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MORPHOLOGICAL DESCRIPTION OF A POSSIBLE NERVE-EOSINOPHILIC GRANULE CELL RELATIONSHIP IN THE RAINBOW TROUT SMALL INTESTINE.

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Master of Science

in the Department of Anatomy and Physiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Mark D. Powell

Charlottetown, PEI.

August, 1991

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20 August 1991

Examiners:

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Dr. G. M. Wright

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25 October 1991

ABSTRACT

Salmonid eosinophilic granule cells (EGC) and mammalian mast cells have been shown to be both functionally and morphologically similar. Mast cells are known to be in contact with non-myelinated nerves in the intestinal tract of mammals. This investigation aimed to examine a possible morphological relationship between the EGCs of the rainbow trout intestine and intrinsic non-myelinated nerves of the intestinal mucosa. Adult fish were injected intraperitoneally with capsaicin, substance P, serotonin, bovine serum albumin or saline vehicle alone (0.5 $\mu g.g^{-1}$ body weight). Fish were killed 30 minutes, 1, 2 and 4 hours post-injection and the small intestine removed and processed for light and electron microscopy. A significant proportion of the EGCs were found to be in close association with non-myelinated enteric nerves in both control and experimental tissues. Both capsaicin and substance P induced ultrastructural changes in EGCs of both the *stratum compactum* and the *lamina propria*, reminiscent of crinophagy of neuroendocrine cells. Acid phosphatase was not found to be present in the crinophagic-like granules confirming that this form of degranulation in EGCs was not a lysosome-mediated autophagic process, but possibly due to a non-lysosomal mechanism of protein degradation. Serotonin, bovine serum albumin and saline had no effect on EGC morphology. It was found that upon stimulation with capsaicin or substance P profound morphological and distributional changes to the EGC population take place. A significant decrease in the number of observable granule cells in the *stratum compactum* was measured. There were no significant changes in the number of granule cells with serotonin or the controls. EGCs could be classified into five cell classes according to their morphology. The proportions of each morphology in the *stratum compactum* or *lamina propria* changed significantly following stimulation with capsaicin and substance P. It was apparent that mature EGCs migrate to the *lamina propria* to fully degranulate. Once degranulated, they appear to return to the *stratum compactum* to regenerate. The fibroblast-like ensheathing cells of the *stratum compactum* may be involved in the maturation and regeneration process. EGCs appear to be closely associated with the intrinsic non-myelinated nerves of the trout intestine being in both close apposition and possibly stimulated by capsaicin. The degranulatory actions of capsaicin and substance P suggest that an endogenous substance P-like compound is the likely mediator since substance P is a putative neurotransmitter of the non-adrenergic, non-cholinergic nervous system. Such a relationship suggests a functional link between the nervous system and the non-specific defense system in fish, somewhat similar to that seen between mammalian mast cells and non-myelinated intestinal nerves.

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To my wife Linda and sons Alex and Matthew.

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1. INTRODUCTION

The rainbow trout (*Oncorhynchus mykiss* Walbaum) is a valued species for aquaculture around the world. Its ability to grow well within the confines of intensification and adapt to the stresses associated with intensive culture make it a model fish for both aquaculture and salmonid research. Despite the research on fish which has been conducted to date, relatively little is known about inflammation and pathophysiology in comparison to mammalian systems. However, care must be taken in extrapolating and conferring advanced mammalian biological concepts on a teleost since it often involves making assumptions with little biological evidence. Thus discretion must be used when comparing piscine and mammalian systems.

1.1. Inflammation and pathophysiology in fish.

The economic significance of pathological processes in commercially viable species, such as trout (*O. mykiss*; *Salvelinus fontinalis*; *S. alpinus*), salmon (*O. keta*; *O. kisutch*; *Salmo salar*), and cyprinids (*Cyprinus carpio*), is spawning research in an attempt to further our understanding of such processes. Like mammals, pathophysiological processes in fish are complex and may involve humoral and cell mediated immune responses, biochemical and non-specific defense responses, as well as complicated behavioral reactions [1]. These various responses may be exacerbated or reduced by stress [2]. If such stresses are allowed to persist untreated they will facilitate immunosuppression [2] with one scenario being invasion of the fish by pathogens.

The entry of such organisms can lead to an acceleration and increased range of pathophysiological reactions and ultimately death. Anaphylactic-like pathophysiological reactions have been successfully induced in the laboratory using channel catfish (*Ictalurus punctatus*) and goldfish (*Carassius auratus*) [3]. Finn and Nielson [4] characterised the nature of cell mediated inflammatory responses in rainbow trout, showing them to be a series of temperature-dependent events [5]. Such inflammatory responses involve leucocytic infiltration(*ie.*, heterophils, macrophages and lymphocytes) into the affected tissue [4, 6]. Although the biochemical responses of fish have not been extensively examined under pathological conditions, recent work has alluded to the role of eicosanoid compounds (*eg.*, prostaglandins, leukotrienes, *etc.*) as potential mediators of the pathophysiological response in fish. For example, prostaglandins have been detected in a variety of fish species (see [7] for review). Thromboxane B_2 has been localised in rainbow trout thrombocytes [8] and in dogfish (*Scyliorhinus canicula*) leucocytes [9]. Leukotrienes B_4 and B_5 have been shown to be synthesised in rainbow trout leucocytes (primarily lymphocytes which make up 59% of rainbow trout white blood cells) and thrombocytes which make up 8% of rainbow trout white blood cells [10]. Prostacyclin (PGI_2) appears to be produced by the macrophage/monocyte population in fish as it is in mammals [11]. It seems possible therefore that these mediators of inflammatory responses could be released following the initiation of a cell mediated inflammatory response as is seen in mammals. The chemotactic attraction of such inflammatory cells (*eg.*, neutrophils, macrophages, *etc*) in mammals is thought to be

induced by the release of eicosanoids from mast cells and resident macrophages. The existence of mast cells in fish remains controversial [12], although it has been suggested that the tissue eosinophil or eosinophilic granule cell in salmonids may be analogous to the mast cell [12].

1.2. Eosinophilic granule cells.

The term "eosinophilic granule cell" (EGC) was coined following its identification during studies on the skin of the European plaice, *Pleuronectes platessa* [13]. Although first described in 1898 by Gulland [14], the function of these cells remained obscure until 1971 when it was speculated that they may be involved in pathological processes [14]. Subsequently, EGCs have been described in a wide variety of salmonid tissues [12, 15, 16]. Similar cells have been identified in a variety of other species besides salmonids. These include cyprinids [17], where the cell is referred to as a "periodic acid Schiff-positive granular leucocyte (PAS-GL)." This cell has histochemical characteristics of both circulating and tissue eosinophils (with strongly acidophilic staining granules) and mast cells (staining metachromatically with toluidine blue) [18]. PAS-GLs are morphologically similar to both EGCs and mast cells in respect of their large membrane-bounded cytoplasmic granules.

1.2.1. Histology and histochemistry.

Histological descriptions of the eosinophilic granule cell are highly contradictory. EGCs have responded to a wide variety of histological stains and assays with little

evidence as to the granule contents. The histochemistry which has been carried out to date is summarised in Table I. There is a general consensus in the scientific literature that EGC granules contain proteins, probably basic in nature [19, 20, 21]. It has been suggested that the granules have an arginine core [15] but this has not been examined further. The granules have also been demonstrated to contain polysaccharides and acid sulphated glycosaminoglycans, the former being more prevalent in immature fish and the latter in older fish [21]. There appears to be little evidence of lipids within the granules, although a positive reaction for phospholipids was detected in Atlantic salmon (*S. salar*) and rainbow trout [15]. There is no evidence of phospholipase enzymes associated with EGCs [19, 21]. However Smith's [15] detection of phospholipid may have been due to staining of the granule membranes. Specialised mast cell stains used by Bergeron [21] yielded inconclusive results. A negative result was also obtained with orthophthaldehyde, a fluorescence technique for demonstrating the presence of histamine [21], conflicting with suggestions that EGCs contain histamine and release the amine upon stimulation by bacterial toxins or histamine liberators [22].

Enzyme histochemistry has demonstrated the presence of an alkaline phosphatase, arylsulfatase and 5-nucleotidase in the EGC granules [15, 23, 24]. Ezeasor and Stokoe [24] obtained a weak reaction for acid phosphatase associated with the granules which contradicted Smith's earlier negative result [15]. EGCs have been tested for several other non-lysosome associated enzymes and the results of the

Table I. Summary of the histochemical analysis of rainbow trout EGCs. Results are classified as + positive result, +/- ambiguous result, and - negative result.

Substance	Reaction/Test	Result	Reference
Neutral Polysaccharide	Periodic acid Schiff	+	19
		+/-	21
		-	24, 15
Glycosamino-glycans	Alcian blue (pH 2.5)	+	24, 21
	(pH 1.0)	+	21
	(pH 0.4)	+	21
General proteins	Mercuric bromophenol blue	+	19, 21
	Sakaguchi	-	15
Basic proteins	Biebrich scarlet	-	24
	Fast green (pH 8.0)	+	21
Neutral lipid	Sudan black	+	19
		+/-	15
		-	21
	Oil-red-O	-	21
Phospholipid	Bakers	+	15
Mast cell specific stains	Gomori aldehyde fuchsin	+	15
Histamine	Orthophthaldehyde	-	21

enzyme histochemistry and cytochemistry which has been conducted to date is summarised in Table II.

1.3. Anatomy of the teleost small intestine.

The trout small intestine can be separated into 3 major histological regions, each with its own characteristics: mucosa, muscularis and serosa (Fig. 1.1). The mucosa consists of three layers, the epithelium, the *lamina propria* and the *stratum compactum*. The epithelium is thrown into a series of finger-like folds with the *lamina propria* extending into the centre (Fig. 1.1). The epithelium consists of mostly columnar cells, (enterocytes, goblet cells and exocrine cells) with apical microvilli. The *lamina propria*, is a loose connective tissue consisting of fibroblasts, collagen fibrils, and infiltrated leucocytes, and is perforated by nerves, lymphatics and blood vessels [25]. The *stratum compactum* is a dense collagenous connective tissue which restricts the extensibility of the gut [25] and contains fibroblasts and a discrete layer of EGCs known as the *stratum granulosum* [24]. EGCs are interdispersed with fibroblast-like ensheathing cells [24]. The muscularis consists of circular and longitudinal smooth muscle separated by a myenteric region rich in nerves and blood vessels [24, 25, 26]. The whole intestine is ensheathed in a serosal connective tissue sheath (Fig. 1.1).

Table II. Summary of enzyme histo/cytochemistry which has been carried out on rainbow trout EGCs. Results are classified as + positive, +/- ambiguous or - negative. Localisation of the enzyme is in the granule (G).

Enzyme	Result	Localisation	Reference
Lipase	-		19, 21
Alkaline phosphatase	+	G	23
	+/-	G	24, 15
	-		19
Acid phosphatase	+/-	G	24
	-		15
Peroxidase	-		19
5-nucleotidase	+	G	24
Arylsulfatase	+	G	24
DOPA oxidase	-		19
Leucine amino-peptidase	-		21

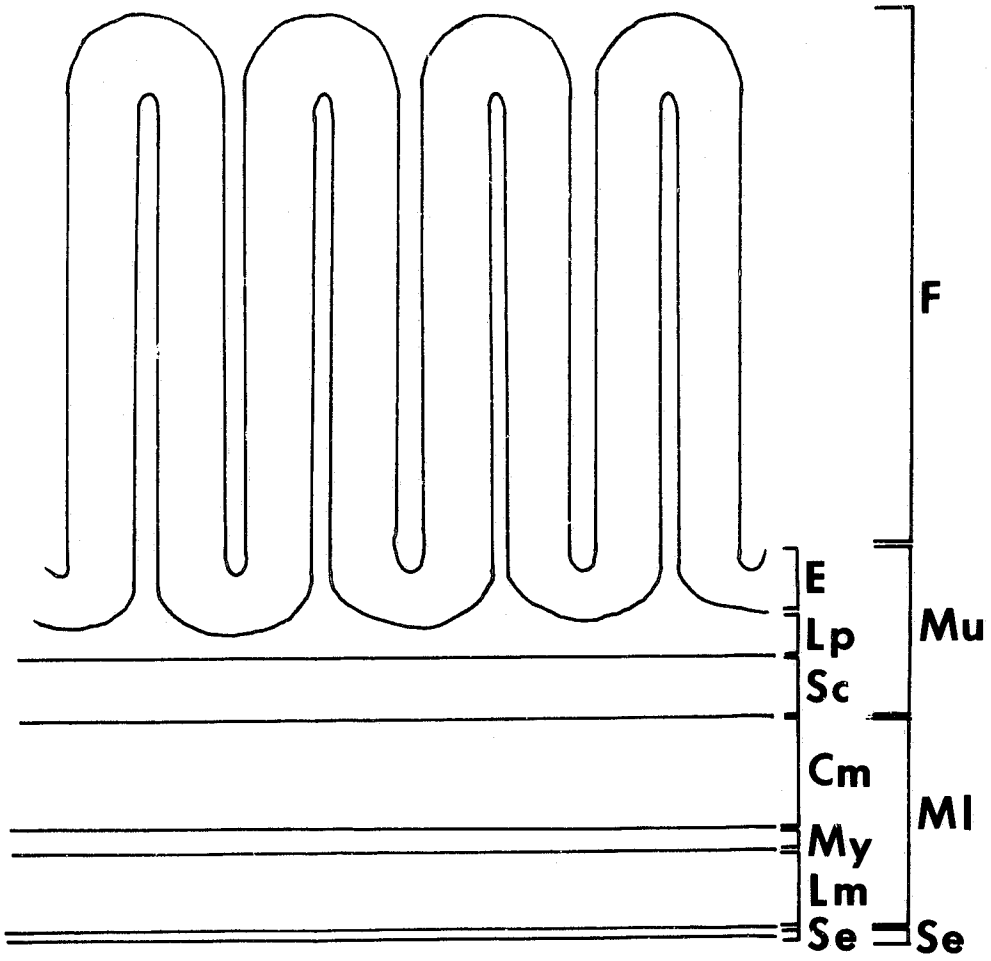


Figure 1.1. Diagrammatic representation of the tissue distribution in a transverse section through the wall of the small intestine of the trout. Note the finger-like mucosal folds which extend into the intestinal lumen (F). Mu= Mucosa; Ml= Muscularis; Se= Serosa; E= Epithelium; Lp= *Lamina propria*; Sc= *Stratum compactum*; Cm= Circular smooth muscle, My= Myenteric plexus; Lm= Longitudinal smooth muscle; Se= Serosa.

1.3.1. Development of the teleost intestinal mucosa.

The EGC layer develops gradually with the age of the fish. Granule cells are first observed scattered throughout the gut mucosa from 16-24 weeks post-hatching [21, 27]. The distribution is similar to that seen in the trout oesophagus where a *stratum compactum* is absent even in adult trout [24]. In the pike (*Esox lucius*) intestine, a *stratum compactum* never develops [28]. Consequently the EGCs remain scattered throughout the intestinal mucosa. Development of the *stratum granulosum*, the layer of EGCs usually 1-2 cells thick, is preceded by development of the collagenous *stratum compactum*. Alignment of the EGCs occurs at 24-28 weeks post-hatching [27]. Bergeron [21] showed a difference in the development time for the *stratum compactum* between fish which were spawned and hatched in the spring and those that were spawned and hatched in the fall. Underyearlings which hatched in the spring all developed a *stratum granulosum* by 28 weeks whereas fall-hatched fish took 32 weeks to fully develop a *stratum granulosum*. Appearance of EGCs and the development of a *stratum granulosum* is concomitant with the development of extensive immunocompetency and sustained immunological memory which occurs by 24-32 weeks (≈4.0 g weight) [29, 30], rather than first feeding which usually occurs at approximately 16-18 weeks [21]. It has been suggested that *stratum granulosum* development facilitates a maturation process for the EGCs [21, 27]. The correlation betwen the appearance of EGCs and with the development of immunocompetency suggested that EGCs were probably involved immunologically similar to the function of the mammalian Paneth cell [13]. Paneth cells are thought to be involved in the

control of intestinal flora [31]. If this is true the more rapid proliferation of EGCs in the *stratum compactum* of spring hatched fish supports the theory that spring is a more susceptible time for pathogen infection [32].

Since the *stratum granulosum* cannot always be destinguished, *ie.*, it exists as a subdivision of the *stratum compactum* and often is difficult to delimit in histological sections, it will not be referred to further in this thesis. In lieu of this and for our purposes the EGCs will be considered to form a layer in the *stratum compactum*.

1.4. The teleost gut as an immunological organ.

The potential for the involvement of the gut in immunological responses is well documented especially with respect to specific immune defense mechanisms [33]. The gut associated lymphoid tissue (GALT), which has been extensively studied in carp (*C. carpio*) [34, 35, 36], has been shown to be of great importance in both specific and non-specific immune response reactions. Rombout *et al.* [34] demonstrated that enterocytes and resident macrophages are responsible for the uptake, processing and presentation of antigens to the immune system. The resident leucocytes in the carp intestine are thought to be involved in local immune responses [36] but, little is known about how leucocytes are attracted and infiltrate infected tissues in fish, providing a defence against pathogen invasion [33]. The potential therefore remains for the EGC, because of its involvement in bacterial and parasitic pathogenesis to play a role in coordinating leucocytic responses in association with

the GALT.

1.5. Mammalian mast cells.

First identified in 1877 by Ehrlich [37], the mast cell has been implicated in many mammalian immunological and physiological responses. A primary role of the mast cell is its involvement in cell mediated non-specific defense mechanisms. This would include inflammatory and type I hypersensitivity responses which release biogenic amines (*eg.*, histamine and 5-hydroxytryptamine) and other vasoactive compounds such as eicosanoids [38].

1.5.1. Mast cell heterogeneity.

Two principal types of mast cells have been identified in mammals: the mucosal mast cell (MMC) of the intestinal mucosa, and the more widely distributed connective tissue mast cells (CTMC) [39]. The latter is widespread throughout the body, usually associated with connective tissue and the lining of the peritoneum, where they are referred to as peritoneal mast cells (PMC). Mucosal and connective tissue mast cells differ histochemically and biochemically (Table III), although they are similar morphologically [40, 41].

1.5.2. Mast cell degranulation.

Both MMC and CTMCs can be degranulated by an immunoglobulin (Ig) E, calcium-dependent mechanism whereby the ligand-IgE complex causes a change in the

Table III. Comparison of some of the major features of mammalian mucosal and connective tissue mast cells, and rainbow trout EGCs. N.D not determined, + substance/activity present, - substance/activity absent.

Comparison	Mucosal MC	Connective tissue MC	EGC	Reference
Size of granules	<0.2 μm	0.2-0.4 μm	0.2-1.5 μm	41
Histamine content	0.1-2.0 pg/cell	1.0-30.0 pg/cell	N.D	41
O-phthaldahyde	N.D	+	-	21, 41
Rat MC protein I	-	20.0-30.0 pg/cell	N.D	41
II	10.0-40.0 pg/cell	-	N.D	41
Chondroitin sulphates	95%	minor	N.D	41
Heparin	5%	major	N.D	41
Alcian Blue	+	-	+	21, 41
Luna's MC stain	+	+	+	21
LT C_4[a] (in rats)	29.0 pg/cell	<0.7 pg/cell	N.D	41
LT B_4[b] (in rats)	12.0 pg/cell	<0.5 pg/cell	N.D	41
PG D_2[c] (in rats)	4.7 pg/cell	7.5-13.0 pg/cell	N.D	41
Degranulation by:				
IgE	+	+	-[d]	41
A23187[e]	+	+	+[f]	42
Compound 48/80	-	+	+	41, 43
Bee venom peptide	-	+	N.D	41
Substance P	+	+	N.D	44

[a] Leukotriene C_4
[b] Leukotriene B_4
[c] Prostaglandin D_2
[d] Rainbow trout do not possess subclass E immunoglobulins.
[e] A calcium ionophore
[f] Powell unpublished observations.

membrane lipid composition, activating voltage-independent calcium channels via a G-protein. The influx of extracellular calcium ions (Ca^{++}) and release of membrane bound intracellular calcium (Ca^{++}_i) from inositol triphosphate (IP_3) turnover facilitates the activation of cytoplasmic proteins (*eg.*, calmodulin and tubulin). The activation of such proteins leads to granule exocytosis and the extracellular release of the vasoactive granule contents [45, 46]. This activation mechanism also initiates the synthesis and release of lipid-derived mediators such as eicosanoids and platelet-activating factor (PAF).

Mucosal mast cells of both the human and rat intestine are located in close apposition to non-myelinated peptidergic nerves [47, 48, 49, 50] suggesting a potential neural control of mast cell degranulation. The neurotransmitter, substance P, has been suggested to be a possible mediator of this degranulation [47, 49, 50]. Substance P and other vasoactive peptides can cause rat mast cell degranulation *in vitro* [45]. The process is rapid and independent of Ig E sensitisation [44]. This degranulation has been shown to occur via the IP_3 second messenger pathway [51] whereby Ca^{++} is released from intracellular calcium stores, similar to immunological degranulation. Connective tissue mast cell histamine release in parasite infected rats is augmented during antigen challenge by vagus nerve stimulation [52] suggesting a functional link between the nervous and non-specific immune system. Capsaicin, a neurotoxin, causes degranulation of CTMCs in rats *in vivo* [53], probably by causing the release of substance P or other neurokinin-like substances from non-myelinated

nerve endings [54].

More recently, substance P has been demonstrated to act directly on G-proteins isolated from calf brain preparations. The activated G-protein then gates a Ca^{++} channel [55]. Similarly this mechanism may occur in mast cells leading to an influx of extracellular calcium, release of intracellular calcium from intracellular stores, and phosphoinositide turnover [55].

1.6. Similarities between EGCs and mammalian mast cells.

Morphologically, mast cells and EGCs are similar, with EGCs being found in fish in the same tissues as CTMCs and PMCs in mammals. EGCs are found in large numbers in skin, intestine, and within the connective tissue of the central nervous system (CNS). Additionally EGCs are found in fish gills [15]. The responses of both EGCs and mast cells to bacterial exotoxin are also similar. Tsuchiya *et al.* [56] demonstrated degranulation of rat CTMCs following injection of *Vibrio cholorae* exotoxin. Ellis [22, 43] demonstrated similar responses of EGCs in rainbow trout injected with extracellular products of *Aeromonas salmonicida.* Degranulation following cholera toxin is thought to occur independent of the phosphoinositide pathway [46], suggesting by default that the adenylate cyclase pathway and production of the second messenger, cyclic adenosine monophosphate (cAMP) may be involved. The intracellular mechanism for degranulation of EGCs remains unknown.

Connective tissue mast cells respond to stimulation by the mast cell secretagogue compound 48/80 [40] (N-methyl-p-methoxy-phenethylamine)(Appendix A) as do EGCs [22, 43]. Compound 48/80-induced CTMC degranulation is calcium dependent, whereby the addition of the calcium ionophore A23187 (Calimycin) (Appendix A) enhances compound 48/80-induced CTMC degranulation [42]. Compound A23187 also induces similar degranulation responses to those demonstrated using compound 48/80 in EGCs [Powell unpublished observations]. The fact that compound 48/80 and A23187 will degranulate both CTMC and EGCs suggests a possible analogy between these two cell types with regard to a calcium-dependent mechanism for degranulation.

1.7. Peptides as neurotransmitters and neuromodulators.

Peptides such as bombesin, somatostatin, substance P, neurokinin A and B, gastrin, vasoactive intestinal polypeptide, and calcitonin gene-related peptide are putative neurotransmitters. As neurotransmitters, neuropeptides, as they are termed, are found in both the central and peripheral nervous system of most animals including fish [see 57 for review]. While their purpose in the central nervous system is not fully understood, they are known to be involved in many diverse functions including spinal transmission of sensory and motor information, including nociception in mammals (*eg.*, substance P) [58]. In the peripheral nervous system the role of neuropeptides is generally attributed to their involvement in the non-adrenergic, non-cholinergic nervous system (NANC). That is they are thought to be involved in the

neurotransmission of nerves which do not contain noradrenalin or acetylcholine as the primary neurotransmitter. As well as being mediators of nociception in mammals, bombesin, substance P and gastrin also control smooth muscle contractility and digestive enzyme release in the gastrointestinal tract, [59] of fish. Of particular relevance to this investigation is the group of peptides referred to as tachykinins. These include substance P, neurokinin A and B, eledoisin, kassinin and physalaemin, which all posess a common C-terminal amino acid sequence (-Gly-Leu-Met-NH_2) [60]. Although the former three compounds are found in mammals and the latter three (*ie.*, eledoisin, kassinin and physalaemin) in amphibians, substance P is the only tachykinin identified in fish [57]. The role of substance P as a putative neurotransmitter of the NANC nervous sytem in fish is discussed in section 1.8.

In addition to being neurotransmitters, neuropeptides are thought to have a neuromodulatory role. The neuromodulatory role stems from their apparent co-localisation with other known neurotransmitters such as catecholamines, serotonin (5-hydroxytryptamine) and acetylcholine in the central and peripheral nervous sysytem of mammals [58]. Additionally they appear to enhance or reduce post-synaptic depolarisation induced by other neurotransmitters [58]. In fish it is not known if peptides are co-localised with other substances or to what extent they modulate neural transmission.

1.8. Innervation of the fish gut.

Intestinal innervation of the piscine class chondrichthyes appears to be tryptaminergic with serotonin immunoreactivity having been demonstrated as the most abundant neurotransmitter in nerve fibres in the gastrointestinal tract of the dogfish *Squalus acanthias* [61] and *Scyliorhinus stellarus* [62] (Chondrichthyes: Elasmobranchii). Similar immunoreactivity has been demonstrated in the holocephalan *Chimaera montrosa* [63]. In both cases, substance P immunoreactive fibres were also detected. The *lamina propria* of the alimentary tracts of fish are rich in substance P-containing nerve fibres [57] being detected in the stomach and upper intestine of salmonids [58], anguillids [64, 65], pleuronectids [57] and the gadids [57]. Substance P immunoreactive fibres are only seen in the intestinal region of cyprinids. Watson [66] demonstrated the presence of serotoninergic-like immunoreactive nerve fibres apparently innervating gut smooth muscle of three marine teleosts (*Myoxocephalus scorpius, P. platessa* and *Clupea harengus*). Anderson [67] demonstrated, using fluorescence histochemistry, the presence of nerve fibres containing amines, most probably serotonin, in the myenteric plexus of the trout intestine. Little fluorescence was seen in the mucosa, reaffirming Burnstock's [25] suggestion that there are regional differences in the innervation of the salmonid intestine. Except for salmonids which have EGCs, the other fish families either possess solely PAS-granular leucocytes [18]) or a combination of PAS-GLs and EGCs as in eels (*Anguilla australis*) [68] and plaice (*P. platessa*) [13] (with the exception of *M. scorpius* and *C. harengus* which do not appear to possess either cell types).

Substance P and serotonin stimulate contractility of gastrointestinal smooth muscle in teleosts [69]. It has since been shown that substance P can act by releasing serotonin from enteric neurons in the stomach of the rainbow trout [70]. The involvement of serotonin as a stimulatory neurotransmitter of the non-adrenergic, non-cholinergic nervous system of rainbow trout stomach has been suggested by Kitazawa [71]. Apparently the amine-containing nerve fibres of the myenteric plexus serve to innervate the circular smooth muscle of the trout [67, 72] *M. scorpius*, *C. harengus* and *P. platessa* intestine [66].

1.9. Capsaicin as a pharmacological tool.

Capsaicin is the pungent ingredient in many *Capiscum spp.*, peppers. It is a derivative of vanillylamide, 8-methyl-N-vanillyl-6-nonenamide (see Appendix A). Capsaicin's use as a pharmacological tool stems from its selectivity for thin afferent neurons in mammals [73], which can be expressed as a short-term stimulation and desensitisation, or a long-term functional and morphological ablation.

1.9.1. Selectivity of capsaicin.

The primary targets of the excitatory action of capsaicin are thin afferent neurons in mammals including some C-fibres (unmyelinated nerve fibres) and Aδ-fibres (thinly myelinated nerve fibres)[for review see 73]. Many of these nerves contain a number of marker peptides including bombesin/gastrin releasing peptide [74], calcitonin gene related-peptide (CGRP) [75], neurokinin A [76], somatostatin [77], vasoactive

intestinal polypeptide (VIP) [78], and substance P [79]. For the purposes of this introduction, discussion shall concentrate on those afferent nerves containing substance P. There are fundamental differences in the selectivity of capsaicin between adults and neonates, some of these will be briefly discussed here and the reader should refer to [73] for a more exhaustive review.

Capsaicin administration to both neonates and adult rats results in dose dependant neurotoxic effects [73]. In neonates there is a rapid (*ie.*, within 30 minutes) degeneration of neurons and depletion of peptides, especially substance P. These effects appear to be long lasting or permanent with no recovery of sensitivity seen within nine months post-treatment [77]. In adults substance P depletion is reversible and the peptide is replenished in the spinal cord 39-60 days following systemic administration of 50-100 $mg.kg^{-1}$ capsaicin. There are other differences between neonates and adults in the non-selective actions of capsaicin on the release of substance P and its effects. For example basal blood flow in the superior mesenteric artery is depressed while in neonate but not adult rats following capsaicin administration [80]. Capsaicin-induced substance P release does not induce histamine release in the spinal cord of neonate rats but does induce release in adults [81]. Peripherally, tissue histamine and serotonin levels in neonatal rats are elevated in the skin which may be related to an alteration of mast cell responsiveness to substance P and bradykinins [82]. In addition to actions on afferent nerves, capsaicin has also been shown to have effects on efferent nerve terminals, although at much

higher local concentrations [73].

1.9.2. Capsaicin effects in non-mammals.

Effects of capsaicin have not been extensively documented in non-mammalian systems. However, it would appear that systemic administration of capsaicin (950 $mg.kg^{-1}$) in pigeons leads to partial depletion of substance P and CGRP from nerves in the myenteric plexus of the small intestine [83]. This is in contrast to the effects of capsaicin in the intestine of mammals [73]. Reptiles may to be more sensitive to the irritant effects of capsaicin than birds because pain reactions can be caused by low concentrations (3.3 nM) in crocodiles [84]. Amphibians do not appear to be as sensitive to the painful effects of capsaicin but 300 μM capsaicin causes a disappearance of most substance P-containing neurons in the intestinal wall of the toad *Bufo marinus* [85]. The effects of capsaicin in fish is not known but the phylogenetic similarities between the peptidergic nervous system of reptiles (*eg.*, caiman *Caiman crocodylus* and crocodile *Crocodylus porosus*) and fish [86] suggests the potential for similarities in the effects of capsaicin in lower vertebrates.

1.10. Research objectives.

The presence of non-myelinated nerves in the *lamina propria* of the intestine suggests that they may serve a function other than mediating contraction of the intestinal smooth muscle which appears to be innervated by the amine-containing fibres of the myenteric plexus [67, 72]. Although the role of the EGC in pathological processes

remains obscure, the morphological and histochemical similarities with mammalian mast cells would suggest that EGCs in fish may be analogous to the mammalian mast cells. In order to examine this analogy further, the present investigation proposes to examine evidence for possible intestinal nerve-EGC interactions in the trout intestine as is seen with mast cells in mammals [47, 48, 49, 50]. The presence of such a relationship would suggest a functional link between the nervous system and the non-specific immune system of defense mechanisms in trout. Systemic administration of the putative non-adrenergic, non-cholinergic neurotransmitter- substance P; a depleter of non-myelinated nerve fibres, capsaicin; and the primary neurotransmitter of the predominant nerve population, serotonin will be used to examine the likelihood of substance P or serotonin as being the mediators of such a nerve-EGC relationship.

The primary objectives of this research subsequently are:

Objective 1: To examine the proximity of intestinal EGCs to non-myelinated nerves in the trout intestine.

Objective 2: To assess the effects of capsaicin, substance P and serotonin on EGCs and the intestinal morphology of the trout mucosa.

Objective 3: To quantify any morphological and distributional changes in trout

intestinal EGCs following stimulation with capsaicin, substance P and serotonin.

2. CHANGES IN EGC MORPHOLOGY INDUCED BY CAPSAICIN AND SUBSTANCE P.

2.1. Introduction.

Neurotransmitters have been implicated in mediating the release of vasoactive compounds from mammalian mast cells [55, 71, 89]. It has also been shown that mast cells are in close association with non-myelinated, peptidergic nerve fibres of the rat [47, 49] and human intestine [50].

The EGCs of salmonids have been implicated as having mast cell properties including:

(a) degranulating in response to bacterial exotoxins [22, 43].

(b) increasing in number in response to ectoparasitic infections and irritation of the epidermis [20], in plerocercoid infections of rainbow trout [89], and in microsporidian infections of the non-salmonid Sergeant-major fish, *Abudefduf saxatilis* [91].

Although EGCs are histochemically and biochemically different from mast cells, certain homologies have been reported between these cells such as the presence of basic proteins and acid (sulphated) glycosaminoglycans in the cytoplasmic granules [19]. Ellis [22] demonstrated an increase in the release of biogenic amines, possibly histamine, from degranulating EGCs following stimulation with *A. salmonicida*

extracellular products. An elevation in histamine concentration is also observed in mammalian intestine following localised diseases such as celiac disease [92]. The source of this histamine is thought to be primarily circulatory eosinophils and resident mast cells.

The presence of non-myelinated peptidergic nerves in the teleost alimentary tract is well documented [57]. Substance P-sensitive serotoninergic neurons have been identified in the stomach of the rainbow trout [70] and are thought to be involved in smooth muscle contraction [71]. The numerous non-myelinated peptidergic-like neurons in the *lamina propria* suggest a function other than or in addition to non-adrenergic, non-cholinergic (NANC) stimulation of gastrointestinal smooth muscle.

Nerve-mast cell interactions are hypothesised to be involved in hypersensitivity reactions induced by stress and anxiety [48]. The histochemical, morphological and distributional similarities between mast cells and EGCs suggest a similar role in fish. The mechanism of EGC degranulation remains obscure, but may involve the release of tachykinins from non-myelinated nerves of the *lamina propria*. To examine this possible relationship, fish were injected with capsaicin, a neurotoxin known to release tachykinins from nerves, substance P, a tachykinin or the putative neurotransmitter and mast cell constituent serotonin. The relationship between non-myelinated nerves and degranulation of the EGCs following possible nerve stimulation was examined

morphologically by electron microscopy since EGC degranulation can only be accurately assessed using such a technique.

2.2. Materials and methods.

2.2.1. Drug preparation.

Stock solutions of serotonin (5-hydroxytryptamine) and capsaicin (Sigma Chemical Company, St. Louis, Mo., USA.), were made up in 0.85% saline solution (w/v NaCl) at a concentration of 1.0 $mg.ml^{-1}$ and stored until required at 4°C (up to 8 weeks). Stock solutions of substance P (Sigma Chemical Company), were made up in polythene vials with 0.1% bovine serum albumin and kept frozen at -70°C [93]. Prior to use all injectates were serially diluted from the stock solutions with 0.85% saline to a concentration of 100 $\mu g.ml^{-1}$.

Controls consisted of injection of 0.85% saline vehicle and bovine serum albumin at the same concentration as the experimental drugs (100 $\mu g.ml^{-1}$).

2.2.2. Fish Holding.

Adult rainbow trout (*O. mykiss*) of mean weight 80.1 ± 3.7 g (Mean ± SD), were supplied by the Fish Health Unit of the Atlantic Veterinary College. Fish were held in aerated fresh water at 10°C ± 0.5°C (Mean ± SD) in a 2 m diameter fibreglass flow-through tank, (approximate flow rate of 20.0 $litre.min^{-1}$), and a stocking density of 35 $Kg.m^{-3}$, under natural photoperiod. All fish were fed *ad libitum* to satiation 5

out of 7 days on a 4 or 5 mm slow sinking commercial pelleted feed (Corey Fundy Choice, Corey Feed Mills Ltd., Frerdricton , N.B., Canada or equivalent).

Prior to experimentation, fish were starved for 24 hours, removed from the main holding facility and transferred to 80 litre flow-through tanks (flow rate of 1.5 $l.min^{-1}$) at a stocking density of 4 fish per tank for a minimum of 2 hours prior to anaesthesia and subsequent injection.

2.2.3. Challenge protocol.

Fish were anaesthetised with MS 222, (tricaine methanesulfonate, 70.0 $mg.l^{-1}$) (Syndel Laboratories, Vancouver, B.C., Canada) and injected intraperitoneally with 0.4 ml of diluted drug (*ie.*, capsaicin, substance P or serotonin) or vehicle control (saline or BSA) to provide a final concentration of 0.5 $\mu g.g^{-1}$ body weight per fish. A total of 20 fish, four fish per each of five treatments were injected in each experimental replicate. A total of 4 replicates were carried out approximately 2 weeks apart. The volume injected was sufficient to provide a dose which, from preliminary trials, would produce a morphological response within the time frame of the experiment, and could be injected intraperitoneally without leakage from the injection site. The injection site was located ventrally, posterior to the pelvic fins. The drug solution was delivered using a 1 ml tuberculin syringe fitted with a 26 gauge 13 mm needle. Following injection, fish were returned to the experimental quarantine tanks to recover from anaesthesia before being killed by spinal severance 30 minutes, one,

two and four hours post-injection. The fish were dissected and a 10 mm section of the intestine, immediately posterior to the spleen was removed and fixed for electron microscopy. The euthanasia and dissection procedures were completed within two minutes so as to minimise *post mortem* cytological changes.

2.2.4. Tissue Preparation.

Tissue was fixed in cold 3% glutaraldehyde in 0.13M Millonig's phosphate buffer (pH 7.3) (Appendix B) for a minimum of 12 hours at 4°C. It was then post-fixed in 1% osmium tetroxide (OsO_4) in the same buffer at 4°C for 1 hour. The tissue was dehydrated in an ascending series of ethanols, cleared and transferred in propylene oxide before infiltration and embedding in an Epon/Araldite resin and polymerised at 65°C.

Ultrathin (70 nm) sections were cut on a Reichart-Jung Ultracut E ultramicrotome using a glass knife and placed on 200 mesh copper grids. The sections were stained with saturated uranyl acetate and Sato lead stain (Appendix C) then examined using a coded blind system on a Hitachi H600 electron microscope at 75 KV.

A random selection of EGCs (25 cells per treatment at each time interval) from fish challenged with capsaicin, substance P, serotonin and controls were examined for their proximity to non-myelinated nerve bundles. EGCs were classified as being approximately >2 EGC cell diameters, 0.5-2 diameters or <0.5 diameters away from

nerves (the diameter was taken to be that of the EGC examined). Only EGCs with nuclei were counted. The proportions of EGCs in close association with nerve bundles were analysed using one way analysis of variance (ANOVA) to compare the differences between time and treatments and Student's t-tests to analyse the significance of each proximity class.

2.3. Results.

2.3.1. Control fish.

Eosinophilic granule cells (EGCs) from control fish showed no evidence of degranulation or irregularities at any of the time periods. The majority of EGCs were found in the *stratum compactum* with few being observed in the *lamina propria*. All the EGCs in the control fish were similar in appearance to those previously described by Ezeasor and Stokoe [24]. They were large ovoid cells characterised by the presence of many large membrane-bounded granules containing a dense homogenous matrix; a large eccentric nucleus; a few profiles of rough endoplasmic reticulum, ribosomes, mitochondria and small, inconspicuous Golgi apparatus with few saccules; and frequently surrounded by fibroblast-like ensheathing cells. EGCs were observed in close association with non-myelinated nerve bundles, often occluded by the ensheathing cells (Fig. 2.1). Reticulated, haloed granules were occasionally (1-5 granules in approximately 1 in 5 cells) observed in EGCs from control fish.

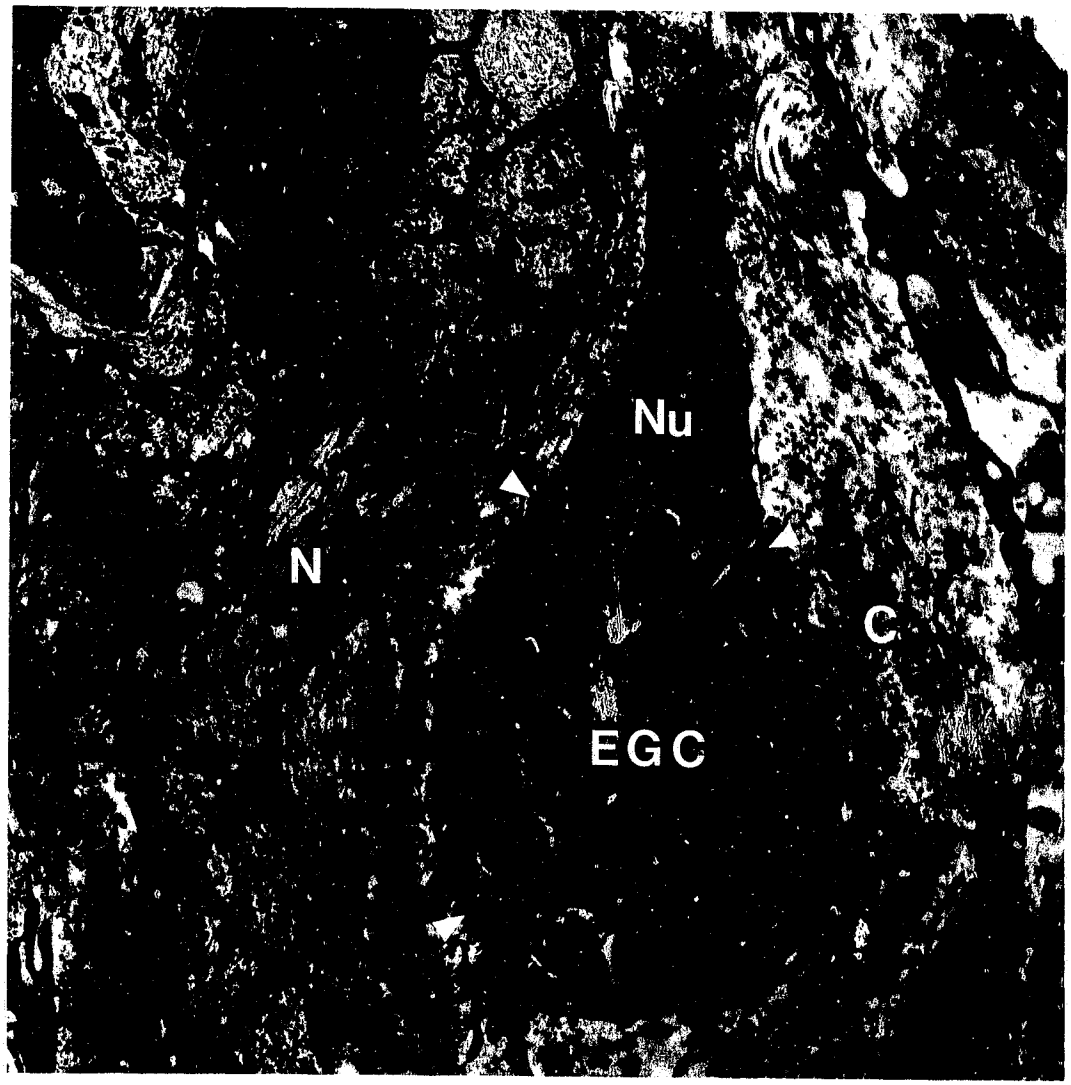


Figure 2.1. Portion of an eosinophilic granule cell (*EGC*) from a control fish surrounded by collagen fibrils of the *stratum compactum* (*C*) in close association with a non-myelinated nerve bundle (*N*) occluded by the fibroblast-like ensheathing cell (*arrowheads*). *Nu* = Ensheathing cell nucleus. (x11 500)

2.3.2. Experimental fish.

There was a significant proportion (H_o: $\mu = 0$, $p < 0.01$) of EGCs in close association (less than 2 cell diameters from nerves) with nerve fibres (mean of all treatments 32.18%), but no significant differences between treatments ($p > 0.01$) and no effect of time ($p > 0.01$).

In experimental cases, there were no definitive time sequences for degranulation events, although a decline in EGC numbers from the *stratum compactum* following capsaicin and substance P administration has been observed with light microscopy (see chapter 3). This decline has been attributed to EGC degranulation. Generally, ultrastructural evidence of degranulation was observed first in those fish treated with substance P (*ie.*, from 30 minutes post-injection onwards), but a time lag in degranulation of approximately one hour was observed with capsaicin, concomitant with the findings of chapter 3.

Serotonin had no effect on the EGC morphology over the duration of the experiment. However, following administration of capsaicin and substance P, signs of partial degranulation were observed in EGCs in the *stratum compactum*. A form of degranulation not previously described in EGCs was observed in the *lamina propria*.

2.3.2.1. *Stratum compactum.*

Following injection of either capsaicin, substance P or serotonin, EGCs maintained their ovoid shape. However with capsaicin and substance P, the matrix of the granules had an extensively reticulated appearance. A prominent halo surrounded most granules. Membrane-bounded electron-lucent channels were observed around the granule periphery (Fig. 2.2). Small dense structures, ranging in diameter between 20 nm and 70 nm, were observed within the perigranular halo and/or associated with the granular contents (Fig. 2.3). No free granules or granule contents were observed within the extracellular matrix. The appearance of the ensheathing cells remained similar to those seen in control tissues.

2.3.2.2. *Lamina propria.*

EGCs of the *lamina propria* and occasionally those at the boundary with the *stratum compactum* exhibited a different morphological appearance than those in the *stratum compactum* in that they had a more irregular shape and numerous cytoplasmic processes (Fig. 2.4). The close apposition of the ensheathing cells seen in the *stratum compactum* was not evident. The cytoplasm of these cells contained numerous smooth walled tubules 40-50 nm in diameter. The matrix of many granules appeared to be sub-divided into vesicular structures some of which contained a prominent dense core material (Fig. 2.5). Fine filamentous material could be seen between the vesicular substructure of the granules. In addition to the numerous membranous-vesicular granules, large numbers of electron lucent vacuoles, often containing fine

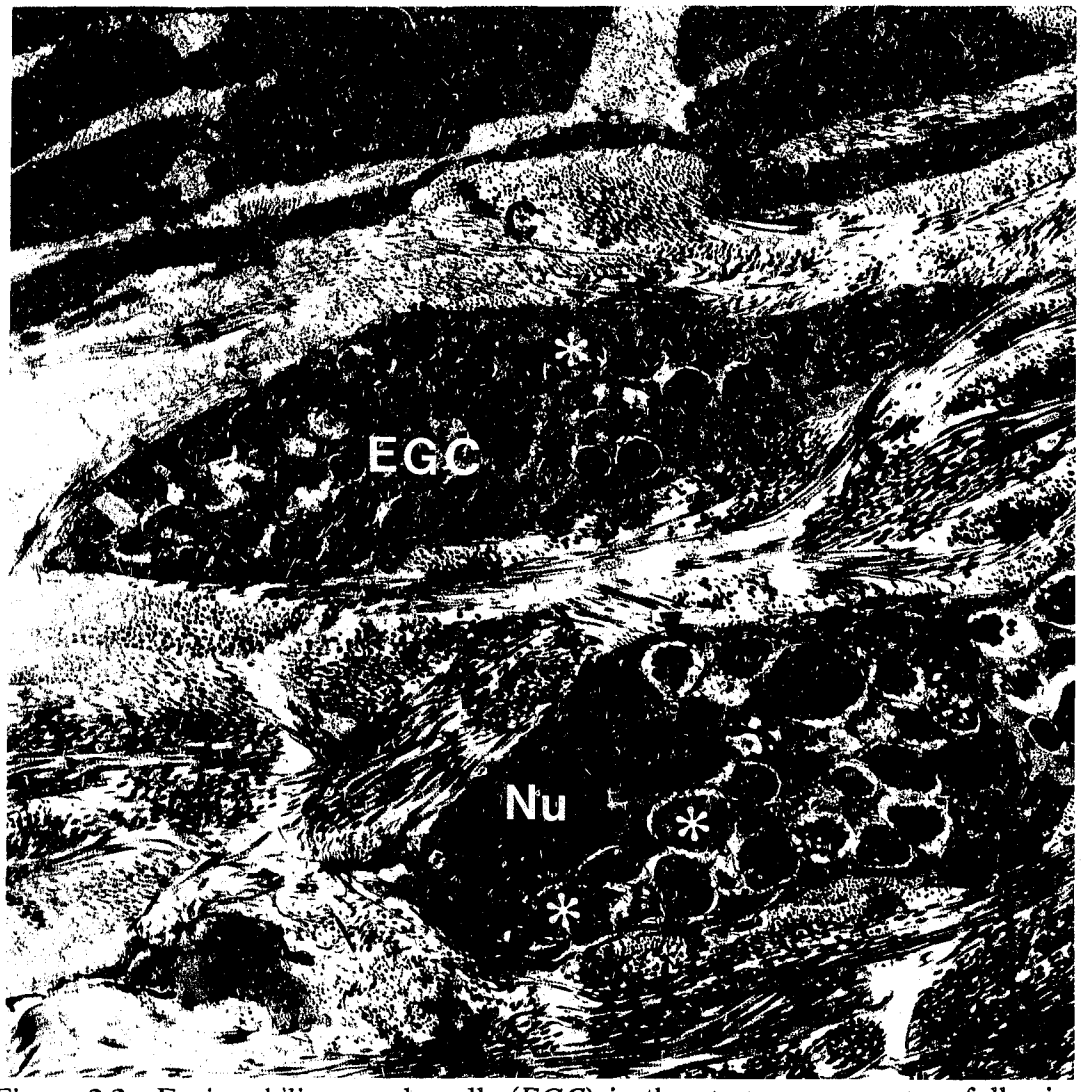


Figure 2.2. Eosinophilic granule cells (*EGC*) in the *stratum compactum* following stimulation with capsaicin (1 hour post-injection) with reticulated granules (*asterisks*), perigranular halos (*arrowheads*) and electron-lucent channels (*arrow*) in the cytoplasm. *Nu* = EGC nucleus; *C* = Collagen fibrils. (x9 600)

Figure 2.3. Granules of an EGC in the *stratum compactum* showing extensive reticulation (*R*) and prominent perigranular halo (*arrow*). Note the small dense structures (*arrowheads*) associated with the EGC granules and regions where membranes surrounding perigranular halos have fused (*asterisk*). (1 hour post-injection with capsaicin). (x25 800)

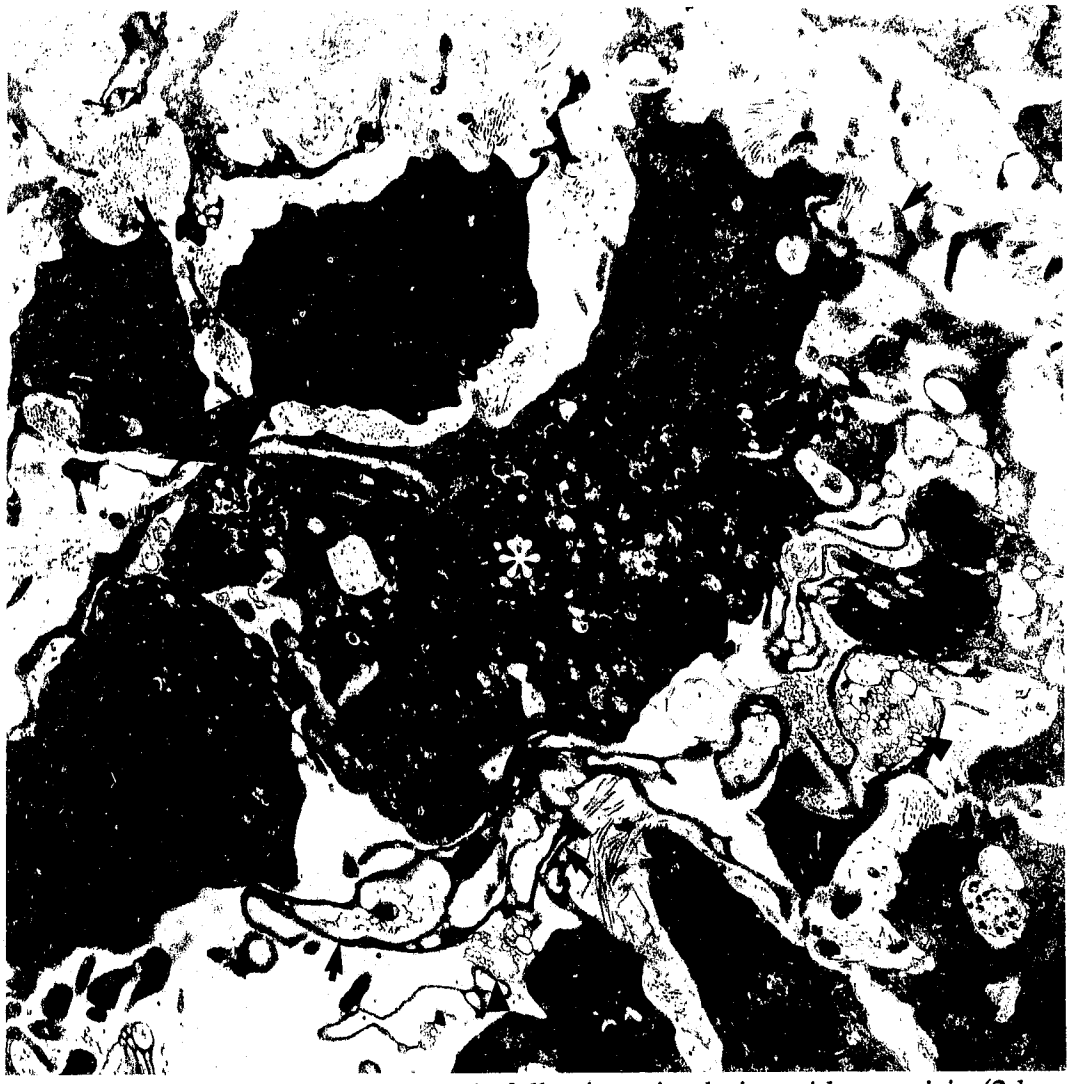


Figure 2.4. EGC of the *lamina propria* following stimulation with capsaicin (2 hour post-injection). Many cytoplasmic extensions (*small arrows*) and extracellular debris (*arrowheads*) are present. The granules are multivesiculated (*asterisk*) with a complex membrane-bounded substructure (*large arrow*). (x9 200)

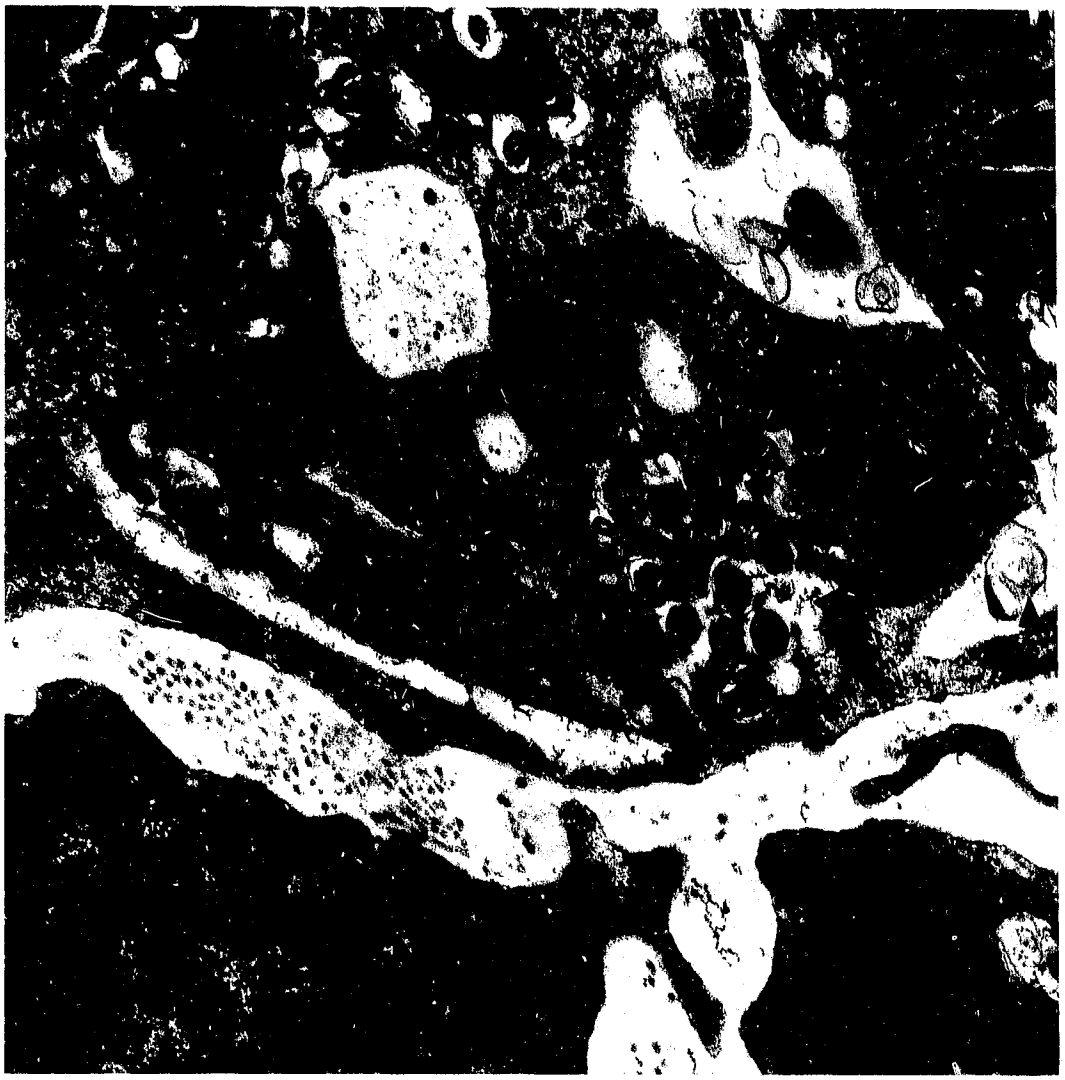


Figure 2.5. A higher magnified portion of figure 4 showing details of the vesicular substructure of the granules (*G*), and associated filamentous material (*arrow*). Extracellular membranous debris (*arrowheads*). Note the extensive smooth tubules within the cytoplasm (*open arrow*). (x34 500)

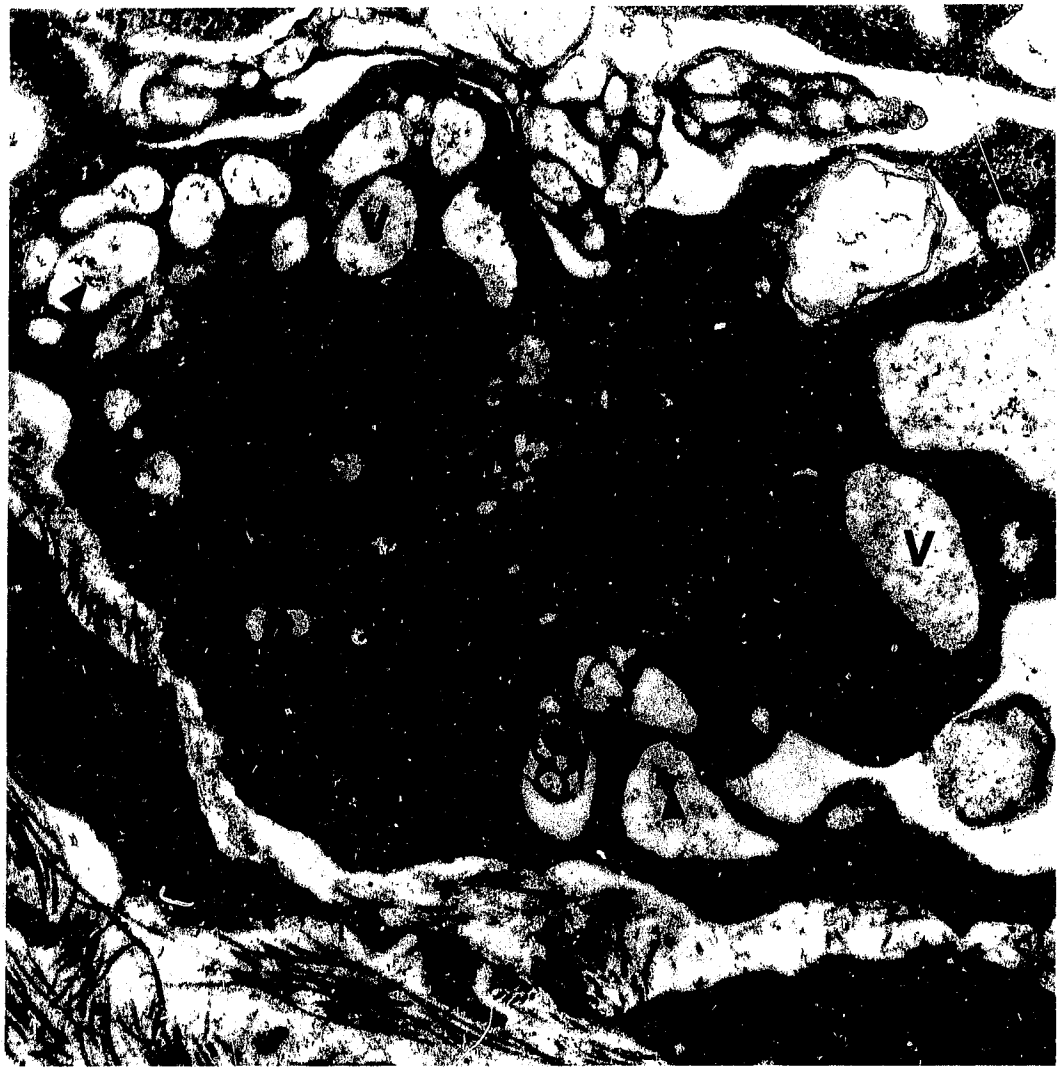


Figure 2.6. A degranulating EGC in the *lamina propria* with a multivesiculated granule and numerous electron lucent vacuoles around the cell periphery (*V*), some containing filamentous material (*arrowheads*). (x23 000)

filamentous material were frequently seen in the peripheral cytoplasm (Fig. 2.6). Similar filamentous material was also present in the extracellular matrix. Serial sections, (Fig. 2.7 a and b) showed that some of the electron lucent vacuoles were continuous with the plasma membrane.

2.4. Discussion.

The putative degranulation of EGCs by capsaicin and substance P observed in the *stratum compactum* was similar to that observed in the same region described by Vallejo and Ellis [43] using *A. salmonicida* exotoxin and compound 48/80.

The labyrinthine channel-like regions are similar to membrane-bounded structures observed within IgE-induced, degranulating mammalian mast cells [94]. In mast cells these channels are thought to originate from the fusion of the granule membranes. Since the haloed EGC granules are also membrane-bounded, fusion of this membrane with other granules would create a membrane-bounded channel. The small dense bodies seen in cells of the *stratum compactum* from fish treated with capsaicin and substance P were similar to structures observed in EGCs of the *stratum compactum* following stimulation with the mast cell secretagogue compound 48/80 in fish pretreated with the antihistamines, cimetidine, and promethazine [43]. In mammalian mast cells, substance P and 48/80 induce histamine release by direct activation of the GTP-binding proteins (G-proteins), which in turn cause exocytosis of granule contents [51]. EGCs of the *stratum compactum* appear to react

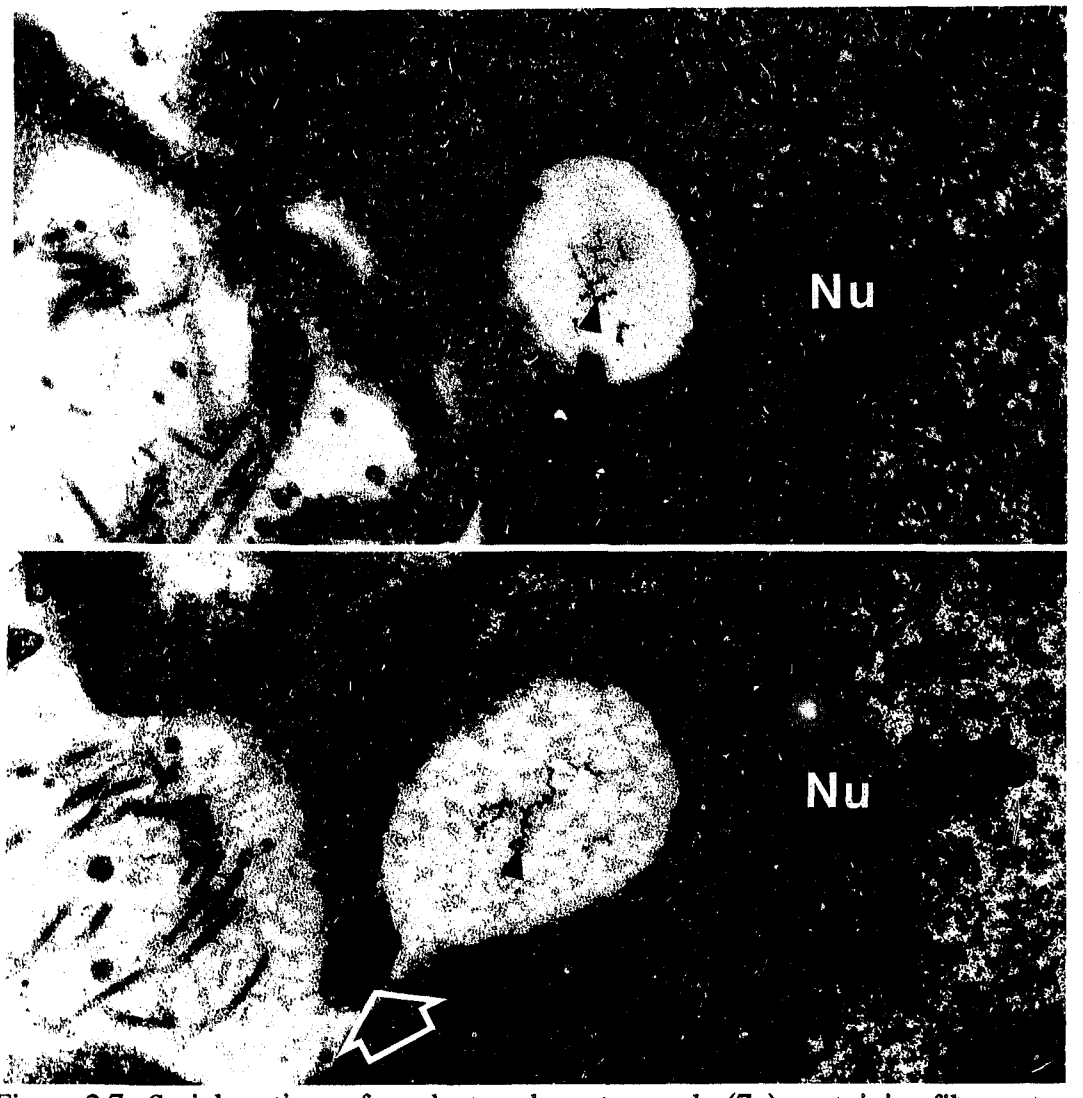


Figure 2.7. Serial sections of an electron lucent vacuole (7a) containing filamentous material (*arrowheads*) and an extracellular opening which is continuous with the plasma membrane (*arrow*)(7b). *Nu*=EGC nucleus. (x58 700)

morphologically in a manner similar to antihistamine-inhibited compound 48/80, capsaicin or substance P stimulation. However as with EGCs treated with antihistamines [43], total degranulation (as indicated by the extracellular presence of free granules and granule contents) does not occur. Although Vallejo and Ellis [52] observed total degranulation in response to compound 48/80 in the *lamina propria*, this was not conclusively demonstrated in the cells of the *stratum compactum*. It is possible that *stratum compactum* EGCs are immature, unable to respond at this stage of development, but will eventually be capable of degranulation when fully mature. Alternatively, these cells may never have the capability of responding fully. Some EGCs migrate into the *lamina propria* from the *stratum compactum* upon stimulation [22, 43]. Cells may mature during this migration and then become capable of degranulation or they may obtain the ability with the change in environment. In our study and that of Vallejo and Ellis [43], total degranulation of EGCs was only observed in those cells within the *lamina propria* or at the boundary with the *stratum compactum*.

The ensheathing cell acts as a physical barrier between the nerve and the EGC. In EGCs from control fish and those in the *stratum compactum* of capsaicin- and substance P-treated fish, the ensheathing cell was directly apposed to the plasma membrane of the EGC. In EGCs of the *lamina propria* of stimulated fish, close apposition of ensheathing cells was not evident, suggesting that the loss of such contact has facilitated total degranulation. The ensheathing cell of the EGCs may

have a similar role in regulating exocytosis by surrounding and occluding the EGC in a manner similar to the folliculostellate cells which appear to regulate secretory cells in the pituitary gland [95].

The morphological changes associated with degranulation of EGCs within the *lamina propria* observed in this study have not previously been described, although similar morphologic changes have been reported in degranulating human lung mast cells following IgE stimulation *in vitro* [96]. The appearance of the numerous cytoplasmic tubular structures is interesting and cannot be readily explained. The appearance of granules in the degranulating EGCs are reminiscent of multivesicular bodies [96, 97] and suggests a lytic-like modification and breakdown of the granule contents which is followed by exocytosis of the degraded products. No evidence of cell death as in necrosis or apoptosis, as defined by Wyllie [99], was observed in the degranulating cells. The degranulation may be a form of crinophagy as exhibited by some endocrine and neurosecretory cells [97, 100]. Ezeasor and Stokoe [24] demonstrated the presence of acid phosphatase and arylsulfatase in association with the periphery of many EGC granules in the *stratum compactum* and *lamina propria* of normal fish. EGC granules appear to be of a storage nature, containing basic proteins, glycosaminoglycans and possibly a biogenic amine. The presence of acid phosphatase and arylsulfatase in the EGC granules suggests that they may normally contain lysosomal enzymes and are slowly being degraded or undergoing proteolytic processing under normal conditions. It is interesting to note that this may be

reflected by the fact that EGCs from control fish occasionally exhibited reticulated and haloed granules, suggesting a low-level, incomplete (partial) degranulation occurs in the unstimulated cells. The extensive reticulation in EGCs of the *stratum compactum* and degranulation in the *lamina propria* following stimulation by capsaicin or substance P may be an acceleration of this low-level processing in the two different areas (*stratum compactum* and *lamina propria*). In the *stratum compactum* this is seen as an increase in the degree of reticulation and in the number of reticulated granules within cells. In contrast, in the *lamina propria*, similar stimulation brings about total degranulation with the formation of multivesicular body-like structures and the presence of extracellular granular material. The large electron-lucent vacuoles, some of which are continuous with the plasma membrane, are suggestive of either exocytosis or endocytosis. The presence of extracellular debris surrounding the degranulating EGCs (which is not observed in the controls) and the presence of the same fine filamentous material in the electron-lucent vacuoles, and within the multivesicular bodies suggests that the processes is exocytotic. The electron-lucent vacuoles are similar to the residual bodies exhibited by degranulating mast cells [94, 96].

In rainbow trout EGCs, total degranulation would appear to be mediated by a substance P-like compound which can be released by capsaicin. The coincidence of the close proximity between EGCs and non-myelinated nerves suggests a possible source of the tachykinin mediator. Since serotonin was apparently ineffective at

degranulating EGCs, it appears that the serotoninergic nerves which innervate the smooth muscle of the rainbow trout gut [67, 71, 72] are not involved with EGC degranulation. Alternatively the 0.5 $\mu g.g^{-1}$ body weight dose of serotonin used in this experiment was insufficient to induce a response. Tachykinins are known to induce mast cell degranulation and histamine release [55], and capsaicin, a tachykinin releaser stimulates rat mast cell degranulation [88]. The mechanism by which substance P degranulates mast cells is thought to be by an influx of extracellular calcium via indirect G-protein-gated calcium channels [51, 55]. A similar mechanism may be present in the degranulating EGCs of the *lamina propria*.

Degranulation, be it partial or total, may be neurally mediated under certain conditions. Different degrees of degranulation have been defined morphologically in EGCs of different regions of the trout gut as being partial or total. The degree to which this degranulation occurs is apparently dependent on the location of the EGC. Although further work is required to clarify the maturity of the EGCs residing in the *stratum compactum*, and the presence of substance P as the likely mediator of EGC degranulation, it is apparent that a nerve-EGC interaction may exist, suggesting that it is one of the alternative control mechanisms of the non-specific cell-mediated immune response of the rainbow trout [101].

3. ACID PHOSPHATASE LOCALISATION IN CAPSAICIN STIMULATED EGCS.

3.1. Introduction.

The eosinophilic granule cell (EGC) of the rainbow trout intestine has many histochemical characteristics similar to both circulatory eosinophils and mammalian mast cells [12]. The mast cell equivalent in fish has also been termed "tissue eosinophil" by Ellis [12]. Capsaicin and substance P induce degranulation of EGCs [102](Chapter 2) through an as yet undetermined mechanism. This results in the multivesicular subdivision of EGC granules in the *lamina propria* and a reticulation of the granules of those EGCs confined to the *stratum compactum*. The multivesicular granule substructure was reminiscent of the multivesicular bodies of autophagic and crinophagic cells [103]. Crinophagy involves the controlled autolysis of stored materials in neuroendocrine and neurosecretory cells [100].

Acid phosphatase is used as an enzymatic marker for the identification of lysosomes in cells [104]. The presence of lysosomes is usually associated with phagocytic or autolytic activities. For example, mammalian eosinophil granules function as lysosomes. They contain acid phosphatase as well as other hydrolytic enzymes (*eg.*, arylsulfatase) as well as peroxidase associated with phagocytosis [105]. Previous studies [24, 102] have suggested that acid phosphatase may not be a normal constituent of EGC granules but rather a feature of their degradation. The present

study was designed to determine whether lysosomes were involved in the crinophagic-like degranulation observed in capsaicin-stimulated EGCs [102] (Chpater 2) by using acid phosphatase as a cytochemical marker.

Materials and methods.

Capsaicin (0.5 $\mu g.g^{-1}$ body weight) was systemically administered by intraperitoneal injection to three yearling rainbow trout (*O. mykiss*) which had been held at 10°C ± 0.5°C in the Fish Health Unit of the Atlantic Veterinary College and fed *ad libitum* to satiation on a commercial pelleted feed. Following injection, fish were held for a 2 hour incubation period prior to euthanasia by spinal severance. A 1 cm piece of intestine immediately posterior to the spleen was promptly excised and fixed overnight in cold 3% glutaraldehyde in 0.1 M sodium cacodylate buffer with 7% sucrose (pH 7.2). Tissue sections (150 µm) were cut on a vibratome (Technical Products Inc., Series 1000) and washed in 0.1 M sodium cacodylate buffer (+ 7% sucrose) followed by 0.1 M acetate buffer (pH 5.0) with 7% sucrose added. The tissue sections were then incubated in excess reaction medium (1mM ß-glycerophosphate, 2mM cerium chloride ($CeCl_3$) (Sigma Chemical Company, Mo., USA.), in a 0.1 M acetate buffer (pH 5.0) (+ 7% sucrose) or a substrate deficient control medium for 2 hours at 37°C. After 1 hour incubation, the reaction and control buffers were drained and replaced with fresh media. After a total of 2 hours incubation, the tissues were washed in cold (4°C) 0.1 M acetate buffer (+ 7% sucrose) in order to stop the reaction and then washed in 0.1M sodium cacodylate

buffer (+ 7% sucrose). The tissue slices were then refixed in 3% glutaraldehyde in 0.1 M cacodylate buffer with 7% sucrose for 1 hour then washed overnight in excess cacodylate buffer. The following day the tissue was post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer (+ 7% sucrose) (pH 7.2) and dehydrated in ascending grades of ethanol. Tissue was then cleared in propylene oxide and embedded in Epon/Araldite resin (see Appendix D and E for details of enzyme cytochemistry protocol and reaction media).

Negative controls consisted of gut slices incubated in ß-glycerophosphate-deficient reaction medium. Three additional fish were not injected with capsaicin to serve as experimental controls to illustrate the localisation of acid phosphatase in unstimulated fish.

Ultrathin (70 nm) sections were supported on formvar (J.B. E.M Services, Montreal, P.Q., Canada) coated 200 mesh copper grids (Appendix F) and viewed unstained with a Hitachi H600 electron microscope at 75 kV.

The localisation of acid phosphatase was identified by the presence of an electron dense deposit in cellular structures where the enzyme was present (Appendix G). Cerium has distinct advantages as a capturing agent for phosphate ions released by acid phosphatase because it produces a finer, more uniform deposit with fewer nonspecific deposits of the reaction products as compared to the more traditionally

used lead [106].

3.3. Results.

3.3.1. Control fish.

EGCs in the *stratum compactum* of control fish appeared similar to those previously described in detail [24, 102] (Chapter2). Perigranular halos were frequently seen around the EGC granules of EGCs located in the *stratum compactum*. Cerium phosphate deposits were seen localised in occasional (*eg.*, 1-2 per cell in 4-5 cells per section) lysosome-like structures (220-350 nm in diameter) in the EGCs in the *stratum compactum*. These could easily be distinguished from small granules by the absence of the homogenous granule matrix seen in all of the EGC granules. There was no evidence of cerium deposits on any of the granules (Fig. 3.1). Similarly acid phosphatase was identified in lysosomes within ensheathing cells associated with the EGCs of the *stratum compactum*. All experimental tissues (*ie:* trout gut incubated with ß-glycerophosphate medium) exhibited a positive reaction for acid phosphatase in lysosomes. No acid phosphatase was detected in the negative control tissues.

Only occasionally were EGCs observed in the *lamina propria* of uninjected fish (2-4 cells per section) and they appeared similar to those in the *stratum compactum* but were not associated with ensheathing cells. EGCs in this region showed no evidence of the complex granule substructure previously observed in EGCs of the *lamina propria* of stimulated fish (Chapter 2) [102].

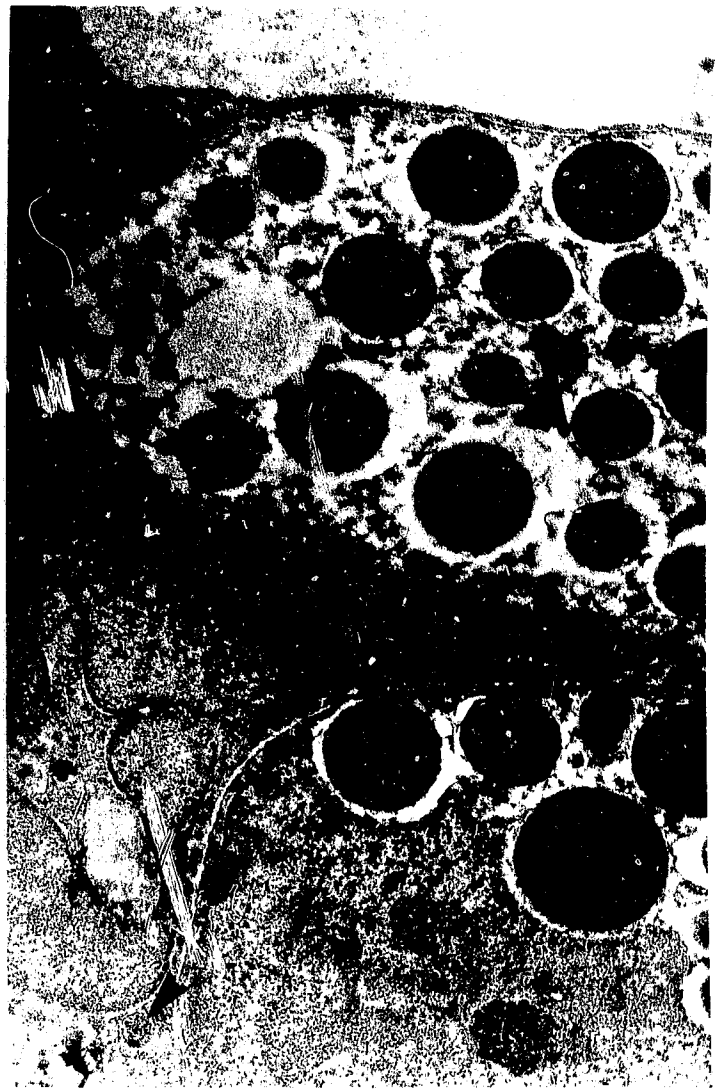


Figure 3.1. Lysosomes (*arrows*) in an EGC and ensheathing cell (*arrowhead*) in the *stratum compactum* of an unstimulated fish. F = Ensheathing cell nucleus, g = EGC granules. (x17 500)

3.3.2. Capsaicin-injected fish.

The EGCs of the *stratum compactum* exhibited a similar morphology to those we have described previously (Chapter 2) [102]. Extensive perigranular halos and reticulated granules were evident (Fig. 3.2). A few lysosomes (1-2 per cell in 4-5 cells per section), identified by the localised reaction product, were present in the cytoplasm of these cells. The incidence of lysosomes did not appear to be any different from those present in EGCs of the same region in control fish. Acid phosphatase was not detected in association with any of the granules whether they were apparently intact, haloed or reticulated. Acid phosphatase was not detected in any cells of the negative control tissues. As in the controls a positive acid phosphatase reaction was evident in the ensheathing cells. Acid phosphatase was observed in occasional cisternae of rough endoplasmic reticulum and lysosomes of these cells.

EGCs observed in the *lamina propria* exhibited a morphology similar to that described previously (Chapter 2) [102]. No evidence of acid phosphatase was observed in association with the multivesicular granules or the smooth tubulo-vesicular cytoplasmic structures (Fig. 3.3). Acid phosphatase was localised in lysosomal structures within epithelial cells which flank the *lamina propria* and in occasional fibroblast-like cells in the *lamina propria* (Fig. 3.4). Acid phosphatase reaction was not observed in epithelial or fibroblast-like cells in any of the negative control tissues.

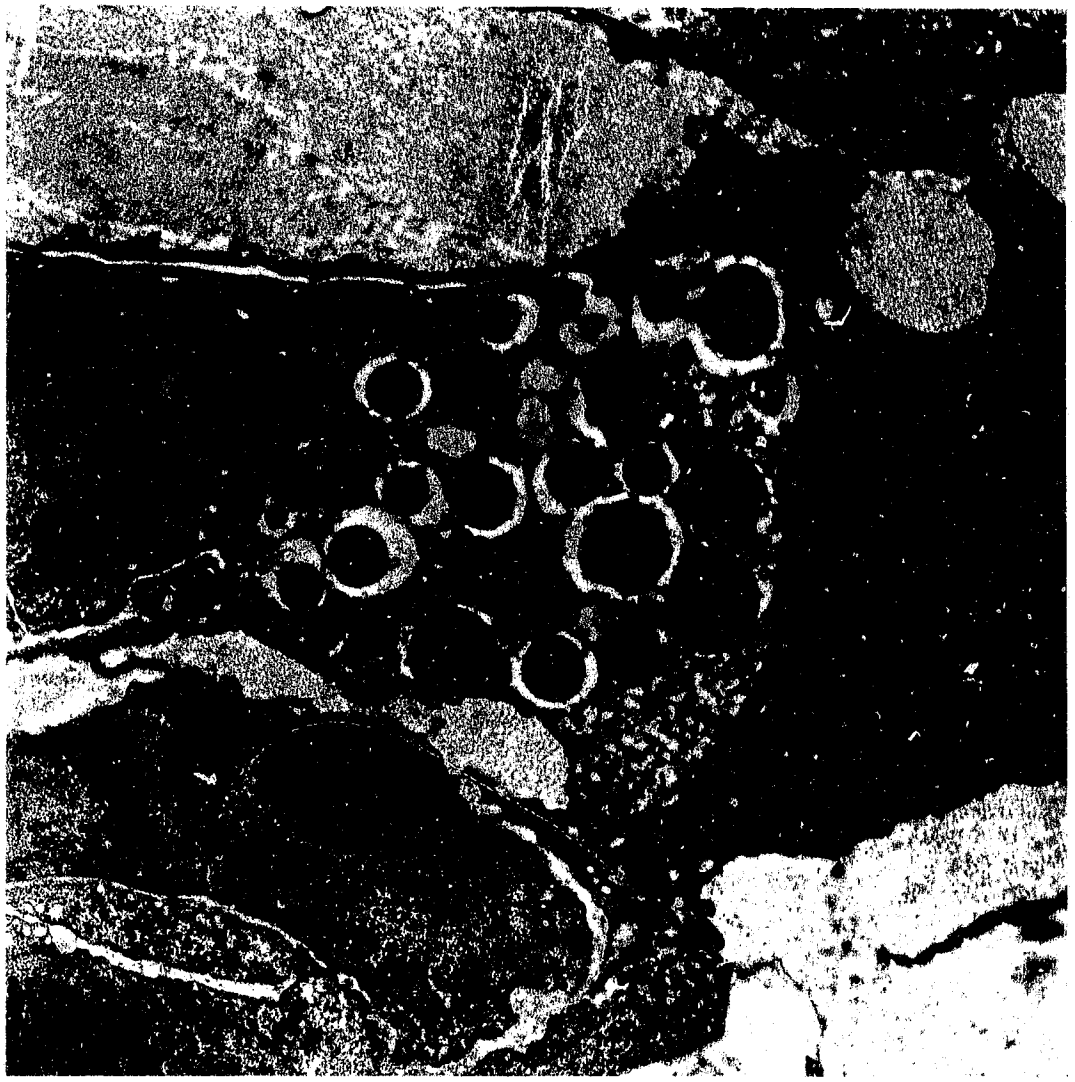


Figure 3.2. Lysosomes (*arrows*) in the ensheathing cells around an EGC in the *stratum compactum* of a capsaicin-injected fish. Note the reticulated EGC granules (*R*). (x12 500)

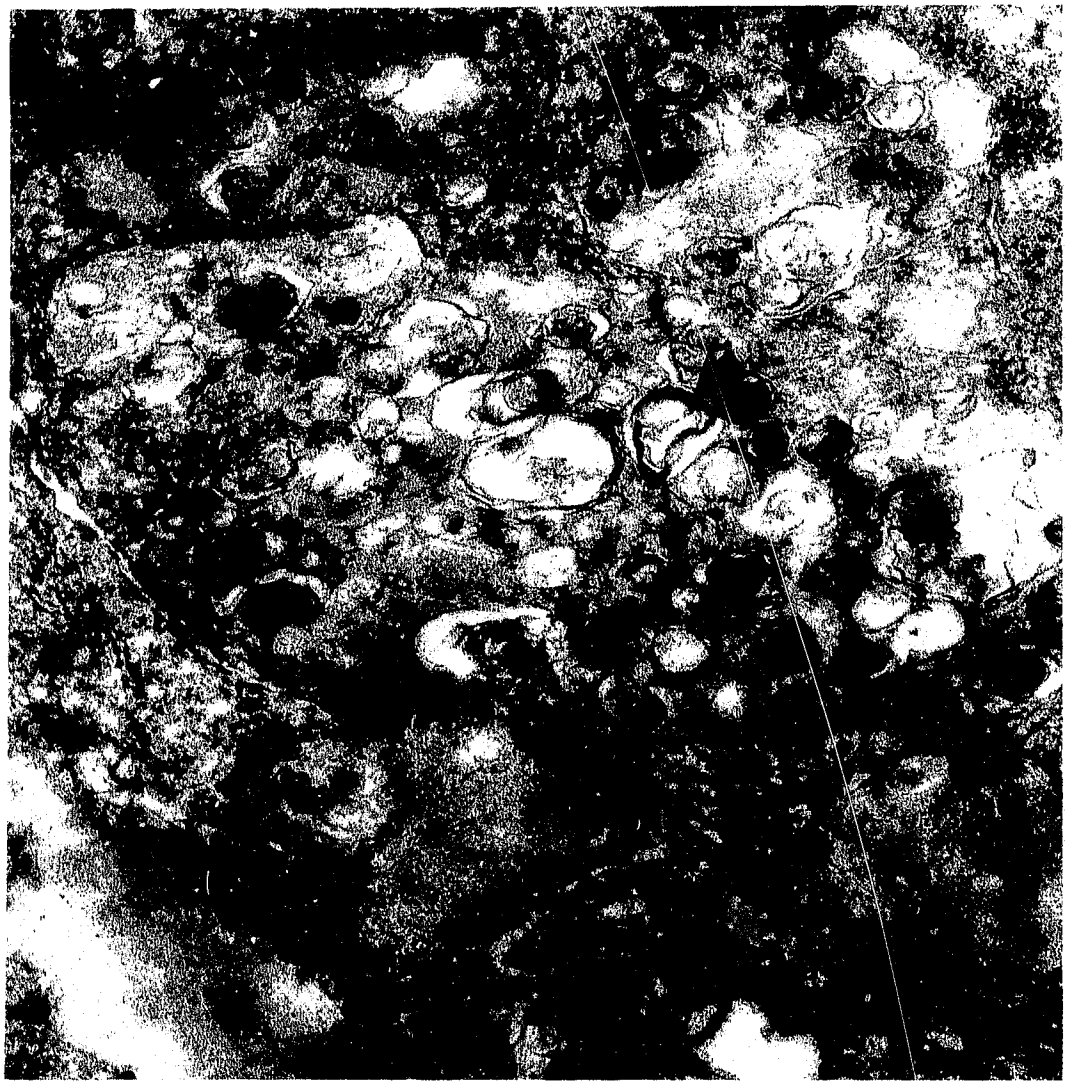


Figure 3.3. Multivesicular substructure of an EGC granule following exposure to capsaicin showing multiple myelin-like figures (*arrows*) and smooth walled tubular structures in the cytoplasm (*arrowheads*). (x50 000)

Figure 3.4. Lysosomes in epithelial cells (*open arrows*) overlying, and fibroblasts (*solid arrows*) within the *lamina propria* (*lp*). (x9 200)

3.4. Discussion.

Acid phosphatase was localised cytochemically in lysosomal structures of EGCs but not within the granules. Lysosomes within the associated ensheathing cells and epithelial cells were also reactive for acid phosphatase. Consequently, it can be concluded that acid phosphatase is not a normal constituent of EGC granules. This absence of acid phosphatase from the EGC granules is interesting as it apparently contradicts a previous study on intestinal EGCs from untreated rainbow trout [24] where the investigators found a weak positive reaction for acid phosphatase present only in the periphery of a few granules. Similarly, there was no reference made in the earlier study of acid phosphatase reactions being observed in lysosomes and rough endoplasmic reticulum within ensheathing cells in the *stratum compactum*. Ezeasor and Stokoe [24] did not report the presence of lysosomes in EGCs. In our study the enzymatic reaction, although non-existent in the EGC granules, was very strong in lysosome-like structures. These discrepancies may be explained by difference in experimental method. Ezeasor & Stokoe [24] used the Gomori's method of acid phosphatase localisation using lead as the marker. It has been shown that lead as a capture agent can give an artifactual deposition of reaction product [107]. Cerium on the other hand does not cause the non-specific precipitation which is a common problem with lead-based techniques [106].

The subdivision of granules observed in the EGCs of the *lamina propria* following stimulation with capsaicin corroborate data obtained by us under slightly different

experimental conditions (Chapter 2) [102]. Interestingly, acid phosphatase was not detected in the multivesicular-like EGC granules, which would discount them from being multivesicular lysosome bodies which are seen in crinophagic neuroendocrine and neurosecretory cells [98]. The absence of acid phosphatase in the multivesicular-like granules coupled with the lack of cytological changes such as swollen mitochondria and disrupted nuclei dispels any suggestion that the cells are undergoing autolysis or cell death as defined by Wyllie [99]. The changes in granule morphology observed in the EGCs of the *lamina propria* in capsaicin-injected fish does not therefore appear to be lysosomally mediated. However, these changes may be coincident with a proteolytic degradation brought about by enzymes other than acid phosphatase which are localised in the granule. This situation is somewhat similar to that seen in mammalian mast cells where acid phosphatase is primarily localised in lysosomes and not normally in the granule [108]. Non-lysosomal mechanisms for protein degradation involves the targeting of susceptible proteins by ubiquitin and their subsequent hydrolysis in the proteosome [109, 110]. While it is not known whether such mechanisms exist in EGCs, it would appear that granule breakdown of these cells in response to stimulation with capsaicin is not under lysosomal control. Rather degranulation is probably due to an as yet undetermined non-lysosomal mechanism. Mammalian mast cell granules have a variety of enzymes such as alkaline phosphatase and arylsulfatase (see [104] for review) that are normally associated with them. EGC granules have been reported to also contain alkaline phosphatase [15, 24] as well as arylsulfatase [24]. This suggests that perhaps

mast cell degranulation and capsaicin-induced EGC degranulation may be forms of non-lysosomal protein breakdown. The localisation of acid phosphatase in EGC lysosomes, and not the granules, provides further support to the theory that the piscine EGC may be analogous to the mammalian mast cell.

It appears that the multivesicular degranulation observed in trout intestinal EGCs following systemic exposure to capsaicin is not an autophagic response. Rather it is a form of degranulation involving non-lysosomal mechanisms for the possible breakdown and release of the granule contents.

4. CAPSAICIN AND SUBSTANCE P INDUCED MORPHOLOGICAL AND DISTRIBUTIONAL CHANGES IN THE INTESTINAL EGC POPULATION

4.1. Introduction.

The eosinophilic granule cell (EGC) of salmonids has been likened to mammalian mast cells [12, 24, 27], having certain similar histochemical and biochemical properties. These include the presence of basic proteins and acid, sulphated glycosaminoglycans [21]. Similar proteoglycans such as heparin are also found in mammalian mast cell granules. Histamine and serotonin are associated with the mast cell granule. Biogenic amines have also been reported to be released from EGCs following degranulation with *A. salmonicida* exotoxin [22].

Subpopulations of mammalian mast cells have been defined in the intestine of both rats and humans, each subpopulation having distinct biochemical and histochemical characteristics [40, 41]. Mucosal and connective tissue mast cells have been shown to be in intimate contact with non-myelinated neurons [47, 48, 49, 50], and respond to stimulation with the neuropeptide, substance P [111]. EGCs in the teleost intestine also appear to be in close association with non-myelinated nerves (Chapter 2) [102]. Trout intestinal EGCs can be degranulated on exposure to substance P [102], a putative neurotransmitter of the non-adrenergic, non-cholinergic (NANC) nervous system of both teleosts [70] and mammals [59], and with capsaicin, a neurotoxin which depletes and releases substance P from sensory nerve endings [112].

In chapter 2 it was demonstrated that EGCs of the rainbow trout intestinal mucosa degranulate in response to stimulation with capsaicin or exogenous substance P. Serotonin, a putative neurotransmitter of the myenteric nerves in teleosts [66, 67, 72] was ineffective at degranulating intestinal EGCs. EGCs in the *lamina propria* appear to degranulate fully, exhibiting a multivesicular granule substructure similar to the crinophagic degranulation of neuroendocrine and neurosecretory cells [97, 100]. EGCs of the *stratum compactum*, however, only partially degranulated. Granules within these cells appeared reticulated with extensive perigranular halos but there was no evidence of exocytosis or the crinophagic-like morphology of cells as seen in the *lamina propria*.

Like the mammalian mast cell, EGCs are thought to be involved in pathological processes. EGCs have been demonstrated to undergo degranulation in response to extracellular products of the fish pathogen *Aeromonas salmonicida* [22, 43]. Parasitic infections of the gut of fish that are known to normally have a population of EGCs (*eg:* salmonids) results in an increase in the frequency of EGCs in the infected area. EGCs are often observed to infiltrate the fibrous encystation of the parasite [90, 91]. Parasite-induced degranulation of EGCs, (as defined by a loss of densely staining granules) has been reported to restrict their detectability with light microscopy [72], necessitating the use of electron microscopy. Non-degranulated EGCs remaining in the *stratum compactum* should still be measureable using light microscopy since they can still be identified by their densely staining cytoplasmic granules.

The aim of this investigation was to:

(a) Assess the effects of systemically administered substance P and capsaicin on the frequency of EGCs in the trout intestinal *stratum compactum* and;

(b) Classify and quantitate morphologically distinct EGCs present in the intestinal mucosa of the rainbow trout following exposure to the above agents. A detailed classification of EGCs based on their morphological appearance using electron microscopy was undertaken in order to better understand the regional variation in EGC morphology.

4.2. Materials and methods.

Adult rainbow trout of average weight 80.1g ± 3.7 g (Mean ± SD) held in the Fish Health Unit of the Atlantic Veterinary College at 10°C ± 0.5°C and fed *ad libitum* to satiation. Fish were anaesthetised with 70 $mg.l^{-1}$ MS 222 and injected with 0.4 ml of capsaicin, substance P, serotonin or BSA and vehicle controls at an absolute dose of 0.5 $\mu g.g^{-1}$ body weight as described in chapter 2. Following a 30 minute, 1, 2 and 4 hour recovery and incubation period, fish were killed and a 1 cm piece of the mid-intestine removed and processed for electron microscopy (as outlined in chapter 2). Each control tissue (*ie:* from fish injected with saline and BSA) was processed blind along with experimental tissues. Semithin (0.55 μm) sections were cut for light microscopy on a Reichart Jung Ultracut E ultramicrotome using a glass knife and stained with 1% aqueous toluidine blue in a 1% borax solution. Light microscopic analysis was conducted on a Nikon Type 104 compound microscope at 1000x

magnification using oil immersion. The EGCs of the *stratum compactum* form an outer layer adjacent to the underlying *muscularis* (Fig. 4.1). The number of cells within this layer that could be viewed under one field (0.18 mm) were counted. A total of six fields were counted for each fish. Ultrathin (70 nm) sections from the same blocks were placed on 200 mesh copper grids and stained with uranyl acetate and Sato lead stain and examined with a Hitachi H600 electron microscope. All sections were examined at random using a coded blind system to minimise observer bias. A total of four fish were used for each treatment and each time interval for light microscopy and three fish for each treatment and time interval for electron microscopy.

4.2.1 Statistical analysis.

4.2.1.1. Light microscopy.

Cell counts of each treatment were analysed at each time interval using a repeated measures analysis of variance followed by a Student-Newman-Kuels *post-hoc* analysis corrected for repeated counts on the same animal (nested data) [113]. Thus differences between treatments could be identified. A p-value of 0.01 was considered to be the cut-off point for statistical significance.

4.2.1.2. Electron microscopy.

The EGCs observed in each section in both the *stratum compactum* and the *lamina propria*) were categorised according to various morphological parameters ie: the

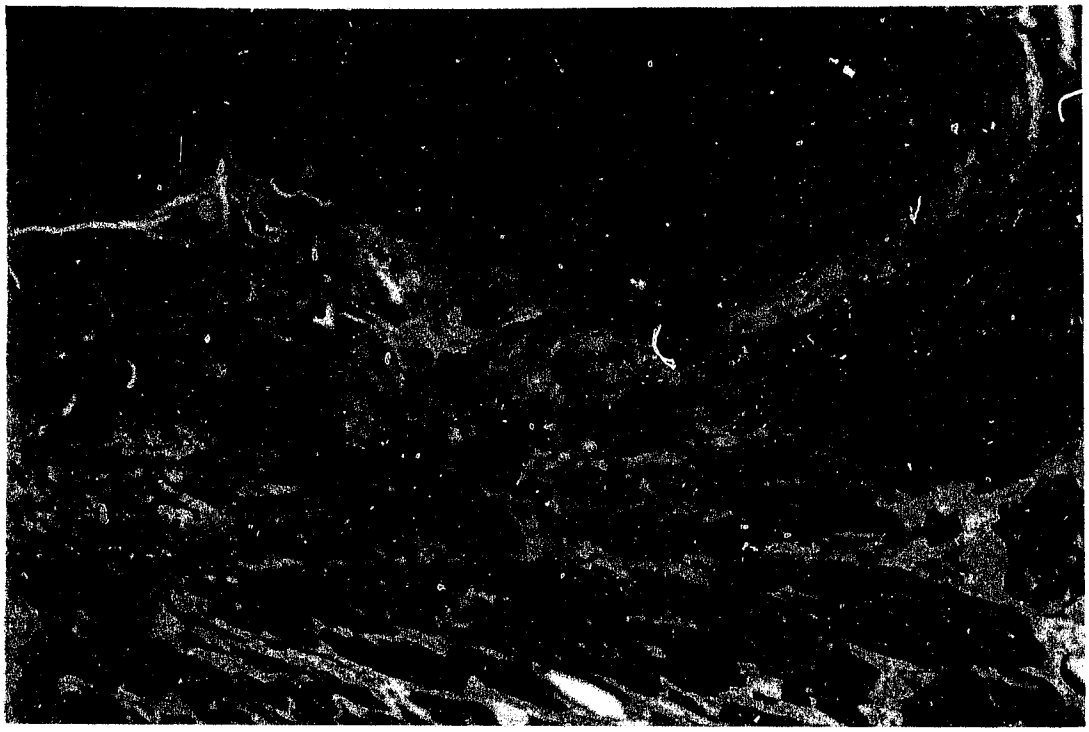


Figure 4.1. Photomicrograph of a transverse section of the trout intestinal mucosa. *E*= epithelium, *lamina propria* (*lp*), *stratum compactum* (*sc*) and *muscularis* (*sm*). *GC*= Eosinophilic granule cells. Semithin section stained with toluidine blue (x2 100).

number, size and appearance of the granules. The number of cells in each category was expressed as a percentage of the total observed cells in the section. The 200 mesh copper grids used to support the section allowed bservation of some 60% of the grid area. Thus, approximately 60% of the tissue area was examined on each section. This corresponded to between 20-40 cells in each section: one section per fish for a total of three fish. All proportions were statistically compared using a General Linear Model (Appedix H) followed by a Student-Newman-Keuls *post-hoc* test (SAS statistics package, SAS Institute Inc., Cary, N.C., USA.), analysing the relative dominance of "normal" cell morphologies and interactions between the different cell classifications, treatment, time and the histological region (*ie.*, *lamina propria* or *stratum compactum*).

4.3. Results.

4.3.1. Light microscopy.

4.3.1.1. Histology.

In all treatments there were no changes in the general microanatomy of the gut from that described by Ezeasor and Stokoe [24] (Fig. 4.1). The incidence of EGCs in the *lamina propria* of control fish and those injected with serotonin was low (1 - 2 cells per section). Fish injected with substance P and capsaicin exhibited more EGCs in the *lamina propria* (5 - 10 cells per section) and generally fewer such cells in the *stratum compactum* which was corroborated by random cell counts (Fig. 4.2).

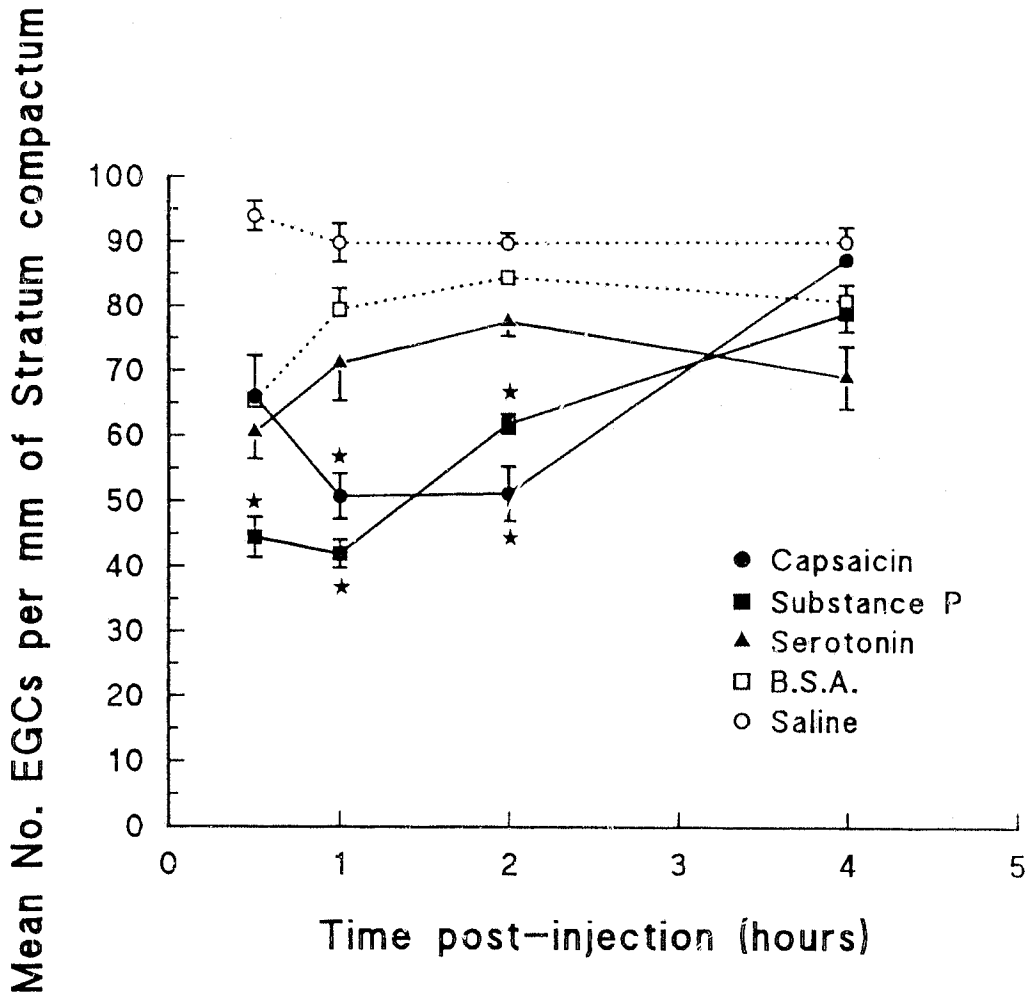


Figure 4.2. The change in the mean number (± SEM) of observable EGCs in the *stratum compactum* of the trout intestine following systemic administration of capsaicin, substance P and serotonin. Values which are significantly different ($p < 0.01$) from both vehicle controls are indicated with a star.

4.3.1.2. Cell frequency.

Repeated measures analysis of variance at each time interval followed by a Student-Newman-Keuls *post-hoc* analysis revealed significant differences between the control treatments and the experimental treatments (*ie.*, serotonin: saline only, capsaicin: saline, and substance P: BSA) ($p < 0.01$). At 30 minutes there was a significant difference between saline and BSA control values. However at other time intervals there was no significant difference between these treatments ($p > 0.01$). The mean number of EGCs in the *stratum compactum* of saline-injected fish remained constant over the duration of the experiment. These values were significantly higher than for all other treatments ($p < 0.01$). A significant difference was observed between the mean number of EGCs in the *stratum compactum* of serotonin-injected fish and saline controls after 30 minutes, one and two hours post-injection (Fig. 4.2). Significant differences were observed between capsaicin-injected fish and controls at one, two and four hours post-injection (Fig. 4.2).

Serotonin- and BSA-injected fish had a low frequency of EGCs at 30 minutes post-injection (60 and 65 cells.mm^{-1} *stratum compactum* respectively). Frequencies increased throughout the duration of the experiment with BSA values reaching those of saline while serotonin values remained somewhat lower.

In fish injected with either substance P or capsaicin there was a decrease in the frequency of cells 30 minutes and 1 hour post-injection respectively (Fig. 4.2). EGC

cell frequencies at 30 minutes post-injection of substance P averaged 44 cells.mm^{-1} *stratum compactum*, considerably lower than that for other treatments. At one hour post-injection the average frequency had dropped further to a minimum of 42 cells.mm^{-1} *stratum compactum* and steadily increased to control values thereafter. Capsaicin similarly caused a decrease in EGC frequency, albeit not so dramatic as with substance P. EGC frequencies were similar to those for BSA controls at 30 minutes post-injection. Unlike the BSA and serotonin curves there was a decrease in EGC frequency at one hour post-injection to a minimum of 51 cells.mm^{-1} *stratum compactum*. EGC frequencies remained low for a further hour before increasing to control values by the end of the experiment. The decrease in EGC frequency in the *stratum compactum* induced by capsaicin appeared to lag behind that for substance P (Fig. 4.2).

4.3.2. Electron microscopy.

It was apparent that several different morphologies of EGC were present in the intestinal mucosa. These could be classified as follows:

Class I: EGCs with > 50% of the granules measuring <0.5 μm in diameter (Fig. 4.3).

Class II: EGCs with apparently "normal" granules. (The criterion for assessing this was based on the appearance of the majority of the control EGCs) (Fig. 4.4).

Class III: EGCs with > 50% of the granules surrounded by or partly surrounded by

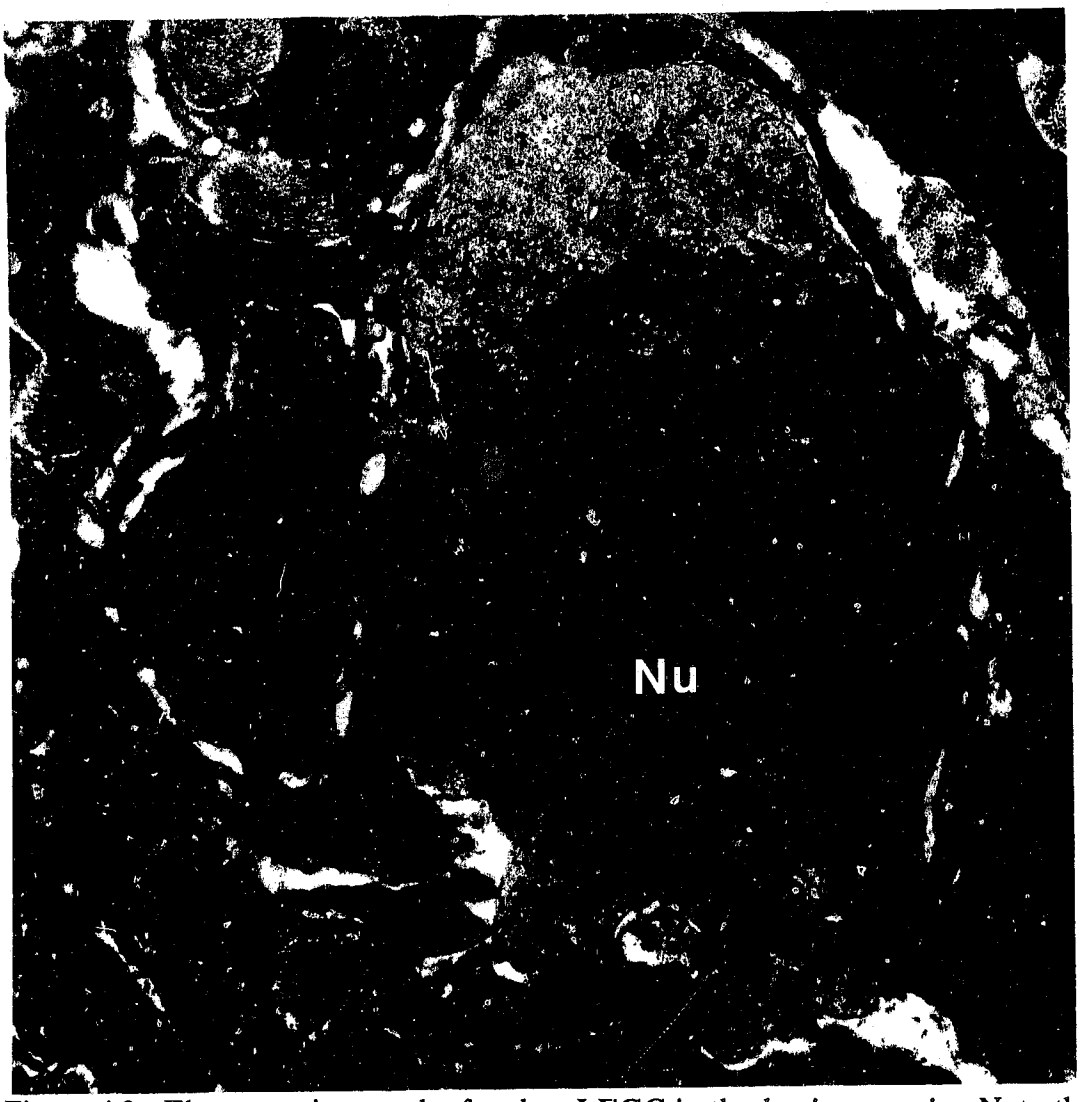


Figure 4.3. Electron micrograph of a class I EGC in the *lamina propria*. Note the small granules (*arrows*) and extensive peripheral cytoplasm. *Nu* = EGC nucleus. (x11 400)

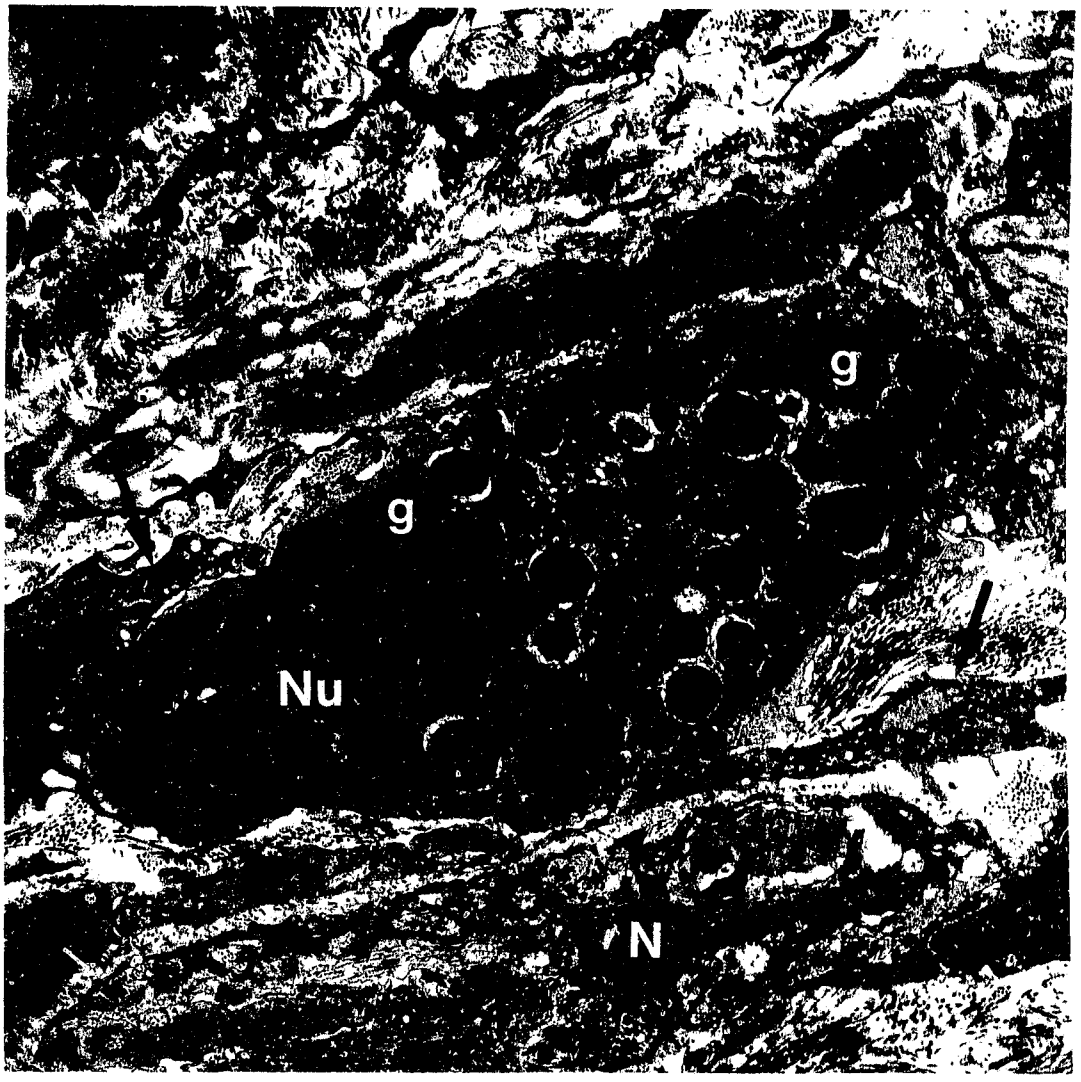


Figure 4.4. Electron micrograph of a class II EGC in the *stratum compactum* in close association with a non-myelinated nerve (*N*). Note the intact granules (*g*) and the fibroblast-like ensheathing cells (*arrows*). *Nu* = EGC nucleus. (x9 975)

a perigranular halo (Fig. 4.5).

Class IV: EGCs with > 50% of the granules having a reticulated appearance (Fig. 4.6).

Class V: EGCs with a degranulating or degranulated appearance as described in chapter 2 (*ie.*, with multivesiculated granules, electron-lucent vacuoles and extensive cytoplasmic processes) [101] (Fig. 4.7).

The proportion of each cell class in each region (*stratum compactum* or *lamina propria*) varied with time post-injection and drug treatment.

4.3.2.1. Control fish.

(a) *Stratum compactum.*

All five classes of EGC were present and observed within the four hour time frame of the experiment. In both of the control groups (saline- and BSA-treated fish), class II cells (normal) dominated (78-97% of observed EGCs). The haloed class III cells were the second most dominant group (3-15% of observed EGCs) ($p = 0.0001$) compared with all other cell classes (compare Fig. 4.8 a & b). Contributions from other cell classes were not significant and there was no significant effect of time ($p = 1.00$).

(b) *Lamina propria.*

There were fewer EGCs observed in the *lamina propria* (ranging from a mean of

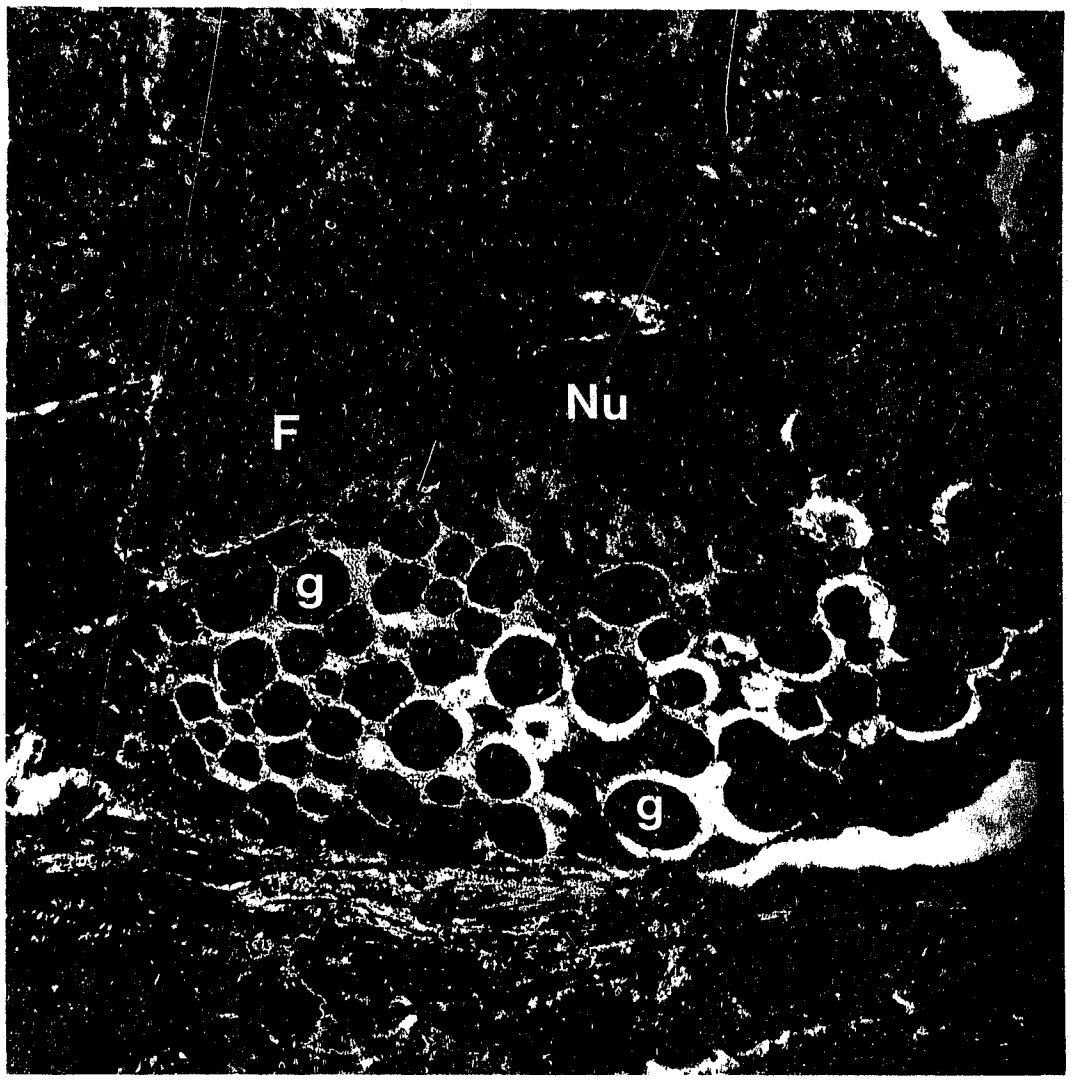


Figure 4.5. Electron micrograph of a class III EGC in the *stratum compactum*. Note the homogenous granules (*g*) surrounded by perigranular halos (*arrows*). *Nu* = EGC nucleus, *F* = Fibroblast-like ensheathing cell nucleus. (x8 800)

Figure 4.6. Electron micrograph of a class IV EGC in the *stratum compactum*. Note the reticulated granules (*asterisks*) and perigranular halos (*arrow*). *Nu* = EGC nucleus. (x15 200)

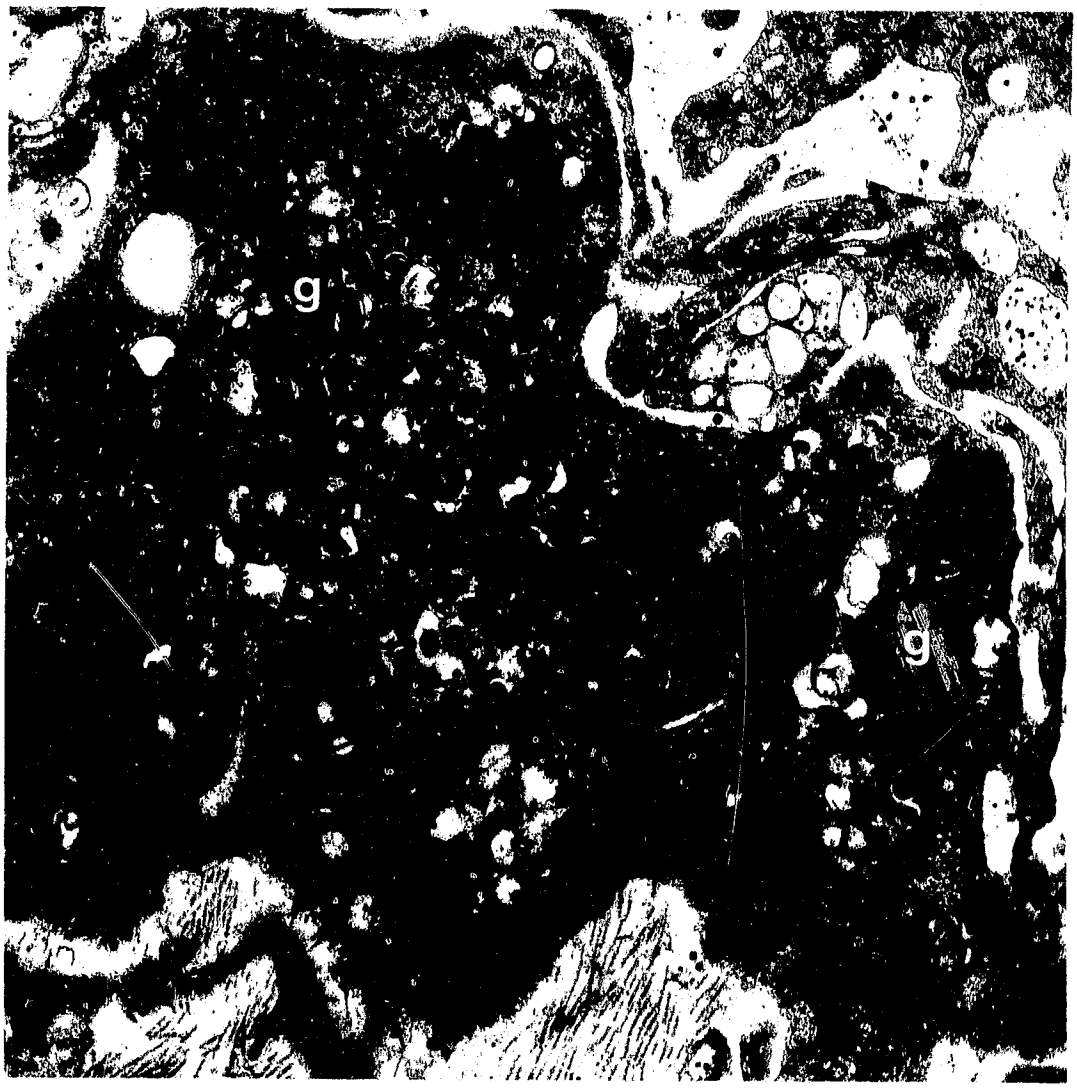
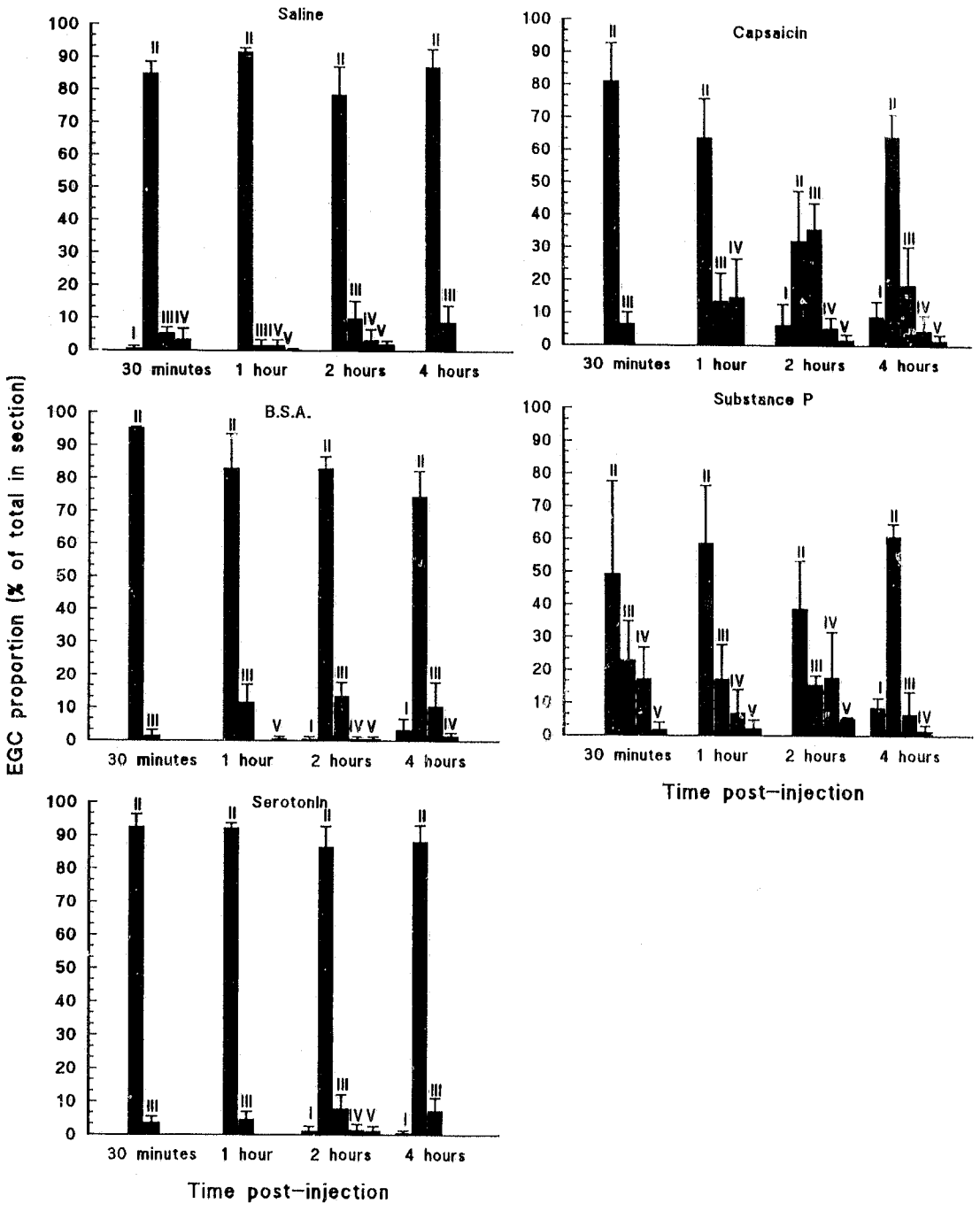


Figure 4.7. Electron micrograph of a class V EGC in the *lamina propria*. Note the multivesicular substructuring of the EGC granules (*g*) and the electron-lucent residual bodies (*arrowheads*). (x23 000).

Figure 4.8 a-e: The distribution of different EGC classes in the *stratum compactum* at four time intervals post-injection for saline- (*a*), BSA- (*b*), serotonin- (*c*), capsaicin- (*d*), and substance P- (*e*) injected fish. Values expressed as mean percentage of the total number of EGCs observed in the tissue section (+ SEM).


Saline
Capsaicin
B.S.A.
Substance P
Serotonin
EGC proportion (% of total in section)
30 minutes
1 hour
2 hours
4 hours
Time post-injection

upto 10% of the total EGCs per section) compared with the *stratum compactum* (upto 95% of the total EGCs per section) (Fig. 4.9 a & b). All five cell classes were not observed over the duration of the experiment. The most dominant cell class observed in the *lamina propria* region was the class II cell compared with all other 4classes (p values: Saline 0.0001, BSA 0.0187) with occasional observations of class V, degranulated cells (1-2 per section) (compare Fig. 4.9 a & b). No significant changes in the cell populations were observed with time (p- values: Saline 0.7977, BSA 0.8943).

4.3.2.2. Experimental fish.

As with the controls, all cell classes were observed during the time course of the experiment although they did not occur at every time interval.

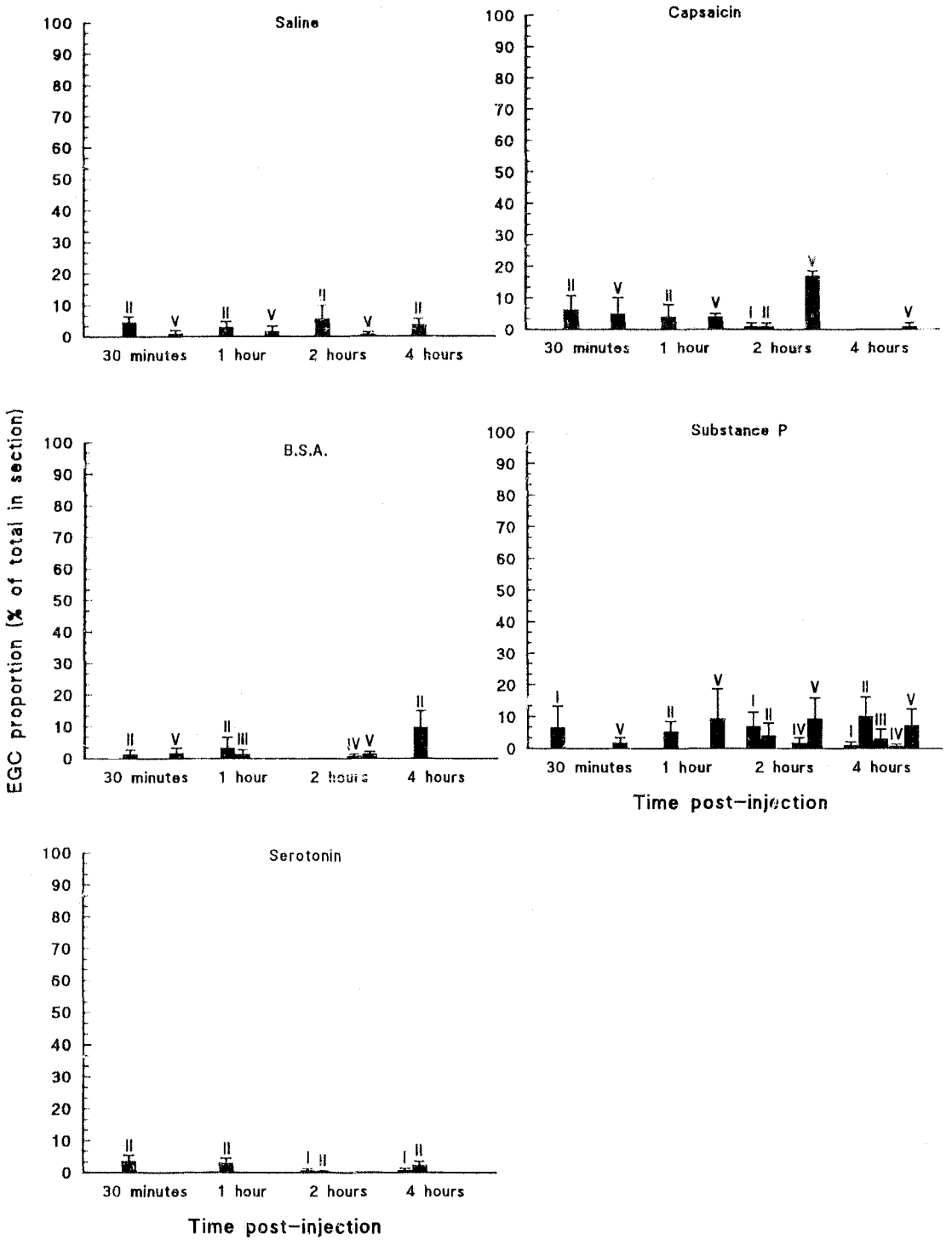
(a) *Stratum compactum.*

Serotonin:

As with the controls there were significant differences in the type of cells present with class II and III being the most dominant classes as compared with all other classes ($p = 0.0001$). There were no significant changes in distribution of the cell classes with time ($p = 0.9999$) as compared to the controls (compare Fig. 4.8 c & a).

In contrast fish injected with capsaicin and substance P exhibited profound changes in the morphology and distribution of cells with time.

Figure 4.9 a-e. The distribution of different EGC classes in the *lamina propria*, at four time intervals post-injection for saline-(*a*), BSA- (*b*), serotonin- (*c*) capsaicin-(*d*), and substance P- (*e*) injected fish. Values expressed as mean percentages of the total number of EGCs in the tissue section (+ SEM).


Saline
Capsaicin
B.S.A.
Substance P
Serotonin
EGC proportion (% of total in section)
100
90
80
70
60
50
40
30
20
10
0
30 minutes
1 hour
2 hours
4 hours
Time post-injection

Capsaicin:

There was a significant change in the cell classes for capsaicin-injected fish over the duration of the experiment compared to saline controls ($p = 0.0001$) (compare Fig. 4.8 a & d). At 30 minutes post-injection there was a predominance of class II cells and a small contribution of class III cells, similar to the controls. At one hour post-injection, the proportion of class II cells was reduced (compared with controls) whereas the proportion of class III and IV cell types was increased. Two hours post-injection the most dominant cell type was class III with the appearance of a significant proportion of both class I (small granule cells) and class V (degranulated cells) ($p = 0.0001$) (Fig. 4.8 d). Four hours post-injection, class II cells reestablished a dominance with class III being the next most dominant cell class. The main difference between the tissues at four hours post-injection with capsaicin and controls was the presence of a proportion of class I and class V cells with capsaicin which was not observed in the saline controls (compare Fig. 4.8 a & d).

Substance P:

The distribution of classes of EGCs was different from the controls although type II cells remained the most dominant class ($p = 0.0001$) (compare Fig. 4.8 b & e). This notwithstanding, the proportion of class II cells in substance P-treated fish were lower than those in the BSA control treatments ($p = 0.0424$). Similarly there were more class III and class IV cell types (haloed granules and reticulated granules respectively) 2 hours post-injection as compared with controls. Four hours post-

injection the proportion of class III and IV cells were similar to control values.

(b) *Lamina propria.*

Serotonin:

Few EGCs were observed in the *lamina propria* of fish injected with serotonin. The only groups observed were class II with occasional class I cells, the proportions of which were not significantly different from the control fish (Fig. 4.9 c).

Capsaicin:

In capsaicin-injected fish at 30 min and one hour post-injection, class II and V cell types were the most significant classes ($p = 0.0002$). At two hours post-injection class V cells were the most dominant cell type in the *lamina propria* (Fig. 4.9 d). However after four hours, only occasional class V cells were seen, the numbers of which were not significantly different from saline controls (compare Fig. 4.9 a & d).

Substance P:

Substance P-treated fish exhibited more cell classes in the *lamina propria* region in comparison to serotonin-treated fish or BSA controls ($p = 0.0591$). In substance P-treated tissues all five cell classes were observed, in comparison to the controls which exhibited only classes I, II, III and V . For substance P, the most dominant cell classes in the *lamina propria* at times 30 minutes, 1 and 2 hours post-injection were class I or V. At four hours post-injection class II and class V cells were most

frequent (compare Fig. 4.9 b & e).

4.4. Discussion.

It was apparent that the saline vehicle used in the drug preparations or the BSA used with substance P had little significant effect on the EGC morphology in either the *stratum compactum* or the *lamina propria*. In controls, EGCs were identical to those described by other workers using untreated fish [24]. Bovine serum albumin has antigenic properties in *Tilapia spp.,* but is a poor antigen in rainbow trout when used in fish immunological studies [114, 115, 116]. The low number of EGCs observed in the *lamina propria* of saline- and BSA- treated fish observed with light microscopy, and class III cells observed in the same sections (*ie.,* cells with haloed granules) may represent a proportion of the unstimulated EGC population in which cells are undergoing the initial stages of degranulation (partial degranulation) or migration. The granules of such cells may be undergoing a proteolytic modification as has been previously proposed (Chapter 2) [102].

The cells identified as EGCs in the *lamina propria* and *stratum compactum* of fish treated with capsaicin and substance P were not infiltrated leucocytes or macrophages. Macrophage responses, although temperature-dependent [4], take considerably longer to develop than the changes in cell frequency observed in this study. Although neutrophil responses are faster than those of macrophages, the trout neutrophil is easily distinguished by its multilobed nucleus and small, generally oval

cytoplasmic granules [12, 26]. These features make the neutrophil quite distinct from the EGCs observed in both light and electron microscopic preparations.

The frequency of EGCs in the *stratum compactum* following injection of serotonin was significantly lower than that for saline controls. Although serotonin at the dose used (0.5 $\mu g.g^{-1}$ body weight) does not appear to cause the characteristic degranulation of capsaicin and substance P [102] (Chapter 2), it does appear to cause an initial reduction in the number of observable EGCs in the *stratum compactum* (see Fig. 4.2). This initial reduction followed by a recovery and stabilisation of frequency of EGCs after one hour with serotonin is interesting and is difficult to explain since there were no morphological or distributional differences between EGCs from sertonin-injected fish and saline controls.

The frequency of EGCs within the *stratum compactum* changed following stimulation with capsaicin and substance P. This change may be due to a loss of the densely staining granules, perhaps due to degranulation, used as the criterion for counting EGCs, making them difficult to identify with light microscopy. Alternatively, the decrease in EGC frequency may be due to a migration of cells from the *stratum compactum* to other regions of the intestine or into the vasculature as observed in the gills [117]. However, EGCs were not frequently observed in the blood vessels or lymphatics of the *lamina propria* or *muscularis*, suggesting that the decrease in EGC frequency is probably due to degranulation. Sharp *et al.* [90] reported that

EGCs were frequently unobservable with light microscopy due to the loss of densely staining granules. Electron microscopic examination confirmed their suspicion, with the presence of numerous EGCs with electron-lucent vacuoles suggestive of granule exocytosis during degranulation. It is interesting that at the dose used, serotonin, a putative neurotransmitter of non-myelinated nerves responsible for the innervation of intestinal circular smooth muscle [67, 68, 71], does not cause an effect similar to substance P. Ultrastructural examination revealed no significant morphological differences in cell classes between fish treated with serotonin and controls (*ie.*, saline), thus confirming previous observations with light microscopy and suggestions that serotonin is ineffective at degranulating intestinal EGCs [102] (Chapter 2). It is indeed possible that serotonin may be a component of EGCs as it is contained in rat mast cell granules [118, 119] and can be released on stimulation of peptidergic nerves [47, 48]. Ellis [22] demonstrated the presence of biogenic amines in gut tissue following degranulation of intestinal EGCs in trout with *A. salmonicida* exotoxins. The presence of biogenic amines in EGC granules is yet to be verified.

There are several scenarios which may help to explain these results. If the decrease in EGC frequency is mediated in part by endogenous neurotransmitters, then it is probably as a result of the presence of substance P or a related tachykinin released from nerves. The source of this compound could be either local, *ie.*, within the trout intestine or central in the spinal cord or brain. If the source of the substance P-like compound is released locally then it may come from nerves other than the

serotoninergic nerves of the myenteric plexus [67, 68]. A possible candidate may be the non-myelinated nerves which have been shown to be in close association with EGCs in the intestinal mucosa. Substance P acted relatively quickly, resulting in a reduction in the number of observable EGCs in the *stratum compactum* within 30-60 minutes post-injection. Capsaicin, however, acted more slowly with the decrease in EGC numbers occurring between one and two hours post-injection. This time lag suggests that capsaicin may indirectly induce degranulation. It appears that under normal physiological conditions endogenous substance P is a possible candidate for initiating EGC degranulation. In the current study exogenous substance P and capsaicin had similar effects on the distribution and morphology of EGCs. It is possible that capsaicin liberates substance P from neurons within the trout intestine which then acts as the mediator of EGC degranulation. Alternatively, capsaicin may be acting centrally and inducing the release of substance P which in turn leads to secondary effects (*eg.*, the release of a hormone or hormones which may then act on EGCs). Capsaicin is known to cause a depletion of several compounds including substance P and adenosine from nerves of the mammalian central nervous system [111]. This would explain the time lag in the effects seen with capsaicin and substance P.

Similar morphological and distributional changes in the EGC population of the trout mucosa occurred for both capsaicin- and substance P-injected fish. There was an apparent depletion of class II (normal) cells in the *stratum compactum* when fish

were stimulated with either capsaicin or substance P. This was compensated for by the increase in other cell types, predominantly classes I, IV and V. The depletion in class II cells occurred within 30 minutes of treatment with substance P and appeared to last the duration of the experiment. For capsaicin the depletion of class II cells did not appear to occ until one hour post-injection (Fig. 4.8 d & e). This apparent time lag is consistent with our previous observations [102] (Chapter 2). This time difference could be explained by the indirect release of substance P, but other factors involving the distribution and action of substance P and capsaicin centrally may be involved, as discussed previously.

The five cell classes observed in this study may represent different stages of granule synthesis of the EGCs. The class I cell type may represent an EGC in which the granules are being newly synthesised, hence their small size. This is somewhat similar to what is observed in regranulating mast cells [108]. The class II cell type possibly represents unstimulated cells with maturing granules. Alternatively, where these cells were seen in tissues of fish challenged with capsaicin or substan P, they may be in a "maturing" or nascent phase and subsequently are unable to degranulate. The class III and IV cells may represent EGCs in which a limited degree of the degranulation process can occur whereby degranulation is incomplete, perhaps due to the absence of a critical stage in the process. The class V cells possibly represent the fully "mature" EGCs which upon stimulation migrate to the *lamina propria* and undergo morphological changes identical to those described previously [102] (Chapter

2).

The apparent increase in the proportion of class I and V cells in the *lamina propria* of capsaicin- and substance P- treated fish suggests that stimulation induces not only morphological changes in the EGC population (*ie.*, degranulation), but also a distributional change. The sequential nature of this change in the proportions of different cell classes in each region (*stratum compactum* or *lamina propria*) is suggestive of cell maturation and migration. The presence of small proportions of class III and IV cells in the *lamina propria* suggests that EGCs of the *stratum compactum* mature and migrate to the *lamina propria* region. EGC migration has been observed in other tissues such as the gill endothelia [117] and following exposure to *V. anguillarum* exotoxin [120].

The presence of predominantly class I and V cells in the *lamina propria* of capsaicin- and substance P-stimulated fish suggests that EGC degranulation occurs primarily in the *lamina propria*. The presence of class I, small granuled EGCs, suggests that this may also be the region of granule resynthesis following degranulation. In substance P-treated fish, class I cells were observed in the *lamina propria* 30 minutes post-injection onward. In the *stratum compactum* of fish under the same treatment, class I cells did not appear until four hours post-injection at which time few were observed in the *lamina propria*. A similar but less well defined pattern was also apparent with capsaicin-treated fish. This suggests that following degranulation, EGCs begin

granule resynthesis and these "regranulating" cells return to the *stratum compactum*. Jamur and Vugman [108] observed rat peritoneal mast cells which, when degranulated with compound 48/80, resynthesised granules that appeared first as small structures that grew with the maturation of the mast cell. The appearance of class I, small granuled EGCs in the *lamina propria* following stimulation with capsaicin and substance P suggests the possibility that a similar regranulation process may be occurring in the EGC.

The apparent migration which has been observed in this study lends support to the theory that the *stratum compactum* acts as a depot or cache for EGCs in the gut [24] but supplements the theory by adding that the *stratum compactum* may be a possible site of EGC maturation. Interestingly, the EGCs are not in intimate contact with the fibroblast-like ensheathing cells in the *lamina propria*, whereas in the *stratum compactum* the ensheathing cell is tightly apposed to the EGC. It would appear likely therefore that the ensheathing cell plays an essential role in EGC maturation. Fibroblasts also play an essential role in the maturation of cultured rat mucosal mast cells [121]. Thus it would appear that the ensheathing cell may play a similar role in the maturation of the mast cell-like EGC in the trout mucosa.

In conclusion, this study has demonstrated that capsaicin and substance P induce changes in the frequency of intestinal EGCs in the *stratum compactum* of trout. Five classes of EGC can now be defined based on morphological differences. These

classes most likely represent different stages in the development and secretion activity of the cell. There are significant changes in the proportion and distribution of the different morphologies when stimulated with capsaicin and substance P. Analysis of the EGC populations has revealed that EGC degranulation is possibly superseded by the appearance of regranulating cells which appear to migrate back to the *stratum compactum*. The ensheathing cell may play a critical role in the response of the EGC to stimuli, similar to the mast cell/fibroblast interactions observed in rat mucosal mast cells [121].

5. GENERAL DISCUSSION

5.1. Changes in EGC morphology.

One of the objectives of this work was to investigate and quantify the relationship between intestinal EGCs and intrinsic nerves. The data presented in the preceding chapters have shown that both substance P and capsaicin have an effect on both the morphology and distribution of the EGCs of the trout intestine. These effects are manifested in an unusual granule morphology and degranulation of EGCs observed in the *lamina propria* of stimulated fish. Similarly a partial degranulation was observed in the EGCs of the *stratum compactum*. The distribution of EGCs was also observed to change with time. Upon stimulation with capsaicin and substance P, there was a decrease in the number of observable EGCs in the *stratum compactum* and an increase in both fully and partially degranulated EGCs in the *stratum compactum* and *lamina propria*. Although it is not possible at this stage to definitively state the endogenous source of the substance P-like compound which is capable of inducing these characteristic changes in EGC morphology, the intrinsic non-myelinated nerves of the intestinal mucosa may be involved. Capsaicin, because of its action on mammalian nerves, possibly depletes substance P from non-myelinated intestinal nerve endings in the trout intestine. Alternatively, capsaicin may act centrally perhaps in the spinal cord and induce a reflexive release of a substance P-like compound which may either act on EGCs directly or indirectly. Serotonin, which is a putative neurotransmitter of the gastrointestinal smooth muscle

of trout [67, 68, 70, 71, 122], had no apparent effect on EGC morphology although it did appear to initially reduce EGC numbers in the *stratum compactum*. This suggests that if these morphological and distributional changes in EGCs are mediated by nerves, the involvement of serotoninergic neurons would be unlikely.

The morphological changes in EGCs following stimulation with capsaicin and substance P have not previously been reported. Vallejo and Ellis [43] induced a process of degranulation somewhat similar to that seen in mammalian mast cells whereby the granules were exocytosed intact. This discrepancy may have been as a consequence of the relatively high doses used as compared with the present study. In the present study the degranulation appears to be a consequence of changes within the granule and the exocytosis of the granule contents following a non-lysosomal mechanism of granule breakdown. The morphological changes in granule substructure appeared to be reminiscent of crinophagy, a specialised form of microautophagy typical of neuroendocrine and neurosecretory cells [103]. The process observed in EGCs differs from crinophagy in that the EGC granules do not contain acid phosphatase from fused lysosomes. In the EGC, acid phosphatase is localised in lysosomes but not found in the subdivided granule matrix of multivesicular granules following exposure to capsaicin (see chapter 3).

5.2. Changes in EGC distribution.

5.2.1. Evidence for EGC migration.

Exposure to capsaicin and substance P produced significant changes in both the EGC population of the trout intestinal mucosa and the mean number of cells within the *stratum compactum*. There was a marked reduction in the number of observable EGCs, attributed to either their moving out of the *stratum compactum* (*ie.*, migration), or to degranulation, breakdown or exocytosis of the densely staining granules. Analysis of the different morphologies of EGCs in the mucosa suggested that cells were not migrating out of the *stratum compactum* and into the blood stream as had been observed in the gills [117] since EGCs were not observed crossing blood vessel endothelia in this study. Rather, EGCs were apparently migrating to the *lamina propria* and degranulating. Diapedesis is a common phenomenon in inflammatory cells (*eg.*, macrophages, eosinophils, neutrophils *etc.*) as it allows a rapid localised immune response. In trout, EGC diapedesis has been observed across gill endothelia [117]. Similarly EGCs have been reported to move to a variety of tissues including the liver and kidney (which do not normally have a population of EGCs) following bacterial infection [120]. It has been suggested that this facilitated a rapid distribution of EGCs throughout the tissue in response to bacterial infections. It would appear however that in capsaicin- or substance P-stimulated fish there is a migration of EGCs to the *lamina propria* in order to degranulate. The *lamina propria*, because of its loose connective tissue environment and perforation by blood vessels and lymphatics, would appear to be a good site for EGC degranulation. The

compounds being released by degranulating EGCs (possibly biogenic amines and eicosanoids) are likely to be vasoactive, that is cause vasodilation or constriction, capillary fenestration and inflammatory cell activation. Consequently it would seem reasonable that these compounds should be released in or near to blood vessels.

The apparent time lag between the reduction in EGC numbers in the *stratum compactum* (Chapter 2) [102] with capsaicin, as compared with the immediate reduction observed with substance P, suggests that capsaicin may be inducing the release of a compound similar to the exogenously injected substance P either locally from the closely associated non-myelinated nerves in the intestinal mucosa, or centrally perhaps in the spinal cord. The same conclusion has been alluded to following statistical analysis of the different cell types observed (based on their ultrastructure) in the different regions of the mucosa (*stratum compactum* or *lamina propria*) in chapter 4. The apparent lag of capsaicin behind substance P may be as a consequence of the effects of capsaicin on non-myelinated peptidergic neurons in the intestinal tract or elsewhere. Capsaicin powerfully depletes tachykinins (*eg:* substance P) and other substances such as adenosine from spinal cord sensory afferent neurons [112] and the gastrointestinal tract of mammals [54]. Thus the effects of this agent may be due to direct actions on peripheral nerves or to sensory actions that ultimately affect target cells, in this case EGCs. Since both scenarios would seem feasible, further studies are needed to verify either the local or central action of capsaicin and substance P in inducing EGC degranulation.

5.2.2. Evidence for EGC maturation.

Analysis of the different cell morphologies induced by capsaicin and substance P is strongly suggestive of the *stratum compactum* being a site of EGC maturation in the trout intestine. This supports earlier suggestions that the *stratum compactum* of the rainbow trout intestine may act as a cache or depot of EGCs which may be distributed about the body in response to infection [24]. It is not possible to confirm from this study that the EGCs residing in the *stratum compactum* are capable of migration to other tissue systems. It is suggested that they migrate to the *lamina propria* to degranulate only when mature. The sequential nature of capsaicin- and substance P-induced morphological changes in EGCs supports the theory of maturation. Partially degranulated EGCs of the *stratum compactum* may not be able to fully degranulate due to the absence of some not yet incorporated enzyme. Alternatively, a restricting influence may be being imposed by either the fibroblast-like ensheathing cell or the micro-environment of the *stratum compactum*.

Mast cells do not appear to migrate to a different histological region when mature but rather mature *in situ*. Rat intestinal mucosal mast cells originate from large lymphocyte-like cells, requiring a T cell factor to differentiate and the presence of fibroblasts to mature [121]. However, human connective tissue mast cells have been shown to develop from blood monocytes [123]. As with mammalian mast cells, EGCs degranulate and appear to begin regranulation by the formation of small granules which appear to become larger with maturation. A similar process has been

reported for compound 48/80-induced degranulated mast cells in rats [108]. Mammalian mast cell maturation requires factors produced by and the physical presence of fibroblasts [121]. Although the ontogeny of EGCs was not the object of this thesis, EGCs may arise from lymphocyte-like cells in the trout gut perhaps the GALT but this requires further investigation. It would appear that fibroblast-like ensheathing cells play a significant role in EGC maturation, especially since small granuled EGCs appear to migrate back to the *stratum compactum* where the ensheathing cells surround them (Chapter 4).

5.3. Significance of a nerve-EGC association.

In mammals, mast cells are involved in hypersensitivity responses. These responses may have psychogenic origins induced by excitement, stress *etc.* The role of nerves in pathophysiologic responses is not well understood but it is thought that neurally-mediated mast cell degranulation may cause the release of compounds independent of ligand-mediated degranulation [47, 48, 49, 50, 124]. The nerve-mast cell interaction would appear to be enhanced during parasitic infections whereby there is a increase in the number of mast cells in close proximity of nerves in parasite infected animals [47, 48, 49, 50]. In the trout intestine we have shown that a spatial relationship exists between non-myelinated neurons and EGCs. Approximately 32% of intestinal EGCs in the trout intestine are in close apposition to nerves (Chapter 2) [102]. Degranulation of EGCs is induced by capsaicin which may release substance P or a similar compound from these closely associated nerves. The

potential exists, therefore, for the release of compounds by EGCs in response to nerve stimulation. The crinophagic-like degranulation shown here in response to capsaicin and substance P, may represent a differential release of compounds compared to the explosive release seen in EGCs exposed to bacterial exotoxins and compound 48/80 [22, 43].

In situ pollen/Ig E-induced degranulation of human nasal mucosal mast cells resulted in a non-explosive release of histamine [125]. Non-explosive degranulation may be a physiological process. A nerve-EGC relationship may provide an alternative pathway for the release of EGC compounds which may act physiologically to maintain the extracellular milieu and be involved in processes such as growth and repair. This may be achieved by the differential release of vasoactive compounds (*eg.*, biogenic amines) from EGCs similar to that shown in mast cells [124]. The more explosive type of degranulation induced by exposure to bacterial exotoxin [22, 43] may be a more pathological response caused by enhanced EGC or mast cell stimulation.

In mammalian mast cells there is often a proportion of cells in which the granules are undergoing a variety of morphological changes although the cells have not been stimulated [126]. This is suggestive of a low-level mediator release. Similar observations were made in trout EGCs in this study, where in the control fish used in this study, cells were observed which were classified as type III or IV (haloed or

reticulated) cells. The presence of these cells in the controls has lead to the suggestion that perhaps there is a low level degranulation occurring even in control fish. Stimulation with capsaicin and substance P appears to exacerbate and accelerate the maturation of EGCs and the degranulation process. This suggests the potential for a neurally mediated enhancement of a variety of processes including immunological defense, tissue growth and repair in which mast cells have been implicated [48, 124]. It would appear from this study that a similar nerve-mast cell relationship may have evolved in the trout.

In conclusion, it has been shown that rainbow trout intestinal EGCs are in close association with non-myelinated nerves and can be degranulated with substance P, the putative neurotransmitter of the non-adrenergic, non-cholinergic nervous system and capsaicin but perhaps not serotonin, the putative neurotransmitter of trout intestinal smooth muscle. Stimulation with capsaicin and substance P appears to enhance the maturation and migration of EGCs in the *stratum compactum* to the *lamina propria* in order to degranulate. Upon degranulation EGCs may to migrate back to the *stratum compactum* to regenerate and mature. This process could be a means of physiologically maintaining the extracellular milieu of the intestine through the release of biogenic amines.

5.4. Future directions.

There are several possible directions in which further research on EGCs must

proceed. For example, the contents of the EGC granule remain obscure despite the extensive histochemistry carried out to date. The nature of the biogenic amine in the granules remains unknown. Is it histamine or serotonin? Similarly the nature of the acid glycosaminoglycans and their function both in the EGC granule and physiologically upon release remains unknown. Although we have alluded that the non-adrenergic, non-cholinergic nervous system may serve as a control of EGC degranulation in the trout intestine, direct stimulation of the intrinsic intestinal nerves would allow confirmation. Similarly the mechanism for capsaicin- and substance P-induced EGC degranulation remains unknown. It is also unknown if EGCs in other regions such as the gills and skin respond to capsaicin and substance P stimulation in the same manner to those of the intestine. Although there are a great deal of questions arising from this study the results presented herein have hopefully laid a foundation for future research on what appears to be a mast-cell analogue in fish.

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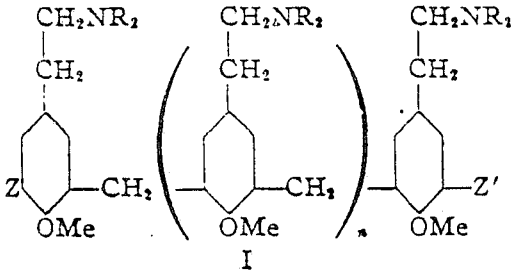
APPENDIX A

Structure of mast cell secretagogues and capsaicin:

Compound 48/80

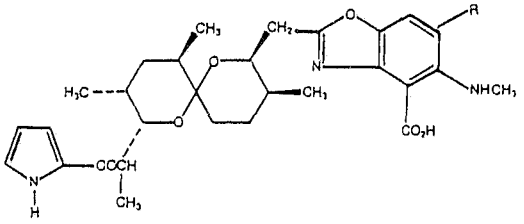


I

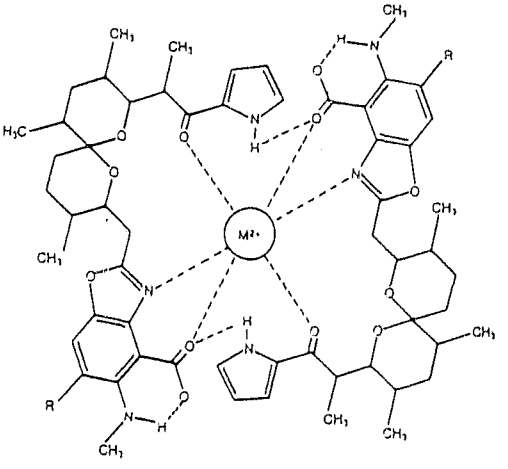
N-methyl-p-methoxy-phenethylamine

Calcium ionophore A23187: Calimycin

6S-[6α(2S* 3S*), 8ß(R*), 9ß, 11α]-5-(Methylamino)-2[3, 9, 11-trimethyl-8-[1-mthyl-2-oxo-2-(1H-pyrol-2-yl)ethyl]-1, 7, dioxaspiro[5-5]-undec-2y1]methyl]-4-benzoxazole carboxylic acid

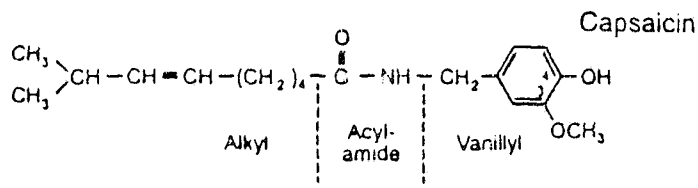


R = H (Free acid)
R = Br (4-Bromo-A23187)



Schematic representation of A23187-metal complex

Capsaicin.



8-methyl-N-vanillyl-6-nonenamide.

APPENDIX B

Tissue processing protocol:

Fix: Overnight in 3% Glutaraldehyde in 0.1M Millonig's Phosphate Buffer (MPB)(pH 7.3) at 4°C.

Wash: 2 x 15 min on rotator with 0.1M MPB.

Post Fix: 1 x 1 hour with 1% Osmium tetroxide (OsO_4) in 0.1M MPB at 4°C.

Wash: 1 x 15 min with distilled water on rotator.

Dehydrate: 1 x 15 min 50% ethanol on rotator.
2 x 15 min 70% ethanol on rotator.
2 x 15 min 95% ethanol on rotator.
2 x 15 min 100% ethanol on rotator.

Clear: 1 x 15 min with propylene oxide on rotator.
1 X 15 min with propylene oxide not on rotator.

Infiltrate: 45 min 50:50 Epon resin:Propylene oxide.
45 min 75:25 Epon resin:Propylene oxide.
90 min Pure Epon resin.

Embed: in labelled moulds at 10 blocks per case.

Polymerise: In vacuum oven overnight at 65-70°C.

APPENDIX C

Double staining for electron microscopy:

Uranyl acetate:

Saturated solution of uranyl acetate in 50% ethanol (5% uranyl acetate in 15 ml ethanol).

Centrifuge at 2500 rpm for 10 min.

Float grids on a drop of uranyl acetate supernatant.

Cover and stand for 30 min.

Wash: dip grids 30 times each in each of 3 beakers of distilled water.

dry on bibulous paper.

Sato lead stain:

To 40 ml distilled water at 40°C dissolve:

1.5 g lead nitrate.
1.5 g lead acetate.
1.5 g lead citrate.
3.0 g sodium citrate.
24.0 ml freshly prepared 1N sodium hydroxide.
40.0 ml distilled water.

Store in 20 ml syringes at 4°C.

Float grids on a drop of 0.22 µm Millipore ™ filtered stain solution for 2 min in a CO_2 reduced atmosphere.

Wash: dip grids 30 times in each of three beakers of fresh distilled water.

dry grids on bibulous paper.

APPENDIX D

Protocol for enzyme cytochemistry: Modified from [106].

Prefix: Overnight in 3% glutaraldehyde on 0.1M sodium cacodylate buffer with 7 % sucrose (pH 7.2) at 4°C.

Wash: 1 x 10 min with 0.1M sodium cacodylate buffer with 7% sucrose.

Sectioning: embed tissue in 7% agar and section at 150 μm on a vibratome containing 0.1 M sodium cacodylate buffer with 7% sucrose.

Wash: 1 x 10 min in 0.1 M acetate buffer (pH 5.0) with 7% sucrose prewarmed to 37°C.

Incubate: tissue slices in 50 ml Erlenmeyer flasks with 25 ml reagent substrate in a water bath at 37°C for 60 min After 60 min. replace with fresh medium and incubate a further 60 min.

Wash: 2 x 10 min 0.1 M cold acetate buffer with 7% sucrose.

Wash: 2 x 10 min cold 0.1 M sodium cacodylate buffer with 7% sucrose.

Refix: 1 x 60 min 3% glutaraldehyde in 0.1 M sodium cacodylate with 7% sucrose at 4°C.

Wash: Overnight in 0.1 M sodium cacodylate buffer with sucrose at 4°C.

Post-fix: for 1 hour at room temperature in 2% osmium tetroxide (OsO_4) in 0.1 M sodium cacodylate buffer (pH 7.2) (+ 7% sucrose).

Wash: 1 x 10 min distilled water.

Dehydrate: 1 x 10 min 50% ethanol.
2 x 10 min 70% ethanol.
2 x 10 min 95% ethanol.
2 x 10 min 100% ethanol.

Clear: 2 x 10 min propylene oxide.

Infiltrate: 90 min 50:50 propylene oxide: epon/araldite resin
90 min 25:75 propylene oxide: epon/araldite resin
overnight in pure resin.

Embed: in labelled moulds, 10 blocks per case.

Polymerise: at 65°C overnight

APPENDIX E

Acid Phosphatase reaction media: [106]

Acetate buffer: Stock A 0.2 M acetic acid
Stock B 0.2 M sodium acetate

for 100 ml: 29.6 ml stock A
70.4 ml stock B
Add 7 % sucrose

Experimental: 1mM ß-glycerophosphate (0.0216 g.100ml^{-1} 0.1 M acetate buffer
2mM cerium chloride ($CeCl_3$)(0.07452 g.100ml^{-1} 0.1 M acetate buffer).

Filter through 0.45µm Millipore™ filter and prewarm to 37°C prior to use.

Control: no ß-glycerophosphate.
2mM cerium chloride (0.07452 g.100ml^{-1} 0.1 M acetate buffer).

Filter through 0.45 µm Millipore™ filter and prewarm to 37°C prior to use.

APPENDIX F

Formvar coating of grids:

Formvar solution: 0.3% formvar in ethylene dichloride (0.9g formvar in 30 ml ethylene dichloride).

Dip: glass slide into coplin jar of formvar solution.

Drain: glass slide in desiccator.

Separate: formvar sheet from glass slide over a clean water surface.

Place: grids dull side down on formvar sheet.

Pick up: grids and formvar sheet with a folded filter paper.

Dry: in a desiccated atmosphere.

APPENDIX G

Reaction chemistry for Acid phophatase reaction:

ß-glycerophosphate ---> ß-glycerol + PO_4^{3-} + $CeCl_3$ ---> $CePO_4$ + $3Cl^-$
precipitate

Acid Phosphatase

APPENDIX H

General Linear model: