and prolongs the action potential in cardiac muscle due to a net loss in cellular Ca^{2+} (Höglund and Gesser, 1987). The plasma pH changes in hypercapnia may be larger than temperature effects which could explain this apparent contradiction. Rainbow trout intestinal smooth muscle will retain contractility over a wide temperature range; maintaining considerable force at 2°C (Burka *et al.*, 1990) while contractility of mammalian tissue is greatly reduced at low temperatures (Blackwood and Bolton, 1993). This discrepancy can be at least partially explained by a higher affinity to Ca^{2+} of the contractile elements of rainbow trout smooth muscle when compared to mammalian tissue (Churcott *et al.*, 1994). It is possible that the functional pH range for rainbow trout intestinal smooth muscle would be wider as well.

1.6 Research objectives

Rainbow trout are hardy animals able to live in a wide variety of habitats and withstand considerable stress during human intervention in the environment. In order to maintain functionality of the systems required for life, the tissues within these animals must perform under a wide range of conditions. These conditions include ranges of internal temperature and pH that are much greater than in mammals. Burka *et al.* (1989, 1993*a*,*b*) have examined the impact of altering temperature on the contractility of the intestinal smooth muscle. These studies showed that fish intestinal segments maintain contractilty at much lower temperatures when compared with mammalian tissues. It is the purpose of this study to expand this research to include the range of pH that these animals tolerate, and to begin the inquiry into the mechanisms by which the tissues regulate internal levels of pH and maintain contractility under conditions of external acid stress.

2. CHAPTER 2 DETERMINATION OF OPTIMUM pH FOR CONTRACTILITY

2.1 Introduction

Altering the pH in the environment of salmonids may cause a change in venous blood pH within the range of pH 7.2 to pH 8.5, as well as a change in the $[HCO_3^-]$: a metabolic acidosis or alkalosis that is related to the $[Ca^{2+}]$ in the water (reviewed by Fromm, 1980; Wood and McDonald, 1982; Wood, 1989; and others). The ionic flux is explained by the Strong Ion Difference for biological membranes (Stewart, 1983) in which changes in $[Ca^{2+}]$ (or other strong ions) alter the plasma $[OH^-]$ and the $[HCO_3^-]$ with a concomitant alteration in $[H^+]$ to maintain electrical neutrality. Acidosis and hypoxia are also associated with an increase in water temperature (Cameron, 1989) as both the ionic dissociation of water and the ability of oxygen to dissolve in water are altered.

One of the symptoms of sub-lethal acid stress is the cessation of eating (Dively *et al.*, 1977). This may be related to a decrease in gastrointestinal motility caused by a generalized stress response and the increased concentration of circulating catecholamines (Atland and Barlaup, 1991, Pickering, 1992). These endogenous chemicals decrease smooth muscle contractility by increasing the production of cAMP thereby exerting a modulating effect on the tissue (Burka *et al.*, 1992). The muscle itself may be sensitive to alterations in pH, either in the intracellular contractile mechanisms, or in the events leading up to the contraction.

Most previous studies of the effects of pH on smooth muscle have focused on mammalian tissues. Löfqvist and Nilsson (1981) reported an increase in K^+ induced tonic contractions of the rabbit taenia coli with increasing Pco_2 or decreasing pH. Both the spontaneous activity (Weinbeck

et al., 1972) and the tension (Aaberg *et al.*, 1967) of guinea pig taenia coli were reduced by decreasing pH.

Since mammalian preparations have yielded such tissue and species specific results, and fish are extremely sensitive to alterations in environmental pH, this study was undertaken to determine the effects of pH alterations on the contractility of a salmonid intestine.

2.2 Methods and materials

2.2.1 Groups of fish

Fish were housed at the Fish Health Unit of the Atlantic Veterinary College (AVC) under the following conditions: water temperature, 10^0C; pH, 7.60; and $[Ca^{2+}]$, 48.64 mg/L. Two groups of rainbow trout were utilized in the study. Group A fish were siblings, approximately 1.25 years post-hatching at the time of purchase, from the Biology Department at UPEI in May 1993. Group B fish were mixed genetic, fall spawning stock obtained from Cardigan Fish Hatchery in May 1994 and were also 1.25 years post-hatching at that time.

2.2.2 Tissue preparation

2.2.2.1 Optimum pH study

Experiments to determine the optimum pH for contractility were conducted on the Group A fish between July and November 1993 when the fish weighed between 300 and 900 grams and were sexually immature.

Fish (n = 6 for each pH) were euthanized by spinal severance and the intestines immediately removed and immersed in modified Krebs-Henseleit solution (KHS) of the following composition: NaCl 118 mM; KCl 4.7 mM; $CaCl_2$ 2.2 mM; $MgSO_4$ 1.2mM; KH_2PO_4 1.2 mM; d-glucose 11.1 mM; HEPES 24.9 mM and brought to pH 7.50 by the addition of 1M HCl. HEPES, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) with a pK_a of 7.5 and

a useful pH range of 6.8 - 8.2, was chosen as the buffer for the control solution. The HEPES was replaced with $NaHCO_3$ or TAPS (N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid), at the same molar concentration in the test solutions. $NaHCO_3$ was used to manipulate the pH by varying the $[CO_2]$, TAPS has a pK_a of 8.4 and a useful pH range of 7.7 - 9.1. Sodium salts of the buffers were used in order to maintain ionic consistency (Table 2.1).

Paired tissues, two proximal and two distal cylindrical segments, approximately 2 cm long, were suspended in pH controlled organ baths at 10°C by a method similar to that described by Kitchen (1984) for guinea pig ileum and Burka *et al.* (1990) for rainbow trout intestine. Tissues were allowed to equilibrate for approximately one hour and maintained under a resting tension of 0.0196 Newtons (isometric tension equivalent to 2.0 gms). Tissue length under tension and weight after being blotted dry were recorded so the developed force could be normalized by cross sectional area (Brink *et al.*, 1981). Longitudinal changes were measured using FT.03 transducers (Grass, Quincy, MA) and recorded on linear chart recorders (Gould, Cleveland, OH). The pH was maintained at pH 7.50 in the HEPES buffered control solution, and decreased in the $NaHCO_3$ buffered solution by increasing the concentration of CO_2 in the aeration of the organ baths. The pH was adjusted by the addition of 1M HCl to the solutions buffered by HEPES and TAPS which were then aerated with 100% O_2. The pH was measured when the buffer solution was mixed, and from the organ bath or the reservoir at approximately 2 hour intervals as the experiment proceeded.

2.2.2.2 Buffer comparisons

Group A fish were euthanized in September or October 1994 and tissues prepared as above, except, where indicated, the intestines were sectioned into three proximal and three distal sections and comparisons

Table 2.1 Buffers used in modified Krebs-Henseleit solution

Buffer	pK_a	Useful Range	Aeration
HEPES	7.5	6.8 - 8.2	100% O_2
TAPS	8.4	7.7 - 9.1	100% O_2
MES	6.1	5.5 - 6.7	100% O_2
Bicarb	Variable	Variable	CO_2 - O_2

HEPES - N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
TAPS - N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid
MES - 2-[N-morpholino]ethanesulfonic acid
Bicarb - Sodium Bicarbonate

made between two buffers against the control. Following the results from the optimum pH experiments, the control solution was adjusted to pH 7.85. Modified KHS of the same buffers and molar concentrations as above were used - with the addition of MES (2-[N-morpholino]ethanesulfonic acid). Comparisons were made at pH 7.5 (HEPES vs TAPS), pH 7.2 (MES - 100% O_2 - and Bicarb - 5% CO_2), pH 6.3 (MES - 100% O_2 - and Bicarb @ 40% CO_2) (Table 2.1).

2.2.3 Chemicals

HEPES, TAPS, MES and 5-HT (5-hydroxytryptamine) were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were obtained from Fisher Scientific Ltd, Oshawa, Ont. Stock solutions were prepared and diluted with distilled water.

2.2.4 Blood chemistry

In September 1994, blood samples were taken from six of the mature Group A fish and six of the immature Group B fish by cardiac puncture immediately following death. Levels of sodium, potassium, chloride, calcium, phosphorus, magnesium, urea, creatinine, glucose and lactate were determined by Diagnostic Services Division of AVC according to standard protocol.

2.2.5 Pharmacological studies

Tissues were contracted by the random ordered application of KCl (70mM), 5-HT (concentrations: 10^{-7}, $3x10^{-7}$, 10^{-6}, $3x10^{-6}$, 10^{-5}, $3x10^{-5}$ and 10^{-4} M) pipetted into a 10 ml organ bath and transmural stimulation (TS) (frequencies: 0.1, 0.3, 1, 3, 10, 30 Hz; voltage: 40V; pulse duration: 5 ms; train length: 15 s) applied to the tissues by platinum electrodes (connected to a Grass S88 Stimulator, Grass, Quincy, MA) suspended on either side of the tissue. After application of the agonist, tissue

2.2.6 Data analysis

The maximum pen deflection over the 5 min of each contraction was measured and standardized as N/m^2 using a Summaplus digitizer (Summagraphics, Fairfield, CT) utilizing a computer program developed by W.P. Ireland (Burka *et al.* 1993*a*). Maximum contractions were used as an index of efficacy. Results were calculated as a percent of paired tissue control. Preliminary analysis on the log transformed data used an analysis of covariance with the Generalized Linear Model (Proc GLM) option of the Statistical Analysis System (SAS, Cary, NC). The model was a nested design, with treatment (KCl, 5-HT and TS) and region (proximal and distal) used as class variables, and the mean square error value for fish (n = 54) nested within pH used as the denominator for the `F' significance test.

In the buffer comparisons, the analysis was treated as a repeated measures design with contrasts within the GLM procedure. In all statistical tests, 5% was accepted as the limit of confidence.

Dose response curves were calculated using MEANCURV® (Carpenter, 1986). The negative base 10 logarithm ($-\log_{10}$) of the 50% effective concentration (EC_{50}) was calculated as was the $-\log_{10}EF_{50}$ (effective frequency). These values were accepted as a measure of potency. Slopes were calculated by using the 30% and 70% values determined by the probit analysis. These values were also analyzed using Proc GLM followed by probability of difference as *post hoc* test. Blood chemistry values were compared between the groups of trout by Student's t test.

2.3 Results

2.3.1 Optimum pH

Paired tissues from the same fish were used to control for seasonal variations known to affect contractility (Burka *et al.* 1993*b*). In the HCO_3^-/CO_2 buffered KHS, the pH was altered by increasing the amount of CO_2 in the aeration of the organ baths. Aeration with 5% CO_2 yielded a solution pH of 7.21, 10% CO_2: 6.98, 20% CO_2: 6.58, 30% CO_2: 6.41, 40% CO_2: 6.32. The pH of 7.85 and the control pH of 7.50 were obtained by the addition of HCl to the HEPES buffered KHS. Adding HCl to the TAPS buffered solution yielded the higher pH values of 8.10 and 8.50. The pH of all solutions was found to remain stable throughout the testing period.

Analysis of the whole data set showed a difference in response between the regions ($p = 0.019$) and a significant interaction term for region and pH ($p = 0.02$). In proximal segments, maximum contractions elicited by all three treatments (5-HT, KCl, and TS) showed a uniform trend. Proximal segments also showed a significant response to changing pH ($p < 0.0001$). The significance of the pH^2 term ($p < 0.0001$) in the model implies the presence of a strong peak contractility at a particular pH. Examination of Fig 2.1 reveals that this peak occurs at pH 7.85 where the maximum contractions elicited by KCl were about 300% of the contractions at the control pH of 7.50; 450% for those produced by TS and 350% following administration of 5-HT. The strength of the contractions falls off rapidly as pH is changed by 0.3 units above or below the optimum value. At a pH of 6.32 contractions were barely discernible with many of the test results equal to 0.

Analysis of the contractions by segments of distal intestine established that the contractility of distal segments increased

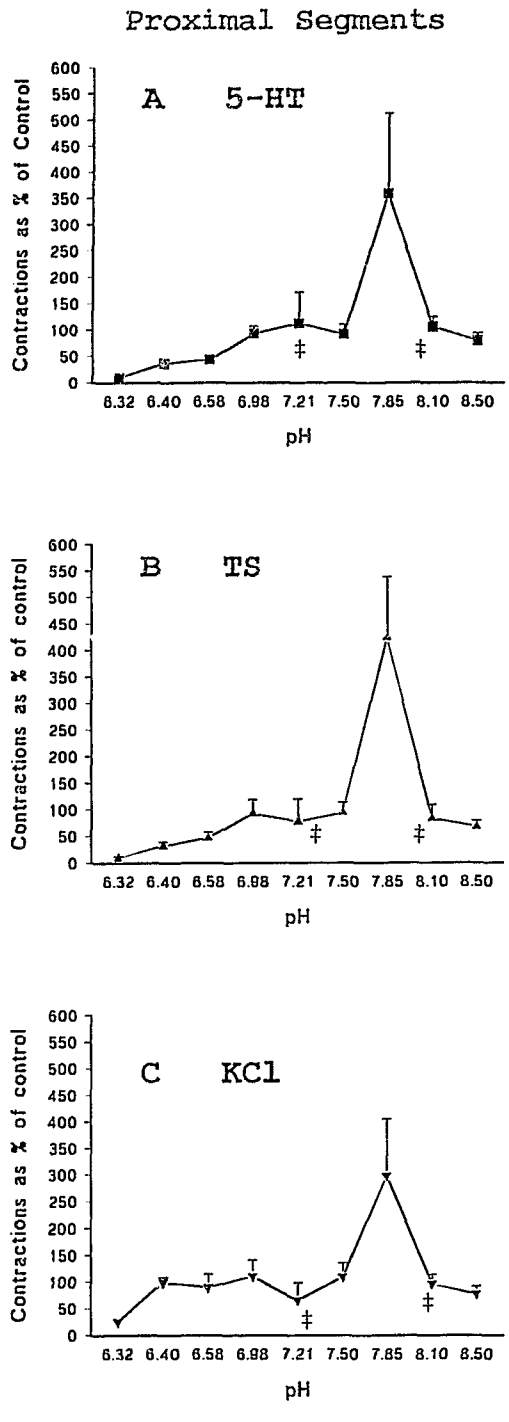


Fig. 2.1. Maximum contractions of proximal intestinal segments expressed as % of control (pH 7.50)at each pH level for A)serotonin(5-HT), B) transmural stimulation (TS) and (C) KCl. Values are means ± SEM (n = 6 at each pH; ‡ denotes n = 5).

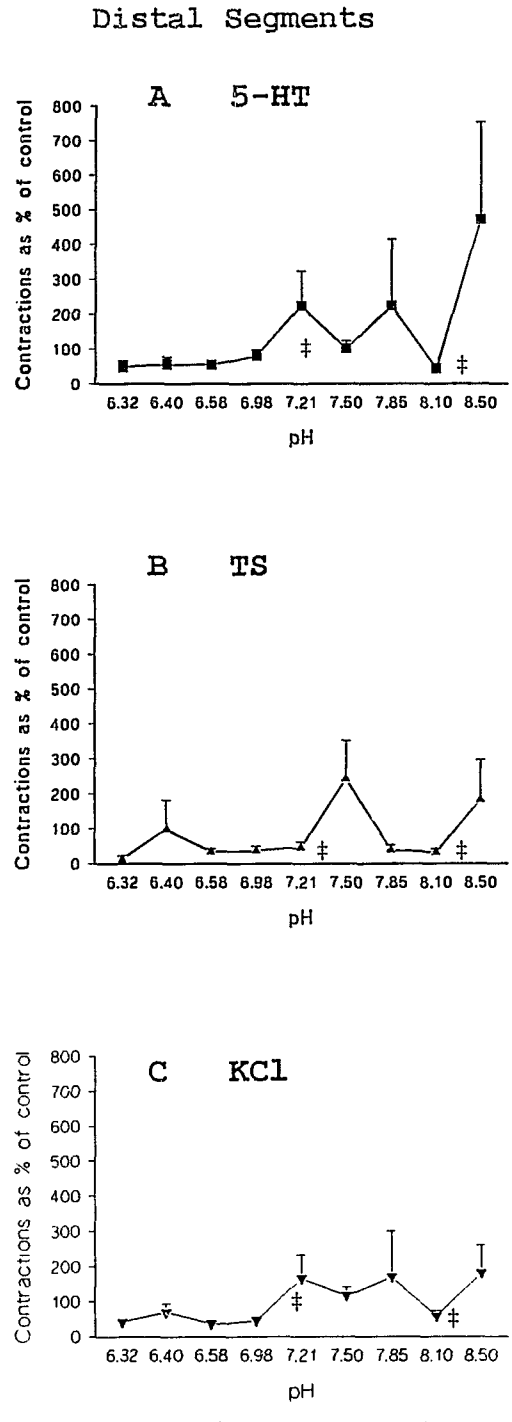


Fig. 2.2. Maximum contractions of distal intestinal segments expressed as % of control (pH 7.50)at each pH level for (A) serotonin (5-HT), B) transmural stimulation (TS) and (C) KCl. Values are means ± SEM ((n = 6 at each pH; ‡ denotes n = 5).

logarithmically as pH was increased (Fig. 2.2). This trend was similar for all stimuli tested, with contractions declining below the minimum physiological pH. An alternative explanation to logarithmic increase is an inhibition of contractions at lower pH values - below pH 7.2 for 5-HT and KCl and below pH 7.5 for TS; and irregular responses above those values. The pD_2, $-\log_{10}EF_{50}$ and the slopes were unaffected by decreasing pH until inhibition approached 100% (pH 6.3), at which point all values approached 0.

2.3.2 Buffer comparisons

Buffer comparisons were performed between July and September 1994, on Group A fish. By this time, the fish were approximately 1.5 years post-hatching and sexually mature. Analysis of the muscle contractions in KHS buffered with TAPS and HEPES at pH 7.5 showed efficacy of all agonists was not significantly different (Fig. 2.3). An examination of MES and CO_2/HCO_3^- at pH 7.2 showed somewhat different results. In both proximal and distal segments (Figs. 2.4 A and 2.5 A) MES as buffer had no effect on the response of any of the tissues at pH 7.2. There was no inhibition in the proximal segments in the 5-HT or KCl initiated contractions in the 5% CO_2/HCO_3^-. Contractions initiated by transmural stimulation, were inhibited by 60% ($p = 0.0249$). For the distal segments (Fig. 2.5 A), MES was not associated with any inhibition. Contractions initiated by KCl were not affected by CO_2/HCO_3^-; however, for 5-HT there was a 75% inhibition ($p = 0.0151$) and for TS an 80% inhibition ($p = 0.0002$) in the CO_2/HCO_3^- buffered solution. Although there appears to be a similar trend with KCl, this was not found to be statistically significant ($p = 0.22$).

At pH 6.5 (Figs. 2.4 B and 2.5 B) the only apparent effect was an 82% inhibition ($p = 0.033$) in the proximal segments in the 20% CO_2/HCO_3^-

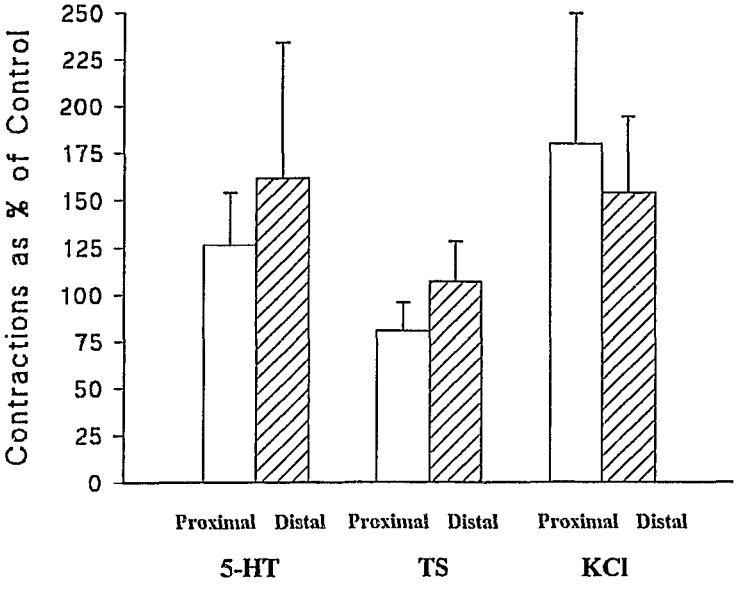


Fig. 2.3. Maximum contractions of proximal [] and distal [/] segments in Krebs-Henseleit buffered with TAPS (pH 7.5) aerated with 100% O_2 as a percent of control (HEPES pH 7.5). Values are means ± SEM (n = 6).

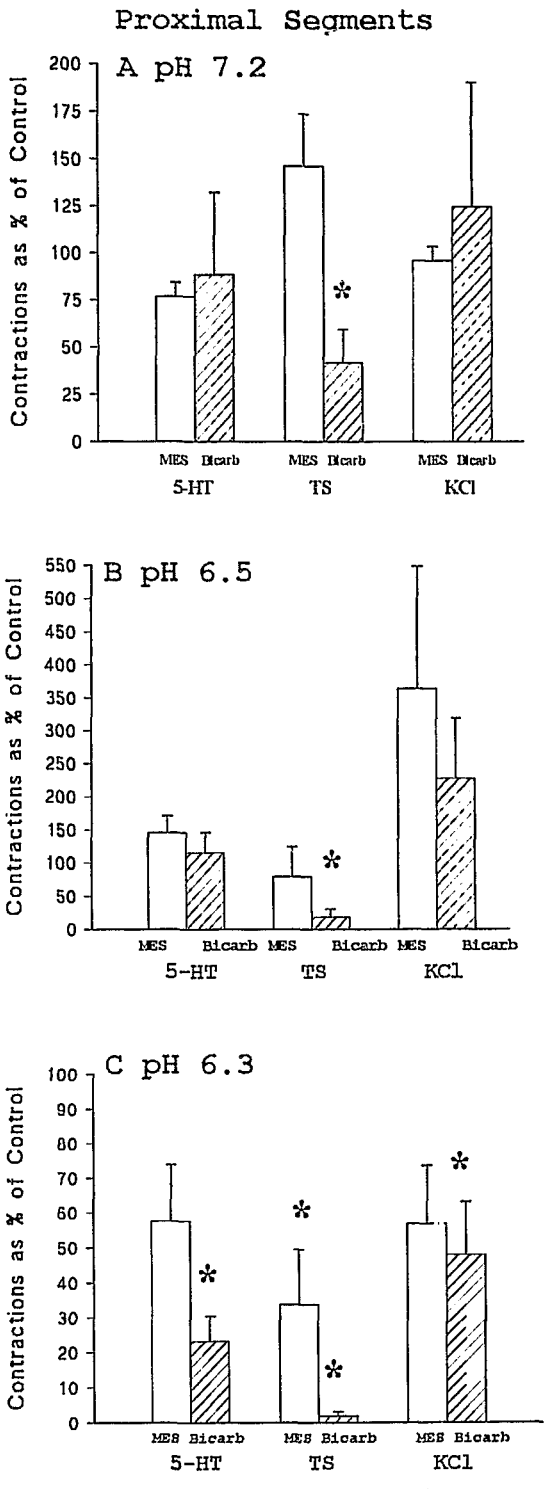


Fig 2.4. Maximum contractions as percent of control (HEPES 100 % O_2; pH 7.85) of proximal intestinal segments at (A) pH 7.2, (B) pH 6.5 and (C) pH 6.3 in solutions buffered with [] MES (100% O_2) and [/] HCO_3^- (5%, 20% and 40% CO_2). Values are means ± SEM (n = 6).* indicates difference from control ($p < 0.05$)

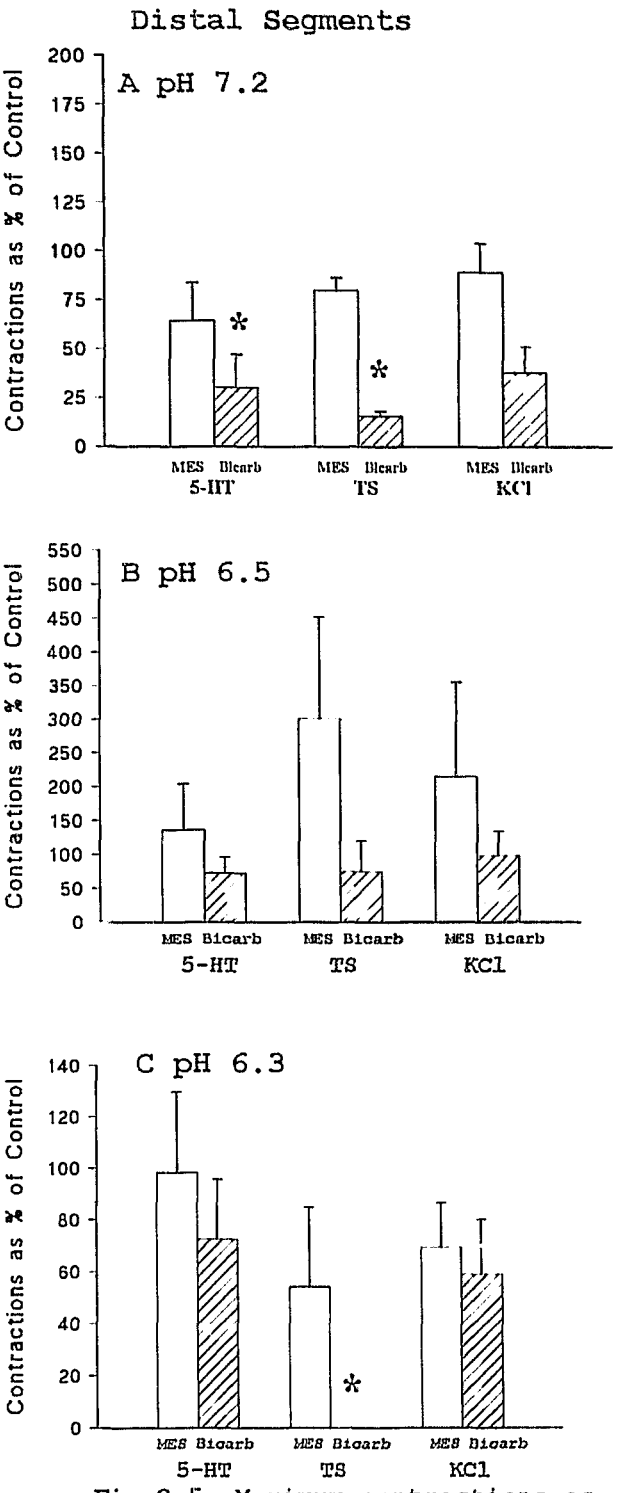


Fig 2.5. Maximum contractions as percent of control (HEPES 100 % O_2; pH 7.85) of distal intestinal segments at (A) pH 7.2, (B) pH 6.5 and (C) pH 6.3 in solutions buffered with [] MES (100% O_2) and [/] HCO_3^- (5%, 20% and 40% CO_2). Values are means ± SEM (n = 6). * indicates difference from control ($p < 0.05$)

buffered KHS. At pH 6.3 (Figs. 2.4 C and 2.5 C), using MES as buffer on the proximal intestine inhibition only occurred when the segments were contracted by transmural stimulation (66% inhibition, $p = 0.008$). Using CO_2/HCO_3^- in the proximal segments inhibition was found with 5-HT (77% inhibition, $p = 0.0001$); transmural stimulation (98% inhibition, $p < 0.001$) and KCl (52% inhibition, $p = 0.019$). In the distal segments there was no inhibition of contractions by the reduction in pH by HCl but contractions to transmural stimulation were completely eliminated in the presence of the high concentration of CO_2.

2.3.3 Group comparisons

As the analysis proceeded, it became obvious that the results were somewhat different than the optimum pH studies (section 2.3.1) the year before where nearly all contractions were eliminated at pH 6.3 (in 40% CO_2/HCO_3^-). Since the control pH had been changed from pH 7.50 to 7.85 based on the results of that study, direct comparisons could not be made. To investigate this disparity, tissues from Group B fish, approximately the same size and age as the ones used in the 1993 optimum pH study, were sacrificed. The results for the younger fish were more similar to those from the original study and quite different from the older fish (Table 2.2 and Fig. 2.6).

In proximal segments from the Group B fish exposed to MES buffered solution, there was a 59% inhibition ($p = 0.04$) in the tissues contracted with 5-HT and a 99% inhibition ($p < 0.001$) with transmural stimulation. Distal segments showed a 95% inhibition ($p < 0.001$) to transmural stimulation. When the solution was buffered with CO_2/HCO_3^-, inhibition was complete in both proximal and distal segments when the tissues were contracted with either 5-HT or transmural stimulation (Fig. 2.6 A and B). Inhibition of KCl-stimulated contractions was approximately 87% ($p = 0.0001$) in both regions (Fig. 2.6 C). Differences between the buffers

Table 2.2. Maximum contraction as % of control (pH 7.85) between Krebs-Henseleit buffered with MES and CO_2/HCO_3^- at pH 6.3

Group	Buffer	Region	Agonist	% of Control	P > F
Group A	MES	Proximal	5-HT	57.64 ± 16.27@	0.052
(UPEI Biol.			TS	33.78 ± 15.71*@	0.008#
Dept.)			KCl	56.90 ± 16.70	0.054
		Distal	5-HT	98.28 ± 31.56	0.960
			TS	54.10 ± 30.65*	0.190
			KCl	69.30 ± 17.10	0.130
	CO_2/HCO_3	Proximal	5-HT	23.21 ± 7.29*@	0.0001#
			TS	1.87 ± 1.30@	0.0000#
			KCl	47.91 ± 15.24*	0.019#
		Distal	5-HT	72.47 ± 23.18*	0.29
			TS	0	0.0000#
			KCl	58.99 ± 21.08*	0.11
Group B	MES	Proximal	5-HT	41.09 ± 11.55@	0.040#
(Cardigan			TS	1.32 ± 1.33*	0.0000#
Fish			KCl	114.86 ± 46.42	0.76
Hatchery)		Distal	5-HT	68.62 ± 23.24@	0.23
			TS	4.60 ± 4.60*	0.0000#
			KCl	79.63 ± 37.18	0.61
	CO_2/HCO_3	Proximal	5-HT	0*@	0.0000#
			TS	0	0.0000#
			KCl	13.26 ± 8.40*	0.0001#
		Distal	5-HT	0*@	0.0000#
			TS	0	0.0000#
			KCl	12.10 ± 6.87*@	0.0001#

Values are means ± SEM (n = 6). Only p values for difference from control are shown.
# indicates difference from control at pH 7.85 ($p < 0.05$)
* indicates differences between groups of fish ($p < 0.05$)
@ indicates difference in tissue response between buffers ($p < 0.05$)

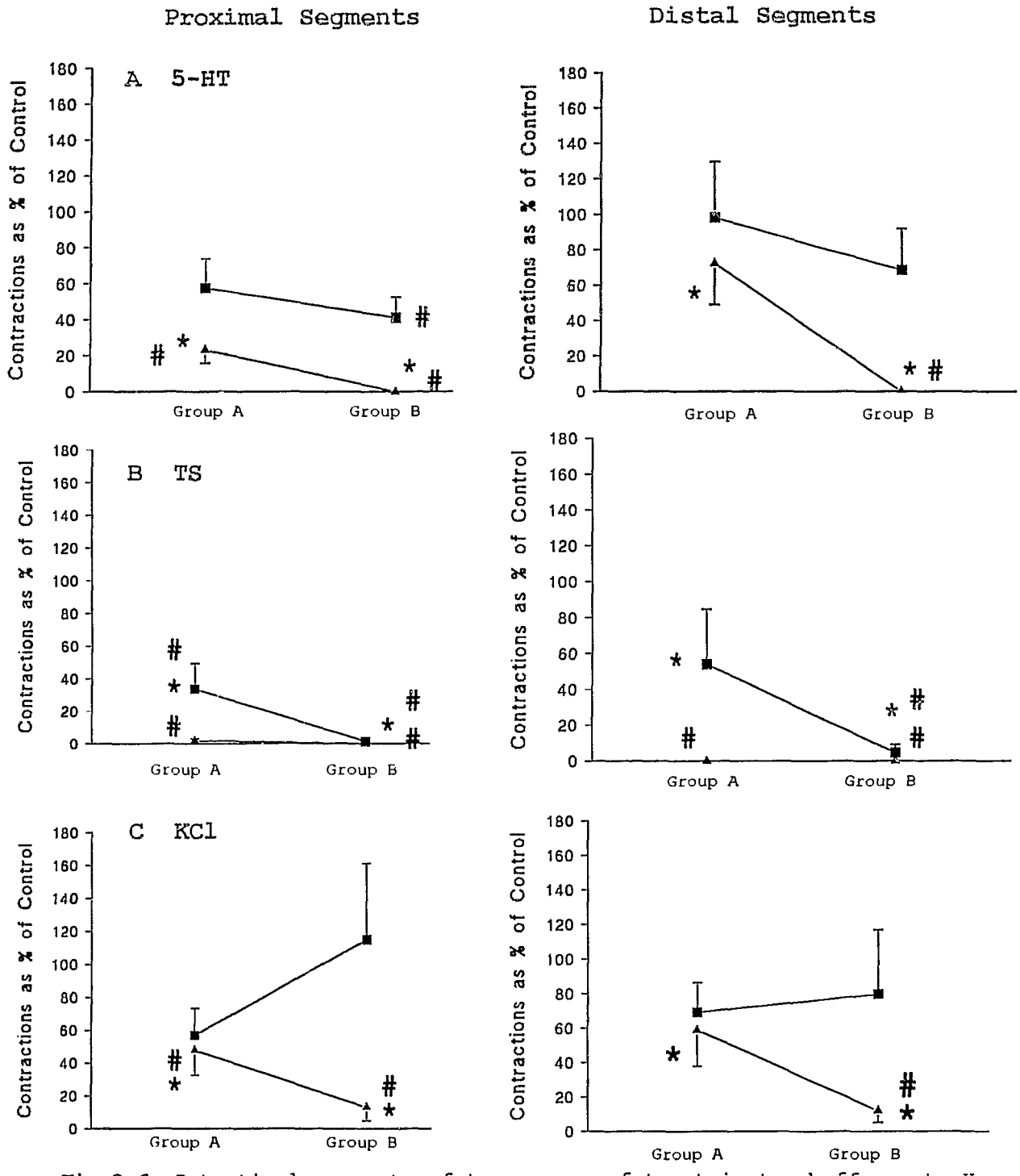


Fig 2.6. Intestinal segments of two groups of trout in two buffers at pH 6.3 (■ MES - 100% O_2; ▲ $NaHCO_3$ - 40% CO_2). (Description of groups of fish in section 2.2.1; buffers in 2.2.2.2). Maximum contractions for both proximal and distal segments to (A) 5-HT; (B) TS; and (C) KCl were calculated as % of control at pH 7.85 (HEPES - 100% O_2). Values are means ± SEM (n = 6). * denotes difference between groups; # difference from control ($p < 0.05$).

were apparent in both regions when the tissues were contracted with 5-HT (proximal - $p = 0.042$; distal - $p = 0.0001$), and in the distal segments contracted with KCl ($p = 0.0195$). In all cases where there was a difference between buffers, the inhibition was greater in the Krebs-Henseleit buffered with 40% CO_2/HCO_3^- than when the buffer was MES aerated with 100% O_2.

For the Group A fish, differences between the buffers were apparent only when the proximal segments were contracted with 5-HT ($p = 0.0489$) and transmural stimulation ($p = 0.034$). As in the Group B trout, inhibition was greater in tissues buffered with CO_2/HCO_3^- .

Pooling the data for both groups, and following analysis, several differences became apparent. When the KHS was buffered with MES, both proximal and distal segments from the Group A fish showed less inhibition at pH 6.3 when the tissues were contracted by transmural stimulation (proximal - $p = 0.0006$; distal - $p = 0.0084$). When the buffer was CO_2/HCO_3^- , there were differences in both the proximal and distal segments contracted with 5-HT and KCl (all $p = 0.0001$). With transmural stimulation, in the distal segments, all contractions were eliminated. In the proximal segments, the contractions were completely eliminated in the Group B fish while a 98% inhibition occurred in the Group A fish. Since the confidence interval included 0 in this sample, statistical analysis was unable to declare a difference; however, there is an obvious physiological difference between the groups as some contractions occurred in the tissues from the Group A fish (Fig. 2.5).

2.3.4 Blood chemistry

Blood from the two groups of fish was analyzed for electrolytes, urea, creatinine, glucose and lactate (Table 2.3). No significant difference was found between the two groups.

Table 2.3. Selected blood parameters for the two groups of trout. Analysis was performed by Diagnostic Services (AVC). Groups are described in 2.2.1.

		Group A Fish	Group B Fish
		Mean ± SEM	Mean ± SEM
Na	mmol/L	162.17 ± 17.24	155.33 ± 4.08
K	mmol/L	24.08 ± 19.38	3.55 ± 1.11
Na:K	ratio	32.33 ± 10.03	62.00 ± 13.57
Cl	mmol/L	121.00 ± 4.13	125.17 ± 5.06
Ca	mmol/L	4.96 ± 1.70	2.67 ± 0.13
P	mmol/L	10.08 ± 5.01	4.44 ± 0.45
Mg	mmol/L	1.85 ± 0.26	1.39 ± 0.14
Urea	mmol/L	1.35 ± 0.34	0.98 ± 0.24
Creat	mmol/L	81.67 ± 22.11	52.40 ± 12.92
Glucose	mmol/L	4.68 ± 0.18	4.23 ± 0.31
Lactate	mmol/L	0.99 ± 0.23	0.80 ± 0.12

Values were not significantly different between both groups of fish (n = 6).

2.4 Discussion

Contractility of rainbow trout intestinal smooth muscle is inhibited by decreasing pH. This inhibition is proportional to the O_2/CO_2 ratio aerating the organ bath, and affects transmural stimulation more than stimulation with either 5-HT or KCl. The inhibition also varies between groups of fish; although, we cannot yet say whether the differences are genetic or age related, or if there are other, as yet unknown, factors involved.

2.4.1 Optimum pH

The plasma pH of rainbow trout held in neutral water is approximately 7.8 - 7.9 (MacDonald *et al.*, 1980); this is inversely proportional to water temperature (Randall and Cameron, 1973, Kieffer *et al.*, 1994). Decreases in plasma pH in rainbow trout in acid waters are associated with high water Ca^{2+} concentrations, but do not occur in soft water (Wood, 1989).

The optimum pH of 7.85 for the proximal segments, as determined by this study, corresponds very well to the plasma pH as described above. It is this pH which is optimal for growth; it is therefore reasonable to assume that the optimum pH for muscle contractility would be close to this value.

Austin and Wray (1993) have shown a nearly linear relationship between intracellular pH (pH_i) of mammalian smooth muscle and organ bath pH. Therefore, it is reasonable to assume that the changes in contractility observed here are related to changes in pH_i. Although the effects of altering pH were different for the proximal and distal segments (Figs. 2.1 and 2.2) the effects within the regions were qualitatively similar for all three agonists. These agonists were chosen because their effects on the smooth muscle of trout intestine have been

researched extensively. 5-HT, which has long been known to be an agonist in intestinal smooth muscle from brown trout, *Salmo trutta* (Burnstock, 1958) and other teleosts including *Pleuronectes platessa* and *Anguilla vulgaris* (von Euler and Östlund, 1957), has more recently been considered an endogenous neurotransmitter in the rainbow trout enteric nervous system (Holmgren, 1983; Kitizawa, 1989) and acts via 5-HT_2 receptors (Burka *et al.*, 1988). This is in contrast to the mammalian enteric nervous system which contains 5-HT_{1A}, 5-HT_{1P}, 5-HT_3 and 5-HT_4 receptors (Wade *et al.*, 1994). KCl bypasses the receptors and directly depolarizes the muscle membrane. Transmural stimulation mimics neuronal transmission as the contractions can be blocked by tetrodotoxin (Burka *et al.*, 1992).

In this study, it appears that the distal intestine has a much greater functional pH range than the proximal segments. In the proximal segments, contractility falls off rapidly on either side of the optimum pH, while in the distal segments, the range is much wider (Figs. 2.1 and 2.2). Active transport is responsible for much of the intestinal absorption of orally ingested ions, and much of this absorption occurs in the posterior region (Evans, 1979, 1993; Gairdaire and Isaia, 1992; Klaren *et al.*, 1993). Thus the regional responses may be related to a differential expression of ion exchangers (e.g. Na^+/H^+, Cl^-/HCO_3^-) along the length of the intestine. Since the posterior intestine is involved in protein digestion (Abaurrea *et al.*, 1993) and amino acids stimulate the Na^+/H^+ exchange (Rhoads *et al.*, 1994), the regional differences may also be a function of greater protein availability in the posterior region.

2.4.2 Buffer comparisons

The differences between the buffers used in the current study are related to the aeration of the solution and the means of stimulation.

There is no difference in any of the tissues immersed in the TAPS, HEPES or MES at pH 7.5 or 7.2 (Figs. 2.3 and 2.4) where the aeration is 100% O_2. Inhibition first occurs at pH 7.2 in the presence of 5% CO_2/HCO_3^-. This contrasts with the situation in rat vascular or uterine smooth muscle where the changes in contractility due to altering pH were independent of the means of effecting the pH change (Austin and Wray, 1993, Taggert and Wray, 1993). In isolated bovine and porcine coronary myocytes (Klöckner and Isenberg, 1994) and snail neuron (Thomas, 1984), substituting HEPES with CO_2/bicarbonate as buffer, caused a decrease in pH_i.

The inhibition of contraction in rainbow trout intestine is more pronounced when the tissues are electrically stimulated (Figs. 2.4 A, B and C; 2.5 A and C; and 2.6 B) suggesting that neuronal transmission is more sensitive to the effects of both increasing the [CO_2] and decreasing the pH. Examination of different tissues has revealed the cellular mechanisms for regulation of pH_i are not the same in all tissues (Roos and Boron, 1981, Thomas, 1984, Aicken, 1994). Three mechanisms of pH_i regulation are commonly recognized. These are the Na^+/H^+ exchange, the HCO_3^-/Cl^- exchange and a sodium dependent Cl^-/HCO_3^- exchange. Although all three have been studied extensively, no evidence yet exists for all three to be present in a single tissue (Thomas, 1984). A greater dependence on the Na^+/H^+ exchange in enteric neurons could explain the increased CO_2-HCO_3^- sensitivity of neuronal transmission over contractions initiated at the membrane.

2.4.3 Group comparisons

The difference in ability of the tissues from the two groups of fish to adapt to the pH alterations is clear (Fig. 2.6). In all cases where differences were apparent, intestinal segments from the less mature Group B fish from the Cardigan Fish Hatchery showed a greater sensitivity to decreasing pH. The two evident differences between the groups are

their genetic background and their age and sexual maturity. In 1976, Robinson *et al.*, using inbred stains of brook trout (*Salvelinus fontinalis*) provided evidence that acid tolerance is hereditary and Dunsun and Martin (1973) found individual differences in survival within the same species (*S. fontinalis*) in a bog fed creek in Pennsylvania. In exercise studies on rainbow trout, Wang *et al.* (1994) found variations in intracellular fluid and electrolyte distribution between two groups of fish examined at different times of year. Other differences between the groups in the study by Wang *et al.* (1994) are not explained.

The process of sexual maturation is associated with hormonal alterations generated largely from the developing gonads. In the female rainbow trout, sexual maturation is associated with an increase in the plasma levels of 17β-oestradiol, testosterone, 11-ketotestosterone and calcium (Scott *et al.*, 1980). In the male, plasma testosterone, 11-ketotestosterone, and 17α-hydroxy-20β-dihydroprogesterone levels are increased (Fostier *et al.* 1987). These hormonal changes in male rainbow trout lead to both cardiac hypertrophy and increased cardiac output (Franklin and Davie, 1992). As an adjunct to sexual maturation, a circannual rhythm, independent of age and sexual maturity, in lipoprotein metabolism has been found in rainbow trout (Wallaert and Babin, 1994). Seasonal variations in the plasma gonadotropin response of Atlantic croaker (*Micropogonias undulatus*) to exogenously applied 5-HT were reported by Khan and Thomas (1994). Circulating thyroid hormones, also associated with maturation in salmonids (Eales *et al.*, 1991), might also impact on membrane function. Seasonal variation in contractile response on rainbow trout intestine was reported by Burka *et al.* (1993*b*). Smoltification is also associated with hormonal and physiological changes including increases in intestinal fluid transport following the surge in thyroid hormones (Collie and Bern, 1982).

Of the variables described above, many could have an effect on the ability of tissues to adapt to pH alterations. Catecholamine regulation of the Na^+/H^+ exchange in red blood cells has been demonstrated in cod (*Gadus morhua*) (Berenbrink and Bridges, 1994), brown trout (*S. trutta*) (Orlov *et al.*, 1994) as well as rainbow trout (Baroin *et al.*, 1984, Motais *et al.*, 1992). Given the ability of hormones to up-regulate Na^+/H^+ exchanger populations (Fliegel and Frölich, 1993), the high concentrations of androgens found by Scott *et al.* (1980) could very well increase the activity of some of the ion exchangers on the intestinal smooth muscle or on the enteric neurons leading to more efficient pH_i regulation in sexually mature fish.

2.4.4 Blood chemistry

Blood chemistry values as determined were all within the ranges reviewed by Folmar (1993) who examined all parameters discussed here with the exception of lactate. The values reported by Kieffer *et al.* (1994) for plasma levels of lactate in resting rainbow trout (0.40mmol/L ± 0.19) are slightly lower than those determined here. This may be explained by a difference in sampling methods; as, in the Kieffer study, fish were cannulated and resting values determined with no chasing whatever. In a study by Riva and Flos (1993), in which the fish were euthanized, the lactate levels determined were more similar (1.26 ± .44 mmol/L) to those found here. Variation in values can also be the result of sampling technique, sample handling methods and time between sampling and analysis (Korcock *et al.*, 1988).

Since no difference was found in the blood parameters between the two groups of fish, the different contractile responses of the tissues from the two groups of fish cannot be explained on the results from the plasma electrolytes examined in this study. Further studies should

include an examination of the thyroid hormone, progesterone and androgen levels.

2.4.5 Conclusions

The contractility of the smooth muscle in intestinal segments from rainbow trout are sensitive to the alteration of organ bath pH. This inhibition is, however, mitigated in some groups of fish. Whether this difference has its basis in genetic variation, or in the hormonal changes related to sexual maturation, is as yet unknown. Following the same group of fish through various life stages would be an aid in answering this question.

It appears that, in intestinal segments from at least some rainbow trout, contractility is affected at pH ranges within the plasma pH range that the fish may experience under extreme conditions. These results suggest that an examination of *in vivo* intestinal motility, under stress conditions that impact on trout acid-base regulation, could lead to a greater understanding of digestion in these animals.

3. CHAPTER 3 EFFECTS OF LACTATE AND ION EXCHANGE BLOCKERS ON CONTRACTILITY

3.1 Introduction

Although fish in their natural habitat rarely, if ever, exercise to complete exhaustion, the human interference of catch and release angling and trucking of live farmed fish can result in extreme stress on the animals (Wood, 1991). Lactic acid is often the end product of the ensuing anaerobic metabolism and, along with increased CO_2, is a source of the acidosis that follows prolonged exercise (Milligan and Wood, 1986*a*).

Examination of exercised rainbow trout have shown that little or no lactate is excreted (Milligan and Wood, 1986*a*), and much undergoes gluconeogenesis within the muscle where it was produced (Turner and Wood, 1983, Milligan and Wood, 1986*a,b*). Plasma levels of lactate following exercise have been measured at 20mM in rainbow trout, with complete recovery to resting levels within 24 hrs (Wood, 1991). For comparison, when plaice (*Pleuronectes platessa* L) were exercised, plasma lactate remained low and mortality was high for those fish when levels exceeded 5mM (Wardle, 1978). Skipjack tuna (*Katsuwonus pelamis*) can tolerate plasma lactate levels as high as 30mM (Weber *et al.*, 1986).

The Cori cycle, by which mammals regenerate glucose or glycogen from lactate delivered to the liver, is not important in any of these species. Benthic species, including plaice (Wardle, 1978) and flathead sole (*Hippoglossus elassodon*) (Turner *et al.*, 1983) retain and metabolize lactate in the white muscle. Tuna utilize the countercurrent heat exchanger to facilitate lactate movement from the white muscle to the red, where oxidation can occur (Weber *et al.*,

1986; Hazel, 1993). Rainbow trout oxidize some lactate *in situ*; the rest enters organs and tissues throughout the body (Milligan and Wood, 1986*b*).

Whether the acid-base disturbance is the result of exercise or environmental insult, whole body acid-base regulation in trout is accomplished with ion exchangers located primarily in the gills. The ion exchangers involved in pH regulation include the Na^+/H^+, Cl^-/HCO_3^-, and an Na dependent Cl^-/HCO_3^- exchanger (Section 1.5). Considerable work has been done to study the exchangers at this level in the organism (McDonald and Prior, 1988; Perry and Laurent, 1993; Goss and Perry, 1994; Perry and Goss, 1994; Iger *et al.*, 1994 and others). Tissues within the animal are also affected by these events, and these same ion exchangers have been found in some tissues in rainbow trout including heart, white muscle, liver and brain. The circulation of sodium amd hydrogen across membranes of both cells and intracellular organelles is accomplished via the Na^+/H^+ exchange (reviewed by Padan and Schuldiner, 1993). Anions involved in these exchange mechanisms include Cl^-, HCO_3^- (may be dependent on Na^+), and lactate (Milligan and Wood 1986*b*).

This portion of the study was undertaken to examine in more detail the effects of different sources of acidification on the rainbow trout intestinal smooth muscle, to determine the means by which these tissues regulate pH_i and to ascertain how these mechanisms impact on the contractility of the muscle.

3.2 Methods and materials

3.2.1 Fish

Experiments to determine the effects of acidification with lactic acid were conducted on the fish from the Cardigan Fish

Hatchery, P.E.I., between September and December, 1994 when the fish were approximately 1.5 - 1.75 years post hatching. At this time, the fish were not sexually mature. The effects of SITS were examined in June 1994, EIPA in December 1994.

3.2.2 Tissue preparation

3.2.2.1 Lactic acid study

Fish were euthanized and tissues prepared as previously described (section 2.2.2.1), and maintained in HEPES or MES buffered KHS aerated with 100% O_2. The pH of the organ bath was manipulated by the addition of lactic acid to the KHS. To obtain a pH of 7.5, 0.67g/L, L(+)lactic acid was added to HEPES buffered KHS (original pH 7.85). The pH of 6.3 was obtained by the addition of 0.67g/L (6.3mM) lactate to MES buffered KHS (original pH 7.2). Adding 1.8g/L (20mM) lactate to the HEPES buffered KHS (pH 7.85) resulted in a solution pH of 5.5. This was then raised to pH 7.2 by the addition of NaOH. Full dose response curves to 5-HT and frequency response curves to transmural stimulation, as well as one sub-maximal contraction to KCl, were calculated (see section 2.2.4).

3.2.2.2 Na^+/H^+ exchange

EIPA (5-(N-ethyl-N-isopropyl)amiloride) was applied to tissues immersed in HEPES buffered KHS (pH 7.85) at three concentrations (10^{-5}, 3 X 10^{-5}, and 10^{-4} M). 5-HT (3 X 10^{-5} M) was applied and the maximum contraction measured as described above (section 2.2.2.1). Baseline measurements were taken on each tissue before application of EIPA to establish tissue viability.

3.2.2.3 Cl^-/HCO_3^- exchange

Preliminary examination of SITS (4-acetamido-4'-isothiocyanto-stilbene-2-2'-disulfonic acid) at concentrations of 10^{-5}, 3 X 10^{-5}, and 10^{-4} M was completed in HEPES buffered KHS (pH 7.85). Further studies were done in KHS buffered with 22.4 mM HEPES and 2.5 mM $NaHCO_3$ so the pH could be manipulated by altering the $[CO_2]$ aerating the organ bath.

The tissues were allowed to equilibrate at pH 7.50 (100% O_2) for approximately 1 hr. SITS (10^{-4} M), a concentration known to inhibit the Cl^-/HCO_3^- exchange in mammals (Duong, 1993), was added to the organ bath. The organ bath aeration was then changed to 20% CO_2 - 80% O_2 which decreased the pH to 6.58. 5-HT (3 X 10^{-5} M) was applied and the maximum contraction over 5 minutes measured. This was repeated on other tissues with 30% CO_2 - 70% O_2 (pH 6.40). After the agonist was rinsed the CO_2 was decreased to 5% and another dose of 5-HT was applied (following the increase to 30% CO_2 only).

3.2.3 Chemicals

In addition to the components of the KHS, and the agonists described in section 2.2.5, EIPA was purchased from Research Biochemicals International, Natick, MA, SITS and L(+)lactic acid from Sigma Chemical Co., St. Louis, MO. SITS and the lactic acid were dissolved and diluted in distilled water. A stock solution of EIPA was prepared in dimethyl sulfoxide (DMSO) which was used as a vehicular control. Dilutions were made with distilled water.

3.2.4 Data analysis

Analyses of the potency and efficacy of the agonists in the lactic acid were conducted as described in section 2.2.6. Maximum contractions to each agonist, the slope of the dose response curve and the $-\log_{10}EF_{50}$ and $-\log_{10}EC_{50}$ were statistically analyzed as a repeated

measures generalized linear model. Contrasts among the concentrations were utilized as *post hoc* tests.

The contractions obtained in each concentration of EIPA were compared as proportions using a one-way analysis of variance followed by a Student-Newman Keuls *post hoc* test. Data from the SITS study were analyzed as paired t-tests within each pH level, or repeated measures design with contrasts between pH levels. All analyses were performed using the Statistical Analysis System (SAS). The level of confidence for all statistical tests was accepted as 5% (except where indicated).

3.3 Results

3.3.1 Lactic acid

Results obtained following acidification with lactic acid are represented in Figs. 3.1 and 3.2. The treatment had no effect on maximum contractions elicited by 5-HT or KCl in either the proximal or distal segments (Fig. 3.1 A and C; Fig. 3.2 A and C). Transmural stimulation in the distal segments also was not affected (Fig. 3.2 B). In all situations with distal segments, there were no significant differences from control (pH 7.85) or between tissues in KHS acidified with HCl or lactic acid. In the proximal segments stimulated electrically, however, tissues acidified to pH 6.3 with both HCl and lactic acid showed inhibition (Fig. 3.1 B). For HCl, the inhibition was 83 ± 9.8% ($p < 0.01$); for lactic acid, 92.1 ± 3.8% ($p < 0.001$). At pH 7.2, with the higher concentration of lactic acid, there was a 61.5 ± 17.7% ($p < 0.05$) inhibition from control. The tissues in the KHS acidified with HCl did not show this inhibition.

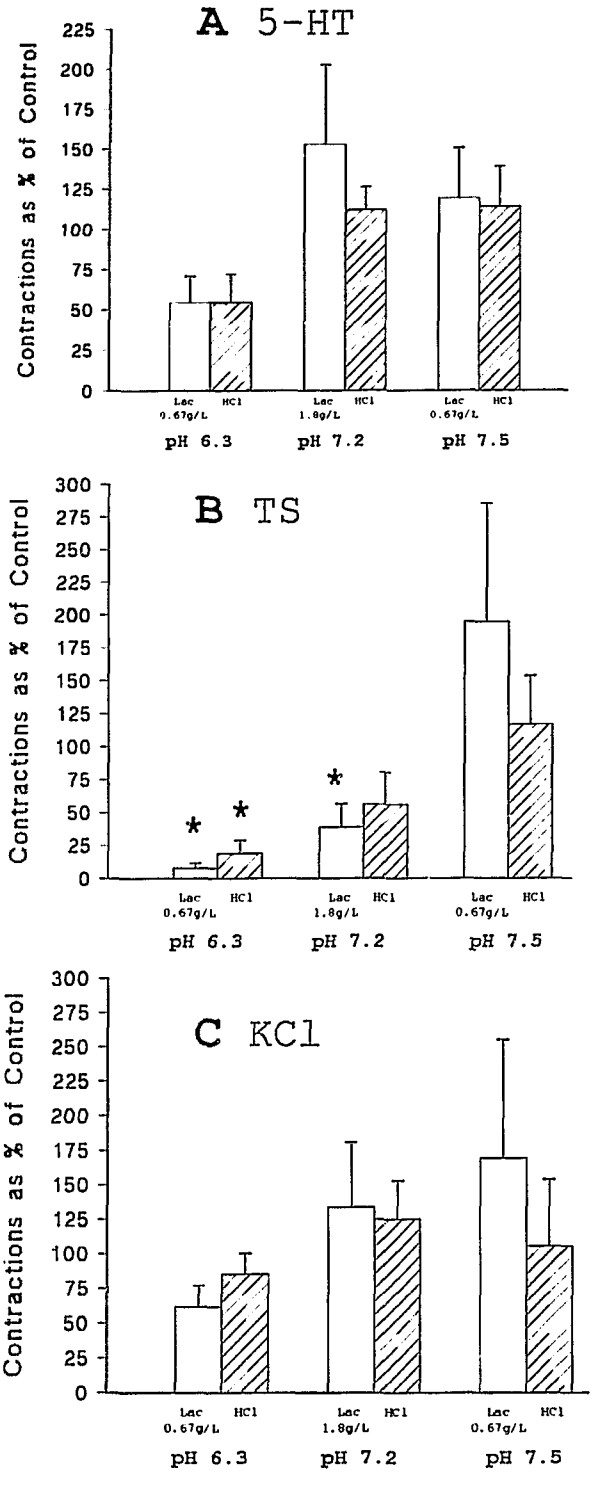


Fig. 3.1. Maximum contractions of proximal intestinal segments in HEPES or MES buffered KHS, acidified with lactic acid [] or HCl [//]. Contractions initiated by (A) 5-HT, (B) TS and (C) KCl. Values are means ± SEM (n = 6). * denotes difference from control (pH 7.85) $p < 0.05$.

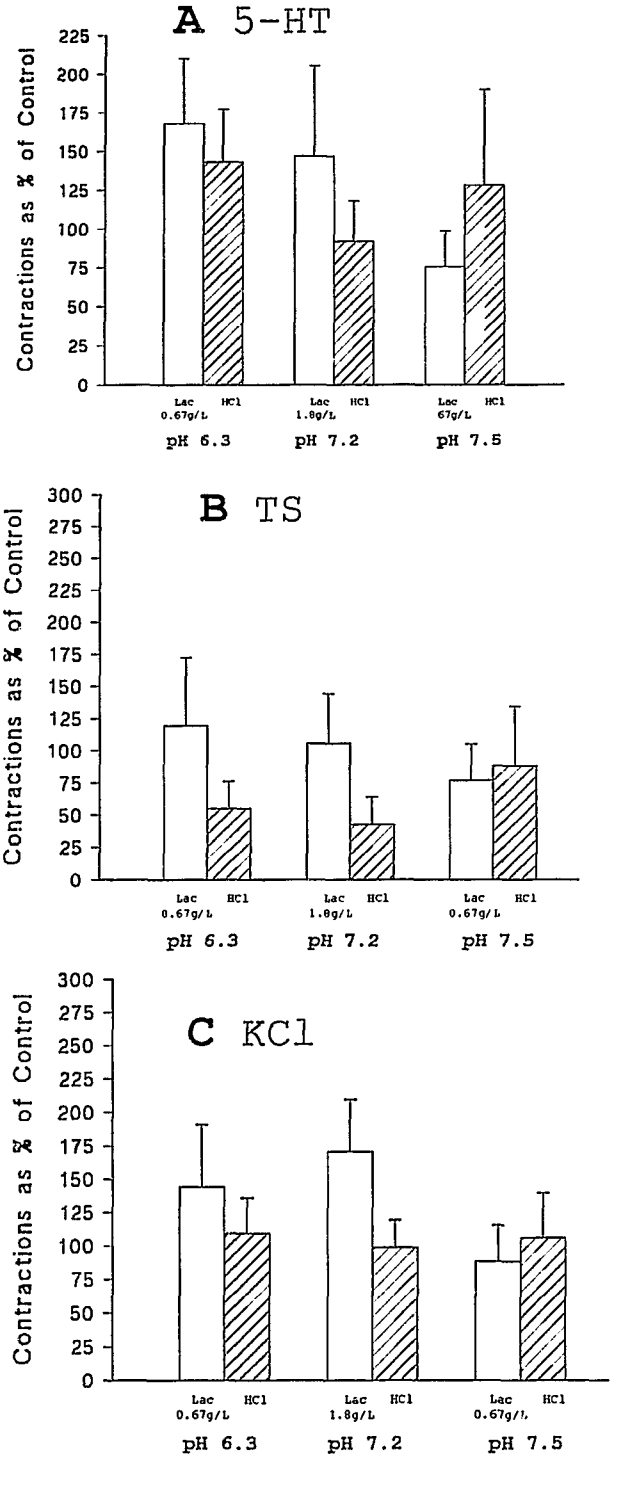


Fig. 3.2. Maximum contractions of distal intestinal segments in HEPES or MES buffered KHS, acidified with lactic acid [] or HCl [//]. Contractions initiated by (A) 5-HT, (B) TS and (C) KCl. Values are means ± SEM (n = 6). * denotes difference from control (pH 7.85)$p < 0.05$.

Analysis of the slope of the dose response curve, as well as the the $-\log_{10}EF_{50}$ and $-\log_{10}EC_{50}$ values, demonstrated that the potency of the agonists were not affected by either method of acidification.

3.3.2 EIPA

Analysis of the contractions showed that EIPA at a concentration of 10^{-5}M had no effect on the tissue. At higher concentrations, however, EIPA had similar inhibitory effects on both the proximal and distal segments (Fig. 3.3). As the EIPA would not wash out, only one contraction with each tissue after the application of EIPA was possible. In the distal segments, in 5 X 10^{-5}M EIPA, contractions to 5-HT were inhibited by 69.4 ± 9.6% ($p < 0.04$), proximal segments by 81.2 ± 7.3% ($p < 0.05$). At 10^{-4}M EIPA, distal segments contractions were inhibited by 91.5 ± 5.7% ($p < 0.01$), while inhibition was 100% in the proximal segments ($p < 0.001$).

3.3.3 SITS

When SITS was applied, and the pH not altered, no variation from control was observed. Increasing CO_2 to 20% for the proximal intestinal segments revealed a trend that did not reach the level of statistical significance. The decrease in pH to 6.58 showed a trend toward inhibition of contraction (58.6 ± 8.3%, $p = 0.07$) (Fig. 3.4 A); the absence of SITS did not affect the inhibition. Decreasing the pH had no apparent effect on the distal segments (Fig 3.4 B).

In 30% CO_2 (pH 6.50) there was a significant inhibition in the presence of SITS, particularly in the distal segments (Fig 3.5 B). decreasing the pH in the presence of SITS caused an inhibition from control of 64.3 ± 12.1% ($p < 0.026$). This inhibition was maintained at 53.1 ± 8.6% as the pH was returned to 7.21. Tissues that were not exposed to the Cl^-/HCO_3^- exchange blocker maintained

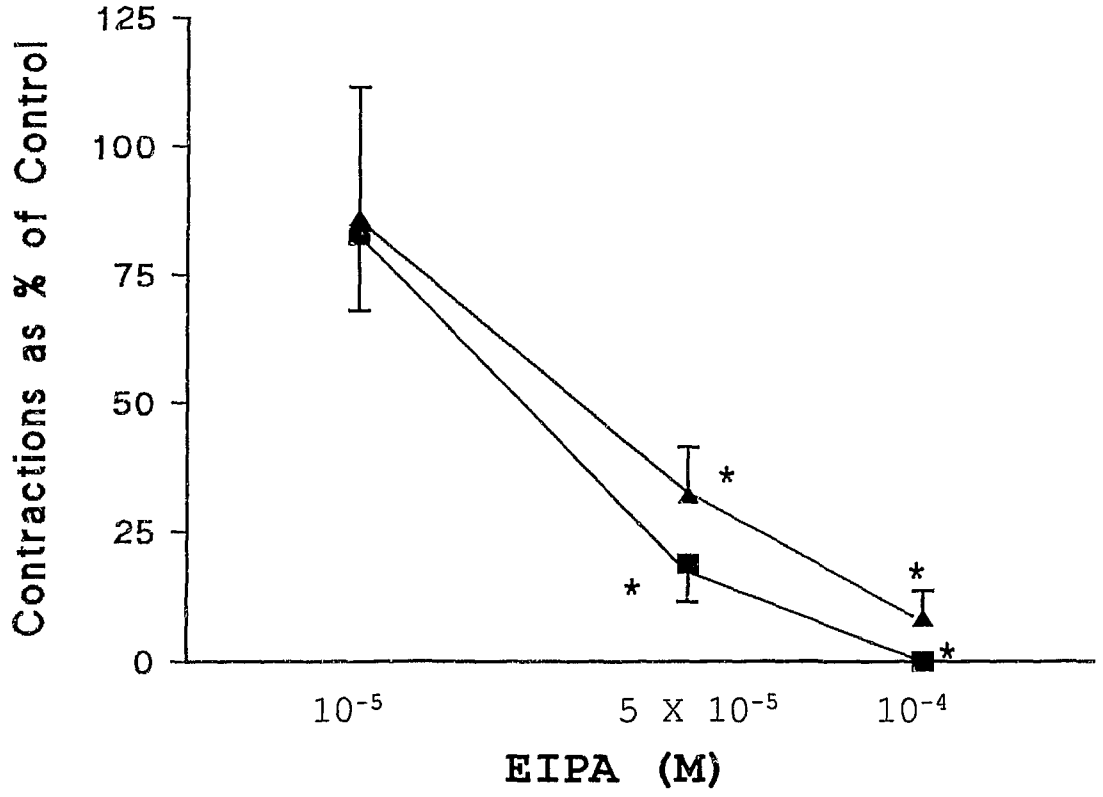


Fig 3.3. Contractions of proximal ■ and distal ▲ intestinal segments exposed to EIPA as percent of control. All contractions elicited by 3 X 10^{-5} M 5-HT at pH 7.85. Values are means ± SEM (n = 6). * denotes difference from control ($p < 0.05$).

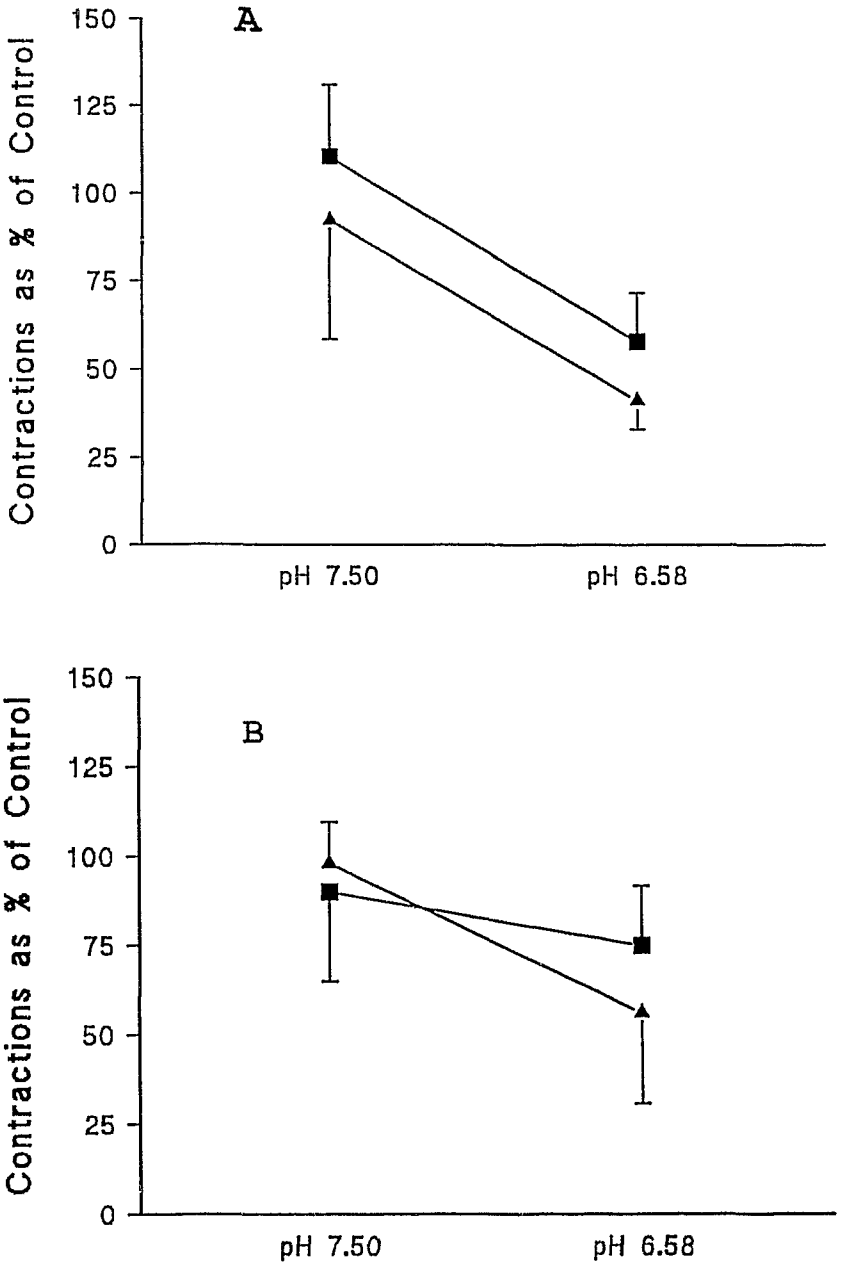


Fig 3.4. Contractions as percent of control (pH 7.85) of proximal (A) and distal (B) intestinal segments to 3 X 10^{-7} 5-HT. The pH was adjusted in the presence ■ and absence ▲ of SITS. Values are means ± SEM (n = 6).

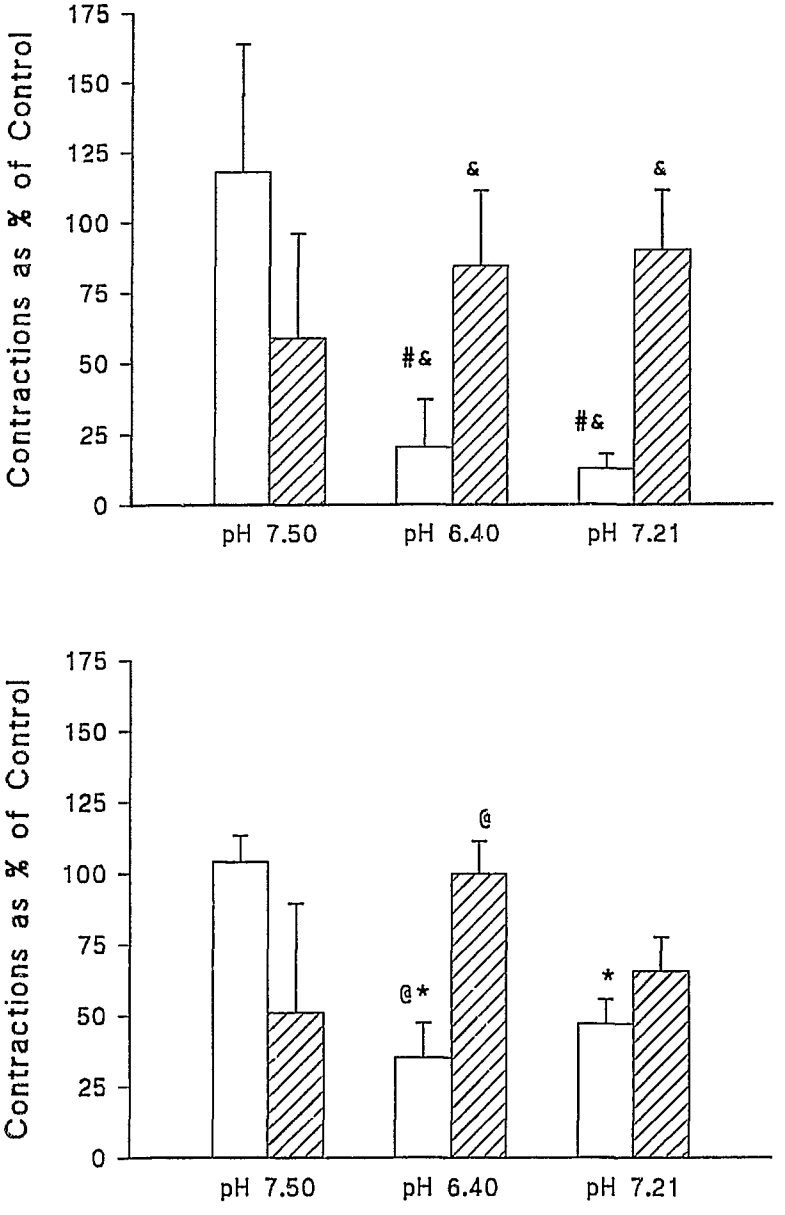


Fig 3.5. Contractions as percent of control (pH 7.85) of proximal (A) and distal (B) intestinal segments to 3 X 10^{-5} 5-HT. The pH was adjusted in the presence [] and absence [//] of SITS. Values are means ± SEM (n = 6). * denotes difference from control ($p < 0.05$); (#: $p = 0.07$). @ denotes difference between treatments ($p < 0.05$) (&: $p = 0.07$).

normal contractions at all pH levels. At pH 6.40, there was a significant inhibition of contractions of the tissues exposed to SITS compared to those not exposed ($p < 0.05$).

Proximal segments did not demonstrate any significanct changes in contractility following the addition of SITS at the 0.05 level, but there were trends obvious with probability values of approximately 0.07 (Fig. 3.5 A). These trends follow the same pattern as the distal segments with the tissues exposed to SITS demonstrating an inhibition as pH decreases; tissues exposed to decreased pH in the absence of SITS showing no inhibition.

3.4 Discussion

In this study, it appears that the efficacy and potency of 5-HT in the rainbow trout intestine are not affected by even relatively low pH values, whether the acidification is caused by an inorganic acid (HCl) or by the physiological L-lactic acid. This contrasts directly with the results reported in section 2.3.1 where the contractility of the proximal segments was much greater at pH 7.85 than at any other pH measured.

The ubiquitous Na^+/H^+ exchanger seems to be present, as does a Cl^-/HCO_3^- exchanger. The extent, however, to which each participates in the regulation of intracellular pH in these tissues is unknown.

3.4.1 Lactic acid

Lactic acid is produced as the end product of anaerobic metabolism in most animals. In cardiac and skeletal muscle, lactic acid production is the result of abnormal conditions such as hypoxia or high metabolic demand. Studies of guinea pig caecum (Ishida and Paul, 1990) and taenia coli (Hellstrand and Vogel, 1985) suggest that lactic acid may be produced in smooth muscle under normal conditions.

Rates of lactic acid production were calculated in these studies, but absolute values were not; however, under hypoxic conditions, the rate of lactate release from guinea pig taenia caeci is linearly correlated with tension development (Ishida and Paul, 1990).

Another potential source for lactate in smooth muscle is the uptake of circulating lactic acid released into the circulatory system during anaerobic skeletal muscle activity. Immediately following exhaustive exercise in rainbow trout both respiratory and metabolic acidosis result, during which whole body pH_i drops to approximately pH 6.7 (Milligan and Wood, 1986*a*). Under these conditions, lactate released from skeletal muscle is not linked to expulsion of H^+ as the level of $lactate^-$ in the blood exceeds the level of H^+ (Turner *et al.*, 1983). Plasma lactate levels have been measured at 20 mM in exhaustively exercised rainbow trout (Wood, 1991). Uptake of circulating lactate occurs in the white muscle, heart, liver and brain of rainbow trout (Milligan and Wood, 1986*b*), and exogenous lactate has been found to be preferred over endogenous fuels in perfused rainbow trout hearts (Milligan and Farrell, 1991). Although fish GI smooth muscle has not been studied in relation to lactate metabolism, both rat skeletal muscles (McDermott and Bonen, 1994) and guinea pig taenia caeci take up L-lactate for utilization as an energy substrate (Endo *et al.*, 1989) . The uptake of lactate without associated proton influx in rainbow trout heart can cause an intracellular alkalosis (Milligan and Wood, 1986*b*).

It is probable that the lactate is taken up into the intestinal smooth muscle in much the same manner as occurs in other tissues. For example lactate uptake in trout hearts is carrier mediated rather than by simple diffusion (Milligan and Farrell, 1991). However, given the lack of inhibition in proximal and distal segments to stimulation by

both 5-HT and KCl (Figs. 3.1, 3.2), it is unlikely that the contractile machinery of the cell is affected by the application of exogenous lactate, at least under aerobic conditions. The acidification did inhibit contractility when the proximal segments were stimulated electrically (Fig 3.1 B). This inhibition was approximately equal whether the decreased pH was the result of adding HCl or lactic acid at pH 6.3. Relatively high concentrations of lactate did inhibit the contractions beyond the inhibition by HCl at pH 7.2, suggesting that neuronal transmission is more sensitive to lactate than the smooth muscle itself. This agrees with mammalian studies where lactic acid has been shown to inhibit neuronal function in rat hippocampal slices (Morimoto *et al.*, 1994) and decrease the electrical activity of guinea pig taenia coli (Aaberg *et al.*, 1967).

In comparing Figures 3.1, 3.2 and the results from Chapter 2 it is apparent that the principal inhibition to contractilty is the result of increasing the concentration of CO_2, and not from an excess of free H^+ ions in the solution. Since CO_2 diffuses readily across membranes, an increase in extracellular CO_2 will combine with H_2O to form H_2CO_3. This rapidly dissociates to H^+ and HCO_3^- decreasing pH_i (Boron, 1989). Weinbeck *et al.* (1972) found, in guinea pig taenia coli, that the inhibitory effects of increasing CO_2 at a constant pH are greater than the effects of decreasing pH by increasing CO_2. A similar effect was found in feline bronchial smooth muscle (Duckles *et al.*, 1972).

3.4.2 EIPA

Amiloride and its analogs are well known antagonists of the major cation exchangers Na^+/H^+ and Na^+/Ca^{2+}. The 5-N substituted analogs, such as the ethyl-isopropyl analog (EIPA), are more selective for the Na^+/H^+ exchange than for the Na^+/Ca^{2+} exchange (Kleyman and

Cragoe, 1988) making them more appropriate for preclusive studies. EIPA is effective in inhibiting the Na^+/H^+ exchange at concentrations of 1μM in ferret hearts (Grace *et al.*, 1993).

Amiloride has been shown to inhibit contractions of smooth muscle in canine trachea (Krampetz and Bose, 1988). The inhibitory effects of amiloride can be attributed to direct inhibition of the myosin light chain kinase and protein kinase C as well as inhibiting cation entry into the cell (Chatterjee *et al.*, 1988). Other effects of amiloride include inhibition of adenylate cyclase and inhibition at muscarinic and α and β adrenergic receptors (reviewed by Kleyman and Cragoe, 1988). Bund *et al.* (1987) demonstrated a dose dependent inhibition of norepinepherine contractions with EIPA in human arterioles.

The inhibition of contractility in trout intestinal segments using EIPA (Fig 3.3 A and B) is consistent with that described above, using similar concentrations as the mammalian preparations. Without further examination, however, it is not possible to determine whether the inhibition in trout intestine is occuring at the level of the Na^+/H^+ exchanger or within the cytoplasm of the cell.

3.4.3 SITS

Isothiocyanate derivatives bind covalently to free amines in the anion exchangers of cell membranes by a chemical process known as the Edman Reaction (Roos and Boron, 1981) and are used extensively as tools in pharmacological perturbations and measurement of intracellular pH. One such compound is 4-acetamido-4′-isothiocyanto-stilbene-2-2′-disulfonic acid (SITS)

Anion flux has been examined in the gills of rainbow trout during acid-base disturbances, induced by changes in water quality

(Wilkie *et al.*, 1994) or by artificial manipulation of the fish itself (Goss and Perry, 1994). In both situations, the Cl^-/HCO_3^- exchange played a major role in maintaining physiological homeostasis. This mechanism also participates in anion exchange of lactate into tissues such as the heart and brain following exhaustive exercise (Milligan and Wood, 1986*b*; Milligan and Farrell, 1991).

In some smooth muscle preparations, such as guinea pig ureter (Aickin, 1994*b*) and porcine coronary arteries (Duong, 1993), the anion exchanger is only involved in recovery from alkalosis. Other tissues, including guinea pig femoral arteries (Aickin, 1994*c*) and rat mesenteric arteries (Aalkjær and Cragoe, 1988), also involve anion exchange in recovery from acidosis. Grace *et al.* (1993) estimated that, in ferret hearts, the Na^+/H^+ and the Cl^-/HCO_3^- exchangers each contribute approximately 50% of the proton efflux in recovery from acidosis. In the present study, no attempt was made to determine the influence of this exchanger on recovery from alkalosis, but it does appear from this data that it is involved in recovery from acidosis.

The addition of SITS at pH levels as low as 6.58 did not affect contractility of the tissues (Fig 3.4). As the pH was decreased to 6.4, however, the tissues exposed to SITS showed a marked inhibition that was statistically significant in the distal segments, and showed a definite trend in the proximal segments (Fig 3.5). The nonreversibility of the Edman Reaction is evident in the continued inability of the tissues to recover from the acidic inhibition after washing and restoring a higher pH. From this we can conclude that blockade of the Cl^-/HCO_3^- anion exchanger prevents the muscle from maintaining or allowing the restoration of a high enough pH_i to retain contractility at control levels following relatively extreme acidic insult.

3.4.4 Conclusions

Lactic acid appears to inhibit contractions of rainbow trout intestinal smooth muscle by inhibiting neuronal transmission, but only in the proximal segments. Inhibition does not occur when the agonists act directly on the muscle itself.

It is likely that both the Na^+/H^+ and the Cl^-/HCO_3^- exchanger are present in the tissues and that both contribute to the removal of excess protons when the tissue is challenged. If the regulation of pH_i is accomplished by the action of both Na^+/H^+ and a Cl^-/HCO_3^- exchangers, then below some critical pH value (between pH 6.58 and 6.4), the action of the Na^+/H^+ exchange alone does not appear to be enough to maintain functionality of the tissue.

4. CHAPTER 4 GENERAL DISCUSSION

4.1 Optimum pH

One of the objectives of this study was to determine the optimum pH required for *in vitro* contractility of rainbow trout intestinal smooth muscle. In Chapter 2 it was shown that the proximal segments have a strong increase in contractility at pH 7.85. It can readily be seen in Figure 2.1 that the contractility decreases rapidly as the organ bath pH is raised or lowered by as little as 0.3 pH units. This optimum pH corresponds to the plasma pH of rainbow trout held in neutral water (MacDonald, 1980). By comparison, mammalian smooth muscle has a pH_i range of 7.1 to 7.2 with a pH_e of 7.4 (Wray, 1988).

Distal intestinal segments (Fig. 2.2) show a much wider functional pH range than the proximal segments, possibly related to the digestive capabilities of the different intestinal regions. Uptake of ions from chyme occurs largely in the posterior intestine (Evans, 1979, 1993; Gairdaire and Isaia, 1992; Klaren *et al.*, 1993) and it is possible that there is a varying expression of the ion exchangers involved.

Although the regional patterns differed, in both the proximal and distal segments, responses to all three agonists (5-HT, TS, and KCl) were similar. The similar responses of electrical stimulation and 5-HT receptor activation and membrane depolarization with KCl suggests that the inhibition of contractility attributable to altering pH is due to direct action on the contractile mechanism within the cell, and does not involve receptor mediated events. This must be questioned, as the subsequent studies described here appear to contradict these results.

4.2 Buffer comparisons

Comparisons were conducted among the buffers used: TAPS (pH 8.1, 8.5); HEPES (pH 7.5, 7.85); MES (pH 6.3 - 7.2) all aerated with 100% O_2 and Na^+/HCO_3 (pH 6.3 - 7.2) aerated with CO_2 (5% - 40%). In these comparisons, inhibition by decreasing pH first appears in both proximal and distal segments when the buffer contains Na^+/HCO_3 and is aerated with 5% CO_2 (Figs 2.4, 2.5). This inhibition increases with the concentration of CO_2. The effect is more pronounced when the tissues are electrically stimulated, implying that neuronal transmission is more sensitive to decreasing pH, particularly when the alteration in pH is accompanied by an increase in CO_2 concentration. In rat vascular (Austin and Wray, 1993) and uterine (Taggert and Wray, 1993) smooth muscle, inhibition of contraction is independent of the means used to induce the acidification. In feline airway smooth muscle, however, CO_2 appears to have a differential effect depending on the agonist used (Duckles *et al.*, 1974).

4.3 Group comparisons

As salmonids mature, many metabolic changes occur. Smoltification requires alterations in ion transport capabilities as the fish prepare for moving to salt water. Many of these changes are related to elevated concentration of circulating thyroid hormones (Eales *et al.*, 1991) and include increases in intestinal fluid transport (Collie and Bern, 1982). Steroid production also increases as the animals become sexually mature, particularly 17β-oestradiol, testosterone, 11-ketotestosterone and calcium in the female (Scott *et al.*, 1980), and, in the male, testosterone, 11-ketotestosterone, and 17α-hydroxy-20β-dihydro-progesterone levels (Fostier *et al.* 1987).

Whether or not any or all of these impacted on the differences between the two groups of trout in this study is unknown. In all instances where differences in contractility at low pH were apparent, tissues from the sexually mature fish were more able to withstand the applied insult (Fig. 2.6). Absolute measures of contractility change seasonally, with the greatest tension occurring between April and June, and the minimum in October (Burka *et al.*, 1993*b*). The fish in this study were sacrificed in late summer within a one month period, minimizing any seasonal variations.

Decreasing pH increases the Ca^{2+} required to initiate a contraction (Austin and Wray, 1994, Churcott *et al.*, 1994). Although other studies have noted an increase in plasma Ca^{2+} associated with sexual maturity (Scott *et al.*, 1980), no differences were apparent between the two groups in this study (Table 2.3).

The other obvious difference between the groups - genetic background - has been found to impart differential resistance to acid stress (Dunson and Martin, 1973; Robinson *et al.*, 1976). Wang *et al.* (1994) found variations in fluid movement between two groups of fish following exhaustive exercise. In the present study, the results obtained from the younger group of fish more closely resemble those obtained in the original determination of optimum pH. Since a different control was used, however, a direct comparison is not possible. This would imply that age and sexual maturity are more important indicators of acid resistance in intestinal tissue than is the genetic strain of rainbow trout used.

4.4 Lactic acid

Lactic acid is produced by at least some types of smooth muscle as part of normal function (Ishida and Paul, 1990; Hellstrand and

Vogel, 1985), as well as under conditions of hypoxia or high metabolic demand. Exhaustive exercise results in a plasma lactate concentration of up to 20mM (Wood, 1991). Since lactate is actively taken up by other tissues in both mammals (McDermott and Bonen, 1994) and trout (Milligan and Wood 1986*b*; Milligan and Farrel, 1991), it is reasonable to assume that uptake by intestinal smooth muscle also occurs. The effect of exogenously applied lactate on contractions initiated by 5-HT and KCl was minimal. Electrical stimulation of the proximal segments, did however, result in an inhibition beyond that of HCl (Fig. 3.1), suggesting that the nerves innervating the region are sensitive to lactate inhibition. Electrical activity of guinea pig taenia coli (Aaberg *et al.*, 1967) and rat hippocampal slices (Moromoto *et al.*, 1994) are inhibited by lactic acid. Why the nerves supplying segments from the distal intestine were not inhibited in the same manner is not known.

4.5 EIPA

Amiloride and its analogs are known inhibitors of smooth muscle contractions in mammals (Bund *et al.*, 1987; Krampetz and Bose, 1988). This inhibition can be attributed both to cytosolic effects as well as cationic inhibition (Simchowitz *et al.*, 1987; Chatterjee *et al.*, 1988). The non-ionic effects include inhibition of myosin light chain kinase, protein kinase C (Chatterjee *et al.*, 1988) and adenylate cyclase (Kleyman and Cragoe, 1988). Inhibition of α and β adrenergic receptors (Kleyman and Cragoe, 1988) also occurs. The dose dependent inhibition by EIPA shown in Fig. 3.3 (A and B) is similar to that shown by Bund *et al.* (1987) in human arterioles. However, it was not possible to determine whether the inhibition is due to disruption of cationic flux or to cytosolic effects.

4.6 SITS

Anion flux in cells is regulated by a Cl^-/HCO_3^- exchanger that may be Na^+ dependent (Thomas, 1984). This exchanger may also be the mechanism by which membrane transport of lactate ions is accomplished (Milligan and Wood, 1986*b*; Milligan and Farrel, 1991). Mammalian tissues may utilize this exchanger in recovery exclusively from alkalosis (Duong, 1993; Aickin, 1994*b*). Recovery from acidosis may also be a function of the Cl^-/HCO_3^- exchanger (Aalkjær and Cragoe, 1988; Grace *et al.*, 1993; Aickin, 1994*c*). It is apparent from this study that the Cl^-/HCO_3^- exchanger in rainbow trout intestinal smooth muscle is involved in protection against, and recovery from, hypercapnic acidosis.

4.7 Conclusions

Intestinal smooth muscle from rainbow trout shows a remarkable resiliency when exposed to extreme values of pH. This becomes more apparent as the fish attain sexual maturity. In immature fish (age 1+), there is a strong peak of intestinal contractility at pH 7.85. As the fish become sexually mature (age 2+), the tissue changes in its capabilities, and contractile differences are not apparent at pH values as low as 6.5.

An increase in the concentration of CO_2 has a greater impact on the contractility of the muscle than an increase in H^+, an inhibition that is also diminished with sexual maturity. Transmural stimulation is more sensitive to all methods of acidification - CO_2, HCl, and lactic acid - than receptor stimulation by 5-HT or depolarization by KCl. With the exception of transmural stimulation, lactate did not affect the smooth muscle any differently than HCl at any of the pH levels examined.

The sensitivity of the tissues to hypercapnia is verified by the importance of the Cl^-/HCO_3^- exchanger in maintenance of homeostasis under low pH/high CO_2 conditions.

4.8 Future directions

This research has answered some questions about the functionality of rainbow trout intestinal smooth muscle under acidic stress, but opens the door to many more. First and foremost, is the applicability of these results to live fish under physiological conditions. One means of establishing this would be to induce acidosis and utilize an *in situ* intestinal preparation for pharmacological examination.

This study has encountered several apparent contradictions in the quantitative response of the tissues to acidification. At what level of pH does inhibition actually occur? This may be affected by age, sexual maturity or genetic strain of the fish under examination. It appears that hypercapnia interferes with functioning, but it is uncertain where in the cascade of events, from stimulation to contraction, the inhibition occurs. Quantitative measurements of intracellular pH and the correlation between pH_i and contraction are important steps in elaborating on the present work. Knowledge of smooth muscle pharmacology in fish is expanding rapidly and it is hoped that this work is an aid in understanding the interactions between pH and physiology.

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